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Abstract

Beekeeping in Central Italy faces significant challenges due to climate change, pathogen spread, and market pressures. Long periods of cold temperatures and continuous rains as well as prolonged periods of drought and high temperatures, habitat loss, and increased exposure to parasites such as *Varroa destructor* and *Vairimorpha spp.* have led to a decline in honeybee colonies, threatening both honey production and pollination services. Additionally, economic difficulties stemming from low honey prices and competition from imported honey bee products have placed further strain on local beekeepers.

The aim of this PhD project, performed in agreement with D.M. n.1061 of 2021 PON resources "Research and innovation" Green topic (action IV.5), is to develop innovative and green strategies that improve the resilience and sustainability of beekeeping practices, facing the problem from different points of view such as protein and mineral nutrition, planned crossbreeding activity and precision beekeeping.

Establishing the ideal number of bees per experimental unit was a necessary step to ensure reliable physiological and behavioral data in subsequent trials. Optimizing colony density in controlled conditions allowed for a more precise evaluation of the effects of nutritional interventions and parasite control strategies, laying the groundwork for the main experimental phase of the study.

The core of this research focuses on the role of supplemental nutrition in counteracting environmental and biotic stressors. Laboratory and field trials were conducted to evaluate the impact of curbicolar pollen diets on honeybee physiology, highlighting their potential as a green and economic solution to provide protein supplementation. Additionally, mineral supplementation, particularly iron-based, was tested as a natural approach to enhance colony resilience against *Varroa destructor* and winter survival. The results suggest that integrating these green supplemental nutritional approaches can improve honey bee health and subsequentially the production, helping the beekeepers to facing the modern apiculture challenges.

Additionally, a crossbreeding program was explored as a potential approach to enhance the adaptability of *Apis mellifera ligustica* to climatic fluctuations. As per the call for PhD course, in

agreement with D.M. n.1061 of 2021 PON resources "Research and innovation" Green topic (action IV.5), these activities were held for a total of 6 months (divided into honey bee active seasons 2022 and 2023) in the host company Azienda Agricola BeeHive. Instrumental insemination and morphometric analyses provided promising insights into the potential of selective breeding for conservation purposes. However, further refinements in breeding protocols are necessary to assess its long-term effectiveness.

The study also evaluates precision beekeeping technologies as a tool for real-time colony monitoring. The integration of sensor-based systems enables beekeepers to optimize management practices, reducing losses and improving productivity. While these technologies offer promising solutions, challenges such as cost-effectiveness and beekeeper training remain barriers to widespread adoption.

Concluding, the combination of these strategies proves to be a feasible approach to addressing the main challenges of beekeeping in Central Italy. By implementing targeted, green, and economically sustainable nutritional strategies, developing resilient genetic lines, and adopting innovative monitoring tools, it is possible to mitigate the effects of climate change, counteract the spread of pathogens, and improve colony survival while simultaneously enhancing economic sustainability.

Riassunto

L'apicoltura nel Centro Italia affronta sfide significative a causa del cambiamento climatico, della diffusione di agenti patogeni e delle pressioni del mercato. Lunghi periodi di basse temperature e piogge continue, così come prolungate fasi di siccità e alte temperature, la perdita di habitat e la crescente esposizione a parassiti come *Varroa destructor* e *Vairimorpha spp.*, hanno portato a un declino delle colonie di api mellifere, minacciando sia la produzione di miele che i servizi di impollinazione. Inoltre, le difficoltà economiche derivanti dai bassi prezzi del miele e dalla concorrenza con prodotti apistici importati hanno ulteriormente aggravato la situazione per gli apicoltori locali.

L'obiettivo di questo progetto di dottorato, realizzato in accordo con il D.M. n.1061 del 2021 nell'ambito delle risorse PON "Ricerca e Innovazione" a tema Green (azione IV.5), è sviluppare strategie innovative e green per migliorare la resilienza e la sostenibilità delle pratiche apistiche, affrontando il problema da diverse prospettive, come la nutrizione proteica e minerale, l'attività di miglioramento genetico mediante inseminazione strumentale e l'apicoltura di precisione.

Determinare il numero ideale di api per unità sperimentale è stato un passaggio necessario per garantire risultati affidabili esenti da variabili esterne non controllabili nei test successivi. L'ottimizzazione della densità delle colonie in condizioni di temperatura e umidità controllate ha permesso una valutazione più precisa degli effetti degli interventi nutrizionali e delle strategie di controllo dei parassiti, ponendo le basi per la fase principale della sperimentazione.

Il focus di questa ricerca si concentra sul ruolo della nutrizione supplementare nel contrastare gli stresso ambientali e biotici. Sono stati condotti studi in laboratorio e in campo per valutare l'impatto delle diete a base di polline curbicolare sulla fisiologia delle api mellifere, evidenziandone il potenziale come soluzione ecologica ed economicamente sostenibile per l'integrazione proteica. Inoltre, la supplementazione minerale, in particolare con composti a base di ferro, è stata testata come approccio naturale per migliorare la resilienza delle colonie contro *Varroa destructor*. I risultati suggeriscono che l'integrazione di questi approcci nutrizionali green possa migliorare la salute delle api e, di conseguenza, la produzione, aiutando gli apicoltori ad affrontare le sfide dell'apicoltura moderna.

Inoltre, è stato testato un programma di incrocio come possibile strategia per migliorare l'adattabilità di *Apis mellifera ligustica* alle fluttuazioni climatiche tipiche degli ultimi anni. Come previsto dal bando di dottorato e in accordo con il D.M. n.1061 del 2021 nell'ambito delle risorse PON "Ricerca e Innovazione" a tema Green (azione IV.5), queste attività sono state svolte per un totale di 6 mesi (suddivisi tra le stagioni apistiche 2022 e 2023) presso l'azienda ospitante *Azienda Agricola BeeHive*. Le attività di inseminazione strumentale e la morfometria hanno fornito risultati promettenti sul potenziale della selezione genetica per la conservazione della sottospecie. Tuttavia, ulteriori perfezionamenti nei protocolli di allevamento sono necessari per valutarne l'efficacia a lungo termine.

Lo studio ha inoltre valutato le tecnologie di apicoltura di precisione come strumento per il monitoraggio in tempo reale delle colonie. L'integrazione di sistemi basati su sensori di vario tipo consente agli apicoltori di ottimizzare le pratiche di gestione, riducendo le perdite e migliorando la produttività. Sebbene queste tecnologie offrano soluzioni promettenti, sfide come la sostenibilità economica e la formazione degli apicoltori rimangono ostacoli alla loro diffusione su larga scala.

In conclusione, la combinazione di queste potrebbe dimostrarsi un approccio valido per affrontare in modo efficace le principali sfide che l'apicoltura deve affrontare nel Centro Italia. Attuando strategie nutrizionali mirate, green ed economicamente sostenibili, sviluppando linee genetiche resilienti e adottando strumenti di monitoraggio innovativi, è possibile mitigare gli effetti del cambiamento climatico, contrastare la diffusione degli agenti patogeni e migliorare la sopravvivenza delle colonie, migliorando nel contempo la sostenibilità economica.

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Chapter 1

General introduction

1.1 The honey bees

Honey bees, classified under the order *Hymenoptera* and the family *Apidae*, are essential pollinators, with *Apis mellifera* being the most notable species, even if some taxonomists also use the name *Apis mellifica* (Linnaeus, 1761).

Honey bees live in colonies that typically range from 60,000 to 80,000 individuals, consisting of a fertile queen, hundreds of drones, and thousands of worker bees (Figure 1.1) (Winston, 1991).

Figure 1.1. Honey bee's castes (Photo: Digital Museum of Natural History).

The honey bees life cycle involves four stages: egg, larva, pupa, and adult. Worker bees take about 21 days to develop, while it takes 15 days for queens, and 24 for drones (Oliver, 2010). The mating process of the queen with drones follows a specific pattern: the virgin queen flies to a congregation area where drones from different colonies gather, even if they are located several kilometers apart. Around fifteen drones deposit approximately 90 million spermatozoa in the queen's oviducts (polyadry). Out of this amount, around seven million spermatozoa are stored in a specialized sac in her body called spermatheca (Zagnoli & Biganzoli Corazza, 2013). The sperm will be released from the spermatheca only when the eggs are fertilized (Heimpel & De Boer, 2008).

The bee egg is a small white cylinder, about 1.5 mm long, attached to the bottom of the cell where the queen lays it. After three days of incubation, a tiny larva hatches and is fed by nurse bees. After five days of feeding, the larva stops eating, isolates itself in a cocoon it has spun, and the worker bees seal the cell with a wax cap. Metamorphosis then begins, transforming the larva into a prenymph and later into a nymph. Eventually, it becomes an adult insect. The timing of this process depends on the bee's future role. Drones and worker bees typically live for about 6-8 weeks during peak activity, though worker bees can survive for up to 6-8 months during winter (David Aston, 2021; Heimpel & De Boer, 2008).

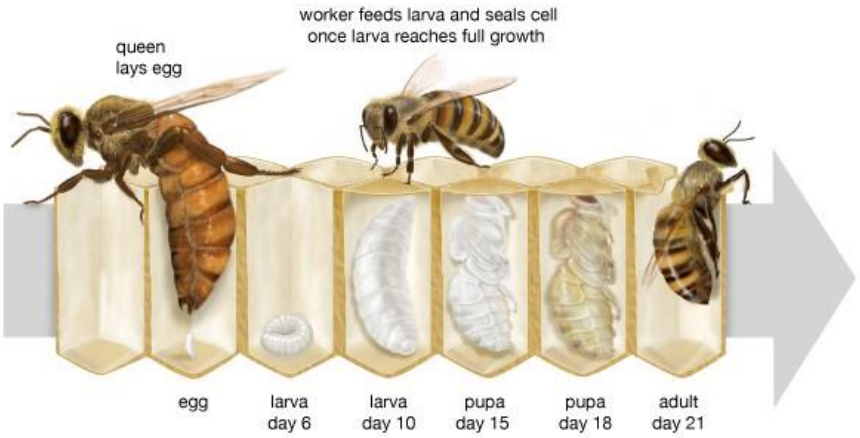


Figure 1.2. Honey bee's life cycle (Photo: https://onlinesciencenotes.com).

Once they emerge, young worker bees start their duties, beginning with cleaning the cells where they were born. Due to their underdeveloped reproductive organs, their roles shift with age. For the first three weeks of life, they stay inside the hive, engaging in tasks like cleaning cells, feeding brood, building honeycombs, storing food, and defending the colony. After this period, worker bees leave

the hive to collect nectar, pollen, propolis, and water, essential for sustaining the colony (Döke et al., 2015).

In Europe, several subspecies of *A. mellifera* are recognized, the most commonly farmed honey bees include *A. m. ligustica* in Italy, *A. m. mellifera* in central and northern Europe, *A. m. carnica* and *A. m. caucasica* in central-eastern Europe, *A. m. sicula* in Sicily, and *A. m. iberiensis* in Spain and Portugal (Spinola, 1806).

1.1.1 Ecological role of honey bees

A significant portion of agricultural crops relies on zoophilic or entomophilous pollination (Vaudo et al., 2015). Bees, along with other insects like Hymenoptera, Lepidoptera, and Coleoptera, are categorized as "pollinators." Through their biological activity, these insects facilitate the sexual reproduction of plants that require entomophilous pollination. In fact, around 300,000 plant species depend on this type of pollination.

Of these 300,000 species, three-quarters of the main crops essential for human nutrition rely on pollinators such as bees. Without them, our diets would be drastically limited, lacking the variety of vital nutrients, including essential vitamins and minerals (ISPRA, 2020).

Bees are the dominant taxonomic group among pollinators, and over 90% of the 107 most important crops for human sustenance globally depend on bee activity. The decline of pollinators is therefore significant, with potential consequences including a 5-8% reduction in global food production (Ponisio et al., 2015).

Insect pollination plays a crucial role in terrestrial ecosystems by providing essential services that support human well-being. A decline in pollinators could result in yield reductions of up to 40% (ISPRA, 2020; Khalifa et al., 2021).

The role of honey bees as pollinators is crucial for preserving natural habitats and maintaining the genetic diversity of local subspecies, along with their essential ecological functions (Fontana et al., 2018; Hung et al., 2018). In natural environments, honey bees are among the most frequent pollinators, accounting for an average of 13% of floral visits. Moreover, 5% of plant species rely exclusively on *Apis mellifera* for pollination (Hung et al., 2018), this underscores the significant role of honey bees in supporting the biodiversity of native flowering plant communities (Fontana et al.,

2018; Mallinger et al., 2017). Honey bees are also valuable for assessing biodiversity, as they can provide insights into the diversity of flowering plants. Pollen richness, often used to estimate the floristic diversity of an ecosystem, can be monitored through honey bees. For example, analyzing the pollen grains collected by honey bees in their pollen baskets offers useful data for biodiversity monitoring (Birks et al., 2016).

Honey bees play an additional role in providing valuable information on environmental pollutants. According to Bromenshenk et al., (1985), *Apis mellifera* as an effective biological monitor for contaminants across large geographic regions dates back to the 1980s. This important function is due to their morphology and behavior, which expose them to a wide range of organic and inorganic pollutants during foraging. Bees encounter pollutants in the air, water, soil, and vegetation. Moreover, these contaminants can infiltrate bee products such as pollen, honey, wax, propolis, and royal jelly.

Using honey bees as a pollution monitoring system offers several advantages compared to traditional methods such as minimal purchase and maintenance costs, self-sustaining biosensors for pollutant collection, and reliable sampling of pollutants. Additionally, being a living organism, honey bees allow researchers to study both lethal and sublethal effects of pollutant exposure on a biological system, providing deeper insights into environmental health.

1.2 Italian honey bee: Apis mellifera ligustica Spinola

The *Apis mellifera ligustica* Spin. (Figure 1.3), one of the most widely used honeybee subspecies globally, is renowned for its characteristics that are highly suited for beekeeping. Native to Italy, this bee is particularly known for its docility, making colony management easier and safer for beekeepers by reducing the risk of stings but it is also less prone to swarming compared to other subspecies, an advantage for maintaining stable colonies and reducing economical loss, also due to its adaptability to climate changes (Dall'Olio et al., 2007).

Figure **1.3**. *Apis mellifera ligustica* Spin. worker bee (Photo: G. Von Wimmer).

In terms of prolificacy, the *Apis mellifera ligustica* is famous for its high egg-laying rates in fact, queens of this subspecies lay large numbers of eggs, resulting in populous colonies that are more efficient in nectar gathering and honey production, this ensures that beekeepers can achieve substantial yields, even in times of limited floral availability (Yang et al., 2021).

One of the distinguishing features of the *Apis mellifera ligustica* is its hygiene behavior. Worker bees are particularly adept at cleaning and removing debris, which helps reduce the incidence of diseases affecting the colony. However, despite this hygienic behavior, the Italian bee is still vulnerable to parasites like the *Varroa destructor* compared to other subspiecies such as the "Primorski Honey bee" (Massaro et al., 2015; Rinderer et al., 2010). Additionally, the chemical composition of propolis produced by this subspecies highlighted its antimicrobial and antioxidant properties, making Apis mellifera ligustica a subject of interest not only for beekeepers but also for medical and nutritional research (Massaro et al., 2015).

Genetic studies conducted on *Apis mellifera ligustica*, particularly those using microsatellite DNA polymorphisms, have revealed notable genetic variability. This diversity enhances the bees' adaptability to different environmental conditions and contributes to disease resistance. However, the introduction and mixing of other subspecies could impact this genetic diversity, necessitating careful management of the breeding populations (Dall'Olio et al., 2007).

These characteristics make *Apis mellifera ligustica* one of the most favored subspecies by beekeepers worldwide, prized for its productivity and relatively easy management compared to other honeybee subspecies.

The *Apis mellifera ligustica* holds a unique legal and biological status as an endemic subspecies to mainland Italy and Sardinia island. This status is supported by both Italian national law and scientific recognition. The Italian law on beekeeping (L 313/2004) defines protections for this subspecies, while the Italian Scientific Committee includes it in the Check List of Italian Fauna specifically acknowledging its taxonomic significance.

From a regulatory perspective, some Italian regions have implemented specific measures to safeguard this subspecies. For example, in 2022, the Lazio Region (central Italy) passed regional law 14/2021, which focuses on the conservation of the *Apis mellifera ligustica* and other pollinators. This law prohibits the breeding of queen bees of any subspecies other than *Apis mellifera ligustica* within the region. Furthermore, the subspicies is registered in the Regional Voluntary Register, where native genetic resources at risk of genetic erosion are safeguarded in line with regional law 15/2000. The protection of this genetic heritage is overseen by two Technical-Scientific Commissions, which cover both plant and animal sectors.

These regulatory efforts complement the biological advantages of the *Apis mellifera ligustica*, ensuring not only its survival but also promoting its pivotal role in agriculture and biodiversity conservation.

1.2.1 Morphometrics and molecular methods for subspecies classification

To date, about thirty *A. mellifera* subspecies have been described. These begin with morphometrical differences, which are summed up by a number of classical studies in this field (Götze, 1930; Ruttner, 1988; Ruttner et al., 1978). These are then supplemented by studies on the variability of the nuclear genome and mitochondrial DNA (mtDNA) (Arias & Sheppard, 1996; Cornuet & Garnery, 1991; Tihelka et al., 2020). These subspecies are divided into five primary evolutionary lineages (A, C, M, O, and Y), three of which are thought to have originated in various parts of Europe (Ruttner, 1988): The African lineage (A) was primarily distributed in Southern Europe (Iberian Peninsula, the nearby Gascony in Southwest France, and several Mediterranean islands, including Sicily); the Italian

peninsula and East of Europe (C); and the northern portion of Western Eurasia (M), which stretches from the British Isles through most of continental Europe.

Morphometric techniques, which rely on a quantitative examination of the morphological traits of the bees, are an essential tool for the identification of *Apis mellifera* subspecies introduced by Ruttner (1988). This method looks at several anatomical measurements, such as the length and width of the insect's fore- and hindwings, the length of its femur, tibia, and metatarsus, and the distances between various body locations, for example the cubital index, which is the ratio of the lengths of two distinct cubital vein segments, is a crucial parameter in morphometric research. The precision of these assessments has been greatly improved by the use of advanced methods such as geometric morphometrics that allows for a deep analysis of morphological variation by describing the shape of anatomical structures using geometric coordinates; it allows, with the use of specialist software (*e.g.* DrawWing, ImageJ), to identify minute variations in digital images of body parts.

Mitochondrial DNA (mtDNA) has already shown its power to reveal the large-scale phylogeography of *Apis mellifera* (Garnery et al., 1998). Through restriction of the entire molecule and sequencing of small fragments, this marker has confirmed the existence of three main evolutionary branches, which coincide rather well with morphometry conclusions (Smith, 1991a, b; Garnery et al., 1992). This method is based on the analysis of the polymorphism of the tRNAleu-COII intergenic mitochondrial DNA locus and makes it possible to determine just the maternal line of bees in fact, using this method, it is impossible to assess the influence of the drone background and identify hybrid colonies.

Reference genomes, whole-genome sequence data, and hundreds of individual genotypes are now accessible for many species thanks to the advancements in high-throughput sequencing and genotyping technologies over the past ten years. High-density single nucleotide polymorphism (SNP) arrays, or “SNP chips” (Jones et al., 2020; Spencer et al., 2009), are a fast, accurate, and efficient technique for genotyping thousands of polymorphisms in high numbers of individuals. Further, a SNP panel was developed for molecular identification and the estimation of introgression levels of the “C” honey bee lineage, with the threatened subspecies *A. mellifera mellifera* (Henriques et al., 2018; Jones et al., 2020). Recently, Momeni et al. (2021), developed a SNPs panel that can discriminate (with a machine learning approach) 14 subspecies or different genetic origins with a mean accuracy of 96.2% ± 0.8 SD: *Apis mellifera ligustica* is also included, but the primary discrimination is always based on morphological analysis of the sample. For this reason, morphometrics and molecular methods are frequently coupled to produce a more reliable and accurate classification.

1.3 Problems of the Italian apiculture

The Italian beekeeping sector is currently facing a severe crisis, endangering the very existence of beekeeping enterprises, even the most structured ones in terms of production and entrepreneurship. The critical issues stem from two main areas. The firs one is the production loss due to climate change: the impact of adverse weather events linked to climate change leads to significant, sometimes total, production losses, as happened with acacia honey in 2019 for example (Osservatorio Nazionale del Miele, 2019). Honey production is concentrated within a very narrow time window, making beekeeping much more vulnerable to weather risks than other agricultural activities. Repeated sharp drops in production are largely caused by extreme weather events, attributed to climate change, often exacerbated by other factors such as environmental pollution due to human activities, incorrect agricultural practices, bee diseases, and hive predators.

Spring is almost characterized by situations of severe difficulty caused mainly by adverse weather conditions, which at various times affect entire regions with different intensity. Long periods of cold temperatures and continuous rains can damage the blooms and hinder the activity of bees, as well as prolonged periods of drought and high temperatures, putting their survival at risk due to lack of food (nectar and pollen) during critical phases of development. Colonies may face starvation, resulting in increased dependence on emergency feeding and frequent transfers which in turn increase production costs for beekeepers. These extra costs have further aggravated the economic damage caused by reduced productivity. According to Osservatorio Nazionale del Miele (2019) the estimated economic loss in 2019 for the failed harvest of just Acacia and Citrus honey amounts to 73 million euros, considering that the entire national production does not exceed 150 million euros.

As regards the main hive product, and greater source of income for beekeepers, there is a significant drop in the prices of italian honey and difficulties in placing the product on the market due to low price imports. The current market trend, which is extremely unfavorable, risks leading to a structural crisis due to unfair competition, often through the importation of low-cost foreign honey, as well as increasingly frequent adulterations, such as the dilution of foreign honey with sugar syrups. Although these products do not meet the qualification of 'honey' under European regulations, they are still marketed as such. The issue of adulteration is becoming increasingly significant, and the available analytical techniques, as well as the high costs of testing, do not adequately address the problem of fraud. After a decline in imports during the pandemic years (2019-2020), the 2022 Figures reached

record levels, and 2023 shows only a slight decline (-2%), while the value saw a more significant drop (-17%) due to falling prices across all markets. For the same reason, the value of exports also decreased. The trade balance shows a deficit of over 54 million euros in value, a significant improvement compared to 2022 (-22%). Consumption remains stagnant, and demand continues to weaken. In the first five months of 2024, volume purchases continued to decline year-on-year, falling by an additional 4.7%, adding to the - 4.3% decline in 2023 (ISMEA, 2024).

This situation highlights the need for targeted interventions to support beekeepers and mitigate the negative effects of climate change on this critical agricultural sector, both by the state and with the help of scientific research.

1.4 Climate change

Climate change is now an undeniable and evident fact; the Earth's surface temperature in the first twenty years of the 21st century was approximately 1°C higher than during the period 1850-1900. High temperatures (including heatwaves) are becoming increasingly frequent and intense in most areas of the globe, particularly in temperate zones, while, on the contrary, extreme cold (including cold waves) occurs less frequently (IPCC, 2021).

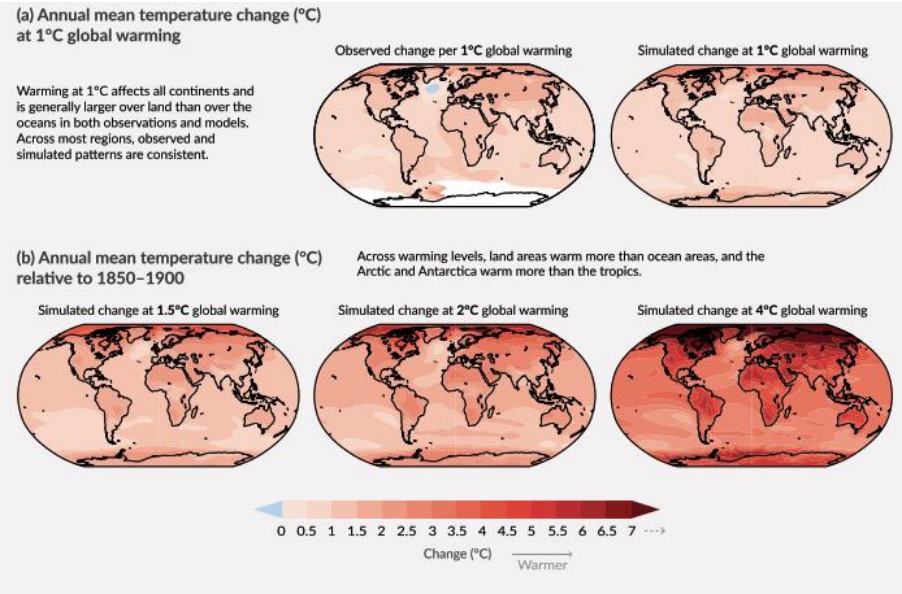


Figure 1.4. Annual mean temperature change (IPCC, 2021).

In recent decades, climate change has generated multiple pressures on agri-livestock production systems (Lamastra et al., 2017), with irregular precipitation and thermal stress being among the most significant. The growing concern for the thermal comfort of livestock is therefore justifiable, not only for countries in tropical zones but especially for those in temperate regions where high temperatures, including heatwaves, are becoming a management challenge (Bernabucci, 2019).

Unfortunately, even pollinator populations are not immune to the effects of climate change (Inouye, 2020). Among these, honey bees (*Apis mellifera* L.) are increasingly affected by climate change, and this leads to increasingly significant and often unpredictable repercussions for the beekeeping sector, which now faces the challenge of managing bees in conditions marked by altered seasonal rhythms and blooming periods.

Among the negative consequences linked to climate change is the increased susceptibility to parasites and pathogens, such as *Varroa destructor*, *Vairimorpha ceranae*, and *Vairimorpha apis*, as well as the introduction into Italy of commercial hybrids or non-native subspecies, such as *A. m. caucasica* or *A. m. carnica*, which are more vulnerable to the challenges posed by climate change.

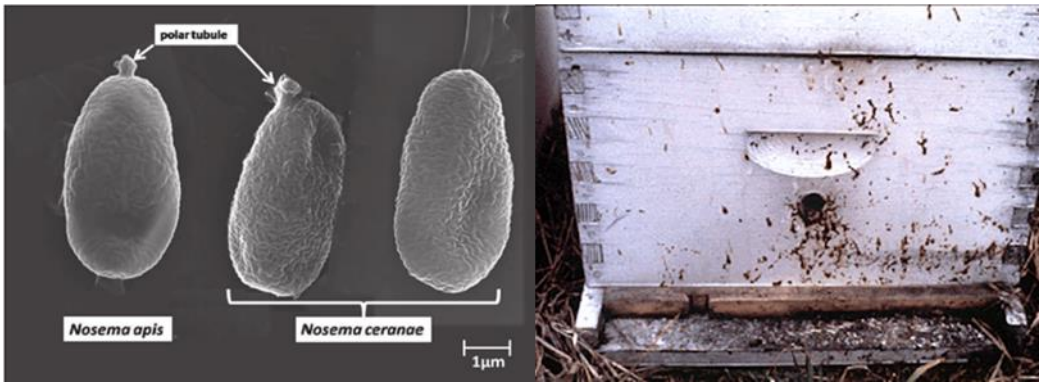
Considering beekeeping as a livestock, heat stress is already a major problem for animal husbandry, costing as much as USD 1.7 billion annually in the USA alone (St-Pierre et al., 2003). For example, currently almost 80% of cattle globally are exposed to conditions exceeding the temperature-humidity threshold (THI) for at least 30 days a year. For global warming above 4°C, heat stress of over 180 days per year emerges in temperate regions, and year-round heat stress expands across all tropical regions by 2100 (North et al., 2023); this scenario could equally impact also the global beekeeping.

1.5 Pathogens and parasites

1.5.1 Vairimorpha spp.

The nosemosis that affects honey bees (*Apis mellifera* L.) is caused by two separate microsporidia: *Vairimorpha ceranae*, which causes nosemosis type C, and the well-known *Vairimorpha apis*, which causes nosemosis type A (Figure 1.5B) (Higes, Raquel, et al., 2010). According to Adl et al. (2005), both microsporidia are obligatory intracellular eukaryotic parasites that are now categorized as fungi. The spore morphology (Figure 1.5A) (Ptaszyńska et al., 2014), genome size (Chen et al., 2013; Pelin

et al., 2015), survival (Higes, García-Palencia, et al., 2010) and spore production (Higes et al., 2007), and effects on the host (Martín-Hernández et al., 2011) are some of the differences between these species (Martín-Hernández et al., 2018).



(A) (B)

Figure 1.5. (A) *Vairimorpha apis* and *Vairimorpha ceranae* spores (Ptaszyñska et al., 2012); (B) Nosemosis symptoms on honey bee hive (Photo: https://www.vita-europe.com/beehealth).

Both parasites first invade the epithelial cells in the posterior region of the ventriculus and in two weeks, they cause a fully developed infection throughout the epithelium. In addition to trophallaxis, which involves bees ingesting contaminated comb material and water, honey storage and crushed infected bees may also contribute to the spread of the disease; in addition, *Vairimorpha* spp. can also be transmitted by air within apiaries (Sulborska et al., 2019). Microsporidian parasites infect host spores by mechanically injecting a polar filament that extends from the germinating spore. The filament physically enters the host cell by penetrating its membrane (ventricular epithelial cells for *N. apis* and *N. ceranae*) (OIE, 2021). The filament allows the infectious sporoplasm to enter the cytoplasm of the host cell, where it replicates and eventually starts to produce spores (Larsson, 1986). Mature spores begin to form in substantial quantities for *N. apis* three days after infection, and for *V. ceranae* around one day later (Forsgren & Fries, 2010).

The presence of *V. ceranae* in the European honey bee Apis mellifera was not discovered until 2005 (Higes et al., 2006). Nevertheless, it is currently present throughout the entire globe (Chen et al., 2008; Fries et al., 2006; Rodríguez et al., 2012). Up until 2005, it was only known to parasitize the Asian honey bee, *Apis cerana* (Fries et al., 1996). By interacting with different environmental pressures, *V. ceranae* can dramatically reduce colony strength and productivity, hence harming colony health (Botías et al., 2012). Although *V. ceranae* has also been found to be the main factor in colony

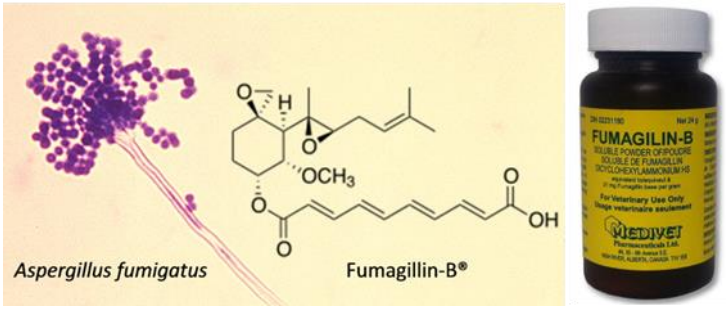
losses in Mediterranean regions, such Spain (Higes et al., 2009), there have also been reports of this parasite's asymptomatic presence (Fernández et al., 2012).

Numerous factors, including colony performance and bee health, are impacted by *Vairimorpha* infection (Martín-Hernández et al., 2018; Rodríguez-García et al., 2021). The impairment of multiple physiological functions, such as metabolic abnormalities sugar metabolism and hormone levels (Martín-Hernández et al., 2024), energetic stress, cognitive deficiencies, bodyweight reduction, and a decrease in life expectancy, are among the pathogenic effects of Vairimorpha infection in honey bees (Antúnez et al., 2009; Mayack & Naug, 2009; Rodríguez-García et al., 2021). Vairimorpha stresses honey bees nutritionally and energetically by using their metabolic resources for their own growth and proliferation, this weakens the bees' immune system. Apoptosis inhibition and substantial downregulation of many immune markers are signs of a compromised or impaired immune system in Vairimorpha-infected bees (Antúnez et al., 2009; Goblirsch et al., 2013; Higes et al., 2013). Due to immunosuppression, *V. ceranae* also makes honey bees more vulnerable to other pathogenic infections, resulting in more severe and complex disorders, in fact the coexistence with other pathogens, such as *Varroa destructor* or *Deforming wing virus*, increases colony mortality (Costa et al., 2011). Infection with *V. ceranae* has been linked, at the colony level, to a decrease in the number of adult bees and their ability to rear their offspring, a decrease in honey output, an increase in queen supersedure, and bee colony losses (Botías et al., 2013; Higes et al., 2009; vanEngelsdorp et al., 2009), also due to the altered flight activity and reduced homing success, resulting in a steady loss of bees in the colony (Martín-Hernández et al., 2024).

1.5.1.1 Methods for Vairimorpha spp. detection and control

The only registered product for nosemosis is the antibiotic fumagillin; however, in the European Union, there is no formulation registered for use in beekeeping (Martín-Hernández et al., 2024; Van Den Heever et al., 2014). The antibiotic fumagillin, isolated in 1949 from the fungus *Aspergillus fumigatus* (Figure 1.6A), has been used to treat microsporidiosis in honey bees for more than 50 years (Bailey, 1953). This substance prevents the methionine aminopeptidase-2 enzyme from acting on *V. ceranae*, which is responsible for the post-translational modification of the parasite's proteins (Williams et al., 2008). Fumigillin treatment of infected hives significantly lowers the infection levels in the colonies and the likelihood of their destruction (Formato et al., 2022; Mendoza et al., 2017).

However, because of its toxicity to mammals and the finding of antibiotic residues in apiculture products after treatment (Van Den Heever et al., 2015), the use of this substance is currently prohibited in Europe (Formato et al., 2022). The issue of bee mass mortality, which hinders the pollination of numerous cultivated and wild plant species, is becoming more and more problematic (Martín-Hernández et al., 2018). To address this, novel and efficient treatments as well as tactics to strengthen bee resilience to microsporidian diseases are needed.



(A) (B)

Figure 1.6. (A) *Aspergillus fumigatus* and Fumagillin-B® molecular structure; (B) Commercial Fumagillin-B® antibiotic (Photos: https://beesciencenews.com).

Some plant and algae extracts have shown good efficacy as well as annual queen renewal, control of other parasites, and avoidance of chronic exposure to pesticides or acaricide can reduce the negative effects of *V. ceranae*, but also gene silencing has also been suggested as a possible control method (Martín-Hernández et al., 2024).

The trend in beekeeping to use fewer antibiotics has resulted in a constant search for natural remedies for illnesses like nosemosis including nutritional supplements. For the treatment of *Vairimorpha* spp., organic compounds or natural treatments are encouraged, much like in the case of Varroa ectoparasite management(Jack & Ellis, 2021). As a constituent of essential oil obtained from *Thymus vulgaris* L., Lamiaceae, and numerous other plant species, thymol (3-hydroxy-p-cymene) is a naturally occurring chemical. Thymol has been used in beekeeping for many years to manage the Varroa destructor mite (Chiesa & D'Agaro, 1991; Imdorf et al., 1995). According to Glavinic et al. (2022), when given to Vairimorpha-infected bees, thymol mostly has favorable impacts on bee health (raising immune-related gene levels and oxidative stress parameter values, and lowering Vairimorpha spore burdens). Furthermore, thymol supplementation had no adverse effects on bees infected with Vairimorpha. On the other hand, it has been demonstrated that it may induce some diseases in Vairimorpha-free bees

(impacting bee survival, lowering oxidative capacity, and downregulating some immune-related gene expressions), suggesting maximum caution in using thymol (Glavinic et al., 2022).

According to a recent study (Branchiccela et al., 2019), nutritional stress is a prominent factor in *V. ceranae* infection. Long-term colony strength may be negatively impacted by this microsporidium, even though it may not induce colony loss right away. Huang (2012) has demonstrated that a spring pollen supplement can reduce the unfavorable symptoms of a *Vairimorpha* spp. infection and act as insurance during inclement weather. It was demonstrated that *V. ceranae* parasitized workers fed with multifloral pollen, lived longer than bees fed with *Cistus* sp. monofloral pollen (Di Pasquale et al., 2013) as well as a global increase of the immuno-competence (*e.g.* increase of the glucose oxidase) has been observed (Di Pasquale et al., 2013).

1.5.2 Varroa destructor

One of the most significant challenges currently facing beekeeping is the *Varroa destructor* mite, a parasitic species that has spread from its original host, the Asian honey bee (*Apis cerana*), to the European honey bee (*Apis mellifera)*. This parasitic mite has a substantial impact on colony declines and production loss (Traynor et al., 2020; Vilarem et al., 2021). Additionally, their pathogenic impacts in the field differ.

Taxonomically, the *Varroa* mite belongs to the family *Varroidae*, within the class *Arachnida*, subclass *Acari* (mites). The genus *Varroa* includes several species of forced ectoparasites, among which we find: *Varroa jacobsoni* Oudemans, a natural ectoparasite of the Asian bee (*A. cerana*), first described by Oudemans in 1904 in Java. Over time, this species adapted to parasitize *A. mellifera* in various parts of the world. The second relevant species is *Varroa destructor* (Figure 1.7A), recognized as a separate species from *V. jacobsoni* in 2000 by Anderson and Trueman, who classified these two species as reproductively isolated and morphologically distinct. While the two species share similar characteristics, the adult females of *V. destructor* are notably larger, exceeding 1 mm diameter (Anderson & Trueman, 2000).

(A) (B)

Figure 1.7. (A) *Varroa destructor* (Photo: apacame.org.br.); (B) Different maturation states of *Varroa destructor* (VETO-PHARMA, 2020).

The *Varroa* mite's mouthparts include two chelicerae, which it uses to cut the cuticle of adult bees, larvae, and pupae, creating a "feeding site" for nutrient access. The chelicerae also help the mite anchor itself to the bee, using small spurs on its pedipalps to grip the cuticle. Being an obligate ectoparasite with no free-living stage its life cycle consists of a phoretic phase on adult bees and a reproductive phase within sealed brood cells, preferring drone (male) cells but also occurring in worker (female) cells, followed by an oviposition phase (Figure 1.7B).

During the reproductive phase, the mite feeds on bee larvae within the brood cell, protected by the operculum, and takes advantage of this environment to reproduce and raise its offspring to adulthood. The timing of mite entry into a brood cell is critical, as infestation occurs a few hours (approximately 24 hours) before capping.

Once the cell is sealed, the oviposition phase begins, with the female *Varroa* laying haploid male eggs. The male mites, once mature, reach the feeding site and mate with the female mites, enabling them to lay diploid female eggs. These female offspring, upon reaching adulthood, can infest other brood cells. After the reproductive phase, the female *Varroa* mites transition to parasitizing adult bees by attaching to them during their emergence (phoretic phase) (Reams & Rangel, 2022).

Figure 1.8. Schematization of the reproductive cycle of varroa within the bee's brood (www.izslt.it/apicoltura/stadi/varroa).

Female *Varroa destructor* mites are transported by adult bees to brood cells for reproduction or disseminated among foraging and swarming bees (Kuenen & Calderone, 1997). During the phoretic phase, the mites hide under the bee's sternites, allowing them to be transported from the adult host to the capped brood cells (De D'Aubeterre et al., 1999).

The parasite inflicts harm on its host by consuming the fat body, a critical tissue responsible for immune function, pesticide detoxification, overwinter survival, and other essential processes for bee health. *Varroa* parasitism generally weakens the bee's immune system, increasing vulnerability to microbial pathogens, facilitating the onset of viral infections, and inducing replication of latent viruses (Johnson et al. 2010; Ramsey et al. 2019; Yves et al. 2010).

Varroa destructor spreads efficiently across territories by using bees as vectors, allowing the mite to infiltrate new hives. A single female mite can initiate an infestation by laying male eggs. Several key mechanisms contribute to *Varroa*'s propagation. First, weakened colonies on the verge of collapse due to *Varroa* are often looted by nearby colonies, leading to mite transfer (Milani, 1999). Second, bee drift occurs when foraging bees or drones accidentally enter neighboring hives, introducing the mites (Goodwin & Van Eaton, 2001). Third, swarming allows phoretic mites to spread as swarms establish themselves in new areas (Paleolog et al., 2023). Finally, routine beekeeping practices, such as transferring brood combs between colonies, can inadvertently spread Varroa (Paleolog et al., 2023).

The introduction of Varroa mite to European bees originated from the movement of *Apis mellifera* colonies into the range of *Apis cerana* in East Asia, particularly in Japan and Korea (Crane & Graham, 1985; Oldroyd, 1999). From there, the mite spread to South and North America (De Jong et al., 2003) and simultaneously reached Europe (Rosenkranz et al., 2010). In Italy, *Varroa destructor* was first detected near Trieste in 1980, with the first confirmed case on the peninsula recorded in 1981 in

Staranzano (Gorizia). Despite preventive efforts, including acaricidal treatments and trade restrictions, the mite spread uncontrollably across Italy by 1984 (Commission of the European Communities, 1988; Frilli, 1983).

Varroa destructor directly harms bees by consuming their fat body, essential for immune function and overwinter survival, and by depleting up to 25% of larvae's nutrient reserves, leading to reduced longevity and weakened bees demands (Garedew et al., 2004). It also produces proteolytic substances that inhibit protein synthesis in pupae and altering its development (Vesco, 2013). Indirectly, the mite suppresses immune responses, reducing vitellogenin levels and impairing wound healing and pathogen defense (Quigley et al., 2019). Additionally, the mite acts as a viral vector, transforming silent infections into symptomatic ones, particularly for *Deformed wing virus* (DWV), which causes wing deformities, shortened abdomens, and altered physiology (de Miranda & Genersch, 2010). The combined action of DWV, *Varroa dustructor*, and abiotic stress, such as cold temperatures, leads to the winter collapse of colonies (Dainat et al., 2012).

1.5.2.1 Methods for Varroa destructor control

The goal of the *Varroa destructor* defense is to lower the number of mites within the colony and, as a result, the viral load in order to limit the rate of infection. Beekeepers have access to a wide variety of acaricidal treatments and the effective application of them necessitates taking into account a number of variables, since the control approach is heavily influenced by regional climate and beekeeping production guidelines. Using the biological control criterion or taking use of the brood block are two more things to think about but determining the extent of Varroa mite infestation in colonies is crucial for devising a suitable control plan. Nowadays, a variety of chemical treatments using synthetic or natural active components and different biomechanical techniques are employed to combat Varroa mites.

Concerning the synthetic acaricides (Figure 1.9A) base their action on their neurotoxicity but the residues persist in honey intended for human consumption beyond the maximum permissible limits for health. The primary active component is Amitraz, an acaricidal active ingredient that has been used in beekeeping for decades; although it is highly effective in reducing infestations, it causes a noticeable start of resistance phenomena even after multiple treatments (Chuda-Mickiewicz et al., 2007; Faucon et al., 2007; Reza et al., 2008; Semkiw et al., 2013) as well as some metabolites may

remain in honey and especially wax (Jiménez et al., 2005; Korta et al., 2003). As for Amitraz, Fluvalinate (neurotoxic) has shown an outstanding effectiveness on Varroa mortality (Ferrer-Dufol et al., 1991; Salima & Haddad, 2015) but also in this case it presents resistance phenomena and problems for the queen as reduced size and deficient locomotor (Frost et al., 2013; Teeters et al., 2012).

Formulations with a low chance of residue accumulation following many treatments in honey or beeswax (Bogdanov et al., 1998; Bogdanov & Martin, 2002) and drug resistance (Milani, 1999) are examples of naturally produced acaricides. Among them are organic acids (Figure 1.9B), and the most widely utilized is the oxalic acid (dihydrate). It acts on phoretic Varroa but is unable to target it in the brood (Gregorc & Planinc, 2001; Imdorf & Berna, 1999; Nanetti et al., 2003). In beehives, oxalic acid can be applied via sublimation, spraying, or dripping (Al Toufailia et al., 2015). As a result, its application is restricted to the time when the brood is blocked, either alone or in conjunction with another slow-release acarid treatment, otherwise it may affect the sealed brood. Oxalic acid has extremely high efficiency of about 90% (Bogdanov et al., 2002; Coffey & Breen, 2016; Nanetti et al., 2003) but when applied in excess, it can have significant harmful effects on the larvae that are exposed to it (Aliano & Ellis, 2009; Ebert et al., 2007). Lactic acid is an organic compound effective in killing Varroa mites (Girisgin & Aydin, 2010; Kraus & Berg, 1994). Like oxalic acid, it is applied by spray and targets phoretic Varroa. However, spraying lactic acid is costly, making it suitable mainly for small-scale beekeepers. Additionally, it can acidify honey, so it should not be used when supers are in place (Imdorf et al., 2003).

Thymol, a monoterpenic phenol, also has good efficacy against Varroa mites (Gashout, 2009; Sabahi et al., 2020). Administered via evaporation due to its volatility, thymol treats both phoretic Varroa and those emerging from the brood, though its effectiveness varies with temperature (Al Naggar et al., 2016; Coffey & Breen, 2016; Leza et al., 2015) and the presence of brood (Giacomelli et al., 2016).

(A) (B) (C)

Figure 1.9. Different methods of *Varroa destructor* control. (A) Synthetic acaricides treatment (Photo: https://www.apicoltoremoderno.it); (B) Organic acid dripping treatment (Photo: https://rbeekeeping.com); (C) Space manipulation with Spazio Mussi® (Author's photo) .

The alternative control methods, which are acaricidal techniques capable of regulating the infestation rate, differ from chemical methods in that they utilize various methods to eliminate the mite, such as mechanical removal (removal of male brood), heat (hyperthermia), or they encourage the mite to fall naturally or following treatment (Figure 1.9C) (Spazio Mussi®, anti-Varroa grid). The removal of the male brood is a varroa control strategy that tries to mechanically remove the mite inside the brood, given the mite's propensity to infest the male brood more (Fuchs, 1990). The brood is extracted using trap frames which specific conformation induces the bees to produce natural honeycombs with male cells.

The anti-varroa grid is a component of the most common beehives; the size of the holes in the grid allows the passage of the varroas, and thus, following a fall of these from the bees (after a treatment or due to a natural fall), the varroas can be removed from the hive (Harbo & Harris, 2004). In the absence of an anti-varroa grid, mites would gather at the bottom of the hive and could readily return to the brood (Harbo & Harris, 2004).

The Spazio Mussi® has a bigger space between the combs than the regular Dadant-Blatt hives. Instead of the 37.5 mm standardized for hives, the Spazio Mussi® has a size of 45 mm; this space would allow to facilitate the hygienic behavior of the bees (or grooming); although its effectiveness is not significantly different from standard spaces, this type of space leads to a greater fall of Varroa. (Manino & Patetta, 2004).

Recent studies (Van Hoorn, M. Ter Horst, 2014) are testing the efficacy of nutritional supplementation of Fe(II) in the form of fumaric acid salt about increased Varroa fall in the phoretic phase and about colony survival to overwintering, on par with copper gluconate. Ferrous fumarate

can stimulate bees to exhibit hygienic behaviors such as cleaning or removing parasitized larvae, when administered to bees in a sugar solution, it has good acaricidal efficacy and is easily obtainable (Van Hoorn, M. Ter Horst, 2014).

1.6 Colony loss

Bees are insects that face a variety of biotic and abiotic risks. Their presence is declining in different parts of the planet, with some of the most common causes being intensive agriculture, monocultures, excessive use of chemical products such as pesticides, and rising temperatures due to climate change. These factors affect both crop production and bee nutrition (Desneux et al., 2007; Paleolog et al., 2023).

In addition to these challenges, bees are also significantly threatened by mites, viruses, and bacteria. *Varroa destructor*, *Paenibacillus larvae* (the cause of American foulbrood), *Melissococcus plutonius* (the cause of European foulbrood), *Aethina tumida*, *Vairimorpha ceranae*, and *Vairimorpha apis* are among the primary agents posing serious threats to bee populations (Krupke et al., 2012; Neumann & Carreck, 2010; Potts et al., 2010; Staveley P.J., 2014).

Analyzing the risk factors for bees is a complex task, as pathological and non-pathological factors can interact, creating not only additive effects but also synergistic or multiplicative ones (Bortolotti et al., 2009; Gray et al., 2020). Stress phenomena, with the resulting impacts on colony loss, can be identified at different times of the year (Bortolotti et al., 2009). For instance, in January-February, when beekeeping activities resume, colony loss can result from orphanhood and/or insufficient honey/pollen stores. In March-April, losses are often associated with spring sowing of some herbaceous crops and pesticide treatments on orchards. Finally, in autumn, high infestations of the *V. destructor* mite combined with the spread of particularly lethal viruses (*e.g. Deforming wing* virus) can compromise the proper development of pre-winter bees.

The critical event that fully represents the effect of climatic factors and pathogens on beekeeping is known as Colony Collapse Disorder (CCD). This phenomenon is characterized by the rapid loss of adult worker bees, but not the queen, resulting in an excess of brood, the absence of dead worker bees both inside and in the immediate vicinity of the hive, and a delayed invasion of hive parasites such as the greater wax moth (*Galleria mellonella*) (vanEngelsdorp et al., 2009).

In Central Italy, colony losses can occur during two main periods of the year: a) during the winter and up to the start of the active season, when sudden population declines are observed without apparent signs of intoxication or pathogenic attacks; b) during the spring, when significant bee mortality coincides with crop treatments using agrochemicals (Porrini et al., 2016).

In light of these facts, the hypothesis of a multi-causal etiology of colony loss is now widely accepted (Maini et al., 2010): the synergistic effect of altered flowering seasons and the nutritional stress induced by climate change with Varroa and/or Vairimorpha infestations, and misdirected bee practices can have a substantial effect on the maintenance of national bee populations.

1.7 Honey bees nutrition

Honey bees rely on flowers to get the nutrients they need. The latter require proteins (amino acids), carbohydrates (sugars), lipids (fatty acids, sterols), vitamins, minerals (salts) and water, and these nutrients must be present in the diet in a defined qualitative and quantitative ratio for an optimal nutrition (Standifer, 1969). Bee nutrition can be classified as macronutrients and micronutrients, just like the nutritional needs of other animals. As the name implies, macronutrients (lipids, proteins, and carbohydrates) are essential for the growth and survival of honey bees and must be consumed in big amount. Even while micronutrients are needed in smaller quantities, they are just as vital. Among the micronutrients that honey bees need include vitamins, minerals, phytochemicals, and phytosterols. Therefore, an ideal balance of all macronutrients and micronutrients is required for a healthy honey bee colony (Tsuruda et al., 2021) growth and immune responses (Alaux et al., 2011; Rosenkranz et al., 2010).

Bees primarily use nectar, which is converted into glucose by invertase enzyme, as a source of carbohydrates for energy but also pollen is a major source of them (Huang, 2010). The harvest of pollen, which contains about 20% of protein on dry matter, provides proteins, especially important amino acids (Thakur & Nanda, 2020), bee bread is subsequently made from pollen and is consumed by newly emerged bees immediately upon their birth (Brodschneider & Crailsheim, 2010; Janmaat & Winston, 2000; Somerville, 2005). Inadequate pollen can hinder brood formation and shorten bee lifespans (Brunner et al., 2014). Pollen, which has an average lipid concentration of 5% to 8% on dry matter, also provides lipids, which are essential for the growth, nourishment, and reproduction of bees (Manning, 2001; Svoboda et al., 1980).

Pollen also contains vitamins, particularly the B and C complexes, nevertheless, because these vitamins are unstable, pollen kept for longer than a year may lose some of their value (Sahinler et al., 2005; Sakla & El-shafeiy, 2022).
In lower amounts, minerals such as potassium, phosphorus (mostly), calcium, iron, and zinc can be found in both nectar and pollen. Specifically, iron helps in direction away from the hive and with maintaining iron homeostasis (Beinert et al., 1996; Kuterbach & Walcott, 1986; Valverde et al., 2023).

1.7.1 Supplemental protein nutrition

Pollen is the only natural source of protein for honey bees. According to Wille et al. (1985), colonies harvest between 10 and 26 kilograms of pollen annually. Crailsheim et al. (1992) calculated the pollen requirements of two 10-frame colonies to be 13.4 and 17.8 kg annually, respectively. Unlike honey, which is kept in large quantities in the colony at all times, pollen stocks in the colony quickly deplete during non-foraging periods (Schmickl & Crailsheim, 2001). With rare exceptions, honey bees find greater nutritional value in bee bread than in freshly collected, frozen, or laboratory-stored pollen (Dietz & Stevenson, 1980; Pernal & Currie, 2000). In order to evaluate the quality of pollen on honey bees, it is necessary to conduct bioassays, of different pollen or protein diets and their effects on brood production (Brodschneider & Crailsheim, 2010; Dietz & Stevenson, 1980), life span (Di Pasquale et al., 2013) using *ex-hive* trials (Manganello et al., 2023) or other physiological parameters.

Colonies without a pollen source can only support brood raising for a brief period before running out of body reserves and using up the bee bread that has been stored (Haydak, 1970). In order to adapt to shifts in the ratio of pollen supply to brood protein need, honey bees can cannibalize brood to obtain protein, which they then utilize to feed other larvae. Older larvae are preserved while younger ones, in which less effort has been put up to that point, are cannibalized (Schmickl & Crailsheim, 2001), therefore there will be no more brood production if the pollen shortage persists. Research conducted in cages has demonstrated that adult honey bees can survive for extended periods on carbohydrates, as these are essential for their energy metabolism, but honey bees feed also with pollen also exhibit longer lifespans (Alqarni, 2006; Manning et al., 2007). The age-specific protein demand of honey bees is remarkable: young adults undergo physiological changes such as the maturation of flight muscles (Tsuruda et al., 2021) or an incomplete and/or deficiency development of hypopharyngeal glands and ovaries (DeGrandi-Hoffman et al., 2010).

It has been demonstrated (Branchiccela et al., 2019) that nutritional stress, induced by lack of pollen, leads to an increase in the *Vairimorpha* spp. spore load in colonies not supplemented with a polyfloral pollen patties in *Eucalyptus grandis* crops. In comparison to supplemented colonies, colonies under nutritional stress (which mostly consumed *E. grandis* pollen) displayed a greater *Vairimorpha* spp. infection level as well as a decreased population of adult and brood bees in the short term. Conversely, the supplemented colonies showed higher infection level with RNA viruses although infection levels were low compared to countries were viral infections have negative impacts. Nutritional stress in pollen has long-term effects on colonies, as the bee population does not recover in spring, as in colonies receiving protein supplementation (Branchiccela et al., 2019).

It has been also observed that additional protein feeding (pollen or other protein forms) increases the number of *Vairimorpha* spp. spores in the intestine of the bee, thus leading to an increase in the pathological condition for the colony (Mortensen et al., 2019). This aspect has also been confirmed by an *in vitro* study which showed that pollen-fed bees had higher levels of *Vairimorpha spp.* infection but also a longer lifespan than bees fed sucrose syrup alone (Jack et al., 2016). Although these studies show the influence of diet on pathogen virulence in the laboratory environment, but nowadays it is not clear how protein supplementation affects the infection of pathogens at colony level in the field.

Alternative protein supplements for honey bee colonies often contain soy or whey rather than pollen. These supplements aim to mimic pollen by promoting nutrient flow and stimulating the hypopharyngeal glands (HPG) of young bees to produce worker jelly. Studies have shown that colonies fed protein supplements can achieve population growth rates similar to those fed pollen (DeGrandi-Hoffman et al., 2008; Mattila & Otis, 2006). Bees fed protein supplements displayed comparable protein levels and HPG development to those fed pollen and reduced *Deformed Wing Virus* (DWV) load (DeGrandi-Hoffman et al., 2010).

Protein supplements can be given to bees in different ways, for example as dry mix containing brewer's yeast or soy meal may be supplied to colonies inside the hive in pocket feeders or outside the hive in trays or containers (Figure 1.10B). An alternative is to provide a protein diet such as a wet cake or candy patty, this is placed in the nest directly on top of the frames (Figure 1.10A), at the center of the glomere. In both cases the addition of 10 to 12% pollen to a substitute feed for bees improves its palatability, instead the addition of 25-30% pollen improves the quality and quantity of essential nutrients required by bees for vital activity (Standifer et al., 1960).

(A) (B)

Figure 1.10. Protein supplementation. (A) Pollen patty inside the hive (Photo: https://rainydaybees.com); (B) Pollen patty outside the hive (Photo: http://www.mieliditalia.it/allevare-le-api).

Summarizing, protein and pollen supplements show effects on protein concentrations and HPG development and can play a significant role in reducing colony losses (DeGrandi-Hoffman et al., 2010).

1.7.2 Supplemental mineral nutrition

Nutritional supplementation based on iron, or other minerals such as copper, can help beekeepers work sustainably and more economically by giving them the opportunity to use non-harmful products.

Copper salts may have acaricidal effects against varroa (Nectoux & Bounais, 1990). A copper salt treatment is done indirectly, the copper salt is administered to bees orally diluted in a sugar solution, then the copper compound is transferred (through the bees) to the phoretic Varroa. The toxic effect of copper is to cause asphyxia, and consequently death, to the varroa mite that feeds at the expense of the fat body of the bee. The respiration of varroa is ensured by particular cells, the hemocyanin, which transport oxygen inside the body, the copper salts bind to the hemocyanin of the mite blocking their activity. At the same time copper salts are harmless to bees, as they do not cause significant symptomatology (Nectoux et al., 1995). Copper salts can also be used with other treatments, copper sulphate ($CuSO_4$) may have synergistic effect when combined with a treatment based on amitraz, as it can increase the biocidal effect towards varroa showing an average efficacy of 83.9% with mortality equal to untreated colonies (Bounias et al., 1994). Copper sulphate is therefore a good acaricidal compound, although the effects are only achievable in the long term and after repeated administration. Furthermore, it is a relatively harmless compound for bees and does not lead to significant increases

in residues in honey (1 mg/kg) when compared with other synthetic acaricides such as amitraz or fluvalinate (Bounias et al., 1994).

Iron salts may have good acaricidal efficacy against Varroa, the acaricidal effect could be to stimulate bees towards grooming and hygienic behaviour towards brood (Danieli et al., 2019; Van Hoorn & Ter Horst, 2014).

Iron (II) salts can be administered to bees in a sugar solution using pocket (Figure 1.11A) or cup feeders (Figure 1.11B) but must be added to solubilize. An iron salt suitable for use as a treatment seems to be the ferrous fumarate ($C_4H_2FeO_4$), since it can have a good acaricidal efficacy, at the same time weakly soluble in water and easy to find. Ferrous fumarate combined with vinasse can increase its effectiveness, as it chelates iron (II) by improving its solubility (Van Hoorn & Ter Horst, 2014).

(A) (B)

Figure 1.11. Mineral supplementation. (A) Pocket feeder (Photo: https://www.latiendadelapicultor.com); (B) Cup feeder (Somerville, 2005).

It has been shown that groups treated with iron fumarate chelated with vinasse have a lower winter colony loss than untreated groups (respectively about 8% versus 40%) and a good fall of the varroa mite (Van Hoorn & Ter Horst, 2014); Danieli et al. (2019) showed that the colonies treated with ferrous fumarate and vinasse showed a higher overall fall of varroa and more damaged adult varroas than the control group. The integration with iron finally seems to promote the increase of bee colonies drastically reducing winter mortality (current losses are on average 40-60%) (IZS Venezie, 2018).

Iron deficiency in bees may be due to the extraction of the element, for example from varroa mite or it may also be due to a reduction in its availability in pollen and/or nectar, this can lead to reduced iron availability for important processes in the body of a bee. In bees, iron is stored largely in the fat body, when the varroa mite parasitizes a bee, the iron is divided between host and parasite, thus

reducing the insect's reserve. If this extraction is prolonged, the iron supply of a bee may run out and only then do bees show deficiency symptoms (Eisenstein & Blemings, 1998).

Thus, the supplemental iron intake seems to positively affect bees in various ways: the percentage of varroa mite fall is higher, bees fly more actively, varroa mite reproduction decreases, bees live longer. Iron could well fit with competing honey bee practices and could be used instead of formic acid, oxalic acid, thymol or synthetic acaricides without any negative effects on queens and reproduction as well as being more economically viable.

1.8 References

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Chapter 2

Objective and Thesis Structure

As per the call for PhD course, this project has been performed in agreement with D.M. n.1061 of 2021 PON resources "Research and innovation" theme Green (action IV.5).

The work addresses some of the major problems currently affecting the world of apiculture in Central Italy, induced by climate changes and nutritional stressors, from the perspective of the beekeepers.

This thesis is organized into several chapters, each addressing a specific topic and representing a green and sustainable approach to solving the challenges faced by apiculture in Central Italy.

The main objective of the project is to develop innovative and green strategies that improve the resilience and sustainability of beekeeping practices, facing the problem from different points of view such as protein and mineral nutrition and planned crossbreeding activity.

Below is an outline of the thesis structure.

- ***Topic 1 – Ex-hive experimental setup optimization.***

  Optimizing Italian honey bee (*Apis mellifera ligustica* Spin.) density in caged laboratory trials.

  This chapter focuses on the efficient management of honey bee populations in controlled environments. The results provide information for an optimal experimental setup with *Apis mellifera ligustica* (Spin.), a necessary step of optimization for topic 2 and 3 discussed in the following chapters.

- ***Topic 2 – Protein nutrition.***

  Physiological responses of young Italian worker bees (*Apis mellifera ligustica* Spin.) induced by different pollen diets.

  This chapter investigates the effects of different pollen diets on the physiological responses of young Italian honey bees (*Apis mellifera ligustica* Spin.) in a suburban environment. The study focuses on how the type and collection period of pollen impact physiological key factors, as well as the bees' resilience to *Vairimorpha* spp. infections.

The main goal is to provide insights into optimizing honey bee nutrition to promote colony health and sustainability in changing environmental conditions.

- ***Topic 3 – Mineral nutrition.***

  Effectiveness of Fe (II) mineral supplementation in Italian honey bee (*Apis mellifera ligustica* Spin.) under field and laboratory conditions.

  This chapter evaluates the use of Fe(II) supplementation as a green and effective method for controlling *Varroa destructor* infestations and promoting colony survival in winter with the aim to reduce the dependency from synthetic/chemicals treatments and providing a non-toxic and benign Fe(II) mineral supply to ensure proper nutrition for colonies under nutritional stress. This sustainable apiculture practices could be economic and easy to apply for beekeepers, promoting its use as well as a viable alternative to commercial products.

- ***Topic 4 – Crossbreeding activities (Host company).***

  Crossbreeding of *Apis mellifera ligustica* Spin. to obtain climate-resilient phenotype.

  In agreement with D.M. n.1061 of 2021 PON resources "Research and innovation" theme Green (action IV.5), the activities presented in this chapter were carried out over a period of 6 months of training (divided into honey bee active seasons 2022 and 2023) in the host company Azienda Agricola BeeHive, located in Gennazzano (Rome, Italy) under the guidance of tutor/beekeeper Paolo Scarpino.

  This chapter deals with breeding strategies, using instrumental insemination and morphometric analysis, to obtain honey bees with high membership belonging to the Italian subspecies (*Apis mellifera ligustica* Spin.). The aim was to develop a phenotype resilient to climate changes, promoting sustainable beekeeping practices in changing environmental conditions.

- ***Topic 5 - Precision Beekeeping.***

  Precision Beekeeping Systems: State of the Art, Pros and Cons, and Their Application as Tools for Advancing the Beekeeping Sector.

  This chapter is a published review article highlighting the adoption of precision beekeeping technologies as a sustainable means for remote colony monitoring. These approaches leverage commercial systems usually equipped with weight, thermal, and flight sensors to enable efficient resource management while supporting the health and

productivity of bee colonies. By providing accurate and continuous monitoring, they allow beekeepers to intervene promptly when necessary.

Chapter 3

Optimizing Italian honey bee (*Apis mellifera ligustica* Spin.) density in caged laboratory trials

3.1 Introduction

Inside the hive, worker bees maintain the high temperature and humidity required for brood rearing through thermoregulation (Jones et al. 2004; Human et al. 2006). The energy expenditure involved in stabilizing these conditions is influenced by the nest's volume and insulating characteristics. In the wild, bees consider these factors when selecting nest sites, often opting for cavities with volumes between 30 and 60 liters. This corresponds to an estimated space per bee of 1.9–3.8 mL, depending on swarm size (Vaudo et al. 2012; McMenamin et al. 2017).

Under laboratory conditions, temperature and humidity can be controlled using temperature-regulated incubators, while nest volume can be simulated with specialized hoarding cages to house groups of honeybees (Williams et al., 2013), these conditions can afford to exclude part of the environmental variability of field trials. An additional critical factor in such studies is the preservation of social interactions within the hive: as eusocial insects, honeybee behavior is intrinsically linked to interactions among colony members (Winston and Michener 1977; Nowak et al. 2010). Key social behaviors include trophallaxis (mutual feeding, reviewed by Crailsheim, 1992), the waggle dance to communicate foraging locations (von Frisch and Lindauer 1956), and group huddling for thermoregulation (Lindauer 1955). These interactions influence individual behaviors, such as nutrient foraging, which is shaped by trophallactic secretions from nurse bees (Schott et al. 2017), and thermoregulation within the brood area (Basile et al. 2008).

Therefore, for behavioral studies, it is therefore vital to house bees in groups to preserve their social interactions, which are essential for maintaining colony temperature, locating forage, and caring for brood. Group size, however, can significantly affect the behavior of caged bees (Hepburn et al. 2014). For instance, in toxicological studies conducted in cages, some bees consume the diet directly, while others are fed indirectly through trophallaxis, mimicking their natural feeding behavior within the hive (Brodschneider et al. 2017).

Although group size significantly impacts bee behavior, yet little attention has been given to how cage design affects honeybee survival and behavior in laboratory settings, in fact survival rates in such conditions are affected by both the dimensions and materials of the cages (Köhler et al. 2013). Previous studies have tested cages of various shapes, sizes, and materials, showing that these factors influence honeybee survival (Williams et al. 2013; Huang et al. 2014). However, the lack of standardization in cage dimensions across studies has resulted in inconsistent space availability per bee, complicating direct comparisons. Additionally, the material used for cages, which can affect ventilation, insulation, and overall behavior, has not been uniformly controlled. Features such as feeders and wax substrates for bee aggregation also contribute to experimental outcomes: usually, feeders are often incorporated (Huang et al. 2014), whereas wax slots is only required for specific experimental designs.

According to Williams et al. (2013), frame and hoarding cages, regardless of their design, should meet specific basic criteria, with adjustments depending on their intended purpose. For instance, cages should either be single-use or thoroughly cleaned and sterilized between uses to minimize contamination from pathogens and chemical residues. In studies on pesticide toxicology, single-use cages are preferred due to the difficulty of completely removing chemical residues. Conversely, reusable cages are appropriate for pathogen research, provided they are constructed from materials like stainless steel or glass that can withstand sterilization processes such as autoclaving or irradiation. Obviously, the sterilization method should be adapted to the study's requirements, for example, exposure to 121°C for 30 minutes is sufficient to eliminate *V. ceranae* spores (Fenoy et al. 2009). Additionally, cages should be made from inexpensive and readily available materials that are easy to manipulate, such as plastic or wood, which facilitate modifications, like the addition of feeding devices. They must also have sufficient air holes to ensure adequate ventilation. To minimize the risk of cross-contamination by pathogens or chemical residues in cages housed within the same incubator, ventilation holes should be covered with filter paper or similar breathable material. If unfiltered vents are used, cages should be positioned facing opposite directions and spaced apart to prevent inter-cage trophallaxis or movement of frass.

Cages should also be designed to allow for easy removal of both live and dead honeybees during experiments while preventing accidental escapes. At least part of the cage should be transparent to allow observation of the bees.

The size of the cage should correspond to the number of bees it will house; for example, a 500 cm^3 (500 mL) cage can accommodate several hundred workers, while a 100 cm^3 cage is suitable for about 30 workers. A general rule of thumb is to maintain a ratio of approximately 3:1 (cm^3/bee) for housing fewer than a few hundred bees (Williams et al. 2013).

Concluding, among the experimental conditions, for practical and scientific reasons, the honey bees' density (HBD) per unity cage of volume can be considered relevant in laboratory trials so the aim of this work is optimizing the honey bee (*Apis mellifera ligustica* Spin.) density per unity cage of volume to have a standard for laboratory trials.

3.2 Materials and methods

3.2.1 Emerging HBs

To obtain the young HBs, a non-experimental B-BOX hive (Beiing Srl, Faenza, Italy) in controlled environmental conditions (25.1 ± 3.2°C) was used (Taber and Owens 1970) (Figure 3.1). To obtain a cohort of 0-24 h old workers, a queen, provided by a queen bee breeder and with the certification of belonging to the subspecies *A. m. ligustica* Spin. according to the official morphometric standard (CREA, 2023), was caged to force laying on the same honeycomb inlaid in a special queen-excluder cage (Erboristeria Apistica, Sondrio, Italy). The development of the brood was monitored daily by manual noting the number of eggs, larvae and capped cells to evaluate the time of the highest laying rate after the queen was caged.

A B

Figure 3.1. B-BOX hive in controlled environmental conditions. A) installation; B) operating status (Author's photo).

3.2.2 Ex-hive trials conditions

About four days before the forecasted emergence of worker bees, the honeycomb was moved in a net cage maintained into an incubator at 33.1 ± 0.8 °C and 72.0 ± 1.5% RH, and young bees were provided with SS50 and tap water *ad libitum* (Figure 3.2). Once all the cages were set up, the trial conditions were modified as follow: 29.9 ± 0.4 °C and 71.1 ± 2.5% RH.

Figure 3.2. Honeycomb in the net cage with newly emerged bees (Author's photo).

Self-built 3D printed cages were designed and produced with fixed measures of 7 × 6 × 4 cm (hight × wide× deep) and a total inner volume of 168 cm^3 (Figure 3.3A). To limit any possible effect of the cage material on the bees, only polyethylene terephthalate (PET) and polylactate (PLA) (Prusa Reseach a.s., Partyzánská, Czech Republic) polymeric filaments were used in the 3D printing process. Each cage used (twelve in total, three cages per honey bee density) had a removable tray at the bottom for monitoring the varroa mite fall and the collection of any debris (*e.g.*, feces). On the left and rights sides there were four holes, two per side, suitably sized to fit 2 mL centrifuge tubes which contained solid diet, pierced (4.5 mm) on the bottom to allow bees to enter. The top of the cage was designed to allow fitting syringes (1 mL) containing tap water or SS50. In the back side of the cage, a special slot fitted with a piece of embossed wax foundation (4 cm × 5 cm) allowed honey bee aggregation.

A four bees' densities trial, in triplicate, was set up with 20 (D1), 30 (D2), 40 (D3) and 50 (D4) 0-24h post-emergence *A. m. ligustica* Spin. workers (11.9, 17.8, 23.8, 29.7 bees/100 cm^3 respectively) collecting through a battery-powered vacuum cleaner from the honey comb and then placed inside the cages. Solid diet, according to Haydak M. (1970) (Recipe: Defatted soybean meal 272.2 g/Kg; Sucrose 272.2 g/Kg; Brewer's yeast (wet) 272.2 g/Kg; Whey powder 90.9 g/Kg; ASTM type I water 90.9 g/Kg.) was added with 2 mL centrifuge tubes.

During two weeks of experimentation, dead bees were counted and removed daily, and the consumption of water and SS50 was recorded by the same operator at the same daily time. The unconsumed solid diet was replaced every two days, dried at 65°C for 24 hours, and the average daily DM intake (DM, mg DM/bee/day) was calculated by difference from the dry weight of diet administered, divided the average number of alive bees. At the end of each trial the bees still alive were narcotized by cooling and stored in freezing conditions for further measurements.

A B

Figure 3.3. A) Self-built 3D printed cages used for trials with SS50 (1), tap water (2), protein diet (3), wax foundation (4) and removable try (5). B) Setup of the incubator during the test. (Author's photos).

3.2.3 Data processing and statistical analysis

At the end of the feeding trial, the HBs mortality rate was calculated, expressed as a percentage based on the number of HBs dead in the fourteen days compared to the initial one. In order to ensure that the parameters which values were percentages could have a normal distribution for the statistical

analysis, the arcsine transformation described by Zar et al. (2014) was applied for mortality variable as percentual fraction of the initial number.

For variables analysis with daily records, such as individual consumption of SS50 (µL/bee/day), water (µL/bee/day), diets (mg DM/bee/day) mortality rate a repeated measures ANOVA was performed including as fixed factor treatments and days. Tukey test was used for pairwise comparisons and were performed with STATISTICA 10 software (Statsoft inc., Tulsa, USA). Significance was always declared at $P \leq 0.05$.

3.3 Results

The effect of HBD resulted statistically significant only for mortality rate: D2 resulted the lowest one compared to the others while the value rate was registered for D3 (Table 3.1). The effect of days, excepted for individual consumption of SS50, resulted always highly statistically significant as well as the interaction HBD × D for IC of H_2O and mortality rate.

Table 3.1. Results of the effect of HBD and days on individual consumptions and mortality rate (Means ± SEM).

						***P*-value**		
	D1	**D2**	**D3**	**D4**	**SEM**	**HBD**	**D**	**HBD×D**
IC_SS50 (µL)	19.62	13.96	23.69	19.18	4.75	0.586	0.080	0.238
IC_H_20 (µL)	7.13	2.05	18.99	1.33	5.82	0.197	0.001	0.008
IC_Diet (mg DM)	0.03	0.19	0.26	0.38	0.19	0.646	<0.001	0.700
Mortality rate (%)	7.8[a]	5.36[b]	19.09[a]	15.58[a]	3.00	0.037	<0.001	0.002

IC: Individual Consumption; HBD: Honey Bee Density; D: Days; [a,b] *P*-value ≤ 0,05

Effect of days on IC of H_2O resulted statistically significant between the first nine days and the last five, in fact HBs consumed almost the double of value of tap water in the last days of trial (Figure 3.4B). Conversely, IC of solid diet was related to the first ten days of trial, with a peak in days 3 and

4 which resulted statistically significant and higher, with a 1.5 fold increase, compared to the other records (Figure 3.4C). Finally, the mortality rate tended to increase with the days, reaching a plateau effect from day 9 (Figure 3.4D).

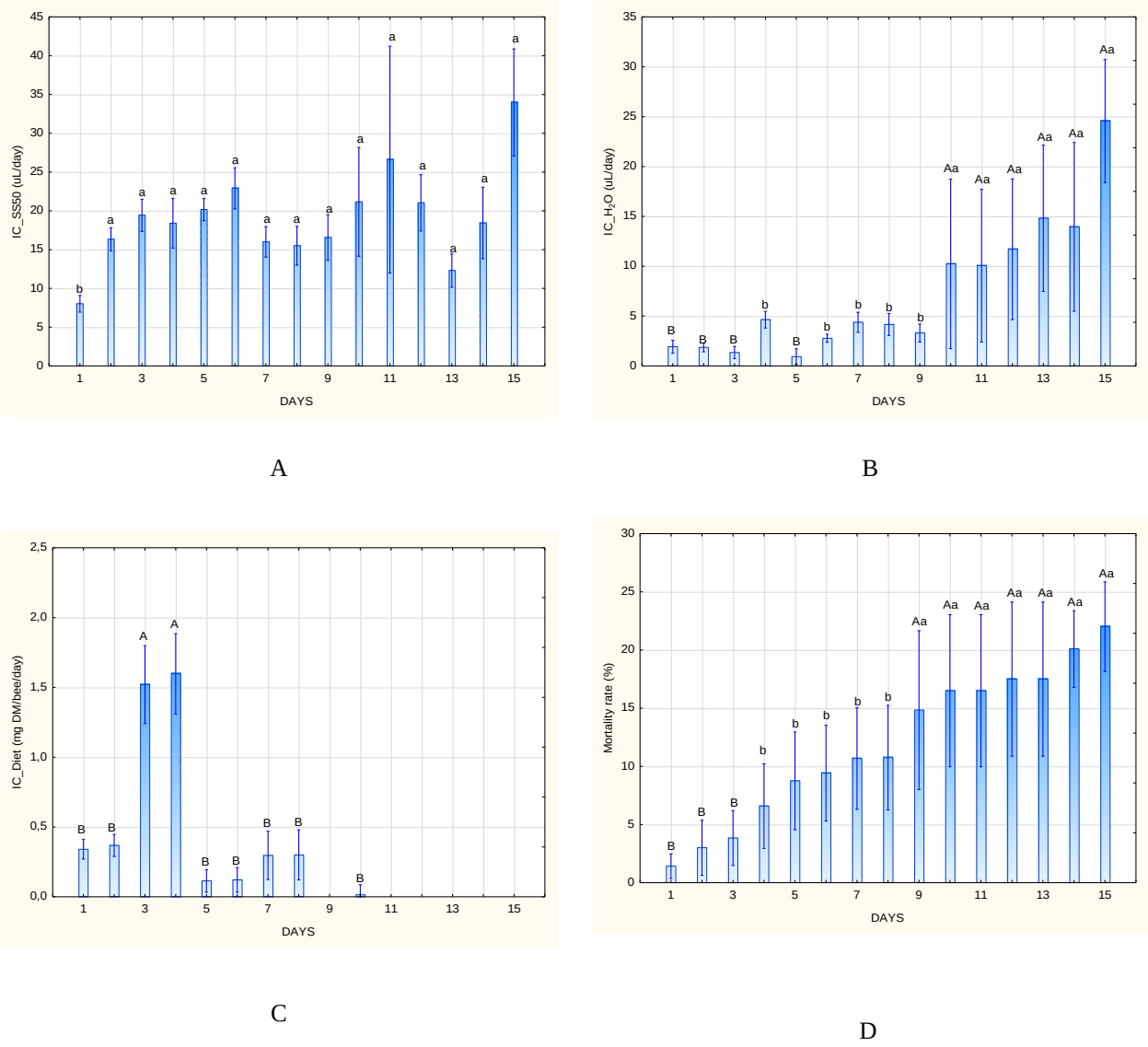
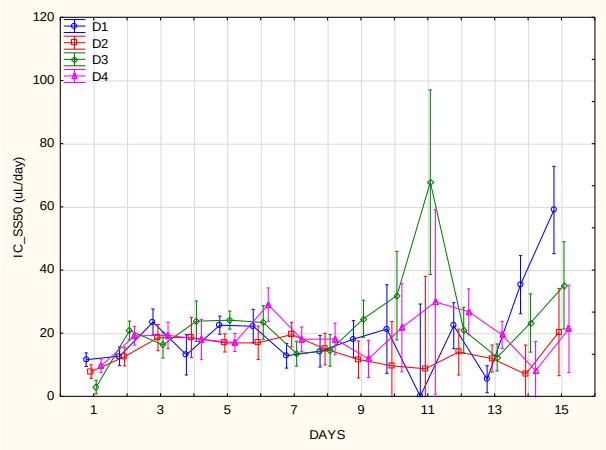


Figure 3.4. Effect of days on IC_SS50 (A), IC_H20 (B), IC_Diet (C) and mortality rate (D) expressed as avarage value per day.

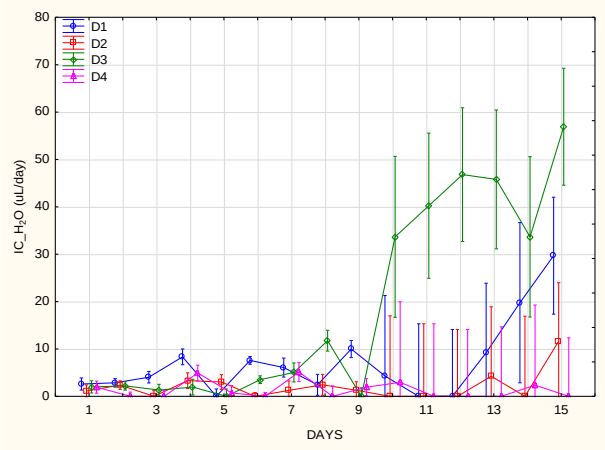
Neither the individual diet intake (Figure 3.5C) nor the individual SS50 consumption in the HBD × D interaction (Figure 3.5A) showed any discernible trends. However, D3 and D1 differed statistically significantly ($P \leq 0.05$) in terms of IC_SS50 on day 11, while, on days three and four, all HBDs for IC_Diet showed highly significant variations from the other time points.

Conversely, for all tests and on the initial days of the same density, H2O individual intake for D3 from day 10 until the completion of the experiment was larger and statistically significant ($P < 0.01$) than other HDBs (Figure 3.5B).

Up until day nine, the mortality rate was similar for all treatments (Figure 3.5D). From day ten onward, it increased with D3, which produced the highest ($P < 0.05$) mortality rate in the final five days of trials when compared to the other time periods. Compared to D3 and D4, D1 and D2 were noteworthy for having lower death rates, falling below the 10% threshold.



A



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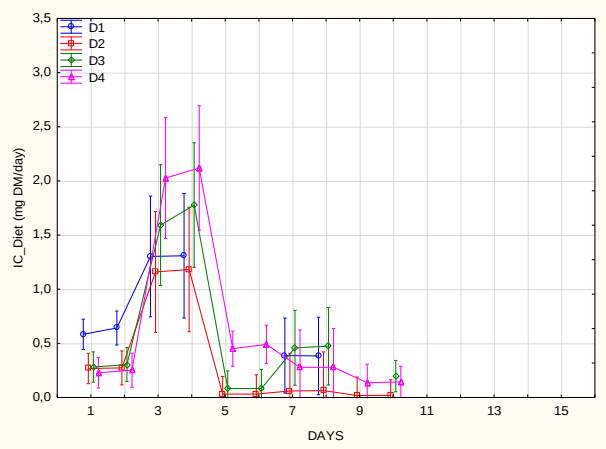
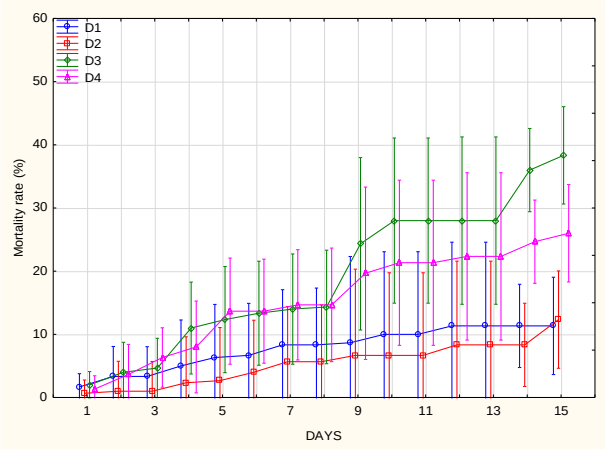


C



D

Figure 3.5. Effect of interaction HBD × D on IC_SS50 (A), IC_H20 (B), IC_Diet (C) and mortality rate (D).

3.4 Discussion

Over the years, laboratory investigations to evaluate the effects of different biotic and abiotic factors on the survival and development of honey bees have made extensive use of cages for keeping adult worker bees under in vitro settings. However, it is challenging to compare research findings across different organizations because different laboratories have created and implemented different cages. Therefore, in addition to optimizing the density of *Apis mellifera ligustica* Spin., the aim of this work is to try to standardize HBD in harvesting and/or challenging tests, not even specified by OECD, (1998), so that different laboratories may use the same protocol for these kinds of testing.

Conditions of high density are simulated by small cages; in the hive setting, high densities can have several benefits, including making thermoregulation and social interaction easier. Younger bees, typically 1–2 days old, exhibit lower thoracic temperatures compared to older bees (Meikle et al. 2018). When clusters form, these younger individuals tend to remain inside the cluster, while older bees are more frequently located on its periphery (Harrison 1987). Workers responsible for the energy intensive task of maintaining hive temperature are generally positioned above the brood cells and depend on food provided through trophallactic exchanges with donor bees (Basile et al. 2008). A higher number of donor bees facilitates faster energy replenishment for these thermoregulating workers, enhancing overall thermoregulatory efficiency. Moreover, the close spatial arrangement of bees within the hive likely promotes these frequent social interactions, including trophallaxis. All HBDs' similar intake of SS50 and solid diet may be explained by these factors, but D1 and D2 could supposed had the lowest mortality rates because there were enough bees to thermoregulate and support trophallaxis without engaging in excessive competition for food. It is important to keep in mind that the amount of consumption per individual bee is determined indirectly and that, even at larger densities, competition may prevent each individual bee from receiving an equal share of the consumption, in fact, according to Bosua et al. (2018) consumption values could be overestimated in cages with high densities of bees.

Given the lower bee mortality under these conditions, it is likely that D2 provides a more stress-tolerant setting for controlled studies than other densities. Indeed, survival was the only studied variable affected by bee density in cage experiments, with higher survival in smaller groups (D1 and D2) than in groups with densities of 23.8 and 29.7 bees/100 cm^3. This result is in agreement with what was found by Bosua et al. (2018) but a different trend has been found by Rinderer and Baxter

(1978). In this case groups of 10-20 individuals had a much lower survival rate than groups of 30-100 individuals, and the survival did not differ significantly between groups once density increased by about 30 bees per cage, suggesting that a minimum density is needed to maintain social interactions that can affect the survival of honey bees. On the other hand, has been highlighted by Huang et al. (2014) that HB caged feed with SS50 plus protein solid diet had lower a mortality rate compared to the one just SS50 provided during twenty days of trial, in particular, when bees fed with only syrup reached almost 60% cumulative mortality at day 20, the cumulative mortality in the group of bees fed with a combination of syrup and bee bread was 20% less on the same date. This aspect should be standardized in caged laboratory trials in order to have not the nutritional influence on the mortality rate.

Under natural hive conditions, a trend of lower consumption in larger groups has been noted, whereby consumption per bee decreased as colony size grew throughout the winter without affecting survival (Free and Racey 1968), furthermore, our findings showed that bees at D2 consume less compared to greater HBDs.

In *Apis mellifera ligustica* Spin. laboratory experiments, a density of 17.8 bees/100 cm^3 (D2) seems to offer several experimental and practical benefits: tasks like replacing diets, counting individuals, and eliminating dead bees are made easier by this density. These findings suggest that D2 would be the ideal honeybee density for laboratory caged experiments, eliminating stress-related confounding variables while balancing practical management requirements.

Concluding, 17.8 bees/100 cm^3 is recommended as a standardized HBD for *A. m. ligustica* investigations. Nevertheless, more investigation is required to ascertain whether this density is as appropriate for other subspecies of honeybees, considering the biological distinctions that exist between them. Future research will concentrate on determining the best HBD for different subspecies with the goal of creating standardized protocols catered to their own unique requirements.

17.8 bees/100 cm^3 density will be used for caged laboratory trials performed in this PhD thesis in order to standardize protocols and try to control as much as possible environmental factors in order to obtain clear and reliable results.

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Chapter 4

Physiological responses of young Italian worker bees (*Apis mellifera ligustica* Spin.) induced by different pollen diets

4.1 Introduction

Adequate nutrition in western honey bee (*Apis mellifera* L.) (HB) is crucial for colonies growth and development (Brodschneider and Crailsheim 2010). Differently from other farmed species, where specific nutritional needs are well known and appropriate diets can be formulated, supporting the HB nutrition is challenging because every colony has specific needs depending on the landscape nutritional opportunities, development stage and its own health condition (Tsuruda et al. 2021). In addition, considering the complex organization of HB colonies, the nutrition can be considered at three levels: colony nutrition, adult nutrition and larval nutrition (Brodschneider and Crailsheim 2010).In addition, foraging activities, and consequently the colony nutrition, is highly dependent on environmental constraints and on the floral composition of the landscape (Donkersley et al. 2014a). While the recently main focus of HB research programs is often on the internal factors of the hive like disease profiles, queen fecundity, varroa mite (*Varroa destructor*) control, overall little effort is made to understand and track the external factors of the hive (DeStefano 2015). For example, poor comprehension we have today on the nutritive value of the pollen and nectar collected by the worker bees according to environmental drivers such as season, location, and climate changes.

It is well known that colony losses, that are occurring worldwide (Requier et al. 2018; Evans and Chen 2021), are the result of the impact of multiple stressors (Neumann and Carreck 2010; Steinhauer et al. 2018). However, it has been proposed that the combination of nutritional stress, infections by pathogens and pesticide exposure are the most important driving forces (Naug 2009; Dainat et al. 2012; Smith et al. 2013; Tosi et al. 2018).

As far as the relationships between HB nutrition and their health status, it is well known how the consumption of pollen and its quality affect lifespan (Di Pasquale et al. 2013), immuno-competence

(Alaux et al. 2010), behavior of the division of labor in the colony (Ament et al. 2010), and resistance to pathogen infection (Alaux et al. 2011; Porrini et al. 2011). Among the pathogens that threaten HB health, *Varroa destructor*, RNA viruses (Dainat et al. 2012), and the microsporidia *Vairimorpha apis* (Zander), formally *Nosema apis* according to Tokarev et al. (2020) and *Vairimorpha ceranae* (Fries)(Fries et al. 1996), formally *Nosema ceranae* according to Tokarev et al. (2020), have the most significant impact on colony losses (Higes et al. 2009; Higes et al. 2013). Huang (2012) has shown that spring pollen supplement can work as an insurance, when weather is poor, and can reduce the negative effects of *Vairimorpha spp.* infection. However, surprisingly, an *in vitro* study found that pollen-fed bees had higher levels of *Vairimorpha spp.* infection but also a longer lifespan than bees fed syrup alone (Jack et al. 2016). These conflicting results highlight the complex relationship between HBs and their parasites/pathogens in the context of other environmental factors (*i.e*., food availability). The impact of pollen intake on bee health is a complex issue that requires considering not only the quantity of pollen consumed, but also the quality and diversity of the pollen diet(Omar et al. 2017) (Radev 2022).

Concerning the nutritional aspect of mono- or multi-floral pollen just a few research has been carried out in the last years. As a matter of fact, prior researches on pollen quality, bee health and foraging behavior relied only on measuring the protein content (Human et al. 2007; Kitaoka and Nieh 2009; Leonhardt and Blüthgen 2012) but, in general, inconsistent insights have been gained. Some studies (Schmidt and Hayden 1984; Schmidt et al. 1995; Alaux et al. 2010; Di Pasquale et al. 2013) have shown that polyfloral pollen is healthier for honey bees than pollens from single plant species. Mixed pollen administered to caged HBs made the bees' live longer than those on a single species of pollen (Huang 2012) suggesting that multi-source pollen has higher value than monofloral pollen to maintiain HBs in good health status.

The diversity of pollen in the HB diet could not be directly associated with improved nursing physiology (Di Pasquale et al. 2013).

According to the increase of the immuno-competence in HBs fed with multifloral pollen, it was also demonstrated that *V. ceranae* parasitized workers fed with multifloral pollen, lived longer than bees fed with *Cistus* sp. monofloral pollen (Di Pasquale et al. 2013)

Overall, in the literature concerning the HB nutrition topic, poor attention was paid on the HB genetics, even though the subspecific gene assortment can play a role on the response of worker bees to their diet. For example, the Saharan bee (*A. m. sahariensis*) (Baldensperger, 1932) and the Tellian bee (*A. m. intermissa*) (Buttel-Reepen, 1906) use pollen differently. In fact, under laboratory conditions, Khedidji et al. (2022) showed that when they receive a protein diet, the Saharan bees promote the development of their hypopharyngeal glands while the Tellian bees use the protein source for optimal development of their ovaries. It seems that from an evolutionary point of view, the use of pollen by a subspecies is oriented towards a very specific physiological function to improve its fitness.

This study aims to evaluate the effectiveness of pollen-based diets collected at different stages of the active season, serving as a protein supplement during critical periods for colonies. The analysis focuses on the physiological effects induced in young Italian worker honey bees (*Apis mellifera ligustica* Spin.) and their resilience to pathogens, particularly *Vairimorpha* spp.

4.2 Material and methods

4.2.1 Pollen harvesting

Curbicolar pollen loads (CPLs) were collected from ten Italian-Dadant bee hives, equipped with pollen traps (Anel, Athens, Greece) and located in the PLANT-B apiary at the Experimental Educational Farm "Nello Lupori" (Tuscia University, Viterbo, Italy) (42.4271454°E; 12,0796127°N; 302 m a.s.l.). From March to August 2021, every three weeks, the pollen traps were activated for 24 h and trapped CPLs from all the hives were collected and then pooled according to the harvesting month. Before pooling, a representative portion of CPL samples were retained for palynological analysis. In the following, the CPL pools were used as pollen diets and named according to the progressive numbers of harvesting months (PD03-PD08).

4.2.2 Diets formulation

For the following feeding trials with caged bee, two controls and six PDs (Table 4.1) were used. The first control diet (SS50) was a sucrose/ASTM type I water solution (50:50 w/w), while the other one (HF) was formulated according to Haydak (1970). To ensure the same moisture of the PDs and HF

control, they were levelled to the one with the lowest dry matter content (CPL pool of March, 610 g/Kg, see section 4.3.2) by adding suitable volumes of ultrapure water (ASTM type I) (Table 4.1).

Table 4.1. Formulation (g/Kg) of the experimental diets used in feeding trials with caged young worker bees.

Diet	Ingredients					
	Defatted soybean meal[a]	**Sucrose**	**Brewer's yeast (wet)[b]**	**Whey powder[c]**	**CPL[d]**	**ASTM type I water**
SS50	-	500.0	-	-	-	500.0
HF	272.7	272.7	272.7	90.9	-	90.9
PD03	-	-	-	-	1000.0	-
PD04	-	-	-	-	954.7	45.3
PD05	-	-	-	-	762.6	237.4
PD06	-	-	-	-	783.4	216.6
PD07	-	-	-	-	774.3	225.7
PD08	-	-	-	-	782.1	217.9

[a]purchased from Bongiovanni Farine (Mondovì, Italy), fat = 10.0 g/Kg, protein = 520.0 g/Kg, carbohydrates = 300.0 g/Kg, data declared by the producer;
[b]purchased from Lesaffre Italia S.p.a. (Trecasali, Italy);
[c]purchased from Fast Ingredients (Nizza Monferrato, Italy), protein = 800 g/Kg, data declared by the producer;
[d]PD03 = CPLs harvested on 26/03/2021, PD04 = CPLs harvested on 30/04/2021, PD05 = CPLs harvested on 21/05/2021; PD06 = CPLs harvested on 11/06/2021, PD07 = CPLs harvested on 02/07/2021, PD08 = CPLs harvested on 23/07/2021 and 12/08/2021, and pooled.

4.2.3 Palynological analysis, proximate composition, and mineral profiling of solid diets

4.2.3.1 Chemicals and Reagents

All the reagents used were of technical grade or better. Distilled water was used if not otherwise specified. For the mineral profiling of solid diets, all the reagents were of analytical grade and ASTM Type-I water was always used. Nitric acid (67-69%) (VWR International, Radnor, USA) was used both for cleaning operation and for serial dilutions. All glassware and plastics were cleaned by overnight soaking in 10% HNO_3 before a final rinse with ASTM Type-I water. The stock solutions (1000 mg/L in 2% NaOH) for calibration were purchased from Carlo Erba Reagents (Milan, Italy) (Na, Ca, K, Fe, Zn) and from VWR International (Radnor, USA) (P, Cu, Mg, Mn).

4.2.3.2 Palynological analysis

The CPLs collected per beehive and sampling date were grouped by color obtaining a total of 228 representative samples in total. A CPL per sample was suspended in 1 mL of distilled water using an

advanced vortex mixer ZX3 (VELP Scientifica, Usmate Velate, Italy), and 20 µL of suspension were dried at room temperature on a microscopic slide. A drop of glycerin jelly was placed on top of the dried pollen grains and then a glass slide was placed atop. Pollen grains were then observed under an B500PPH optical microscope (OPTIKA S.r.l., Ponteranica, Italy) at 400× magnification. For each representative CPL, about 500 pollen grains were counted according to the "transect" method (Tamic et al. 2011). Pollen grains were identified at the lowest possible taxonomic level according to the available literature (Ricciardelli d'Albore 1998; Bucher 2004; El-Labban 2020) and the PalDat (2000) palynological databases. The identified pollens were classified following the "pollen types" nomenclature proposed by Persano Oddo and Ricciardelli d'Albore (1989).

4.2.3.3 Proximate composition of solid diets

Representative aliquots of solid diets (Table 4.1) were dried in an oven at 65°C to constant weight, grinded through a ZM 200 Ultra Centrifugal Mill (Retsch, Haan, Germany) to pass a 0.5 mm screen and then stored in sealed polyethylene bottles until analysis. The dry matter content (**DM**, g/Kg) was determined by gravimetry according to the AOAC method n. 934.01 (AOAC 2000). The gravimetric determination of the ash content (**ASH**, g/Kg DM) was obtained after sample incineration by a muffle furnace (AOAC method n. 942.05) (AOAC 2000). Crude protein (**CP**, g/Kg DM) and ethereal extract (**EE**, g/Kg DM) contents were measured according to the AOAC methods n. 978.04 and 920.39 (AOAC 2000). In addition, as a measure of potentially undigestible carbohydrates in solid diets, the neutral detergent fiber corrected for the ash content (**aNDF$_{om}$**, g/Kg DM) (Van Soest et al. 1991) was determined by boiling 0.5 g of sample for 1 h in 100 mL of neutral detergent solution (Carlo Erba Reagents, Milan, Italy) with 0.05 mL of heat-stable α-amylase (Astori Tecnica s.r.l., Poncarale, Italy) and 0.5 g of sodium sulfite. Then, the non-fibrous carbohydrates content (NFC, g/Kg DM), was calculated as follows (Bernabucci et al., 2009)

$$NFC = 1000 - ASH - CP - EE - NDF \quad \text{(Eq. 1)}$$

4.2.3.4 Solid diet samples mineralization

Aliquots of 0.7 g of solid diet samples were digested with 6 mL concentrated HNO_3 and 1 mL water using a Multiwave GO Plus microwave oven (Anton Paar Italia S.r.l., Italy). A Reagent Laboratory Blank (RLB) composed of HNO_3 2% was included in each mineralization cycle (one out of eight

positions per run). The samples' mineralization program consisted of a 20 min ramp followed by 10 min at 100 °C ; a 10 min ramp followed by 5 min at 180 °C, and finally a 5 min temperature ramp down to room temperature. After each mineralization run, a standard cleaning procedure for the digestion Teflon vessels was performed by filling them with 4 mL of HNO_3 and 1 mL of water and by running a 10 min ramp up to and internal temperature of 180 °C, that was then held for 5 minutes. Once the cleaning procedure was completed, the vessels were emptied and then left to dry in a stove at 65°C for two hours. Before mineralisation, all the samples and the reagents were handled with non-metallic materials carefully washed with 10% HNO_3.

4.2.3.5 Mineral profiling by atomic absorption spectrophotometry (AAS)

The Na, K, Ca, Mg, Mn and Zn contents in solid diets were determined by flame atomic absorption spectroscopy (F-AAS) using a Shimadzu AA 7000 spectrophotometer equipped with a flame atomisation unit and an ASC-7000 autosampler (Shimadzu Corporation, Kyoto, Japan). Acetylene 6.0 grade (AirLiquide Italia SpA, Milan, Italy) was used as combustible gas and compressed air produced by an oil-less compressor as comburent. The elements Fe, Cu, Mn and P were analysed by graphite furnace atomic absorption spectroscopy (GF-AAS) using a Shimadzu GFA 7000A (Shimadzu Corporation, Kyoto, Japan) with an ASC-7000 autosampler (Shimadzu Corporation, Kyoto, Japan). For the graphite furnace measurements, argon 6.0 grade (AirLiquide Italia SpA, Milan, Italy) and pyrolytic coated graphite tubes with platform (Shimadzu Corporation, Kyoto, Japan) were used. Multi-element (for Fe, Cu, K, Na, Mg, Cr, Cd, Pb) or single element (for Zn and P) hollow cathode lamps (Hamamatsu Photonics K.K., Shizuoka, Japan), were used as radiation sources. A deuterium lamp (Shimadzu Corporation, Kyoto, Japan) was used for background correction for both the atomization systems. For the AAS calibration, working standard solutions were daily prepared by diluting the standard stocks to 10 mg/L, then further diluted in HNO_3 (67%) to obtain the from three to five calibrators depending on the element. For F-AAS analysis, the calibration ranges varied between 0.1 – 0.5 mg Mg/L and 1.25 - 5.0 mg Ca/L, with a minimum linear fitting (R^2) of 0.994 (Ca). For the elements analyzed by GF-AAS, the calibration ranges varied between 0.5 - 4.0 µg P/L to 1.25 – 10.00 µg Fe/L, with the lowest linear fitting (R^2) recorded for iron of 0.997. Each analytical data was the average of three consecutive AAS runs performed on the same sample, calibrator or RLB.

Due to the lack of a suitable pollen Standard Reference Material (SRM) allowing to assess the whole-method's accuracy for the investigated elements, non-fat milk powder (SRM 1549) from the National

Institute of Standards & Technology (Department of Commerce, USA) was used following the same mineralisation/analysis procedure adopted for the solid diets' samples. From two replicates, the average recoveries were calculated (Table S4.1). The data on mineral content of solid diets, were then corrected for the respective average recoveries.

4.2.4 Feeding trials with young workers

4.2.4.1 Queen caging and emerging workers' management

To obtain the young worker bees, a healthy colony housed in a B-BOX hive (Beiing Srl, Faenza, Italy) and maintained in an all-year round facility (Taber and Owens 1970) at the University of Tuscia under controlled environmental conditions (25.1±3.2°C) was used. To obtain a cohort of 0-24 h old workers, the laying queen, provided by a *A. m. ligustica* Spin. queen bee breeder (CREA, 2023), was caged forcing her to lay on an honeycomb inlaid in a plastic queen-excluder cage (Erboristeria Apistica, Sondrio, Italy). The brood development was monitored daily by manual noting the number of eggs, larvae, and capped cells. As the whole experimentation was carried out in duplicate, the first queen caging took place on 05/02/2022, and the second one took place on 10/03/2022. In case of outside adverse weather conditions during queen caging, the colony was fed at 100 mL sucrose solution (50:50) per day. About four days before the forecasted mass emergence of worker bees, the honeycomb was moved in a net cage maintained into a laboratory incubator (Carbolite Gero Ltd, Hope, UK) at 33.6±1.2 °C and 71.6±3.4% RH. Emerging bees were provided with SS50 and tap water *ad libitum*.

4.2.4.2 Feeding trials set-up

Groups of 30 emerging workers were collected through a battery-powered vacuum cleaner and then placed inside self-built 3D printed cages (6 cm × 4 cm × 7 cm; inner volume 168 cm^3) at a density of 17.8 bees/100 cm^3 (Bosua et al. 2018; Manganello et al. 2023). To limit any possible undesired effect of the plastic material on the bees, only polyethylene terephthalate (PET) and polylactate (PLA) filaments (Prusa Reseach a.s., Partyzánská, Czech Republic) were used in the 3D printing process. A total of twenty-four (three cages per diet/control) cages were used equipped with a removable bottom tray for monitoring the varroa mite fall and the collection of any debris (*e.g.*, feces). Each cage had two hole per side, suitably sized to fit 2 mL centrifuge tubes which contained the diet to be tested; the tubes were pierced (4.5 mm) on the bottom to allow for the bees to enter. The top of the cage was

designed to allow fitting 1 mL graduated syringe barrels containing water or SS50. In the back side of the cage, a removable panel was fitted with a piece of embossed wax foundation (4 cm × 5 cm) to allow monitor the wax produced by the bees.

Each trial lasted two weeks along with dead bees were counted and removed daily. The daily consumption of water (**WI**; µL/bee/day) and SS50 (**SSI**; µL/bee/day) was also recorded. The unconsumed solid diet was replaced every two days, dried at 65°C for 24 hours, and average daily dry matter intake DM (**DMI**, mg DM/bee/day) was calculated by difference from the dry weight of diet administered divided the average number of alive bees. At the end of each trial the bees still alive were narcotized by cooling and were stored in freezing conditions for further measurements. Also, at the end of the trial the wax removable panels were taken and weighed, and the amount of wax daily produced in 14 days (**DWP**, mg/cage/day) was obtained by subtracting the initial weight of each wax panel and dividing the increment by trials days.

4.2.5 Worker bees' body proximate composition

4.2.5.1 Ethereal extract content

Eleven HBs from each cage, thirty-three per treatment, were randomly selected from the end-trial alive ones. Each HB was dissected with a surgical steel scalpel respectively in head (H), torax (T) and abdomen (A); after that, the respective body part groups were dried in an oven at 65°C to constant weight to determine the dry DM by gravimetry (AOAC method n. 934.01) (AOAC 2000). Subsequently, the samples were moved to 2 mL centrifuge tubes (Eppendorf, Enfield, USA) and crushed (3 minutes at 2500 oscillation per min) by using a Mini-BeadBeater 24 homogenizer (BioSpec Products, Inc., Bartlesville, USA) with one 4 mm diameter magnetic beads (Biosigma, Cona, Italy). Each sample, representative of a body part, was quantitative moved and weighted (w1), by an analytical balance OHAUS Explorer® Pro (Ohaus GmbH, Switzerland), inside a lipid-free filter bag (Astori Tecnica s.r.l., Poncarale, Italy), previously weighed (w2) with the same balance, and then sealed. The samples were then submitted to with a Soxhlet extraction according to AOAC

method n. 920.39 (AOAC 2000). The EE, separately of HBs' heads (EEH), thoraxes (EET) and abdomens (EEA) (g/Kg DM), was calculated as follows:

$$EE = \frac{w1+w2-w3}{w1} \times 1000 \quad \text{(Eq. 2)}$$

Where *w3* was the sample dried weight (105°C, 30 minutes) after the extraction. The method accuracy was estimated by a recovery exercise. Due to the lack of a suitable Standard Reference Material, whole milk powder (SRM 8435) from the National Institute of Standards & Technology (Department of Commerce, USA) was used. From three daily replicates, the average recovery was calculated to be from 110% to 112%. The data obtained in different working days were corrected for the respective average recoveries. The total body EE content (**EE_{TB}**; g/Kg DM) was estimated as weighted mean based on the single body part weight.

4.2.5.2 Crude protein content

Due to limited amount of sample material, the CP content of HB body parts was assessed through the Kjeldahl method (AOAC method n. 978.04) (AOAC 2000) after the ethereal extraction. Sulphuric acid (0.1 N) was used to titer the total nitrogen of the samples by using a Flash automatized titrator (Steroglass, Perugia, Italy) to end-point pH 4.2. To consider the nitrogen content of the filter bags used, three empty filter bags for each mineralization cycle, were analyzed daily and the mean value subtracted from the titrant volume used to reach the endpoint; CP content of head (**CP_H**), thorax (**CP_T**) and abdomen (**CP_A**) samples (g/Kg DM) was estimated as follows:

$$CP = \frac{(V_s - V_0) \times 0.0014}{w1} \times 6.25 \times 1000 \quad \text{(Eq. 3)}$$

Where V_s is volume of H_2SO_4 added to reach the endpoint for a sample, V_0 is the mean H_2SO_4 volume needed for empty bags, *w1* is the sample dry weight and 6.25 is a conventional factor applied to the net total nitrogen (Danieli et al. 2023) as in Eq. 2. As in the case of the EE analysis, whole milk powder (SRM 8435) from the National Institute of Standards & Technology (Department of Commerce, USA) was used to assess the method accuracy. From three daily replicates, the average recovery was calculated to be from 97% to 117%, data obtained were corrected for the respective

average recoveries. The total body CP content (**CP_{TB}**; g/Kg DM) was estimated as weighted average based on the CP content measured for each body part.

4.2.6 Vairimorpha spp. spore load

At the end of each feeding trial, two survived HBs per cage were randomly selected and managed as a sample. The DNA extraction was performed on the samples with the commercial kit QIAmp® DNA Mini (QIAGEN, Velno, Netherlands) according to Babin et al. (2022). Aliquots of 1 µL of the DNA extract were analysed using a Quant Gene 9600 RT-PCR (Bioer Thecnology, Hangzhou, China) with WizPure qPCR Master (SYBR) (Wizbiosolutions Inc, Seongnam, South Corea) and customs primers described by Chen et al. (2009) with the following amplification cycle: 5 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 30 s at 56 °C. As confirmation, six DNA samples from as many HBs pairs, in double (two reference samples and four HB samples which tested positive by RT-PCR) were subjected to the SANGER amplicon sequencing, performed by BioFab Research s.r.l. (Rome, Italy).

To perform an absolute quantification of the bees' spore load, calibrations with single positive (*V. ceranae*) and double positive (*V. ceranae* and *V. apis*) reference samples, gently provided by Centro de Investigación Apícola y Agroambiental (CIAPA-IRIAF, Marchamalo, Spain), were performed. At first, the spore contents of the reference samples were quantified by using a Burker chamber (Marienfeld GmbH & Co., Lauda-Königshofen, Germany) under a Primo Star optical microscope (Carl Zeiss, Jena, Germany) at 400× magnification. According to the Texas Apiary Inspection Service (accessed 14/10/2024), Fries et al. (1996-2013) and Oliver (2007), the quota of *V. apis* spores in the double positive reference sample (Figure 4.1) was microscopically assessed on three technical replicates by the same operator as 34.9±8.5%.

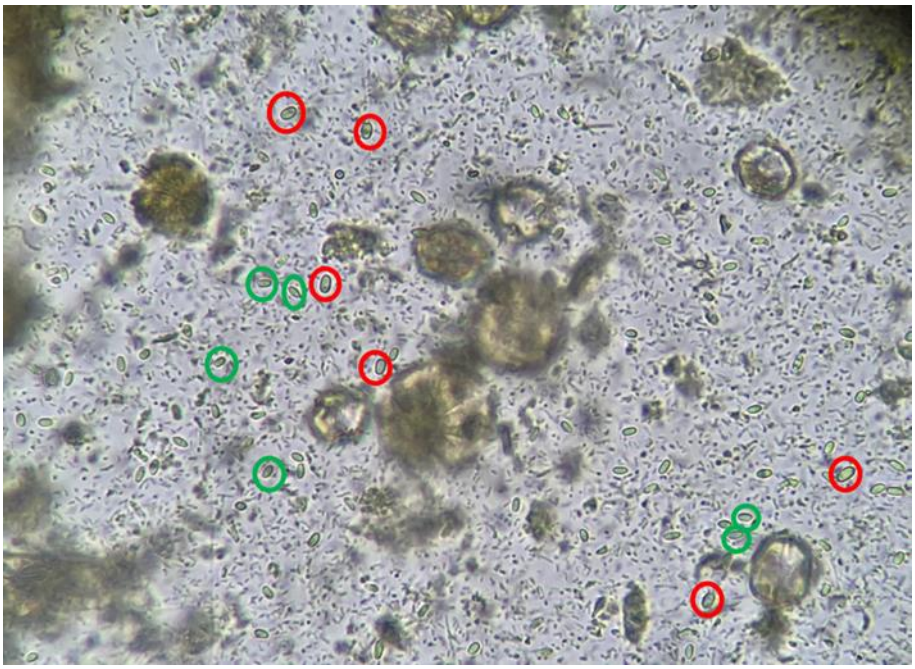

Figure 4.1. Reference sample with *V. ceranae* and *V. apis* spores respectively highlighted in green and red (optical magnification 400×).

After counting, the reference samples were then serially diluted from 1:1 to 1:256 in PCR grade water (spore densities ranging from 1.12×10^5/ mL to 3.96×10^2/mL and from 3.56×10^4/mL to 1.38×10^2/mL, for *V. ceranae* and *V. apis* respectively) and then the total DNA was extracted. By using diluted reference samples as calibrators, three daily Real Time PCR (RT-PCR) calibration runs were performed with the same experimental set-up. Dilutions were considered positive after checking the melting curve (Chen et al. 2009) and the LOD was established for $C_t \geq 42.59$ (dilution 1:8 for V.apis). Finally, the spore number detected was referred to a single bee. For *V. ceranae* (Eq. 4) and *V. apis* (Eq. 5) two logarithmic calibrations were obtained with R^2 of 0.98 and 0.96, respectively:

$$C_{t_c} = -4.18 \ln(SN) + 74.08 \quad \text{(Eq. 4)}$$

$$C_{t_a} = -3.12 \ln(SN) + 63.14 \quad \text{(Eq. 5)}$$

Where C_{t_c} referred to *V. ceranae*, C_{t_a} referred to *V. apis,* and *SN* was the spore number obtained by manual count combined with the dilution factors adopted.

The limit of quantification (LOQ), which is the lowest DNA concentration detected in ≥ 95% of replicates, was assessed on 9 replicates of the lowest concentration of the calibrators, evenly distributed on three independent RT-PCR runs. The LOQ was valid if a specific amplification signal was detected (*i.e.*, >LOD) in at least in 8 replicates (Babin et al. 2022). For *V. ceranae* a LOQ of C_t=40.86 (sample dilution of 1:8), and for *V. apis* a LOQ of C_t=42.59 (sample dilution of 1:8) were estimated (Table 2). Method's LOD coincided with *V. apis*' LOQ (C_t=42.59) according to Armbruster and Pry (2008). As with the reference samples, the DNA from the unknown ones was extracted and amplified by RT-PCR as above mentioned and the spore load (**SL**; n/bee) for *V. ceranae* (**SL_c**) and *V. apis* (**SL_a**) were extrapolated from Eq. 4 and Eq. 5, respectively. Relative spore load per bee (**RSL,** %) was calculated considering the initial spore load found in a pool of two emerging workers per trial as follow:

$$RLS_c = \left(\frac{SL_c - ISL_c}{ISL_c}\right) \times 100 \quad \text{(Eq. 8)}$$

$$RLS_a = \left(\frac{SL_a - ISL_a}{ISL_a}\right) \times 100 \quad \text{(Eq. 9)}$$

$$RLS_{ca} = \left(\frac{SL_{ca} - ISL_{ca}}{ISL_{ca}}\right) \times 100 \quad \text{(Eq. 10)}$$

Where ISL_c, ISL_a and ISL_{ca}, were the initial spore loads for *V. ceranae* and *V. apis*, respectively. Prevalence of *V. ceranae* (**P_c**), *V. apis* (**P_a**) and co-infection cases (**P_{ca}**) was expressed in percentual term of positive bees' samples per diet.

4.2.7 Worker bees' ovary and hypopharyngeal gland development

Eight frozen HBs per cage were shipped and analyzed by the VALCORE Laboratory at the UMBB University (Boumerdes, Algeria). Each worker was dissected, and the weights of heads, thorax and abdomen were recorded using an analytical balance OHAUS Explorer® Pro (Ohaus GmbH, Nänikon, Switzerland).

The ovary development was studied under a binocular microscope using the classification of developmental stages. Five stages were differentiated: the two first stages (sizes 1 and 2) corresponded to undeveloped ovaries and the last three (sizes 3–5) to developed ovaries (Table 4.2).

Table 4.2. Development stages classification of the ovary (Velthuis 1970; Khedidji et al. 2022).

Development stage	Classification factors
Stage 1	Inactive thread-like ovarioles without vitellus
Stage 2	Swollen ovarioles without vitellus
Stage 3	Swollen ovarioles with visible vitellus but without distinct oocytes
Stage 4	The ovarioles contain distinct, but immature oocytes
Stage 5	Fully activated ovaries with distinct mature oocytes

The development of the hypopharyngeal glands (HPG) was assessed by calculating their weight. For this, HPG were removed with forceps through an incision in the front part of the head and put on a microscope slide in a droplet of sodium chloride solution. The HPG were removed with a fine clip and dried for 4 hours at 110°C (Fluri et al. 1982). The dry weight of the individual HPG was determined by subtracting the weight of glass blade to the weight of dried HPG fixed on it.

4.2.8 Data processing and statistical analysis

Shannon (H') and Shannon Evenness (E) indexes were also calculated to appraise the floral biodiversity of the pollen diets. The molar Ca/P and Cu/Fe ratios were calculated in order to consider possible interactions among these elements (Fadlalla 2022). For the daily recorded variables, such as **SSI**, **WI**, **DMI**, individual intake of CP (**CPI**; mg CP/bee/day), EE (**EEI**; mg EE/bee/day), and NFC (**NFCI**; mg NFC/bee/day), but also the HBs mortality rate (**MR;** %) was calculated, at the end of trial, as the percentage of dead bees in fourteen days on the initial number per cage and the varroa mite fall (**VMF**; n/day/cage) a repeated measures ANOVA was performed including diet (**D**; SS50, HF, PF03-PD08), time (**T**; 1-14 days), and diet × time interaction as fixed factor. The Tukey's test was used for pairwise comparisons, and significance was always declared at $P \leq 0.05$. Linear contrast analysis were performed for daily recorded variables, significance was declared at $P \leq 0.05$.

At the end of each feeding trial, the average daily gain (**ADG**; mg/day/bee) was calculated by the difference between the average weight of the alive bees at 14 days and the average weight of the bees at the beginning of the trial, divided by the trial days.

For the **ADG**, the average daily wax produced (**DWP**; mg/day), the HB body part and total body DM (g/Kg), CP and EE contents (see Eq. 2 and Eq. 3), **HPGw** and head weight (**Hw**) (mg DM/cage) and the **HPGw/Hw** ratio, a General Linear Model was performed using **D** as fixed factor. To test any possible effect of daily recorded variables, on the end-trial variables, average SSI, WI, DMI, CPI,

EEI and NFCI were entered as covariates in the model. The Tukey's test was used for pairwise comparisons, and significance was always declared at $P \leq 0.05$.

As the ovary development (**OD**, five stages), and the **RSL** for *V ceranae*, *V. apis,* and *V. ceranae* + *V. apis* were not normally distributed, the effect of the diet was tested by the non-paramentric Kruskal-Wallis test followed by the *post-hoc* Dunn's test for pairwise comparisons; significance was always declared at $P \leq 0.05$. The binomial output of $\boldsymbol{P_c}$, $\boldsymbol{P_a}$ and $\boldsymbol{P_{ca}}$ were used to create a contingency table and tested with non-parametric independent test for qualitative categorical variables (Chi-square and Wilks' G^2). Pearson's moment correlation analysis was performed for all the physiological variables. In order to ensure that the parameters which values were percentages could have a normal distribution for the statistical analysis, the arcsine transformation described by Zar et al. (2014) was applied for mortality variable as percentual fraction of the initial number. All the analysis were performed with STATISTICA 10 software (Statosoft inc., Tulsa, USA).

4.3 Results

4.3.1 Palynological analysis of pollen diets

Pollen biodiversity resulted high and quite stable in pooled CPLs from April to July (*i.e.*, PD04-PD07) (Figure 2), with the H' ranging from 2.39 and 2.65. The lowest CPL botanical diversity was observed in August (*i.e.*, PD08), with only 6 pollen types observed (H' = 1.64). The Evenness peaked in April CPLs (PD04; E = 0.92), then decreased in May (PD05; E = 0.88), before rising again at the end of the season with August CPLs matching the value of April. Overall, H' and E followed similar patterns during the season, excepted for August CPLs, due to the lower number (six) of pollen types almost evenly represented (Figure 4.2).

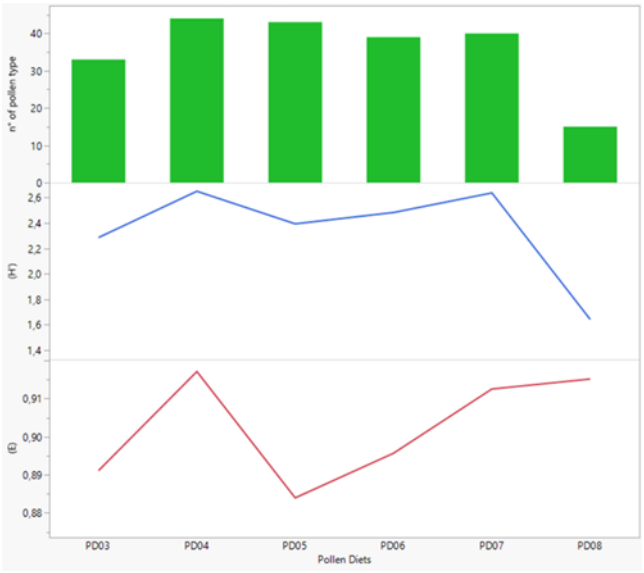

Figure 4.2. Biodiversity of pollen diets assessed by the number of different pollen types found (top), and by the Shannon (middle), and the Evenness (bottom) indexes.

4.3.2 Proximate composition and mineral profile of pollen diets

Among the PDs, the highest CP level was found in PD06 and the lowest in PD08 (Table 4.3). However, the HF control had about 53% more CP than the average value of PDs (239.4 g/Kg DM). The highest EE content in pollen diets was found in PD04, which was around 10% higher than PD03 and more than twice the diet with the lowest EE content (PD07) while the lowest value was recorded for HF. The PD06 had the highest fiber content assessed as the aNDFom, followed by HF control, and the lowest value was found in PD08. The NFC peaked in PD05, while PD07 and PD08 reached respectively 95% and 90% of this maximum, otherwise HF diet showed the lowest value of NFC. By contrast, the lowest NFC level in PD06 reflected the high aNDFom content of this diet. The ASH content was higher in PD03 to PD05 than in PD including CPLs harvested later in the season (*i.e.*, PD06-PD08).

The mineral profile differed across the diets. In particular, the PD04 had the highest Na content and the PD08 had the lowest. The Ca, Mg and K contents tended to be steady in all the pollen diets with exception of a 5 % lower level of K in PD08 with respect to the average value of the other PDs (11.3±3.9 g/Kg); conversely, K content in HF was 97 % higher than the average value of PDs (11.3±1.0 g/Kg). Zinc, manganese (that showed the highest content in PD07) and copper concentrations were noticeably low in HF diet and in PD08. Except for HF, which had the highest

content, sodium was from 2 to 3 times higher in PD03 and PD04 than in other PDs (63.7±20.4 mg/Kg).

Table 4.3. Proximate composition (g/Kg DM), macro (Ca, K, Mg, P; g/Kg DM), micro-mineral (Na, Zn, Mn, Fe, Cu; mg/Kg DM) and trace elements (Cd, Cr, Pb; mg/Kg DM) profiles of solid diets.

	HF	**PD03**	**PD04**	**PD05**	**PD06**	**PD07**	**PD08**
CP	367.4	249.4	247.4	209.1	279.5	255.0	196.1
EE	18.5	60.8	62.4	47.6	63.9	30.5	77.6
$aNDF_{om}$	78.1	57.6	44.7	34.4	114.3	70.5	33.1
NFC	502.1	601.4	615.1	679.9	519.8	620.4	669.4
ASH	33.9	30.7	30.4	29.0	22.4	23.6	23.8
Ca*	1.2	1.4	1.3	1.3	1.3	1.4	1.3
K*	22.3	10.6	11.8	11.3	13.2	10.3	10.7
Mg*	1.1	1.0	1.1	1.2	1.2	1.1	0.8
P*	1.4	1.7	1.8	1.9	2.0	2.0	1.1
Na	229.1	183.0	135.1	61.1	52.1	97.5	44.0
Zn	29.9	61.2	48.3	51.3	55.3	63.2	25.1
Mn	4.1	10.5	9.5	9.7	12.4	14.9	5.1
Fe	3.3	5.2	4.2	3.5	4.8	4.6	3.9
Cu	3.6	5.2	5.5	5.1	5.5	5.4	3.4
Ca/P**	0.7	0.6	0.6	0.5	0.5	0.5	0.9
Cu/Fe**	1.0	0.9	1.2	1.3	1.0	1.0	0.8
Cd	0.0079	0.0123	0.0093	0.0081	0.0107	0.0128	0.0129
Cr	0.0068	0.0134	0.0075	0.0046	0.0049	0.0068	0.0068
Pb	0.001	0.062	0.051	0.053	0.034	0.039	0.045

CP: Crude Protein; EE: Ethereal Extract; aNDFom: Neutral Detergent Fiber corrected for the ash content; NFC: Non-fiber carbohydrates; * g/Kg; ** molar ratios

4.3.3 Physiological variable analysis

Both the factors diet and time affected the SSI, WI, DMI, CPI, EEI, NFCI, but their interaction was always significant as well (Tab. 4.4). As far as the SSI, though any pairwise comparison was significant. Higher values were observed during the first seven trial days for SS50, HF compared to other PDs (Figure S4.1A), even though the contrast analysis, extended to the whole experiment (Table S4.3), did not support a differential intake of SS between control and PD fed bees.

The WI resulted comparable across all groups except for PD07 (Table 4.4), but the contrast between controls (0.62±0.00 µL/day) and PDs (0.36±0.05 µL/day) was highly significant ($P < 0.001$) (Table

S4.3), due to an overall higher water intake in controls compared to PDs during the first three trial days (Figure S4.1B).

Concerning the DMI, consistent increases were observed across all PD groups, with pairwise comparisons showing significant differences ($P<0.01$) compared to the HF-fed groups (Table 4.4). Additionally, significant contrasts ($P<0.001$) were found when comparing the DMI (2.71±0.17 mg/day) of PD fed bees to those of the HF counterparts (Table S4). The CPI reflected the same pattern of DMI for the PD groups (Table 4.4), as well as for significant contrast analysis ($P<0.001$) between HF and PD fed bees (0.64±0.05 mg/day) (Table S4.3).

The EE intake resulted higher ($P<0.01$) in PD fed bees compared to the HF fed ones, with the highest values recorded for PD03, PD06 and PD08, while PD07 showed an intermediate value (Table 4.4). Contrast analysis of PDs against HF, PD03, and PD07 highlighted a significant lower ($P<0.001$) trend throughout the entire trials of these diets compared to the other PD groups (Table S4.3).

The NFC intake was comparable for the first three pollen diets, while higher values were recorded respectively for PD07 and PD06 ($P<0.01$); even in this case the bees fed on HF showed the lowest intake compared to the PD fed bees ($P<0.01$) (Table 4.4). Contrast analysis highlighting that HF and PD08 diets were consistently lower ($P<0.001$) compared to the other PD groups (Table S4.3) throughout the entire trials.

As far as the mortality rate, it was affected by the diet and time, but the D × T interaction was not significant (Table 4.4). Notably, the MR in PD fed bees was always higher than in HF groups ($P<0.05$), except for the PD08 one.

Table 4.4. Effect of treatment on physiological variables analyzed by ANOVA repeated measure (Means ± SEM).

Variable	Diet (D)								SEM	P-value		
	SS50	HF	PD03	PD04	PD05	PD06	PD07	PD08		D	T	D×T
SSI	21.88	22.43	19.89	21.37	22.43	21.1	19.36	19.10	1.28	<0.001	<0.001	<0.001
WI	0.62^{a}	0.62^{a}	0.56^{a}	0.34^{a}	0.45^{a}	0.40^{a}	0.17^{b}	0.26^{a}	0.09	0.003	<0.001	<0.001
DMI	nd	0.41^{B}	1.97^{A}	2.93^{A}	3.27^{A}	2.99^{A}	2.48^{A}	2.60^{A}	0.35	<0.001	<0.001	<0.001
CPI	nd	0.15^{B}	0.49^{A}	0.72^{A}	0.68^{A}	0.83^{A}	0.63^{A}	0.51^{A}	0.09	<0.001	<0.001	<0.001
EEI	nd	0.01^{C}	0.12^{AB}	0.18^{A}	0.15^{AB}	0.19^{A}	0.07^{B}	0.20^{A}	0.02	<0.001	<0.001	<0.001
NFCI	nd	0.03^{D}	0.11^{C}	0.13^{C}	0.11^{C}	0.34^{A}	0.17^{B}	0.09^{C}	0.02	<0.001	<0.001	<0.001
VMF	0.54	0.07	0.19	0.31	0.17	0.23	0.00	0.24	0.13	0.193	<0.001	0.297
MR	9.5^{a}	2.0^{b}	6.3^{a}	3.2^{a}	4.4^{a}	5.4^{a}	8.6^{a}	2.4^{ab}	1.5	0.007	<0.001	0.220

SSI: daily individual intake of SS50 (µL/day); WI: daily individual intake of water (µL/day); DMI: daily individual intake of DM of solid diet (mg/day); CPI: daily individual CP intake (mg/day); EEI: daily individual EE intake (mg/day); NFCI: daily individual NFC intake (mg/day); VMF: Varroa Mite Fall (n); MR: Mortality Rate (%); SEM: Standard Error of the Mean (pooled); T: Time; ^{A,B} P≤0.01; ^{a,b} P≤0.05.

The diet played a role on the ADG, Hw and OD measured at the end of the trials, but not on the other physiological variables (Table 4.5). No one of tested co-variables (average SSI, WI, DMI, CPI, EEI and NFCI) had significant effect. As far as the ADG, the PDs fed bees exhibited a higher (P<0.05) weight gains compared to the HF and SS50 counterparts (Table 4.5). Head weight, used also as marker of HPG development, showed a significant variation among diets with the bees fed on PD04 to PD07, but on SS50 too, that exhibited higher values compared to the HF controls (P<0.01). Concerning the bees' ovaries, the SS50, HF and PD04 fed bees showed lower development (P<0.05) than the PD06-08 fed counterparts (Table 4.5).

Table 4.5. Effect of diet on physiological traits calculated at the end of the trial.

	SS50	HF	PD03	PD04	PD5	PD06	PD07	PD08	SEM	P-value
ADG	0.21^{b}	0.13^{b}	1.18^{a}	1.36^{a}	1.5^{a}	1.28^{a}	1.42^{a}	1.11^{a}	0.25	<0.001
DWP	2.46	1.46	3.63	1.00	2.29	4.24	3.18	2.67	0.74	0.066
Hw	10.1^{A}	9.4^{B}	10.5^{AB}	10.9^{A}	10.9^{A}	10.9^{A}	11.1^{A}	10.1^{AB}	0.3	0.002
HPGw	0.20	0.20	0.21	0.22	0.23	0.19	0.22	0.20	0.02	0.654
OD	1.02^{b}	1.04^{b}	1.13^{ab}	1.04^{b}	1.38^{a}	1.38^{a}	1.13^{ab}	1.21^{a}	0.10	0.014
HPGw/Hw	0.02	0.021	0.02	0.021	0.021	0.017	0.02	0.02	0.01	0.697

ADG: Average Daily Gain (mg AI); DWP: Daily Wax Production (mg DM); Hw: Head weight (mg DM); HPGw: Hypopharyngeal glands weight (mg DM); OD: Ovary development (stage n.); SEM: Standard Error of the Mean (pooled); ^{A,B} $P \leq 0.01$; ^{a,b} $P \leq 0.05$.

4.3.4 HB body composition analysis

The effect of the diet on the DM, CP and EE contents of the different HBs' body parts resulted always significant except for the DM_T and the EE_H (Table 4.6).

As far as the DM content in heads, only the PD07 fed bees showed a higher value ($P<0.01$) compared to the controls, but as far as the abdomen DM content, it was also higher in PD04 and PD05 than in SS50 and HF fed bees. The contrast of HF vs. PDs, was not significant for DM_H (Table S4.7).

The CP_H and CP_T contents in all PDs fed bees resulted higher compared to controls ($P<0.05$), but as far as the CP_A content it was not the case for PD05 and PD06 (Table 4.6). However, the total body CP content of all the PD fed bees (348.2±20.2) was higher ($P<0.01$) than SS50 and HF groups (230.1±11.8).

Concerning the abdomen, the bees fed on PD04 and PD08 showed the highest EE content (Table 4.6), followed by the PD05 bees ($P<0.01$) and then by the PD07 fed bees that not differed from the controls ($P<0.01$). Except for PD07 fed bees, the total body EE of all PD groups was higher in comparison to the controls ($P<0.01$).

Conversely, the EE_T was higher in controls than in PD fed bees, with the lowest value recorded for PD08. Pairwise comparisons and contrast analysis did not result statistically significant (Table 6).

Table 4.6. Effect of diet on HBs' body composition.

	SS50	HF	PD03	PD04	PD05	PD06	PD07	PD08	SEM	P-value
DM_H	308.6^{B}	302.4^{B}	328.3^{AB}	336.3^{AB}	342.8^{AB}	330.7^{AB}	355.1^{A}	319.8^{AB}	7.8	<0.001
DM_T	303.9	298.2	331.0	298.4	339.9	339.8	341.8	290.4	18.4	0.081
DM_A	161.7^{B}	140.2^{B}	227.0^{AB}	238.4^{A}	243.3^{A}	216.8^{AB}	267.1^{A}	207.9^{AB}	14.9	<0.001
CP_H	543.9^{b}	567.1^{b}	640.9^{a}	661.2^{a}	647.8^{a}	629.1^{a}	603.5^{a}	626.1^{a}	22.7	0.007
CP_T	629.8^{b}	657.6^{b}	715.1^{a}	764.1^{a}	778.1^{a}	715.02^{a}	707.9^{a}	716.1^{a}	28.7	0.018
CP_A	327.8^{B}	383.3^{AB}	409.3^{AB}	421.2^{AB}	464.1^{A}	460.0^{A}	385.3^{AB}	410.1^{AB}	23.3	0.007
CP_{TB}	218.3^{B}	241.9^{B}	332.3^{A}	352.5^{A}	384.8^{A}	357.8^{A}	337.2^{A}	323.3^{A}	15.9	<0.001
EE_H	122.9	124.9	123.7	113.5	119.6	97.2	104.7	114.6	14.2	0.902
EE_T	56.4	50.9	49.0	47.6	36.2	37.3	34.1	34.0	5.4	0.034
EE_A	132.4^{C}	155.9^{C}	232.4^{AB}	245.1^{A}	200.6^{B}	194.8^{BC}	123.5^{C}	258.8^{A}	9.2	<0.001
EE_{TB}	62.2^{C}	71.4^{C}	128.8^{A}	140.5^{A}	117.0^{A}	105.9^{B}	77.2^{C}	133.8^{A}	5.3	<0.001

DM_H, DM_T, DM_A: Dry Matter content of the Head, Thorax and Abdomen respectively (g/Kg); CP_H, CP_T, CP_A, CP_{TB}: Crude Protein content of the Head, Thorax, Abdomen and Total Body (g/Kg DM); EE_H, EE_T, EE_A, EE_{TB}: Ethereal Extract content of the Head, Thorax, Abdomen and Total Body (g/Kg DM).

4.3.5 V. apis and V. ceranae infection: prevalence and relative spore load

The 100% of newly emerged bees used in starting the trials and tested for Vairimorpha spp. infection, were positive for V. ceranae, and 50% of them were also positive for V. apis.

At the end of the trials, nine HB pools out of forty (22.5%) resulted co-infected, 20 (50.0 %) were positive for V. ceranae only, and 11 resulted positives for V. apis (27.5 %) only. For V. ceranae (Figure 4.3), the prevalence ranged from 0% in bees fed on HF up to 100% in bees fed on PD08, while V. apis prevalence ranged from 17% in PD05 up to 67% in PD06 groups (Figure 4.3). The frequency of double positive cases was overall low except for PD06 (50% of the total) and PD08 (Figure 4.3) and it was nil in the controls (SS50 and HF).

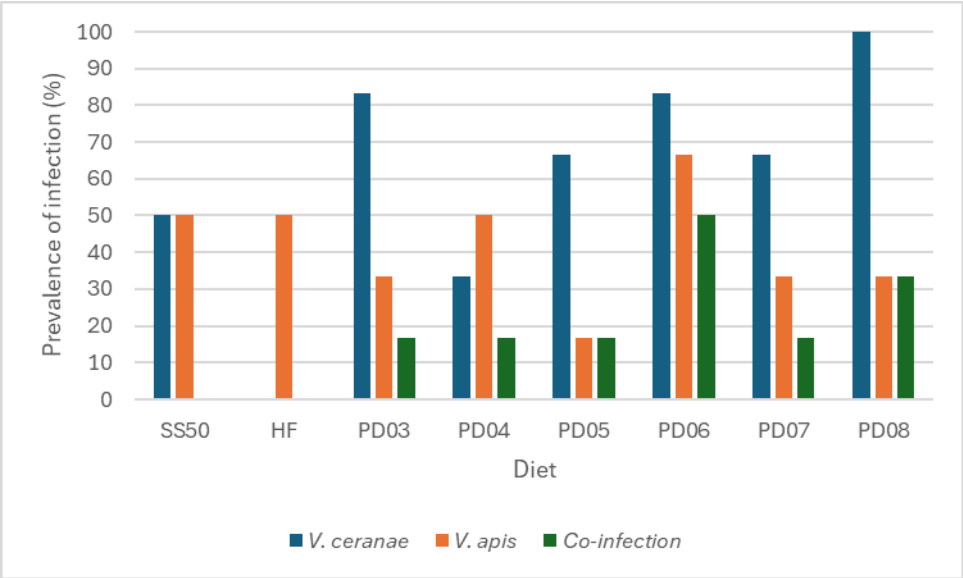

Figure 4.3. Prevalence of infection of *V. ceranae*, *V. apis* and co-infection.

However, the independence test between infection state and diets did not result statistically significant (Table 4.7).

Table 4.7. Effect of diets on *Vairimorpha* spp. relative spore loads (%).

	Observed	Critical	DF	alpha	*P*-value
Chi-square	23.741	36.415	24	0.05	0.476
Wilks' G²	28.705	36.415	24	0.05	0.231

With the exception of coinfection (RLS_{ca}), the RSL for the cases of *V. ceranae* or *V. apis* infection were negative. However, the effect of the diet was significant just for RSL_c ($P<0.05$) with total remission of spore load for HF-fed bees ($P>0.01$), though not significantly different from diets PD03-PD05 and the SS50 control (Table 4.8).

Table 4.8. Effect of diets on *Vairimorpha* spp. relative spore loads (%).

Variable	SS50	HF	PD03	PD04	PD05	PD06	PD07	PD08	SEM	P-value
RSL_c	-50.09 ^{AB}	-100.00 ^{B}	-37.75 ^{AB}	-79.99 ^{AB}	-61.97^{AB}	-21.41^{A}	-24.46 ^{A}	-19.11^{A}	18.56	0.032
RLS_a	-39.54	-48.15	-62.91	-38.75	-89.91	-41.47	-36.47	-49.61	28.01	0.891
RLS_{ca}	0.00	0.00	10.60	11.82	11.53	37.82	18.91	31.81	13.40	0.421

RSL_c: *V. ceranae* relative spore load; RLS_a: *V. apis* relative spore load; RLS_{ca}: relative spore load in co-infection cases; SEM: Standard Error of the Mean (pooled).

4.4 Discussion

Adequate nutrition is essential for honey bee colony growth and stress resistance (Brodschneider and Crailsheim 2010). Though the honey bee nutrition strongly depends on seasonal and floral diversity, yet researches often overlooks these environmental factors (Donkersley et al. 2014a; DeStefano 2015; Tsuruda et al. 2021). Multifloral pollen diets are reported enhancing longevity, immunity, and pathogen resistance in honey bees (Schmidt et al. 1995; Alaux et al. 2010; Di Pasquale et al. 2013), even though pollen diversity, among other factors, is strongly affected by seasonality (Malagnini et al. 2022).

In this study, pollen biodiversity of CPLs was high in the first part of the production season (April-June) but markedly decreased in August (Figure 4.2). Accordingly, both H' and E followed similar patterns during the season, excepted for August CPLs, due to the low number of pollen types (six) that resulted almost evenly represented (Figure 4.2). These results are roughly in line with Dimou et al (2007) who collected the highest number of different pollens in Greece between April and June (though almost double than in this study) and with Malagnini et al. (2022), that in Northern Italy observed the higher pollen biodiversity of CPLs in March–May, though with 50% lower value of H' compared to our findings. It is interesting to notice that three pollen types in this study (*i.e.*, *Rubus*, *Quercus*, and *Echium* types) were always well represented in CPLs harvested from April to June, collectively representing 22% of the total pollen types of that period. Specifically, *Rubus* pollens accounted for 10.2%, *Echium* for 6.3%, and *Quercus* for 5.5%. Though the specific nutritional value of these pollen types are not fully documented in literature, their consistent availability could represent a reliable foraging base for the honey bee colonies in the region, as it has been highlighted in a previous study (Donkersley et al., 2014)

Previous researches on pollen gathered by the honey bees, reported protein contents ranging from 20 to 600 g/Kg, and fat contents between 10 and 200 g/Kg (Roulston et al. 2000; Roulston and Cane 2000; Ruedenauer et al. 2019; Vaudo et al. 2020). Though the CP content of PDs, the average level was assessed as 239.4 g/Kg DM, aligned with the mentioned data. Moreover, CP content (Table 4.3) exhibited a positive but not significant association with pollen biodiversity (H') ($r = 0.70$, $p = 0.116$, data not shown).

The EE and ash contents of PDs, were consistent with literature findings (Roulston et al. 2000; Roulston and Cane 2000; Campos et al. 2008; Thakur and Nanda 2020; Vaudo et al. 2020). In particular, all PDs, but also the HF control, for both the constituents (Table 4.2) were close to the lower limit of the reported range, none of them showed any noteworthy correlations with other tested variables (data not shown).

To date, no specific data are available for the NFC content in CPLs as assessed in this study. However, Herbert and Shimanuki (1978) found starch and pectin contents in corbicular pollen of 18.0 g/Kg DM and 16.0 g/Kg DM respectively. In another study (Bayram et al., 2023), total carbohydrate in Italian CPL samples ranged from 256.6 g/Kg DM to 613.0 g/Kg DM, whereas in Croatian samples it ranged from 433.9 to 479.0 g/Kg DM (Prdun et al. 2021). Even if total carbohydrates in literature data didn't consider the $aNDF_{om}$ contribution, NFC content of our solid diets (Table 4.3) were consistent with mentioned data, except for PD05 PD07 and PD08 which resulted higher the maximum detected in literature.

The ash content of CPLs, according to Campos et al. (2008) and Thakur et al. (2020), can be variable from 20 and 60 g/Kg. Our data, ranging from 22.4 to 33.9 g/Kg DM, reflected the literature ones and showed a decreasing trend as the season progressed, with the highest rate recorded for the HF control diet (Table 4.3).

Concerning the macro element profile, the data on CPL of this study confirms literature data as the average values of 12.88 g/kg DM for K and 4.81 mg/kg DM while, for microelements, Cu fall within the reported ranges (K: 2.2–38.0 g/kg DM; Cu: 3.0–42.0 mg/kg DM)(Somerville and Nicol 2002; Valverde et al. 2023). Conversely, the average Na content in PDs (114.6 mg/kg DM, Table 4.3) was approximately twice the maximum value reported by Somerville et al. (2002). In contrast, the average Fe content in solid diets (4.2 mg/kg DM) was about 70% lower than the minimum detected by Somerville et al. (2002) (range: 14.0–520.0 mg/kg DM), and approximately 92% lower than the values reported by Valverde et al. (range: 58–134 mg/kg DM).

Differently from the main compositional traits, where only for CP was found a slight association with H' that was not statistically supported, moderate-to-strong positive associations of the variable content of P ($r = 0.93$, $p = 0.007$, data not shown), Mg ($r = 0.84$, $p = 0.036$), Cu ($r = 0.96$, $p = 0.002$), Mn ($r = 0.82$, $p = 0.047$), and Zn ($r = 0.81$, $p = 0.048$) in CPLs were found with the variation of the

Shannon index. It is known that phosphorus and magnesium play an essential roles in regulating hemolymph osmotic pressure and maintaining inter- and intracellular fluid balance in honey bees (Valverde et al. 2023). On the other side, elements such as copper, manganese, and zinc have been recognized as crucial for bees' growth, development, and reproductive functions (Valverde et al. 2023). Based on the reported physiological effects of such macro and micro minerals on honey bee, it can be speculated that the positive association found in this study could, though partially, explain the widely recognized correlation in the literature (Schmidt et al. 1995; Alaux et al. 2010; Di Pasquale et al. 2013) between pollen diversity and the health of honey bee colonies.

Furthermore, the chemical composition analysis of the diets showed that the highest protein content was observed in the June diets (PD06), while the lowest was in August (PD08). PD08 was also poor of the same mineral elements correlated with H' (Mg, P, ZN, Mn and Cu) just like HF diets; this results agreeing with previous studies emphasizing the importance of pollen quality in honey bee health (Di Pasquale et al. 2013). Finally, since the protein content of pollen is a reliable and direct measure of pollen quality (Pernal and Currie 2000), we can say that, together with the mineral elements mentioned above, a high biodiversity of pollen is an indicator of higher-quality pollen diets.

Pollen fed groups showed higher intake of DM, and therefore of CP, EE and NFC, than the HF controls (Table 4.4). The low DMI of HF controls could be due to a poor palatability caused by the matrix structure or the absence of nectar in the formulation. Pollen is typically preferred by honey bees over pollen replacements (Ghramh and Khan 2023; Kim et al. 2024), not because it is more rich in nutrients but rather because it contains specific carbohydrates, lipids, and amino acids (Bilisik et al. 2008) in suitable compositions that make it highly appealing to the honey bees (Huang 2012). In addition, foraging workers normally add regurgitated nectar or honey when packing pollen into their corbicula with further positive effects on its palatability (Boch 1982; Lan et al. 2021).

Diet is a crucial factor in honey bee development, directly influencing body composition, physiological functions, and overall health (Di Pasquale et al. 2013; Arien et al. 2020). Total body composition data for PD fed bees of this study were aligned with previous findings by Kunert and Crailsheim (1988) and Ghosh et al. (2016), who reported to vary from 250 to 780 g/Kg DM for CP, and from 69 to 160 g/Kg DM for the EE content. Notably, not only the CP-free (SS50) but also the HF fed bees showed lower total body CP contents than the PD counterparts (Table 4.6). Although HF

was higher in protein than PDs, the lower intake (Table 4.4) was most probably the main limitation of the CP accumulation in bees (Ghramh and Khan 2023; Kim, Frunze, Maigoro, et al. 2024).

The low lipid content of HF, combined with the reduced DMI (Table 4.4), resulted in a very low end-of-trial lipid accumulation in bees similarly to SS50-fed bees (Table 4.6). On the contrary, the higher EEI in PD-fed bees (Table 4.4) reflected in a higher total body fat content, though some differences among the PD groups were detected.

The evidence for CP and EE accumulation in the body of young workers paralleled the average daily gain of PDs-fed bees which were higher than SS50 and HF controls, especially for diets during the central months of the season (PD04-PD07; Table 4.5), reflecting exactly the trend of DM intake (Table 4.4).

As far as the allocation of main nutrients (CP, EE) in the bees' body, CP in bees' heads and thoraxes was twice or more than in the abdomen, that in turn was the main site of EE accumulation followed by the bees' head, though with no difference among the experimental groups (Table 4.6).

Despite a similar dry matter intake across all pollen diets (Table 4.4), the lower lipid content of PD07 (Table 4.3) resulted in a lower EEI compared to the other PD groups (Table 4.4), leading to a reduced lipid deposition in the bee's abdomen and thereby in whole body (Table 4.6). It is well known that the fat body develops more extensively in bees fed pollen diets, especially multifloral ones (Alaux et al. 2010).

Also regarding PD07, the high protein content of the diet (Table 4.3), combined with a high CP intake (Table 4.4), promoted a greater accumulation of proteins in the head and thorax (Table 4.6). These findings highlight how diet composition influence the body composition of developing bees.

The role of macro- and micronutrients was also evident in the positive associations between protein and lipid contents in HB total bodies and the phosphorus and magnesium concentrations in pollen diets. In particular CP presented strong correlation with Mg ($r=0.608$, $P<0.001$) and ($r=0.697$, $P<0.001$) while lipid content showed positive, but less stronger compared to CP ones, correlations for magnesium and phosphorus (respectively $r=0.396$, $P<0.001$ for Mg and $r=0.419$, $P<0.001$ for P) (data not shown). As in honey bees magnesium is involved in muscle contraction and relaxation and participate in ATP metabolism (De Sousa et al. 2022) as well as for P that may function as energy

sources for ATP synthesis (Hsu et al. 2007), our data confirm the importance of nutrient balance for bee health, as previously suggested by Brodschneider and Crailsheim (2010).

Proteins play a crucial role in the development of HPGs (DeGrandi-Hoffman et al. 2010; Sun et al. 2021; Khedidji et al. 2024), and it is known that honey bees with limited pollen intake or those fed protein-deficient pollen exhibit less developed HPGs (Crailsheim and Stolberg 1989; Khedidji et al. 2022). Our data suggest that multifloral diets demonstrated the ability to promote greater development of hypopharyngeal glands in PD fed bee compared to HF control (Table 4.5), as indicated by increased head weight (Hrassnigg and Crailsheim 1998; Rahman et al. 2014; Rinkevich et al. 2016), but not by HPG_w maybe due by the suboptimal sensitivity of the protocol adopted. In multifloral pollen fed bees has been observed, by Alaux et al. (2010), higher glucose oxidase (GOX) activity which may partially explain the positive outcomes observed in HPG development, in diets PD06 and PD07, as well as for a slightly increase in OD although they are always at the inactive stage (Table 4.5). GOX is produced mainly within the hypopharyngeal glands (Ohashi et al. 1999) and facilitates the transformation of b-D-glucose into gluconic acid and hydrogen peroxide, thus playing an important role in bees immunity. These findings support the hypothesis that a multifloral diet can enhance immune functions, in agreement with Alaux et al. (2010).

Vairimorpha spp. infection and pollen nutrition is a widely discussed topic (Martín-Hernández et al. 2011; Porrini et al. 2011; Jack et al. 2016). Since the microsporidia have a limited ability to produce ATP and the absence of most primary metabolite genes increase the metabolic cost for the host (Williams 2009; Azzouz-Olden et al. 2018), infected bees may compensate by feeding more, as energetic stress is shown to rise with higher spore doses (Martín-Hernández et al. 2011).

Even if no clinical symptoms of *Vairimorpha* spp. infection were observed, spore loads of 561±741 and 2,850 ±2,784 were detected for *V. apis* and *V. ceranae*, respectively, with maximum values reaching 2,455 and 10,950 spores per bee (data not shown). SL_c were consistent with data reported by Bourgeois et al. (2010) while SL_a resulted always higher (*V.apis* = 303±38; *V.ceranae* = 12,768±8,106), these spore loads could be sufficient to cause infection in honey bees (Malone and Stefanovic 1999).

The statistical analysis failed to reveal a significant relationship between diet type and *Vairimorpha* spp. prevalence (Table 4.7). This result may reflect the complexity of interactions between nutrition

and infection by the microsporidians, as previously noted by (Jack et al. 2016), who found higher infection levels in pollen-fed bees (prevalence range 23-35% compared to 50% of prevalence of *V.ceranae* and 27.5% of *V.apis* in our trials) but also greater longevity compared to those fed sugar syrup. The not relationship between diet type and *Vairimorpha* spp. prevalence (Table 4.7) underscores that while pollen may not directly prevent infection, it can enhance bees' physiological responses by supporting their metabolic and immune functions. Interestingly, pollen diets contributed to reducing RSL_a and RSL_c (Table 4.8), consistently showing a negative reduction percentage. Notably, for RSLc, diets PD03, PD04, and PD05 exhibited reduction values comparable to those of the HF group, which achieved a total reduction in spore load between the start and end of the test (Table 4.8). The reduction in sporal load observed in pollen diets could have contributed to promoting a high survival rate at the end of the trials, which was 94.77±6.38% (data not shown), as reported in previous studies by Di Pasquale et al. (2013) and Khedidji et al. (2022). Nevertheless, in this study the mortality rate of the PD groups (5.23±2.60%), with the exception of the PD08 one, was higher compared to the HF controls (Table 4.4). Mortality highlighted just in pollen diets may be explained by the evidences that curbicolar pollen could serve as vector of contaminants like pesticides (de Oliveira et al. 2016; Ruiz-Toledo et al. 2018), organic pollutants (Roszko et al. 2016) and heavy metals (Morgano et al. 2010). The higher dry matter intake from PD compared to HF (Table 4.4), along with the corresponding accumulation of CP and EE in the total body (Table 4.6), could result in an increased intake of these toxic contaminants by bees. For example, Pb content in the pollen diets (Table 4.2) may have played a role, although no direct correlation was observed ($r=-0.10$, $P=0.495$). To support the pollen contaminants hypothesis, the mortality rate is only slightly related to RLS_c ($r=0.259$, $p = 0.075$) (data not shown). Interestingly, this study reports the presence of *V.apis* in *A. m. ligustica*. The methodological refinements applied in this study allow us to assert its persistence in tested honey bee colonies. Specifically, the use of species-specific primers selected from Chen et al. (2009), amplification of positive samples, Sanger sequencing of the resulting amplicons, and alignment with the NCBI database provide robust evidence supporting this finding, warranting further investigation on a wider scale.

In summary, the data confirm that diverse and high-quality composition and biodiversity pollen diets, not only support the physiological development of honey bees but can also improve the physiological response to infections by *Vairimorpha spp*, especially *V.ceranae*, and nutritional stressors (*e.g.* harsh summer temperatures, drought conditions, lower forage availability). Furthermore, curbicolar pollen

collected during the central months (April-June) of the beekeeping season, with high quality composition and botanical biodiversity, seems to be more effective to induce positive physiological responses in young *Apis mellifera Ligustica* (Spin.) honey bee: this kind of pollen supplementation could be a valid approach for beekeepers to challenging the modern apiculture issues.

However, the complexity of interactions between nutritional and pathological stressors requires further studies (*e.g.* challenging test with instrumental infection of *Vairimorpha* spp. in *Apis mellifera ligustica* supplemented with mono- and multifloral pollen diets) to fully understand these relationships and optimize colony management strategies, particularly in increasingly challenging environmental contexts.

4.5 References

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4.6 Supplemetary materials

Table S4.1. Calibration details for the Atomic Absorption Spectrometry analysis.

Element	UoM	Atomization System	Calibration range	Fitting (R^2)*	LOD	LOQ	Percent Recovery
Ca	mg/kg	FL	1.25-5.00	0.9944	0.02	0.07	93.8
K	mg/kg	FL	0.25-2.00	0.9998	0.002	0.007	90.8
Mg	mg/kg	FL	0.10-0.50	0.9992	0.0007	0.002	95.3
P	mg/kg	GF	1.00-4.00	0.9992	0.01	0.05	106.6
Na	mg/kg	FL	0.20-1.00	0.9999	0.0006	0.002	104.6
Zn	mg/kg	FL	0.25-1.00	1,0000	0.002	0.007	94.8
Mn	mg/kg	FL	0.05-0.25	0.9999	0.006	0.02	109.9
Fe	µg/kg	GF	1.25-10.00	0.9976	0.06	0.20	103.3
Cu	µg/kg	GF	1.25-5.00	0.9977	0.03	0.10	103.2
Cd	µg/kg	GF	0.50-2.00	0.9985	0.004	0.01	109.3
Cr	µg/kg	GF	0.50-4.00	0.9982	0.01	0.05	107.6
Pb	µg/kg	GF	1.00-20.00	0.9995	0.05	0.20	95.2

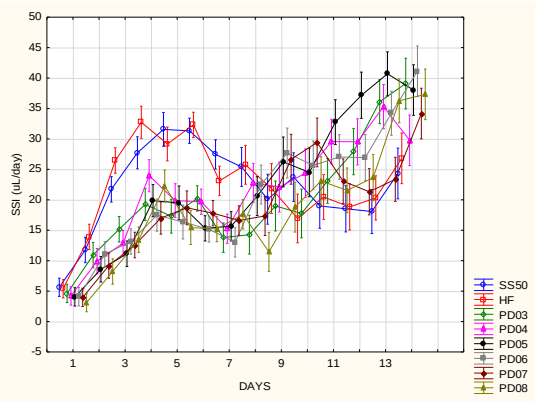
*All the fittings were represented by a linear model

Table S4.2. Raw data of the RT-PCR calibration runs.

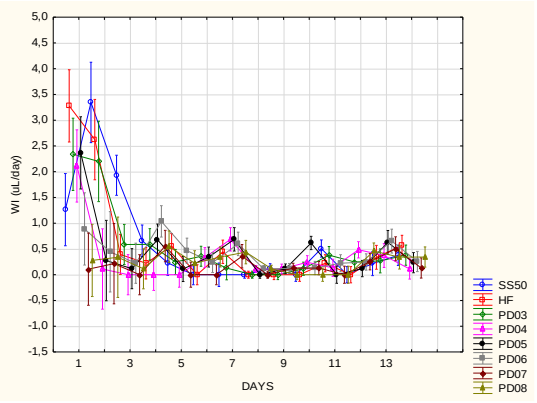
Sample Name	Run 1			Run 2			Run 3		
	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
CER_1:1	24.88	25.09	25.17	28.82	30.82	31.12	29.47	31.28	31.83
CER_1:2	27.88	28.04	28.26	34.38	33.7	34.26	34.01	33.97	34.36
CER_1:4	31.23	30.39	30.24	36.01	34.71	35.7	36.44	35.7	35.51
CER_1:8	33.89	34.57	34.58	39.12	<LOD	40.86	39.75	40.28	38.93
CER_1:16	<LOD	35.9	35.73	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CER_1:32	38.49	<LOD	38.48	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CER_1:64	<LOD	37.87	37.23	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CER_1:128	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CER_1:256	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
APIS_1:1	31.39	31.32	31.23	32.89	33.08	32.65	33.88	33.74	33.64
APIS_1:2	33.58	34.12	33.69	34.54	34.42	35.86	36.24	36.1	36.36
APIS_1:4	35.25	35.54	34.7	37.05	37.04	35.74	<LOD	36.6	36.82
APIS_1:8	38.87	38.37	37.75	37.77	39.87	42.59	40.88	<LOD	39.90
APIS_1:16	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
APIS_1:32	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
APIS_1:64	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
APIS_1:128	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
APIS_1:256	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

Table S4.3. Contrast analysis for variables analyzed with repeated measure ANOVA.

Item	SS50	HF	PD03	PD04	PD5	PD06	PD07	PD08	Type	F	P-value
SSI	-1.5	-1.5	0.5	0.5	0.5	0.5	0.5	0.5	Linear	2.36	0.132
WI	-1.5	-1.5	0.5	0.5	0.5	0.5	0.5	0.5	Linear	12.96	<0.001
DMI	nd	-1.71	0.28	0.28	0.28	0.28	0.28	0.28	Linear	37.22	<0.001
CPI	nd	-1.71	0.28	0.28	0.28	0.28	0.28	0.28	Linear	28.14	<0.001
EEI	nd	-1.14	-0.14	0.86	0.86	0.86	-1.14	0.86	Linear	50.18	<0.001
NFCI	nd	-1.14	0.86	0.86	0.86	-1.14	-1.14	0.86	Linear	26.76	<0.001
VMF	-1.75	0.25	0.25	0.25	0.25	0.25	0.25	0.25	Linear	6.7	0.013
MR	-1.75	0.25	0.25	0.25	0.25	0.25	0.25	0.25	Linear	8.95	0.004



A



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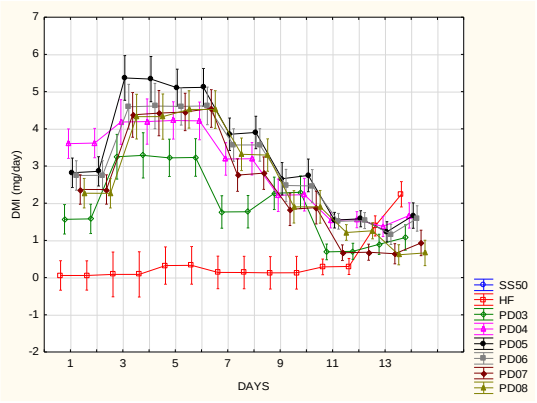

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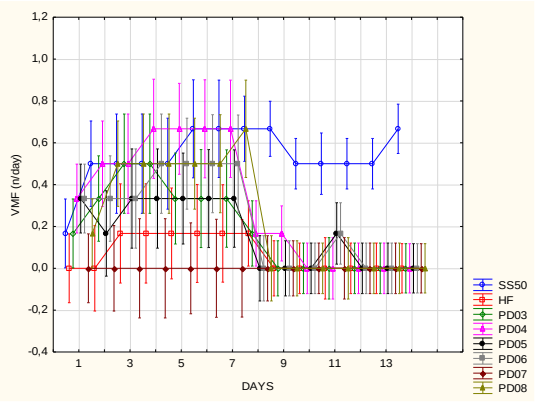

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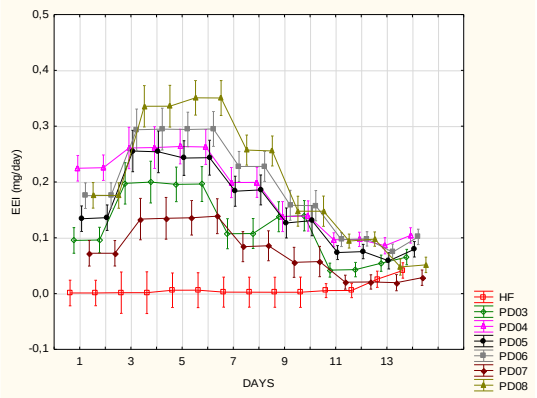

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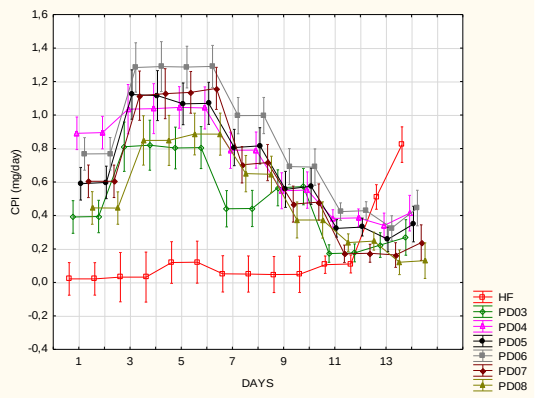

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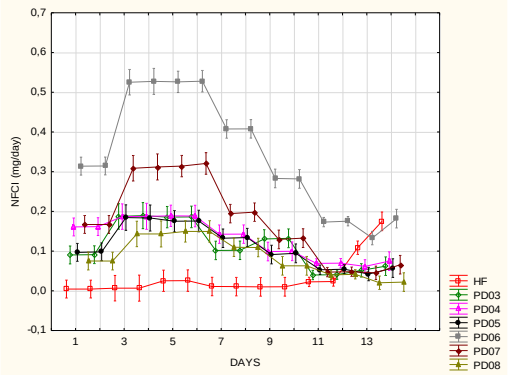


G

Figure S4.1. Diet × Time interactions for A) DSSI; B) DWI; C) DMI, D) VFM, E) EEI, F) CPI G); and NFCI

Table S4.4. Palinological analysis' results. Number of observations of pollen type per month.

Pollen Type	March	April	May	June	July	August	September	Total
Aesculus		6						6
Agrimonia				1				1
Apiaceae A/H-form				5		1		6
Apiaceae H-form				1		1		2
Boraginaceae		1						1
Cannabaceae					4			4
Caryophyllaceae	5				2	1		8
Castanea					8			8
Chenopodiaceae							1	1
Clematis		2		1				2
Compositae A-form			2	1				3
Compositae H-form		2					1	3
Compositae S-form			2					2
Compositae T-form	3	5			2	9	2	20
Convolvulus					1			1
Crucifarae	2	5		7	7			21

Echium		5	3	1		9
Euphorbiaceae				1		1
Fabaceae			2	2		4
Filipendula				4		4
Fraxinus	3					3
Gleditsia			3			3
Helianthenum			1			1
Hypericum				1		1
Knautia					1	1
Labiatae L (Lamium type)			1			1
Laurus	4	1				5
Leguminosae					1	1
Liliaceae	1	2	1			4
Liliaceae (Asparagus)	1					1
Liriodendron			1	1		2
Magnolia				2		2
Malus/Pyrus group		3				3

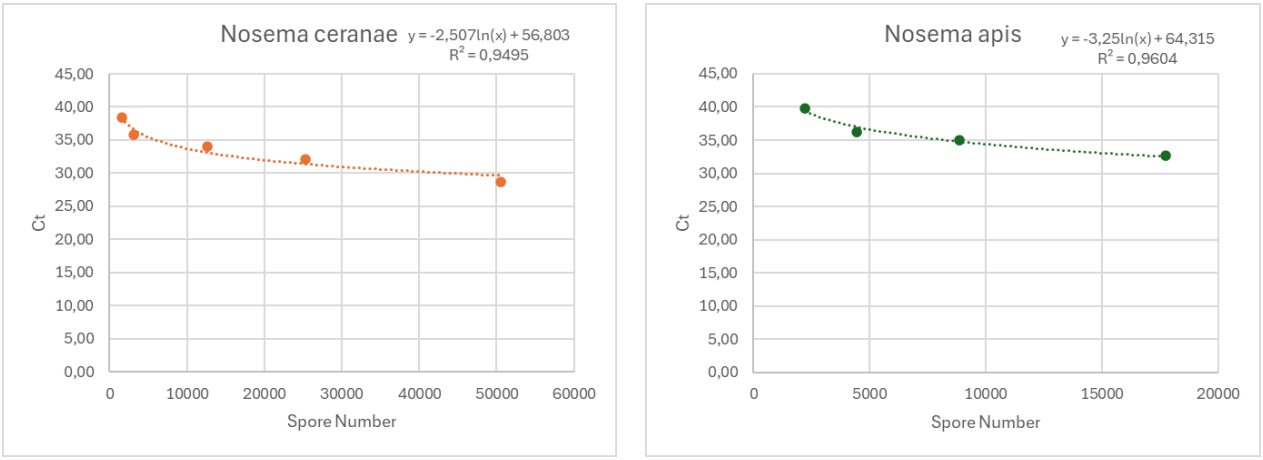
Malus/Pyrus type	3						3
Medicago				1			1
Melilotus - Group			1				1
Papaver			7	7			14
Papaveraceae					1		1
Pinus spp	2						2
Plantaginaceae						2	2
Prunus type	1						1
Quercus		5	2				7
Quercus spp		3					3
Ranunculaceae			1				1
Ranunculus	1						1
Rhamnaceae	6	3	2				11
Rosaceae		1	1				2
Rosaceae (Rosa)			1	1			2
Rosaceae (Rubus)					1		1
Rubus group	1	1	5	7	3		17

Rubus type	1					1
Salix	2					2
Spore (Puccinia?)	1					1
T. incarnatum	5	1				6
T. incarnatum group			1			1
T. pratense	1					1
T. pratense group			1			1
T. repens	1			1		2
T. repens group			1			1
Verbascum				3	2 1	6

Table S4.5. Ovaries' stage development (N. of observation).

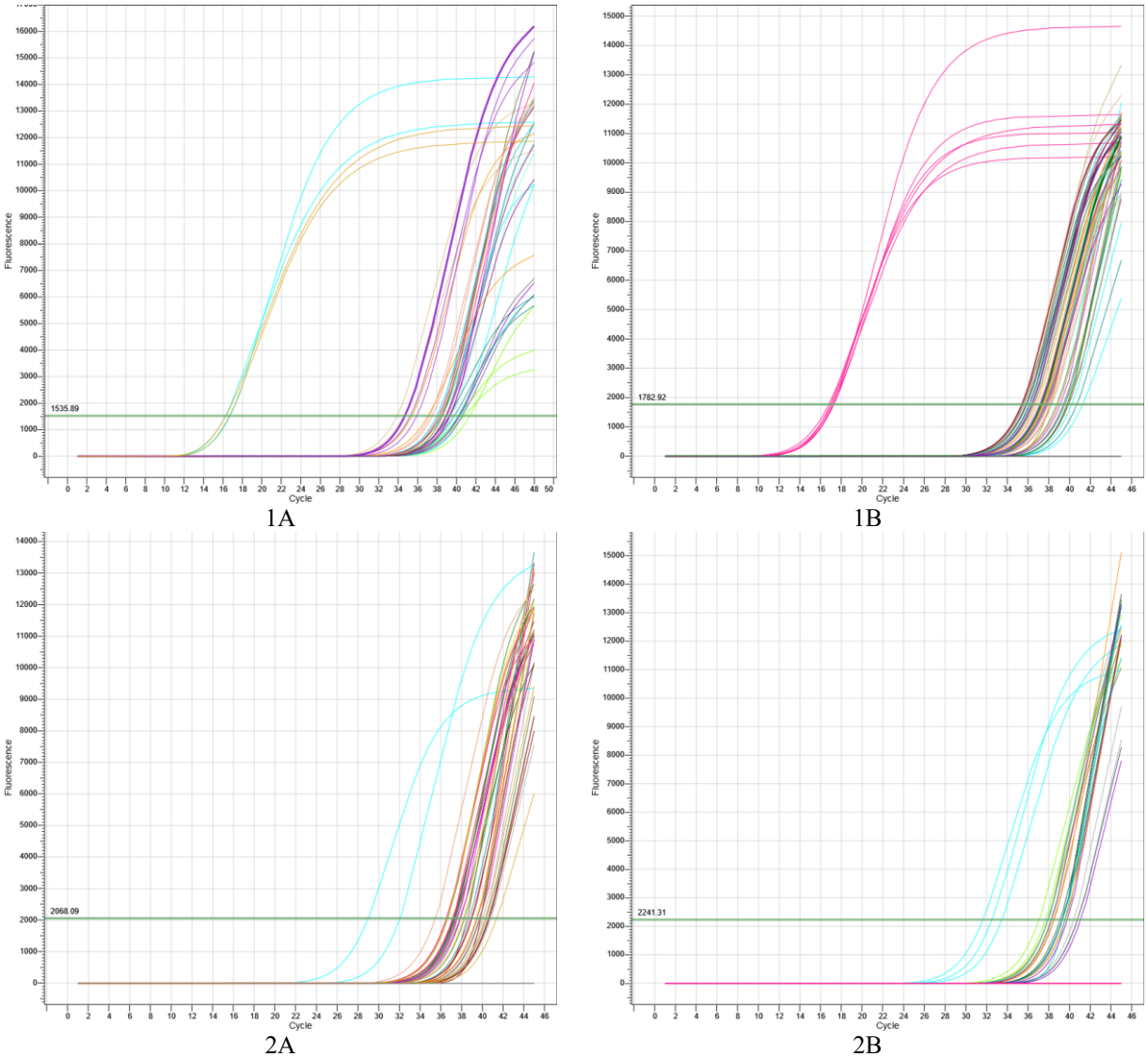
Ovaries' stage	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
NE	48	0	0	0	0
SS50	47	1	0	0	0
HF	46	2	0	0	0
PD03	44	2	2	0	0
PD04	47	0	1	0	0
PD05	37	5	5	1	0
PD06	34	12	0	2	0
PD07	43	4	1	0	0
PD08	38	10	0	0	0
Total (n)	384	36	9	3	0
Total (%)	89.1	8.2	2	0.7	0

NE: Newly Emerged; HF: Haydak formulation



A B

Figure S4.2. Calibration curve for *V.ceranae* (A) and *V. apis* (B)



1A 1B

2A 2B

Figure S4.3. Amplification plot of *V. cerane* run 1 (1A) and 2 (1B) and *V. apis* run 1 (2A) and 2 (2B). Each color represents a sample in triplicate.

Table S4.6A. Correlation matrix (Pearson values).

	Replicate Hw (mg)	Replicate HPGw (mg)	OD	Total Consumption of SS50 (mL)	Total Consumption of H2O (mL)	Total Consumption of Diet (g SS)	VMF (n)	ADG (mg)	MR (%) mean/cage	Wax production (mg)	DWP (mg)	SL_c (n)	SL_a (n)	RSL_c (%)	RSL_a (n)	EE_{TB} (g/Kg)	CP_{TB} (g/Kg)	Ash (g/Kg)	EE (g/Kg)	CP(g/Kg)	aNDFom (g/Kg)	NFC (g/Kg)	Cd (mg/Kg)	Cr (mg/Kg)	Pb (mg/Kg)	Ca (mg/Kg)	Cu (mg/Kg)	Fe (mg/Kg)	K (mg/Kg)	Mg (mg/Kg)	Mn (mg/Kg)	Na (mg/Kg)	P (mg/Kg)	Zn (mg/Kg)
Replicate Hw (mg)	**1**	**0,374**	0,226	0,073	**-0,287**	**0,503**	-0,032	**0,464**	0,235	0,248	0,248	0,113	-0,031	-0,022	-0,055	0,175	**0,550**	0,034	0,236	-0,016	0,125	0,235	0,223	0,047	**0,417**	0,245	**0,374**	0,276	-0,176	0,255	**0,517**	-0,212	**0,393**	**0,442**
Replicate HPGw (mg)	**0,374**	**1**	-0,054	0,128	**-0,318**	0,097	0,068	-0,034	0,069	-0,019	-0,019	-0,246	-0,076	-0,127	0,091	0,137	0,142	0,105	0,040	-0,011	-0,125	0,145	0,053	0,078	0,201	0,099	0,125	0,061	-0,043	0,097	0,125	0,028	0,131	0,140
OD	0,226	-0,054	**1**	-0,243	0,034	0,131	-0,054	0,217	0,093	**0,397**	**0,397**	-0,007	0,088	-0,190	-0,007	0,016	0,232	-0,053	0,090	0,018	0,194	0,014	0,104	0,015	0,068	0,064	0,098	0,124	-0,044	0,057	0,178	-0,106	0,106	0,143
Total Consumption of SS50 (mL)	0,073	0,128	-0,243	**1**	0,080	-0,206	0,115	-0,260	-0,091	-0,172	-0,172	0,031	**-0,314**	-0,007	**-0,354**	0,025	0,039	0,081	-0,076	0,072	-0,017	-0,018	-0,146	-0,115	-0,129	-0,041	-0,028	-0,105	0,153	0,059	-0,138	0,075	-0,022	-0,103
Total Consumption of H2O (mL)	**-0,287**	**-0,318**	0,034	0,080	**1**	**-0,305**	-0,104	-0,084	0,133	0,090	0,090	-0,045	-0,164	0,036	-0,183	-0,216	-0,203	-0,093	**-0,316**	-0,085	-0,111	**-0,353**	**-0,411**	-0,077	**-0,319**	**-0,328**	**-0,291**	**-0,308**	0,029	-0,212	**-0,391**	0,178	**-0,299**	-0,282
Total Consumption of Diet (g SS)	**0,503**	0,097	0,131	-0,206	**-0,305**	**1**	-0,214	**0,702**	**-0,295**	0,251	0,251	0,042	-0,044	-0,061	-0,070	**0,462**	**0,744**	**0,369**	**0,660**	0,235	0,237	**0,638**	**0,542**	0,232	**0,687**	**0,603**	**0,628**	**0,560**	0,094	**0,553**	**0,612**	-0,150	**0,631**	**0,576**
VMF (n)	-0,032	0,068	-0,054	0,115	-0,104	-0,214	**1**	-0,203	0,000	0,050	0,050	-0,128	0,051	0,040	-0,090	-0,132	-0,242	**-0,356**	-0,266	**-0,335**	-0,244	**-0,370**	**-0,340**	-0,266	-0,238	**-0,381**	**-0,349**	**-0,354**	**-0,292**	**-0,360**	-0,265	-0,202	**-0,343**	**-0,299**
ADG (mg)	**0,464**	-0,034	0,217	-0,260	-0,084	**0,702**	-0,203	**1**	-0,051	0,279	0,279	0,117	-0,067	-0,057	0,021	**0,317**	**0,576**	0,260	**0,478**	0,139	0,156	**0,487**	**0,448**	0,250	**0,599**	**0,469**	**0,509**	**0,464**	-0,014	**0,405**	**0,555**	-0,104	**0,513**	**0,525**
MR (%) mean/cage	0,235	0,069	0,093	-0,091	0,133	**-0,295**	0,000	-0,051	**1**	0,187	0,187	**0,323**	-0,038	0,259	-0,003	-0,228	-0,059	**-0,428**	**-0,312**	**-0,366**	-0,128	**-0,351**	-0,215	-0,185	-0,101	**-0,315**	-0,207	-0,214	**-0,448**	**-0,314**	0,056	-0,263	-0,179	-0,029
Wax Produc (mg)	0,248	-0,019	**0,397**	-0,172	0,090	0,251	0,050	0,279	0,187	**1**	**1,000**	0,067	-0,018	-0,220	-0,092	-0,074	0,253	-0,103	0,127	-0,022	0,223	0,013	0,177	0,078	0,123	0,076	0,091	0,175	-0,114	0,017	0,230	-0,157	0,103	0,188
DWP (mg)	0,248	-0,019	**0,397**	-0,172	0,090	0,251	0,050	0,279	0,187	**1,000**	**1**	0,067	-0,018	-0,220	-0,092	-0,074	0,253	-0,103	0,127	-0,022	0,223	0,013	0,177	0,078	0,123	0,076	0,091	0,175	-0,114	0,017	0,230	-0,157	0,103	0,188
SL_c (n)	0,113	-0,246	-0,007	0,031	-0,045	0,042	-0,128	0,117	**0,323**	0,067	0,067	**1**	-0,259	**0,565**	-0,113	-0,004	0,158	-0,207	0,118	-0,146	0,065	-0,018	0,155	-0,022	0,096	0,006	-0,016	0,078	-0,228	0,104	0,153	-0,279	-0,008	0,063
SL_a (n)	-0,031	-0,076	0,088	**-0,314**	-0,164	-0,044	0,051	-0,067	-0,038	-0,018	-0,018	-0,259	**1**	-0,102	**0,782**	-0,070	-0,156	-0,090	-0,050	-0,012	0,053	-0,098	-0,017	-0,045	-0,113	-0,062	-0,056	-0,029	-0,026	-0,075	-0,019	-0,010	-0,068	-0,059
RSL_c (%)	-0,022	-0,127	-0,190	-0,007	0,036	-0,061	0,040	-0,057	0,259	-0,220	-0,220	**0,565**	-0,102	**1**	-0,013	**0,328**	-0,177	-0,221	-0,251	-0,096	0,022	-0,227	-0,131	-0,176	-0,239	-0,187	-0,143	-0,148	-0,113	-0,161	-0,024	-0,074	-0,131	-0,098
RSL_a (%)	-0,055	0,091	-0,007	**-0,354**	-0,183	-0,070	-0,090	0,021	-0,003	-0,092	-0,092	-0,113	**0,782**	-0,013	**1**	-0,079	-0,202	-0,082	-0,092	-0,077	-0,129	-0,020	0,037	-0,017	-0,035	-0,041	-0,074	-0,049	-0,103	-0,113	0,002	-0,024	-0,075	-0,052

EE_{TB} (g/Kg)	0,175	0,137	0,016	0,025	-0,216	**0,462**	-0,132	**0,317**	-0,228	-0,074	-0,074	-0,004	-0,070	**-0,328**	-0,079	**1**	**0,563**	**0,468**	**0,848**	0,182	0,002	**0,617**	**0,519**	**0,515**	**0,787**	**0,564**	**0,492**	**0,541**	0,093	**0,396**	**0,301**	0,063	**0,420**	**0,384**
CP_{TB} (g/Kg)	**0,550**	0,142	0,232	0,039	-0,203	**0,744**	-0,242	**0,576**	-0,059	0,253	0,253	0,158	-0,156	-0,177	-0,202	**0,563**	**1**	**0,430**	**0,633**	0,280	0,273	**0,660**	**0,553**	**0,318**	**0,734**	**0,639**	**0,691**	**0,610**	0,122	**0,609**	**0,673**	-0,062	**0,697**	**0,669**
Ash (g/Kg)	0,034	0,105	-0,053	0,081	-0,093	**0,369**	**-0,356**	0,260	**-0,428**	-0,103	-0,103	-0,207	-0,090	-0,221	-0,082	**0,468**	**0,430**	**1**	**0,517**	**0,892**	**0,508**	**0,861**	**0,696**	**0,741**	**0,504**	**0,881**	**0,812**	**0,789**	**0,841**	**0,884**	**0,491**	**0,760**	**0,783**	**0,666**
EE (g/Kg)	0,236	0,040	0,090	-0,076	**-0,316**	**0,660**	-0,266	**0,478**	**-0,312**	0,127	0,127	0,118	-0,050	-0,251	-0,092	**0,848**	**0,633**	**0,517**	**1**	**0,347**	**0,290**	**0,745**	**0,753**	**0,552**	**0,804**	**0,735**	**0,624**	**0,737**	0,222	**0,531**	**0,469**	0,014	**0,560**	**0,506**
CP(g/Kg)	-0,016	-0,011	0,018	0,072	-0,085	0,235	**-0,335**	0,139	**-0,366**	-0,022	-0,022	-0,146	-0,012	-0,096	-0,077	0,182	0,280	**0,892**	**0,347**	**1**	**0,796**	**0,705**	**0,662**	**0,600**	0,207	**0,809**	**0,769**	**0,765**	**0,948**	**0,872**	**0,513**	**0,763**	**0,753**	**0,628**
aNDFom (g/Kg)	0,125	-0,125	0,194	-0,017	-0,111	0,237	-0,244	0,156	-0,128	0,223	0,223	0,065	0,053	0,022	-0,129	0,002	0,273	**0,508**	**0,290**	**0,796**	**1**	**0,419**	**0,556**	**0,349**	0,082	**0,615**	**0,680**	**0,695**	**0,673**	**0,713**	**0,627**	**0,401**	**0,681**	**0,630**
NFC (g/Kg)	0,235	0,145	0,014	-0,018	**-0,353**	**0,638**	**-0,370**	**0,487**	**-0,351**	0,013	0,013	-0,018	-0,098	-0,227	-0,020	**0,617**	**0,660**	**0,861**	**0,745**	**0,705**	**0,419**	**1**	**0,882**	**0,659**	**0,754**	**0,965**	**0,860**	**0,866**	**0,587**	**0,857**	**0,674**	**0,375**	**0,850**	**0,749**
Cd (mg/Kg)	0,223	0,053	0,104	-0,146	**-0,411**	**0,542**	**-0,340**	**0,448**	-0,215	0,177	0,177	0,155	-0,017	-0,131	0,037	**0,519**	**0,553**	**0,696**	**0,753**	**0,662**	**0,556**	**0,882**	**1**	**0,749**	**0,695**	**0,924**	**0,791**	**0,936**	**0,473**	**0,706**	**0,729**	**0,335**	**0,762**	**0,751**
Cr (mg/Kg)	0,047	0,078	0,015	-0,115	-0,077	0,232	-0,266	0,250	-0,185	0,078	0,078	-0,022	-0,045	-0,176	-0,017	**0,515**	**0,318**	**0,741**	**0,552**	**0,600**	**0,349**	**0,659**	**0,749**	**1**	**0,645**	**0,734**	**0,642**	**0,803**	**0,437**	**0,534**	**0,480**	**0,710**	**0,567**	**0,653**
Pb (mg/Kg)	**0,417**	0,201	0,068	-0,129	**-0,319**	**0,687**	-0,238	**0,599**	-0,101	0,123	0,123	0,096	-0,113	-0,239	-0,035	**0,787**	**0,734**	**0,504**	**0,804**	0,207	0,082	**0,754**	**0,695**	**0,645**	**1**	**0,714**	**0,702**	**0,722**	0,000	**0,531**	**0,667**	0,055	**0,668**	**0,724**
Ca (mg/Kg)	0,245	0,099	0,064	-0,041	**-0,328**	**0,603**	**-0,381**	**0,469**	**-0,315**	0,076	0,076	0,006	-0,062	-0,187	-0,041	**0,564**	**0,639**	**0,881**	**0,735**	**0,809**	**0,615**	**0,965**	**0,924**	**0,734**	**0,714**	**1**	**0,934**	**0,959**	**0,652**	**0,911**	**0,766**	**0,472**	**0,914**	**0,842**
Cu (mg/Kg)	**0,374**	0,125	0,098	-0,028	**-0,291**	**0,628**	**-0,349**	**0,509**	-0,207	0,091	0,091	-0,016	-0,056	-0,143	-0,074	**0,492**	**0,691**	**0,812**	**0,624**	**0,769**	**0,680**	**0,860**	**0,791**	**0,642**	**0,702**	**0,934**	**1**	**0,926**	**0,571**	**0,951**	**0,888**	**0,438**	**0,990**	**0,948**
Fe (mg/Kg)	0,276	0,061	0,124	-0,105	**-0,308**	**0,560**	**-0,354**	**0,464**	-0,214	0,175	0,175	0,078	-0,029	-0,148	-0,049	**0,541**	**0,610**	**0,789**	**0,737**	**0,765**	**0,695**	**0,866**	**0,936**	**0,803**	**0,722**	**0,959**	**0,926**	**1**	**0,554**	**0,842**	**0,824**	**0,467**	**0,891**	**0,889**
K (mg/Kg)	-0,176	-0,043	-0,044	0,153	0,029	0,094	**-0,292**	-0,014	**-0,448**	-0,114	-0,114	-0,228	-0,026	-0,113	-0,103	0,093	0,122	**0,841**	0,222	**0,948**	**0,673**	**0,587**	**0,473**	**0,437**	0,000	**0,652**	**0,571**	**0,554**	**1**	**0,758**	0,239	**0,751**	**0,562**	**0,377**
Mg (mg/Kg)	0,255	0,097	0,057	0,059	-0,212	**0,553**	**-0,360**	**0,405**	**-0,314**	0,017	0,017	-0,104	-0,075	-0,161	-0,113	**0,396**	**0,609**	**0,884**	**0,531**	**0,872**	**0,713**	**0,857**	**0,706**	**0,534**	**0,531**	**0,911**	**0,951**	**0,842**	**0,758**	**1**	**0,754**	**0,506**	**0,957**	**0,837**
Mn (mg/Kg)	**0,517**	0,125	0,178	-0,138	**-0,391**	**0,612**	-0,265	**0,555**	0,056	0,230	0,230	0,153	-0,019	-0,024	0,002	**0,301**	**0,673**	**0,491**	**0,469**	**0,513**	**0,627**	**0,674**	**0,729**	**0,480**	**0,667**	**0,766**	**0,888**	**0,824**	0,239	**0,754**	**1**	0,159	**0,906**	**0,959**
Na (mg/Kg)	-0,212	0,028	-0,106	0,075	0,178	-0,150	-0,202	-0,104	-0,263	-0,157	-0,157	-0,279	-0,010	-0,074	-0,024	0,063	-0,062	**0,760**	0,014	**0,763**	**0,401**	**0,375**	**0,335**	**0,710**	0,055	**0,472**	**0,438**	**0,467**	**0,751**	**0,506**	0,159	**1**	**0,388**	**0,370**
P (mg/Kg)	**0,393**	0,131	0,106	-0,022	**-0,299**	**0,631**	**-0,343**	**0,513**	-0,179	0,103	0,103	-0,008	-0,068	-0,131	-0,075	**0,420**	**0,697**	**0,783**	**0,560**	**0,753**	**0,681**	**0,850**	**0,762**	**0,567**	**0,668**	**0,914**	**0,990**	**0,891**	**0,562**	**0,957**	**0,906**	**0,388**	**1**	**0,951**
Zn (mg/Kg)	**0,442**	0,140	0,143	-0,103	-0,282	**0,576**	**-0,299**	**0,525**	-0,029	0,188	0,188	0,063	-0,059	-0,098	-0,052	**0,384**	**0,669**	**0,666**	**0,506**	**0,628**	**0,630**	**0,749**	**0,751**	**0,653**	**0,724**	**0,842**	**0,948**	**0,889**	**0,377**	**0,837**	**0,959**	**0,370**	**0,951**	**1**

Values in bold are different from 0 with a significance level alpha=0,05

Table S4.6B. Correlation matrix (P-values).

Replicate Hw (mg)	Replicate HPGw (mg)	OD	Total Consumption of SS50 (mL)	Total Consumption of H2O (mL)	Total Consumption of Diet (g SS)	VMF (n)	ADG (mg)	MR (%) mean/cage	Wax production (mg)	DWP (mg)	SL_c (n)	SL_a (n)	RSL_c (%)	RSL_a (n)	EE_{TB} (g/Kg)	CP_{TB} (g/Kg)	Ash (g/Kg)	EE (g/Kg)	CP(g/Kg)	aNDFom (g/Kg)	NFC (g/Kg)	Cd (mg/Kg)	Cr (mg/Kg)	Pb (mg/Kg)	Ca (mg/Kg)	Cu (mg/Kg)	Fe (mg/Kg)	K (mg/Kg)	Mg (mg/Kg)	Mn (mg/Kg)	Na (mg/Kg)	P (mg/Kg)	Zn (mg/Kg)	Replicate Hw (mg)
Replicate Hw (mg)	**0**	**0,009**	0,122	0,624	**0,048**	**0,000**	0,829	**0,001**	0,108	0,089	0,089	0,445	0,833	0,882	0,710	0,234	**<0,00 01**	0,819	0,107	0,917	0,396	0,108	0,128	0,750	**0,003**	0,093	**0,009**	0,057	0,231	0,081	**0,000**	0,148	**0,006**	**0,002**
Replicate HPGw (mg)	**0,009**	**0**	0,714	0,384	**0,028**	0,513	0,644	0,820	0,642	0,899	0,899	0,092	0,607	0,390	0,540	0,355	0,336	0,479	0,786	0,943	0,398	0,324	0,721	0,596	0,170	0,501	0,398	0,681	0,770	0,512	0,398	0,850	0,373	0,343
OD	0,122	0,714	**0**	0,096	0,816	0,376	0,716	0,138	0,528	**0,005**	**0,005**	0,965	0,551	0,196	0,962	0,914	0,112	0,723	0,542	0,905	0,187	0,925	0,483	0,920	0,646	0,666	0,508	0,402	0,768	0,699	0,225	0,473	0,475	0,333
Total Consumption of SS50 (mL)	0,624	0,384	0,096	**0**	0,590	0,161	0,437	0,074	0,536	0,242	0,242	0,834	**0,030**	0,964	**0,013**	0,864	0,793	0,585	0,609	0,625	0,906	0,904	0,323	0,436	0,384	0,781	0,850	0,478	0,298	0,690	0,348	0,612	0,879	0,484
Total Consumption of H2O (mL)	**0,048**	**0,028**	0,816	0,590	**0**	**0,035**	0,481	0,569	0,369	0,543	0,543	0,760	0,265	0,807	0,214	0,141	0,166	0,530	**0,029**	0,567	0,452	**0,014**	**0,004**	0,603	**0,027**	**0,023**	**0,045**	**0,033**	0,843	0,147	**0,006**	0,226	**0,039**	0,052
Total Consumption of Diet (g SS)	**0,000**	0,513	0,376	0,161	**0,035**	**0**	0,145	**<0,00 01**	**0,042**	0,085	0,085	0,779	0,769	0,680	0,639	**0,001**	**<0,00 01**	**0,010**	**<0,00 01**	0,108	0,104	**<0,00 01**	**<0,00 01**	0,112	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	0,525	**<0,00 01**	**<0,00 01**	0,308	**<0,00 01**	**<0,00 01**
VMF (n)	0,829	0,644	0,716	0,437	0,481	0,145	**0**	0,166	0,999	0,734	0,734	0,387	0,733	0,786	0,541	0,370	0,098	**0,013**	0,068	**0,020**	0,094	**0,010**	**0,018**	0,068	0,103	**0,008**	**0,015**	**0,014**	**0,044**	**0,012**	0,069	0,168	**0,017**	**0,039**
ADG (mg)	**0,001**	0,820	0,138	0,074	0,569	**<0,00 01**	0,166	**0**	0,731	0,054	0,054	0,429	0,653	0,701	0,887	**0,028**	**<0,00 01**	0,074	**0,001**	0,345	0,289	**0,000**	**0,001**	0,086	**<0,00 01**	**0,001**	**0,000**	**0,001**	0,922	**0,004**	**<0,00 01**	0,481	**0,000**	**0,000**
MR (%) mean/cage	0,108	0,642	0,528	0,536	0,369	**0,042**	0,999	0,731	**0**	0,203	0,203	**0,025**	0,798	0,075	0,984	0,119	0,691	**0,002**	**0,031**	**0,011**	0,385	**0,015**	0,142	0,208	0,495	**0,029**	0,159	0,145	**0,001**	**0,030**	0,704	0,071	0,225	0,845
Wax Produc (mg)	0,089	0,899	**0,005**	0,242	0,543	0,085	0,734	0,054	0,203	**0**	**<0,00 01**	0,650	0,901	0,132	0,532	0,616	0,083	0,486	0,391	0,883	0,128	0,931	0,229	0,598	0,406	0,606	0,538	0,235	0,439	0,908	0,117	0,285	0,487	0,202
DWP (mg)	0,089	0,899	**0,005**	0,242	0,543	0,085	0,734	0,054	0,203	**<0,00 01**	**0**	0,650	0,901	0,132	0,532	0,616	0,083	0,486	0,391	0,883	0,128	0,931	0,229	0,598	0,406	0,606	0,538	0,235	0,439	0,908	0,117	0,285	0,487	0,202
SL_c (n)	0,445	0,092	0,965	0,834	0,760	0,779	0,387	0,429	**0,025**	0,650	0,650	**0**	0,075	**<0,00 01**	0,443	0,979	0,283	0,157	0,423	0,323	0,663	0,904	0,293	0,882	0,516	0,969	0,915	0,597	0,118	0,481	0,298	0,054	0,959	0,671
SL_a (n)	0,833	0,607	0,551	**0,030**	0,265	0,769	0,733	0,653	0,798	0,901	0,901	0,075	**0**	0,491	**<0,00 01**	0,636	0,291	0,544	0,734	0,938	0,719	0,507	0,910	0,761	0,443	0,674	0,704	0,844	0,861	0,612	0,899	0,947	0,644	0,690
RSL_c (%)	0,882	0,390	0,196	0,964	0,807	0,680	0,786	0,701	0,075	0,132	0,132	**<0,00 01**	0,491	**0**	0,931	**0,023**	0,230	0,130	0,085	0,515	0,882	0,121	0,374	0,230	0,101	0,202	0,331	0,314	0,446	0,274	0,869	0,619	0,374	0,508
RSL_a (%)	0,710	0,540	0,962	**0,013**	0,214	0,639	0,541	0,887	0,984	0,532	0,532	0,443	**<0,00 01**	0,931	**0**	0,593	0,169	0,581	0,534	0,602	0,383	0,895	0,804	0,910	0,812	0,782	0,618	0,741	0,485	0,445	0,988	0,874	0,614	0,725
EE_{TB} (g/Kg)	0,234	0,355	0,914	0,864	0,141	**0,001**	0,370	**0,028**	0,119	0,616	0,616	0,979	0,636	**0,023**	0,593	**0**	**<0,00 01**	**0,001**	**<0,00 01**	0,215	0,988	**<0,00 01**	**0,000**	**0,000**	**<0,00 01**	**<0,00 01**	**0,000**	**<0,00 01**	0,530	**0,005**	**0,038**	0,672	**0,003**	**0,007**
CP_{TB} (g/Kg)	**<0,00 01**	0,336	0,112	0,793	0,166	**<0,00 01**	0,098	**<0,00 01**	0,691	0,083	0,083	0,283	0,291	0,230	0,169	**<0,00 01**	**0**	**0,002**	**<0,00 01**	0,054	0,060	**<0,00 01**	**<0,00 01**	**0,027**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	0,410	**<0,00 01**	**<0,00 01**	0,677	**<0,00 01**	**<0,00 01**
Ash (g/Kg)	0,819	0,479	0,723	0,585	0,530	**0,010**	**0,013**	0,074	**0,002**	0,486	0,486	0,157	0,544	0,130	0,581	**0,001**	**0,002**	**0**	**0,000**	**<0,00 01**	**0,000**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,000**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,000**	**<0,00 01**	**<0,00 01**	**<0,00 01**

EE (g/Kg)	0,107	0,786	0,542	0,609	**0,029**	**<0,00 01**	0,068	**0,001**	**0,031**	0,391	0,391	0,423	0,734	0,085	0,534	**<0,00 01**	**<0,00 01**	**0,000**	**0**	**0,016**	**0,046**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	0,130	**0,000**	**0,001**	0,924	**<0,00 01**	**0,000**
CP(g/ Kg)	0,917	0,943	0,905	0,625	0,567	0,108	**0,020**	0,345	**0,011**	0,883	0,883	0,323	0,938	0,515	0,602	0,215	0,054	**<0,00 01**	**0,016**	**0**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	0,158	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,000**	**<0,00 01**	**<0,00 01**	**<0,00 01**
aNDF om (g/Kg)	0,396	0,398	0,187	0,906	0,452	0,104	0,094	0,289	0,385	0,128	0,128	0,663	0,719	0,882	0,383	0,988	0,060	**0,000**	**0,046**	**<0,00 01**	**0**	**0,003**	**<0,00 01**	**0,015**	0,579	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,005**	**<0,00 01**	**<0,00 01**
NFC (g/Kg)	0,108	0,324	0,925	0,904	**0,014**	**<0,00 01**	**0,010**	**0,000**	**0,015**	0,931	0,931	0,904	0,507	0,121	0,895	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,003**	**0**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,009**	**<0,00 01**	**<0,00 01**
Cd (mg/K g)	0,128	0,721	0,483	0,323	**0,004**	**<0,00 01**	**0,018**	**0,001**	0,142	0,229	0,229	0,293	0,910	0,374	0,804	**0,000**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,001**	**<0,00 01**	**<0,00 01**	**0,020**	**<0,00 01**	**<0,00 01**
Cr (mg/K g)	0,750	0,596	0,920	0,436	0,603	0,112	0,068	0,086	0,208	0,598	0,598	0,882	0,761	0,230	0,910	**0,000**	**0,027**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,015**	**<0,00 01**	**<0,00 01**	**0**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,002**	**<0,00 01**	**0,001**	**<0,00 01**	**<0,00 01**	**<0,00 01**
Pb (mg/K g)	**0,003**	0,170	0,646	0,384	**0,027**	**<0,00 01**	0,103	**<0,00 01**	0,495	0,406	0,406	0,516	0,443	0,101	0,812	**<0,00 01**	**<0,00 01**	**0,000**	**<0,00 01**	0,158	0,579	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0**	**<0,00 01**	**<0,00 01**	**<0,00 01**	1,000	**0,000**	**<0,00 01**	0,712	**<0,00 01**	**<0,00 01**
Ca (mg/K g)	0,093	0,501	0,666	0,781	**0,023**	**<0,00 01**	**0,008**	**0,001**	**0,029**	0,606	0,606	0,969	0,674	0,202	0,782	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,001**	**<0,00 01**	**<0,00 01**
Cu (mg/K g)	**0,009**	0,398	0,508	0,850	**0,045**	**<0,00 01**	**0,015**	**0,000**	0,159	0,538	0,538	0,915	0,704	0,331	0,618	**0,000**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,002**	**<0,00 01**	**<0,00 01**
Fe (mg/K g)	0,057	0,681	0,402	0,478	**0,033**	**<0,00 01**	**0,014**	**0,001**	0,145	0,235	0,235	0,597	0,844	0,314	0,741	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,001**	**<0,00 01**	**<0,00 01**
K (mg/K g)	0,231	0,770	0,768	0,298	0,843	0,525	**0,044**	0,922	**0,001**	0,439	0,439	0,118	0,861	0,446	0,485	0,530	0,410	**<0,00 01**	0,130	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,001**	**0,002**	1,000	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0**	**<0,00 01**	0,101	**<0,00 01**	**<0,00 01**	**0,008**
Mg (mg/K g)	0,081	0,512	0,699	0,690	0,147	**<0,00 01**	**0,012**	**0,004**	**0,030**	0,908	0,908	0,481	0,612	0,274	0,445	**0,005**	**<0,00 01**	**<0,00 01**	**0,000**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,000**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0**	**<0,00 01**	**0,000**	**<0,00 01**	**<0,00 01**
Mn (mg/K g)	**0,000**	0,398	0,225	0,348	**0,006**	**<0,00 01**	0,069	**<0,00 01**	0,704	0,117	0,117	0,298	0,899	0,869	0,988	**0,038**	**<0,00 01**	**0,000**	**0,001**	**0,000**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,001**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	0,101	**<0,00 01**	**0**	0,282	**<0,00 01**	**<0,00 01**
Na (mg/K g)	0,148	0,850	0,473	0,612	0,226	0,308	0,168	0,481	0,071	0,285	0,285	0,054	0,947	0,619	0,874	0,672	0,677	**<0,00 01**	0,924	**<0,00 01**	**0,005**	**0,009**	**0,020**	**<0,00 01**	0,712	**0,001**	**0,002**	**0,001**	**<0,00 01**	**0,000**	0,282	**0**	**0,006**	**0,010**
P (mg/K g)	**0,006**	0,373	0,475	0,879	**0,039**	**<0,00 01**	**0,017**	**0,000**	0,225	0,487	0,487	0,959	0,644	0,374	0,614	**0,003**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,006**	**0**	**<0,00 01**
Zn (mg/K g)	**0,002**	0,343	0,333	0,484	0,052	**<0,00 01**	**0,039**	**0,000**	0,845	0,202	0,202	0,671	0,690	0,508	0,725	**0,007**	**<0,00 01**	**<0,00 01**	**0,000**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**<0,00 01**	**0,008**	**<0,00 01**	**<0,00 01**	**0,010**	**<0,00 01**	**0**

Table S4.7. Correlation matrix for pollen H'.

	Var iabl es	**H'**	**ASH**	**CP**	**EE**	**aNDF**	**NFC**	**Ca**	**K**	**Mg**	**P**	**Na**	**Zn**	**Mn**	**Fe**	**Cu**	**Ca/P**	**Cu/Fe**	**Cd**	**Cr**	**Pb**
Pearson value	H'	**1**	0,214	0,707	-0,673	0,415	-0,429	0,234	0,294	**0,840**	**0,932**	0,353	**0,815**	**0,818**	0,270	**0,964**	**-0,895**	0,637	-0,426	-0,094	-0,111
p-value	H'	**0**	0,684	0,116	0,143	0,413	0,396	0,655	0,572	**0,036**	**0,007**	0,493	**0,048**	**0,047**	0,605	**0,002**	**0,016**	0,174	0,399	0,860	0,834

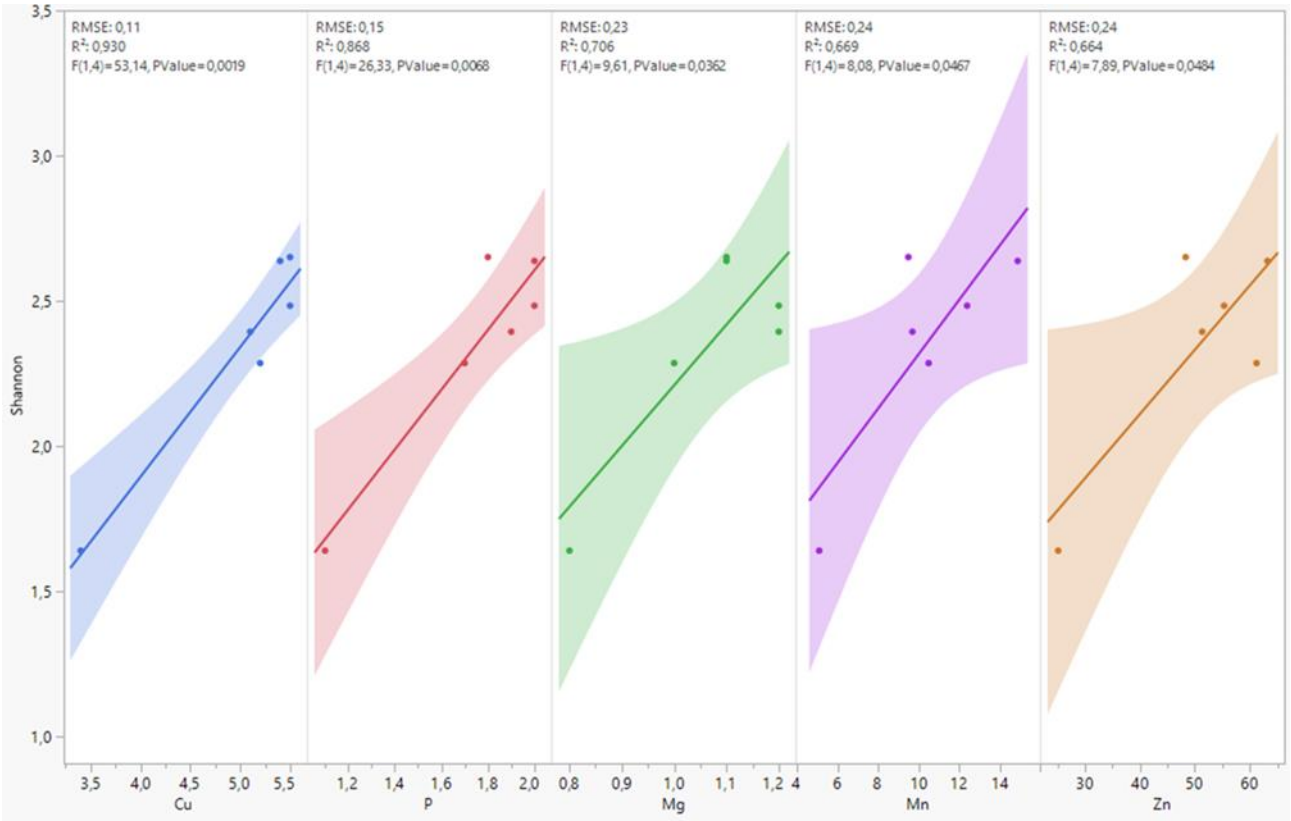


Figure S4.4. Correlation plot for pollen H'.

Table S4.7. Linear contrast analysis for the body composition variables analyzed with ANOVA.

Item	SS50	HF	PD03	PD04	PD5	PD06	PD07	PD08	F	P-value
DM_H	-1.55	-1.55	0.44	0.44	0.44	0.44	0.44	0.44	0.61	0.523
DM_T	-1.55	-1.55	0.44	0.44	0.44	0.44	0.44	0.44	0.38	0.176
DM_A	-1.55	-1.55	0.44	0.44	0.44	0.44	0.44	0.44	0.28	0.228
CP_H	-1.55	-1.55	0.44	0.44	0.44	0.44	0.44	0.44	0.61	0.604
CP_T	-1.55	-1.55	0.44	0.44	0.44	0.44	0.44	0.44	0.38	0.366
CP_A	-1.55	-1.55	0.44	0.44	0.44	0.44	0.44	0.44	0.28	0.228
EE_H	-1.55	-1.55	0.44	0.44	0.44	0.44	0.44	0.44	0.61	0.569
EE_T	-1.55	-1.55	0.44	0.44	0.44	0.44	0.44	0.44	0.38	0.372
EE_A	-1.55	-1.55	0.44	0.44	0.44	0.44	0.44	0.44	0.28	0.345

Chapter 5

Effectiveness of Fe (II) mineral supplementation in Italian honey bee (*Apis mellifera ligustica* Spin.) under field and laboratory conditions

5.1 Introduction

Due to its crucial function in pollinating agricultural crops, the western honey bee (*Apis mellifera* L.) is an important species with substantial economic and cultural significance on a global (Berry et al. 2021; Manzano Sánchez et al. 2021; Bartlett 2022). Despite its significance, the beekeeping sector faces many obstacles that affect honey bee colony health and productivity. Numerous stresses have been implicated in the global decline in honey bee populations over the past few decades (Potts et al. 2010; Paudel et al. 2015; Shumkova et al. 2021). One of the most serious of them is the ectoparasitic mite *Varroa destructor*, which cause the collapse of a large number of bee colonies every year (Annoscia et al. 2012).

Varroosis, a disease caused by *Varroa destructor*, has been associated with up to 15% higher winter mortality rates in European colonies and is a major cause of colony collapse (Brodschneider et al. 2018; Gray et al. 2020; Reams et al. 2022). The parasite increases the viral load of infections like the *Deformed Wing Virus* and changes strain diversity, which worsens colony loss (Annoscia et al. 2012; Martin et al. 2012). The parasite's ability to become resistant to common acaricides, such as acrinathrin, flumethrin, fluvalinate, amitraz, and coumaphos, makes managing *Varroa destructor* a major challenge for beekeepers (Pietropaoli and Formato 2019; Bahreini et al. 2020; Allabergenova et al. 2021; Ward et al. 2022).

Additionally to resistance problems, seasonal variance, the active ingredients employed, and the general health of the colonies all affect acaricidal effectiveness (Giacobino et al. 2015). Given the reduced risks of residue accumulation and resistance, this situation emphasizes the necessity of

integrated management strategies that incorporate the use of natural alternatives such as organic acids (lactic, oxalic, and formic acids) and essential oils (Bahreini et al. 2020; (Milani 1999; Bogdanov et al. 2002). However, these natural treatments are subject to limitations, including restricted application periods and variable efficacy (Lodesani and Costa 2008; Maddaloni and Pascual 2015; Pietropaoli and Formato 2019).

Oxalic acid, in particular, is widely used and recognized for its efficacy, though its effectiveness is not absolute (Llorente and Suárez 1999; Rademacher and Harz 2006). According to recent research, oxalotrophic bacteria linked to *Varroa destructor* may break down oxalic acid, lessening the parasite's toxicity (Maddaloni and Pascual 2015).

Additionally, it is known that the varroa mite's parasitosis may lead to a nutritional deficit of mineral elements like iron by the extraction of hemolymph in the pupal stage (Eisenstein and Blemings, 1998). Iron is mostly stored in the fat body of bees, where it serves as both orientation and mineral nutrition. Through the use of magnetoception, bees use the iron that has collected in their bodies to find their location in space. Therefore, when a bee is parasitized by a varroa mite, iron is shared between the parasite and the host, lowering the insect's reserve and influencing its regular physiological levels. Long-term extraction may deplete a bee's iron supply, at which point the bees will exhibit signs of a shortage (Eisenstein and Blemings 1998). In the end, this may result in less iron being available for critical bodily functions. By enabling beekeepers to employ non-toxic products, nutritional supplements based on iron or other minerals like copper can help them operate more responsibly and economically.

The potential of iron (FeII) as an alternative treatment has also been investigated in recent studies (Van Hoorn and Ter Horst 2014; Danieli et al. 2019).

Iron (II) salts can be provided to bees in sugar solutions via pocket or cup feeders, as they require dissolution for effective use. Acaricidal characteristics, limited water solubility, and widespread availability make ferrous fumarate ($C_4H_2FeO_4$) an attractive therapy choice. Since vinasse functions as a chelating agent and increases the solubility of iron (II), its combination with ferrous fumarate can increase its effectiveness (Van Hoorn and Ter Horst 2014). According to Van Hoorn et al., (2014), bee colonies treated with ferrous fumarate chelated with vinasse have a noteworthy decrease in varroa mites and a significantly lower winter death rate (around 8% against 40% in untreated colonies).

Furthermore, compared to untreated controls, colonies receiving this therapy showed a larger overall mite drop and more severely damaged adult mites, according to Danieli et al. (2019).

In light of these findings, the present study aimed to evaluate the effectiveness of Fe(II) salts, specifically ferrous fumarate, for Varroa mite control through oral application, while testing its impact on honeybee survival and over-wintering. Both laboratory and field conditions were employed to evaluate the efficacy of this method.

The field tests were previously conducted by the author, outside of the PhD thesis period, but their results were analysed during the PhD fellowship in order to have a solid base to construct a fine tuned approach for laboratory trials with the aim to assess the right dosage of treatment to propose to beekeepers.

5.2 Material and methods

5.2.1 Chemical reagents

For laboratory and field treatments, solutions based on Fe(II) fumarate 94% (AlfaAesar, Ward Hill, USA) was used and chelated in diluited vinasse (DV). All the solutions were dissolved in 50:50 (w/w) sugar syrup (SS50) with 0.4% citric acid (99.8% purity) (Carlo Erba reagents, Milan, Italy) to promote the sucrose inversion.

5.2.2 Apiary trials

The two-years study was carried out from October to February 2020 and 2021 in the Plant-B apiary (PBA) located at the Experimental Teaching Farm “N. Lupori” (Tuscia University, Viterbo, Italy) (42.4271454°E, 12.0796127°N, 302.2 m EASL). The apiary consisted of 8 Italian-Dadant hives for 2020 trial, and 8 hives for the 2021 trial, this in order to use hives as much homogeneous as possible as far as colony strength and stores (Table 5.1). Half of the hives were equipped with Mussi-type spacers® (45 mm of distance between the centers of the adjacent honeycombs) (WS), and half equipped with regular spacers (37.5 mm spaced) (RS). The Mussi-type was tested to evaluate whether the type of hive, with the same treatment, could have an additional effect on the fall of varroa.

Table 5.1. Experimental setup for apiary trials.

Year	Treatment	Negative control	Hives number	WS	RS
2020	Fe(II)	SS50+DV	8	4	4
2021	Fe(II)	SS50+DV	8	4	4

SS50: Sugar Syrup 50:50 (w:w); DV: Diluited Vinasse; WS: Wider Spacers hive; RS: Regular Spacers hive

The hives were divided into two groups: control and FeFUM supplementation divided between RS and WS. The supplementation solutions were prepared as follows, according to the number of bees in the various hives. For the FeFUM treatment, 6.4 mg FeFum was administered per sixth of bees (approximately 206 bees) chelated with 0.4 ml of 1/100 ml diluted vinasse and diluted in 50 ml of SS50, with a final concentration of 1 mg/Kg of HBs. The control group, on the other hand, was administered a 1/100 (4 ml per sixth of bees) diluted vinasse solution in a 50:50 (DV) sugar solution.

The solutions were administered in November of each year of the experiment. The protocol comprised three main phases, a pre-supplementation phase (PRE), a 4-week supplementation treatment phase (TREAT), and a 12-week follow-up phase (POST).

The solutions, prepared in the hours immediately prior to treatment in the laboratory, were administered twice a week during the TREAT phase, in the cup feeders with which each hive was provided.

Before, during, and after the treatment, hive inspections were performed and data on the number of bee sixths (**BS;** n), storage sixths (honey + pollen) (**SS**; n) were recorded (Imdorf and Berna 1999). Hive mortality (**HM**; n) was assessed by using the underbasket traps (Porrini 2007) which were checked for dead bees every 7 days for a total of six weeks. The dead bees and bee parts were collected, counted, weighed, and then frozen for further evaluations. The VM fall was carried out through 35 × 50 cm adhesive paper sheets placed just beneath the anti-VM screen of each hive. The sheets were changed every 48 hours for the first two weeks. In the following four weeks they were placed once a week for 48 hours. Mature (**MVM**; n) and immature (**IVM**; n) VM specimen were automatically counted using the Bee Varroa Scanner V.2 (BeeVS) (Apisfero APS, Italy), using high-

resolution images and a residual convolutional neural network including 3 convolutional and 2 fully connected layers developed in Python v.3.7 (2018), in order to discriminate MVM and IVM.

Figure 5.1. Plant-B Apiary (PBA) used for field test (Author's photo).

5.2.3 Laboratory Trials

5.2.3.1 Queen caging and emerging HBs management

To obtain the young HBs, a healthy colony housed in a B-BOX hive (Beiing Srl, Faenza, Italy) and maintained in an all-year round facility (Taber and Owens 1970) at the University of Tuscia under controlled environmental conditions (29.1 ± 2.2°C) was used. To obtain a cohort of 0-24 h old workers, the laying queen, provided by a queen bee breeder and with the certification of belonging to the subspecies *A. m. ligustica* Spin. (CREA, 2023), was caged forcing her to lay on the same honeycomb inlaid in a plastic queen-excluder cage (Erboristeria Apistica, Sondrio, Italy). The brood development was monitored daily by manual noting the number of eggs, larvae, and capped cells. As the whole experimentation was carried out in triplicate, the first queen caging took place on 12/04/2022, the second one took place on 27/01/2023 while the third started from 15/05/2023. In case

of outside adverse weather conditions during queen caging, the colony was fed at 100 mL SS50/day. About four days before the forecasted mass emergence of worker bees, the honeycomb was moved in a net cage maintained at 30.2 ± 1.1 °C and 70.6 ± 1.4% RH into a laboratory incubator (Carbolite Gero Ltd, Hope, UK). Emerging bees were provided with SS50 and tap water ad libitum.

5.2.3.2 Ex-hive trials conditions

Groups of 30 emerging workers were collected through a battery-powered vacuum cleaner and then placed inside self-built 3D printed cages (6 cm × 4 cm × 7 cm; inner volume 168 cm^3) at a density of 17.8 bee/100 cm^3 (Bosua et al. 2018; Manganello et al. 2023). To limit any possible effect of the plastic material on the bees, only polyethylene terephthalate (PET) and polylactate (PLA) (Prusa Reseach a.s., Partyzánská, Czech Republic) filaments were used in the 3D printing process. Cages had a removable tray at the bottom for monitoring the varroa mite fall and the collection of any debris (*e.g.*, feces). Each cage had two hole per side, suitably sized to fit 2 mL centrifuge tubes which contained a protein diet according to Haydak (1970); the tubes were pierced (4.5 mm) on the bottom to allow for bees to enter. The top of the cages was designed to allow fitting 1 mL graduated syringe barrels containing tap water or SS50. In the back side of the cage, a special pre-weighed slot fitted with a piece of embossed wax foundation (4 cm × 5 cm) allowed to monitor the wax produced by the bees.

Each trial lasted three weeks along while dead bees were counted and removed daily. The daily consumption of water and SS50 was also recorded. The unconsumed solid diet was replaced every two days, dried at 65°C for 24 hours, and average daily dry matter intake DM (**DMI**, mg DM/day) was calculated by difference from the dry weight of diet administered divided the average number of alive bees. At the end of each trial the bees still alive were narcotized by cooling and were stored in freezing conditions for further measurements. Also, the wax slots were taken and weighed. The amount of wax produced (**DWP**, mg) per cage was obtained by subtracting the initial weight of each wax slot.

The experimental scheme included: SS50 and SS50+VINASSE as controls, and Fe(II) fumarate in SS50 at concentrations of 0.4, 0.7, 1, 1.3, 1.6, 1.9, 2.2, 2.5 mg/Kg of HBs in SS50+DV, named increasing with the dose from D1 to D8.

5.2.3.3 Data processing and statistical analysis

At the end of each *ex-hive* trial, the HBs mortality rate (**MR**, %) was calculated as the percentage of HBs dead in fourteen days on the initial number per cage. The average daily gain (**ADG**, mg/day/bee) was calculated by the difference between the average weight of the alive bees at 21 days and the average weight at the bees at the beginning of the trial. This difference was then divided by the number of test days. The dead varroa mite **(DVM**, %), was calculated as the percentage of varroa mite fall dead in twenty one days on the alive mite number per cage at the end of the trials.

For the daily recorded variables, such as the daily individual intake of SS50 (**SSI**; µL/bee/day), water (**WI**; µL/bee/day), DM of solid diet (**DMI**; mg DM/bee/day), the **MR** and the varroa mite fall (**VMF**; n/day/cage) a repeated measures ANOVA was performed including Fe (II) dose (**D;** SS50, SS50+DV; D1; D2; D3; D4; D5; D6; D7; D8), time (**T**; 1-21 days), and dose × time interaction as fixed factor. Per cage, the average SSI, WI and DMI, values were also tested as covariates. The Tukey's test was used for pairwise comparisons, and significance was always declared at $P \leq 0.05$.

Linear contrast analysis were performed for daily recorded variables, significance was declared at $P \leq 0.05$.

For physiological variables calculated at the end of the trial, such as the **ADG**, the average daily wax produced (**DWP**; mg/day) and **DVM**, an ANOVA was performed using **D** as fixed factor. Per cage, the average SSI, WI and DMI values were also tested as covariates. The Tukey's test was used for pairwise comparisons, and significance was always declared at $P \leq 0.05$.

Concerning apiary trials, a General Linear Model was performed using hive-type (**H;** WS, RS), treatment (**TR**; Control, FeFum) and experimental phase (**P**; PRE, TREAT, POST) and their interaction (H×TR, H×P, TR×P) on tested variables BS, SS, MVM, IVM and HV.

In order to ensure that the parameters which values were percentages could have a normal distribution for the statistical analysis, the arcsine transformation described by Zar et al. (2014) was applied for mortality rate and dead varroa mite variables as percentual fraction of the initial number. All the analysis were performed with STATISTICA 10 software (Statosoft inc., Tulsa, USA).

5.3 Results

5.3.1 Apiary trials

The effect of phase is the only resulted statistically significant on SS (P = 0.029) (Table 5.2) with the TREAT phase that resulted higher compared to the POST one (P ≤ 0.05) (Table 5.3). Effect of hive, phase and their interaction resulted high statistically significant (P ≤ 0.01) on mature varroa mite fall (Table 5.2); as for SS, TREAT phase that resulted higher compared to the POST one (P ≤ 0.01) (Table 5.3). Moreover, also immature varroa mite fall was influenced by H (P = 0.040) and P (P = 0.049) but not by their interaction (Table 5.2); pairwise comparisons didn't result statistically significant for both of them (Table 5.3). Finally, the effect of treatment (P = 0.002) and phase (P ≤ 0.001) resulted highly statistically significant on hive mortality (Table 5.2); in particular the mortality was higher (P ≤ 0.01) for colonies supplied with FeFum and during the pre- and post-treatment phase (Table 5.3). No covariates resulted statistically significant for all the tested variables.

Table 5.2. Effect of Treatment (TR), Hive type (H) and Phase (P) on field variables.

Variable	P-value					
	TR	**H**	**P**	**H×TR**	**H×P**	**P×TR**
BS*	0.864	0.342	0.194	0.18	0.174	0.367
SS*	0.761	0.125	0.029	0.207	0.158	0.470
MVM*	0.543	<0.001	<0.001	0.767	0.006	0.482
IVM*	0.705	0.040	0.049	0.179	0.689	0.442
HM*	0.002	0.473	<0.001	0.318	0.827	0.032

TR: Treatment; H: Hive type; P: Phase; BS: Bee Sixths; SS: Storage Sixths; MVM: Mature Varroa Mite; IVM: Immature Varroa Mite; HV: Hive Mortality; ^{A,B} P≤0.05; ^{a,b} P≤0.01

Table 5.3. Descriptive statistics and pairwise comparisons (within factor) on field variables.

Factor	Level	BS		SS		MVM		IVM		HM	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
H	WS	24.49	1.76	25.74	2.07	4.45^{B}	0.39	0.62	0.07	3.41	0.37
	RS	26.44	2,00	29.61	2.74	7.78^{A}	0.87	0.87	0.11	3.84	0.5
TR	Control	25.48	1.77	27.36	2.28	5.84	0.55	0.68	0.07	2.79^{B}	0.22
	FeFum	25.44	2.03	28.14	2.65	6.62	0.93	0.84	0.13	4.93^{A}	0.69
P	PRE	22.83	2.23	26.70^{ab}	2.69	8.29^{AB}	2.7	0.31	0.15	4.94^{A}	1.58
	TREAT	28.79	1.71	34.00^{a}	2.82	10.70^{A}	1.89	0.64	0.21	1.43^{B}	0.16
	POST	25.42	2.7	22.96^{b}	3.15	5.03^{B}	0.41	0.82	0.07	4.11^{A}	0.38

H: Hive type; WS: Wide Spacers; RS: Regular Spacers; TR: Treatment; SS50+DV: Sugar Syrup 50:50+Diluited Vinasse; FeFum: FeFum 6.4 mg/sixth of bees; P: Phase; PRE: Pre-treatment phase; TREAT: Treatment phase; POST: Post-treatment phase; BS: Bee Sixths; SS: Storage Sixths; MVM: Mature Varroa Mite; IVM: Immature Varroa Mite; HV: hive Mortality ^{A,B} $P \leq 0.05$; ^{a,b} $P \leq 0.01$; pairwaise comparisons were performed within factor

5.3.2 Ex-hive trials

Dosage had no effect on SSI but the interaction between it and time resulted highly statistically significant (P = 0.001) as well as the linear contrast (P = 0.006) of D5 and D7 (32.3 ± 1.0 µL/day) against other treatments (22.0 ± 5.5 µL/day) (Table 5.4). Time had effect only on mortality rate (P ≤ 0.001), it raised from 4.84 ± 0.79 in day one to 83.04 ± 2.30 in the last day; MR was also influenced by SSI covariable (P = 0.002).

The dosage also had a significant effect on VMF (P = 0.030), with SS50 yielding the highest value compared to the D7 and D8 dosages (P ≤ 0.05). Contrast analysis, conducted to further investigate the statistically significant interaction between D and T (P ≤ 0.001), revealed a highly significant difference (P ≤ 0.001) between controls and Fe(II) fumarate treatments. Specifically, the controls mean value (6.81 ± 0.85) was significantly higher than that of the Ds (1.89 ± 0.45) throughout the entire period.

Except for SSI on MR previously cited, no one of covariable affected the in-trial variables analyzed.

The average daily gain was not significantly affected by dosage; however, pairwise comparisons revealed negative or negligible values for most treatments, with the exception of D5 and D7, which showed higher values compared to the lower dosages, particularly D2 ($P \leq 0.05$).

Conversely, dosage had a significant effect on wax production ($P = 0.021$), with D6 resulting in the lowest and negative production compared to D1 and the SS50 control ($P \leq 0.05$), which exhibited the highest daily wax production.

Dosage had a significant effect ($P \leq 0.001$) also on dead varroa mite (Table 5.4) with SS50 and SS50+DV controls that showed the highest values compared to the lowest (D1 and D2) and highest dosages of D7 and D8 ($P \leq 0.05$).

No one of covariables did not affect the end day variables.

Table 5.4. Effect of dosage on ex-hive trials variables.

Variable	Dosage (D)										SEM	P-value					
	SS50	SS50+ DV	D1	D2	D3	D4	D5	D6	D7	D8		D	T	D×T	SSI	WI	DMI
SSI*	23.84	24.62	17.24	13.33	19.47	22.99	31.25	25.00	33.28	21.46	5.16	0.417	0.213	0.001	-	-	-
WI*	5.12	8.67	8.03	3.56	4.79	6.77	8.68	10.34	11.74	8.50	2.60	0.725	0.515	0.225	-	-	-
DMI**	1.43	2.88	5.50	0.93	0.66	1.15	3.40	2.12	1.67	15.29	3.13	0.898	0.13	1,000	-	-	-
MR***	51.04	56.23	52.61	62.70	54.42	45.98	52.50	43.26	51.67	53.68	6.88	0.964	<0.001	0.159	0.002	0.792	0.689
VMF****	7.66^{a}	5.96^{ab}	2.24^{ab}	1.62^{ab}	2.36^{ab}	3.49^{ab}	2.12^{ab}	2.29^{ab}	1.08^{b}	1.53^{b}	1.31	0.030	0.593	<0.001	0.851	0.893	0.561
ADG$	-0.48^{ab}	-0.03^{ab}	-1.13^{ab}	-5.58^{b}	0.00^{ab}	-0.41^{ab}	0.69^{a}	-0.72^{ab}	0.41^{a}	-1.46^{ab}	0.41	0.136	-	-	0.286	0.376	0.753
DWP$	5.21^{a}	3.94^{ab}	4.99^{a}	2.24^{ab}	3.94^{ab}	2.37^{ab}	1.95^{ab}	-0.12^{b}	1.24^{ab}	1.89^{ab}	0.35	0.021	-	-	0.893	0.764	0.286
DVM***	88.88^{ab}	93.12^{a}	42.59^{d}	43.70^{d}	56.66^{abc}	61.66^{abc}	47.77^{bc}	53.14^{abc}	25.55^{d}	31.48^{d}	9.65	<0.001	-	-	0.962	0.961	0.691

SSI: daily individual intake of SS50; WI: daily individual intake of water; DMI: daily individual intake of DM of solid diet; MR: Mortality Rate; VMF: Varroa Mite Fall; ADG: Average Daily Gain; DWP: Daily Wax Production; DVM: Dead Varroa Mite; * (µL/day); ** (mg/day); *** (%); **** (n); $ mg; a,b $P \leq 0.01$

5.4 Discussion

Honey bees are essential for pollinating agricultural crops, consequently managing *Varroa destructor* in *Apis mellifera Ligustica* colonies sustainably is essential to reducing honey bee losses. Alternative options, such as supplementing with mineral salts like Fe(II), have been investigated because to resistance to traditional acaricides and the need for less hazardous methods (Van Hoorn and Ter Horst 2014; Danieli et al. 2019). The efficacy of ferrous fumarate (FeFum) was assessed in this study in order to ascertain its potential as a novel treatment for this parasite.

In the apiary trials, the fall of mature mites (MVM) was generally lower in FeFum-treated groups compared to the controls (Table 5.3) as well as the VMF in laboratory tests was also lower than in the controls (Table 5.4). Similarly, DVM showed higher values in the controls compared to the treated groups, although comparable values to the controls were obtained at intermediate doses such as D3, D4, D5, and D7 (Table 5.4). Intermediate treatments, such as D5 (1.6 mg/Kg of HBs), appeared to represent a better compromise between tolerability and negative effects, but the benefits remain limited. This finding suggests that the treatment might not have been sufficiently effective, contrary to expectations.

The higher mortality of colonies treated with FeFum, which is especially noticeable in the post-treatment stages (Table 5.3), is an important aspect. Two treated colonies died with wide spacers (WS) at the conclusion of the second apiary test in the spring of 2022, which accounted for 25% of the colonies examined that year and 50% of the treated colonies (data not shown). Laboratory test also showed that pattern, with substantial mortality rates at higher dosages (D7 and D8) (Table 5.4). The relationship between field and laboratory mortality suggests the possibility that the treatment may directly harm bees or interfere with their physiological processes in a dose-dependent manner: a previous study by He et al. (2021) demonstrated how the Fe(II) overload, could be a critical factor for activating oxidative stress and aggravating mortality in honey bees. The dose effect was especially noticeable in our laboratory experiments, where higher dosages had a negative impact on variables like daily wax production and average daily gain (Table 5.4).

The temporal dynamics of sugar syrup consumption (SSI), which demonstrated a strong interaction between dosage and time (Table 5.4), is another noteworthy aspect. This implies that the availability and absorption of the mineral diet may be impacted by the behavioral and physiological responses of

bees. It is known that iron absorption from the diet requires uptake by intestinal cells, transport from the apical to the basal membrane of the intestinal epithelium, and subsequent transfer to the hemolymph. Once in the hemolymph, iron must be distributed to individual cells for utilization or storage for future metabolic needs (Casey et al. 1988). The orientation of bees is influenced by changes in magnetic field intensity and polarity, attributed to the presence of magnetite crystals (Fe_3O_4) within their bodies. These crystals function as components of the sensory receptors for magnetic fields (Wiltschko and Wiltschko 2005). In bees, iron is primarily stored within trophocytes, specialized cells located in the abdomen, which contain a significant number of iron-rich granules (Gould et al. 1980). Trophocytes are, therefore, plausible candidates for serving as sensory receptors for magnetic fields. Additionally, these iron granules may have other roles, such as serving as an iron reserve for periods of increased demand or acting as ballast during flight (Lowenstam 1962). Consequently, the temporal dynamics of Fe(II) uptake through sugar solutions could be a critical factor in determining the efficacy of treatments involving iron supplementation.

Winter survival is a crucial aspect as well as the effectiveness of anti-varroa treatments. FeFum therapy may have an undesirable impact on colony resilience throughout the winter, as demonstrated by the 2021 apiary trial loss of two treated WS colonies. These losses imply that the use of the treatment could harm the bees' capacity to survive the winter, which is already a crucial time for colony survival.

From the perspective of overall colony health, the results suggest that FeFum supplementation could poses some issue, such as increased mortality and a reduction in wax production, which might lead beekeepers to reconsider the cost-benefit balance of the treatment. To reduce side effects and maintain a balance between safety and efficacy, FeFum's use as a possible treatment needs to be carefully optimized.

In the future, optimizing the concentration and frequency of administration will be essential to balance efficacy and tolerability. Furthermore, long-term monitoring of the treatment’s impact on colony productivity and the quality of bee products, such as honey and wax, and bee’s physiology will be necessary to propose an environmentally and economically sustainable and acceptable approach to beekeepers. Revising the treatment formulation, potentially combining FeFum with other integrated pest management (IPM) such as the use of different spacers or organic treatment such as oxalic acid, could represent a decisive step toward developing a viable strategy for controlling *Varroa destructor*.

Effective validation of protocols in long-term field conditions is necessary to ensure that the treatment meets the needs of beekeepers while safeguarding bee welfare and the sustainability of beekeeping activities.

In conclusion, preliminary results indicate that FeFum, in its current formulation, does not guarantee the required levels of effectiveness for controlling Varroa destructor and winter survival. The practical implementation of this strategy requires significant modifications and long-term studies especially in field.

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Chapter 6

Crossbreeding of *Apis mellifera ligustica* Spin. to obtain climate-resilient phenotype

Company activities

6.1 Introduction

As per the call for PhD course, in agreement with D.M. n.1061 of 2021 PON resources "Research and innovation" theme Green (action IV.5), were held n. 6 months of training activities (divided into honey bee active seasons 2022 and 2023) in the host company Azienda Agricola BeeHive, located in Gennazzano (Rome, Italy) under the guidance of tutor/beekeeper Paolo Scarpino.

The aim of these activities was to obtain honey bees with a potentially climate change-resilient phenotype within the Italian subspecies (*Apis mellifera ligustica* Spin.), by producing and instrumentally inseminating virgin queens.

6.2 Instrumental insemination protocol

On 4 and 5 July 2022, a course on instrumental insemination of queen bees was held at the host company Azienda Agricola BeeHive (Gennazzano Italy), held by the professional in the field, Mrs. Agata Cierlinska. The protocol used were acquired during this course and the actions carried out are reported below.

6.2.1 Queen cell preparation and management

Starting at the end of February, initial transfers can begin. The preparation phase for queen cells and orphaned nuclei is essential to ensure the success of later stages. Specifically, creating nuclei with brood frames is recommended to facilitate the acceptance of the new queen. Alternatively, allowing the orphan nucleus to develop queen cells, which are then removed on the seventh day, two days

before introducing the selected cell, is another option. The selected cell is placed inside a queen cage (Figure 6.1A), which enables contact between the workers and the future queen.

Six days after emergence, the virgin queen is removed to be anesthetized: the cage containing her is placed inside a plastic bag (Figure 6.1B) for the administration of CO_2 for 2 minutes, after which she is returned to the hive. This procedure will accelerate the passage of sperm into the spermatheca, allowing egg-laying to start sooner typically within 7-10 days instead of approximately one month after insemination. On the seventh day, she is collected for instrumental insemination. The optimal age of the queen for this procedure should be between 7 and 14 days of adult life.

A B

Figure 6.1. A) Two queen cage types for the sequestration of queen; B) CO2 narcois of caged queens (Author's photos).

6.2.2 Drone selection and capture

To obtain sexually mature drones (aged between 14 and 21 days) for semen extraction, it is essential to prepare the “paternal families” at least three weeks prior to larval transfer for queen production. Drones that are too young (<14 days) produce semen that is too fluid with a low concentration of spermatozoa, while drones older than 21 days have denser semen that does not easily separate from mucus. Additionally, drone quality strongly depends on the amount of nurse bees and pollen present, as well as on the strength and health of the parent colony. Spermatozoa vitality and activity are influenced by the temperature around the male brood, which must be maintained between 33 and 35°C by the bees.

For drone collection, specialized cages (Figure 6.2A) can be used to isolate them. When positioned on the open hive at the optimal time of day (afternoon), these cages allow drones to fly up, gathering at the top edge for easy capture. Alternatively, if no cage is available, drones can be collected by hand (Figure 6.2B) early in the morning or in the evening when they are not flying, by removing them directly from the frames or using 3D self-printed cages (properly developed during the PhD period) placed at the entrance of the hive that allow the workers passage but not the drones one which remain trapped in the upper part of the cage that can be easly removed (Figure 6.2C) . Once collected, they are transferred to a small cage (Figure 6.2D) for transport to the laboratory. This cage, with two compartments separated by a metal mesh, allows the simultaneous presence of workers and drones, ensuring ideal maintenance until semen extraction.

To verify drone maturity directly in the apiary, quick tests such as palpation for firmness or checking for vibratory response when lightly squeezed between the fingers can be conducted.

A B C D

Figure 6.2. A) Cage for capturing drones in flight. (Photo by Agata Cierlinska, 2021); B) Drone catched by hand (Author's photo); C) 3D self-printed cage (Author's photo); D) Cage for collecting and transporting drones to the laboratory. The red arrow indicates the compartment in which to introduce the drones, while the green arrow indicates the in which to insert the worker bees (Author's photo).

6.2.3 Materials

The equipment needed to perform instrumental insemination is the following.

A micromanipulator (Figure 6.3A) equipped with queen holder with CO_2 diffuser; probes (hook probe on the left (Figure 6.3B) and a second probe with an hole on the right (Figure 6.3C), to hold the

stinger) with micrometric adjustment for blocking and manipulating the queen equipped with syringe holder with micrometric regulator and a syringe with micrometric plunger.

A B C

Figure 6.3. A) Micromanipulator; B) Ventral probe; C) Dorsal probe (Photo http://www.aparatdoinseminacji.pl/descrizione.php).

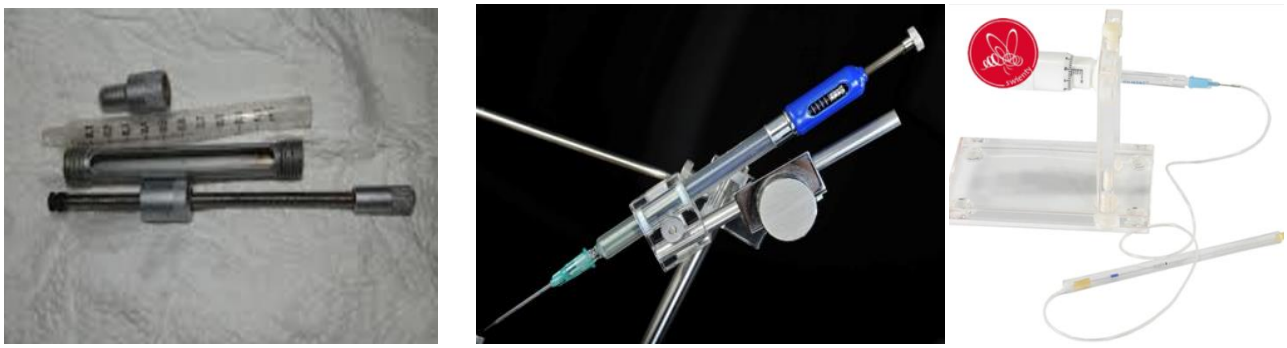
A stereomicroscope (Figure 6.4A), possibly with a 14× magnification, integrated light and the possibility of rotating the optical block on the opposite side in order to allow the positioning of the micromanipulator under the optics and a CO2 cylinder with flow regulated by a pressure reducer with a manometer and bubbler placed between the cylinder and the micromanipulator with a flow approximately of two bubbles per second (Figure 6.4B).

A B

Figure 6.4. A) Stereomicroscope equipped with the micromanipulator; B) CO_2 cylinder equipped with bubbler. (Author's photos).

Two types of syringes are available for aspirating two doses of semen, each measuring 8 µl: one type is made entirely of glass (Figure 6.5A), while the other uses insulin syringes (Figure 6.5B). In both cases, the plunger must be replaced with one that allows micrometric adjustment, and the needle must be substituted with a glass capillary. Additionally, there are syringes on the market equipped with a micro-selector, enabling precise control over the semen dose to be aspirated and injected (Figure 6.5C).

The syringes and capillaries are sterilized in alcohol the evening before use, allowing complete evaporation by the time they are needed. Before use, the syringe is loaded with a commercially available, sterile isotonic saline solution to facilitate semen aspiration. An air bubble is left between the saline solution and the semen to prevent dilution of the latter.



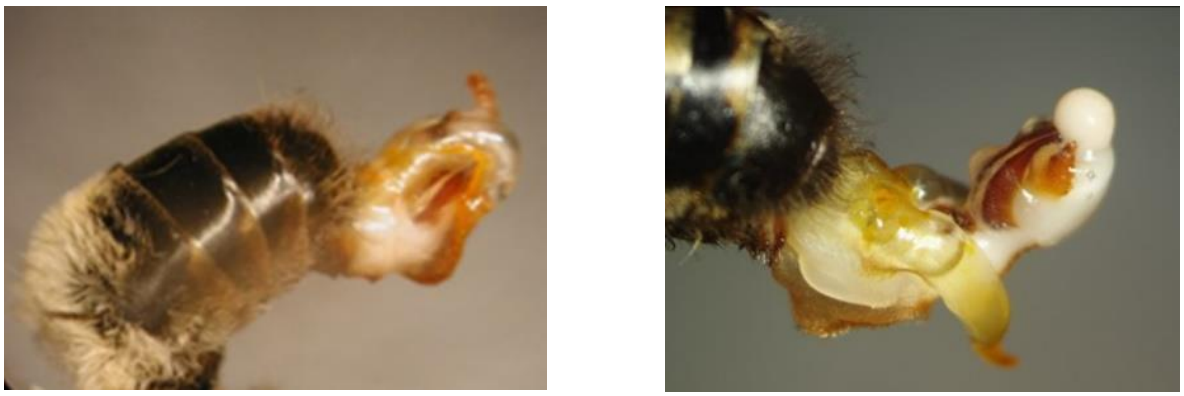
A B C

Figure 6.5. A) glass syringe used for male semen aspiration; B) plastic insulin syringe; C) syringe equipped with a micro-selector for aspiration of more than two doses. (Photo by Agata Cerlinska, 2021)

6.2.4 Instrumental insemination

6.2.4.1 Drones sperm collection

For correct manipulation, the drone must be held between the head and the thorax using the index finger and thumb and then suppressed with a light pressure on the head. With a finger of the other hand, it must be push on the upper part of the abdomen to allow, with an initial compression, the partial exit of the endophallus (Figure 6.6A) and, with a second pressure, the total exit and that of the semen inside it (Figure 6.6B).



A B

Figure 6.6. A) Partial release of the endophallus of a mature drone, following a first compression; B) Complete release of the endophallus and the semen inside it, following a second pressure (Photo: Cobey et al., 2013).

The sperm is then collected using the syringe (Figure 6.7) under a stereomicroscope. The semen appears as a yellowish-white substance with a marbled character, positioned over a more abundant quantity of white mucus and with a higher density. During the aspiration, care must be taken not to collect the mucus as well, which will cause the syringe to clog, and not to mix the collected semen with the saline solution present inside the tip, always maintaining an air bubble between the two. Once the sperm of the first drone has been recovered, the collection of another drone is carried out, until a quantity of 8 μL of collected semen is reached, avoiding the formation of air bubbles inside it. Approximately 15 drones are necessary to reach the desired quantity of sperm.

Figure 6.7. Representation of the drone endophallus during semen collection (Photo: Cobey et al., 2013).

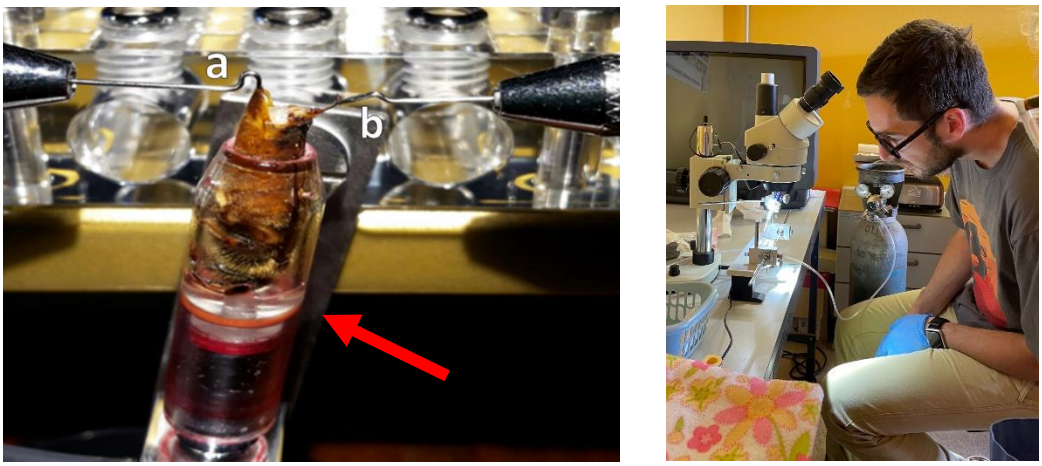
6.2.4.2 Insemination of the queen

Before proceeding with the insemination phase, the queen's vigor must be checked. By placing her inside a glass, check whether she can easily climb the walls and whether she has quick and lively movements. The latter, in fact, are an indication of excellent vitality, while the integrity of the nails and the suckers located at the end of the leg allow the queen to adhere and walk on vertical surfaces, thus allowing more effective movement on the frames for laying. If, from the assessment carried out, a non-optimal state of health emerges, insemination is not carried out, as the queen could have future laying problems.

Subsequently, the queen is inserted into the appropriate support, equipped with a CO2 diffuser, inclined approximately 30° to the right, with the ventral part towards the left of the operator, leaving

approximately two tergites protruding from the support, thanks to the insertion of the latter on a base of the micromanipulator, which allows its adjustment. After a few deep breaths, where the belly is observed to swell and relax again, the queen will remain still sedated and it will be possible to proceed with the insemination.

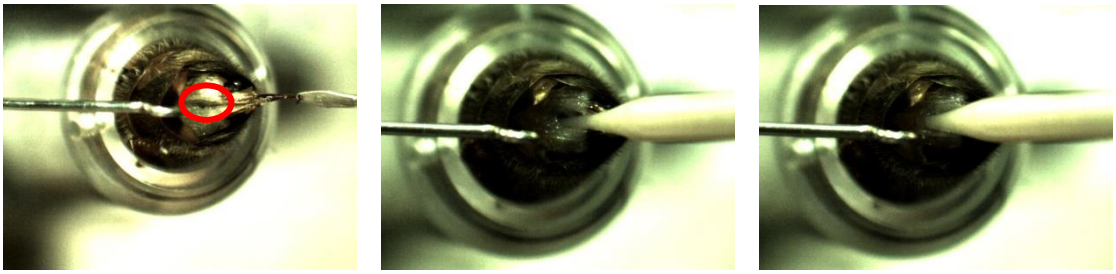
With the hook-shaped probe in the ventral position, the tip of the tergite is hooked (a) (Figure 6.8), while the stinger is passed inside the hole present in the dorsal probe in position (b) (Figure 6.8).



A B

Figure 6.8. A) Queen hooked with the two probes and ready for insertion of the syringe, the red arrow indicates the adjustable base of the micromanipulator in which to position the queen. B) Author performing instrumental insemination (Author's photos).

Once both probes are hooked, they are gently pulled outwards until the vaginal canal is visible (Figure 6.9A). After removing any air present in the needle, the needle is gently inserted into the vaginal canal up to about half of the fine part (Figure 6.9B). At this point the tip of the needle will be supported by the vaginal valve. To go around it and get close to the oviducts, the syringe is slightly tilted using the lever that controls it (Figure 6.3A), so as to move the needle towards the operator's left. At this point, the remaining part of the needle can be inserted (Figure 6.9C), up to about three-quarters of the fine part.



A B C

Figure 6.9. Phases for insemination of the queen: A) Hooking with the probes and identification of the vaginal canal (circled in red); B) Insertion of the distal end of the syringe; C) Maneuver to move the vaginal valve and complete insertion of the syringe (Photo by Agata Cierlinska, 2021).

During the entire insertion process (Figure 6.10), no resistance should be felt and no movement of the tissues involved should occur, indicating incorrect movements. Once you have reached the position described in Figure 6.9C, slowly release the seed by acting on the micrometric screw that controls the syringe plunger until the entire dose is emptied, taking care not to insert air. Once insertion is complete, gently remove the needle by lifting the syringe, to then allow the tergite and sting to come together again and the ventral and dorsal probes to be released.

At this point, with the queen still sedated, she is removed from the support and marked with adhesive clips to keep track of the crossbreeding just carried out.

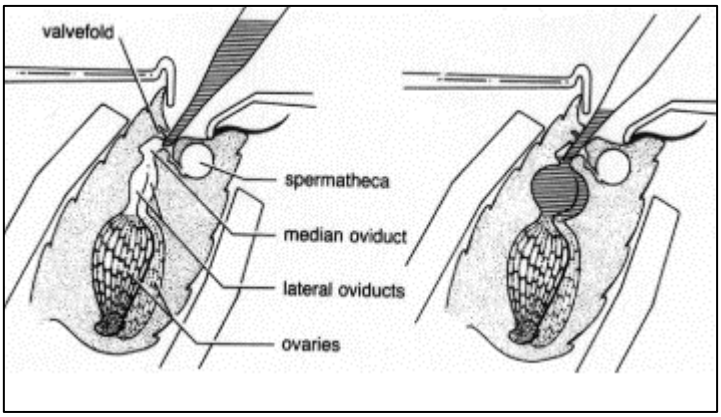


Figure 6.10. Illustration of the capillary movements for the displacement of the vaginal valve (circled in red) and the release of the semen into the two oviducts (Illustrations adopted from Cobey et al., 2013).

6.2.4.3 Reintroduction of the queen into the hive

The inseminated and marked queen is placed in queen cages (Figure 6.11A) that allow entry and contact with the worker bees. There are two possibilities for reintroduction: the first is to place the caged queen in a "queen bank" (Figure 6.11B) for 48 h and then transfer the cage (opened and plugged with candy) to the destination hive. The second possibility is to directly place the closed cage with the queen in the destination hive or nucleus and release it after 48 h as in the first option. Acceptance nuclei should be prepared with frames without fresh brood so that the workers have no opportunity to build their queen cells. If the above process has been successful, the inseminated queen should start laying after 7-10 days; it is advisable to check her only after this period. If, after the check, she has not started laying, a further short anesthesia of about two minutes with CO2 can be performed. If the pre-insemination narcotization was not performed, it can be repeated 24-48 h post-insemination with the same methods illustrated above.

In post-insemination inspections, it is essential to verify the absence of abnormal behavior by the workers (aggressiveness and nervousness), to verify that the recently inseminated queens are recognized and avoid rejection and/or replacement of the same. The queen can be considered effectively accepted only when the bees deriving from her eggs have emerged.

A B

Figure 6.11. A) Queen cage with an inseminated and marked queen inside, reintroduced into the colony for the acceptance phase; B) Queen bank for their maintenance during the first 48 h after insemination (Photo by Agata Cierlinska, 2021).

6.3 Morphometric protocol for membership determination

The morphometric approach for classifying subspecies of *Apis mellifera* remains the reference method to this day. Biometric analyses of wing morphology, tergite banding, and hind leg length were conducted on honey bee samples (Cresta et al., 2023; Danieli et al., 2022).

To determine the degree of membership to the subspecies *Apis mellifera ligustica* of the bees derived from the inseminated queens, morphometric analyses were conducted on the corresponding F1 generations.

The method involves the collection of 20 worker bees per colony, chosen randomly. In the laboratory, the bees' relevant body parts (wings, tergites, and hind legs) are dissected with the aid of a stereomicroscope and then prepared for measurement. For bilateral body parts (wings, legs), measurements are taken on both the right and corresponding left sides, then averaged for each bee. Therefore, 40 measurements were taken for each bilateral morphometric trait.

The acquired data are directly compared with available "breed standards," or pre-processed for subsequent comparison with reference values. Separately for each biometric trait analyzed (18 in total), the statistical hypothesis H_0 (the sample does not differ from the reference population) is tested, and the overall membership level of the sample to the reference population is determined by calculating the ratio of matching traits to the total number of traits. Colonies were considered to be subspecies of A*pis mellifera ligustica* if they showed a membership greater than or equal to 50%.

20 honey bees (generation F1 of instrumental inseminated queens) per hive were sampled in near frames with predominantly capped brood, when possible, to minimize the chance of sampling worker bees not belonging to the colony (i.e., drifting foragers, robbers) and immediately stored at -20°C in falcon tubes. Samples were thawed in ethanol and air dried and checked that the wings and legs are present and in good condition (there must be no breaks, cuts or fraying of the wings) before the analysis.

6.3.1 Morphometric analysis of the wings

The first pair of wings were removed using tweezers and mounted, side by side, inside a slide holder with 24 x 36 mm anti-Newton glass (e.g. GEPE brand). On each slide, 5 pairs of wings are

conveniently arranged (Figure 6.12A). Before placing the wing on the slide, they were immersed in ethanol for a few seconds in order to clean it and make it adhere to the slide.

A B

Figure 6.12. A) Slide holder being set up with forewings of workers arranged in pairs (Right + Left); B) Closed slide holder complete with identification codes of the colony and individual bees with progressive numbers from 1 to 20 (Author's photos).

For wing morphometry, a commercial scanner (Canon 9000 F Mark II) provided with slide holder, frames was used. Hight resolution scans (4800 dpi) were obtained for each slide from which all single wings were resized and saved with a unique code including hive, individual and L/R.

Wings images were uploaded in DrawWing[1], a software that converts an image of an insect wing into a list of coordinates (X;Y), derived from feature points such as vein junctions (Figure 6.13). Coordinates values were exported in .CSV format and the corner formed by each vein junction and derivate variable were calculate and compared to the breed standards values.

[1] https://www.drawwing.org/node/2

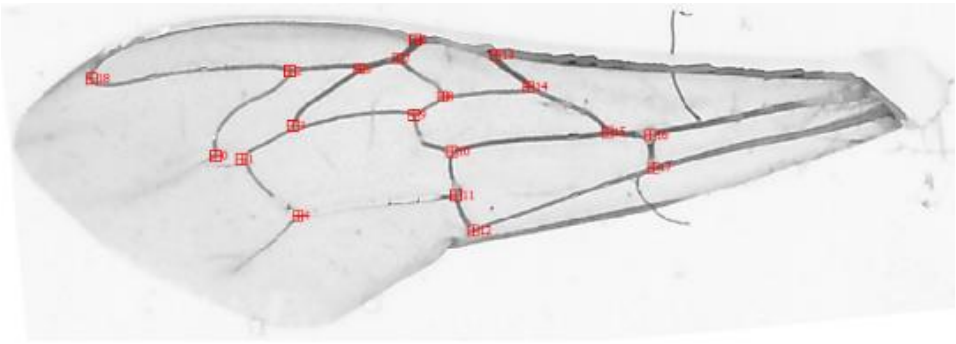


Figure 6.13. DrawWing software, wing with the 19 crosshairs placed on the grain joints (Author's photo).

6.3.2 Morphometric analysis of the legs

The hind legs were detached with tweezers by grasping them in the innermost part, at the level of the coxa. The paws were both placed on a sheet of graph paper fixed to the base of the stereoscope (ZEISS Stemi 305) provided with a digital camera (Axiocam ERc 5s); the femur (F) was oriented upwards and the tarsus (T) downwards, with the internal side of the paw facing upwards (Figure 6.14), as this surface had less hair, reducing interference with the identification of the junction points between the paws segments.

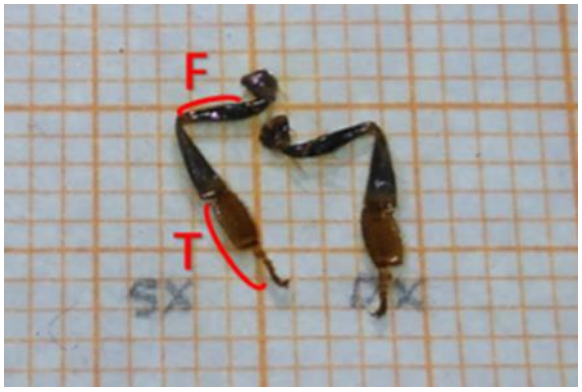
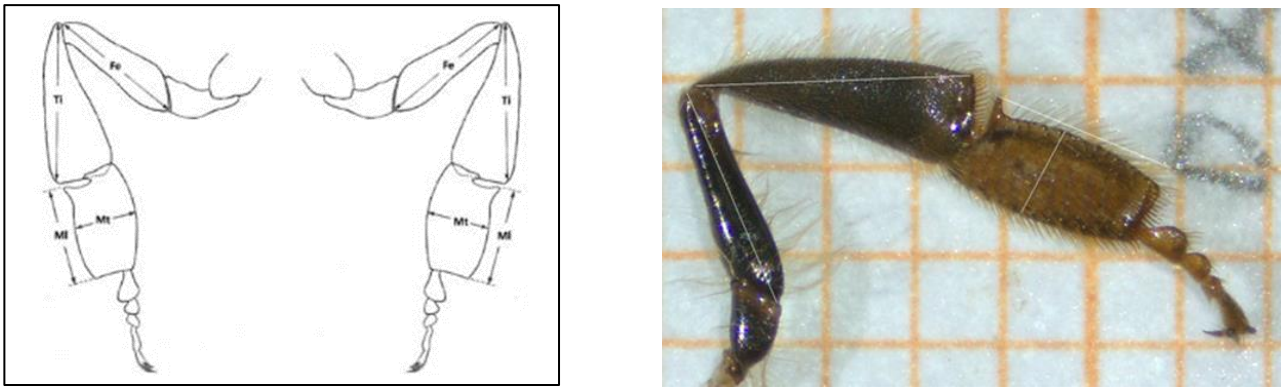


Figure 6.14. Pair of hind legs of a worker on the stereomicroscope plane, oriented with the femur (F) upwards and the tarsus (T) downwards (Author's photo).

A photo of each pair of legs were taken using ZEN software (Zeiss, Oberkochen, Germany) adjusting brightness, magnification and focus to obtain a high-resolution photo saved with a unique code name, as well as for the wings, in . JPG format.

The acquired photos are imported into ImageJ[2] software to quantify the following traits: femur length (Fe), tibia length (Ti), metatarsal length and width (Ml and Mt) (Figure 6.15). The measurements are quantified in millimeters and recorded in a database and compared to the standard breed values.



A B

Figure 6.15. A) Illustration of the segments to be measured on a pair of legs; B) Segments relating to the 4 measurements to be taken on each paw (Author's photos).

6.3.3 Analysis of tergites

For this type of analysis, the second and third tergites (T2 and T3) are used (Figure 6.16). Extraction is performed with well-pointed tweezers, inserted under the third tergite and brought forward until reaching the second. The tergites are delicately lifted by moving the tweezers to the right and left so that they separate from the abdomen, after that they were cleaned in ethanol and then placed on a small cylinder to be read.

[2] https://imagej.net/ij/download.html

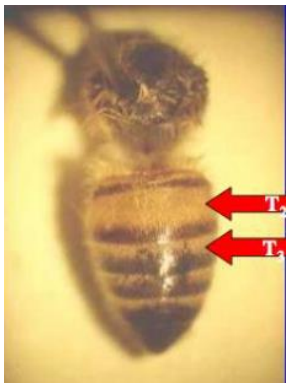


Figure 6.16. Photo indicating the two tergites to be extracted. (Author's photo).

The reading is carried out using the stereomicroscope comparing the right or left end of the extracted tergites with that of the ten models represented in the Goetze scale (Figure 6.17) and assigning a value to each tergite. The assigned T2 and T3 values are reported on the same database used for wings and legs and compared to the breed values.

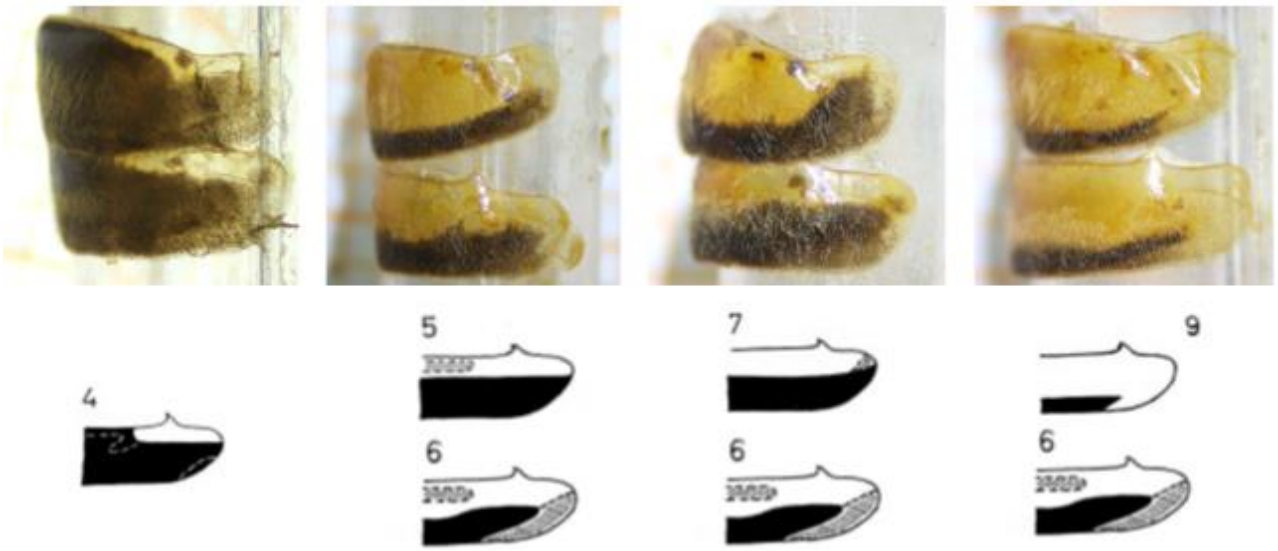


Figure 6.17. Photo of tergites and their subjective interpretation based on the Goetze scale.

6.4 Crossbreeding

6.4.1 Crossing activities 2022

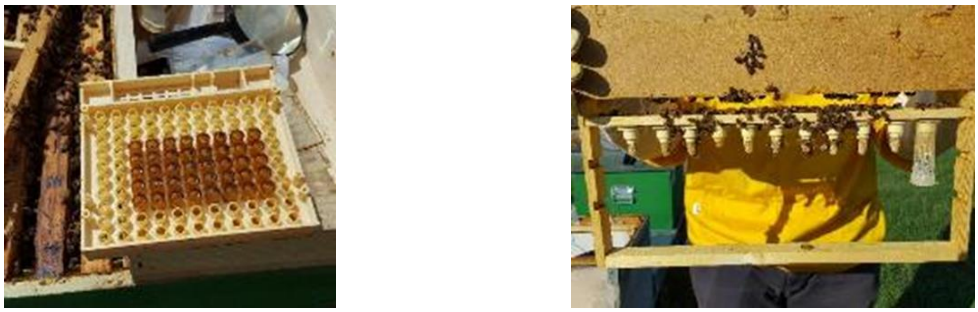
In the host company (Gennazzano Apiary, GA), controlled production of queens of *Apis mellifera* L. was carried out in two consecutive cycles during June and July 2022, with a view to producing virgin queens for instrumental insemination.

Two colonies harbored in special hives known in Italy as “cassone tipo-francese” (Figure 6.18A and 6.18B) were used to producing virgin queens. This type of hive is internal divided in two parts and allow to have an orphan swarm were rising queen cells and a mother colony were managing the oviposition of the queen.

A B C

Figure 6.18. A);B) "cassone tipo-francese" (Photo: https://mitoitalia.it) ; C) Author and colleague working in GA (Author’s photo).

Oviposition is managed in special cages named Nicot cages (Figure 6.19A) where the queen can lay her eggs in special cups. These cups, once hatched the larvae (typically after 3 days), were moved in the orphan side of “cassone francese” using special frames where workers could build the queen cells (Figure 6.19B).



A B

Figure 6.19. A) Nicot cage; B) special frame with queen cells (Author's photos).

Previously, by the morphometric analyses described above and by a phenotypic characterization assessed by the beekeepers carried out with questionnaires (*e.g.* amount of storages and brood, tendency to swarm, honey production, docility), 3 colonies of interest were identified among those available with high membership belonging to subspecies *A.m. ligustica* (Table 6.1).

Table 6.1. Membership to *A.m. ligustica* of colonies selected for instrumental insemination for 2022 beekeeping season.

ID Mother colony	Membership (%)
1C	38.9
4C	61.1
2G	50.0

From these colonies all possible crosses were programmed (n = 7) each would have to be replicated for 4 times (Table 6.2).

Unfortunately, the adverse climate conditions that characterized the spring/summer 2022 (*e.g.* the delay in spring leads to the subsequent onset of harsh summer temperatures and drought conditions), didn’t allowed the expected production of queens.

Only three virgin queens were inseminated in total: two were lost in supersedure events in the following weeks after the reintroduction in the hive, while a third survived and deposed: the results

of morphometric analyses on the resulting F1 generation showed a membership of 33%, which is lower than the membership of the parent colonies (Table 6.2).

Table 6.2. Programmed instrumental inseminations for 2022 beekeeping season and relative results.

♀	♂	♀ Membership (%)	♂ Membership (%)	Planned Replicates	Effective Replicates	Laying Queens	ID Inseminated queen	F1 Membership (%)	End season queen status
2G	4C	50.0	61.1	4	1	0	2I		SUPERSEDURE
4C	2G	61.1	50.0	4	1	0	4I		SUPERSEDURE
4C	4C	61.1	61.1	4					
1C	4C	38.9	61.1	4					
4C	1C	61.1	38.9	4					
2G	1C	50.0	38.9	4					
1C	2G	38.9	50.0	4	1	1	1I	33.3	ALIVE
Efficiency					3 (10.7 %)	1 (33.3 %)			

6.4.2 Crossing activities 2023

The same protocols for selecting the colonies to be crossed and for queen production used in 2022 were also applied in the 2023 beekeeping season, which proved to have more favorable weather conditions for the purpose at GA.

Seven colonies were selected (Table 6.3) and a scheme of 8 crossings (three replicate each) was conceived (Table 6.4).

Table 6.3. Membership to *A.m. ligustica* of colonies selected for instrumental insemination for 2023 beekeeping season.

ID Mother colony	Membership (%)
BAS32	67,7
BNT08	47,8
CA36	54,4
DO23	57,0
RUM1	57,0

The replicates that took effectively place were a bit less than 48% of the planned ones, with 36.4% of mated/laying queens (Table 6.4). The F1 workers produced had a satisfactory membership degree always above 50% and therefore belonging to the subspecies in question as stated in section 6.3 of this chapter. Specifically, the four F1 colonies analysed showed a membership of 50.0%, 51.2%, 62.0% and 64.7%. Unfortunately, two of the did not overwintering and *were lost (Table 6.4).*

Table 6.4. Programmed instrumental inseminations for 2023 beekeeping season and relative results.

♀	♂	♀ Membership (%)	♂ Membership (%)	Planned Replicates	Effective Replicates	Laying Queens	ID Inseminated queen	F1 Membership (%)	End season queen status
BAS32	RUM1	67,6	57,0	3	0	0	-	-	-
RUM1	BAS32	57,0	67,6	3	2	1	R1	50.0	ALIVE
BAS32	DO23	67,6	57,0	3	2	1	B6	62.0	ALIVE
DO23	BAS32	57,0	67,6	3	1	0	-	-	-
RUM1	DO23	57,0	57,0	3	1	0	-	-	-
DO23	RUM1	57,0	57,0	3	1	0	-	-	-
DO23	CA36	57,0	54,4	3	2	1	DO4	51.2	DEAD
BAS32	CA36	67.6	54,4	3	2	1	B4	64.7	DEAD
Efficiency					11 (45,8%)	4 (36,4%)			

6.5 Conclusions

The crossbreeding project of *Apis mellifera ligustica* Spin. developed in this PhD period yielded significant results despite the challenges encountered during the 2022 and 2023 beekeeping seasons. The primary objectives, which included the production of virgin queens and their instrumental insemination to develop honey bee colonies resilient to climate change, were partially achieved. The training course on queen instrumental insemination, held at the host company Azienda Agricola Beehive, provided a foundation for the practical application of protocols. However, it also highlighted the need to further refine the skills of the author and all operators involved, given the complexity of the process itself.

The 2022 season, marked by adverse climatic conditions, limited the number of queens produced and inseminated. Only three queens were subjected to instrumental insemination, with just one F1 colony surviving, showing a membership level of 33%, which was below expectations. In contrast, the 2023 season benefitted from more favorable weather conditions and greater operator experience, resulting in improved efficiency. The F1 generations achieved a membership level above 50%, meeting the standards of the *A. m. ligustica* subspecies.

Morphometric analyses played a pivotal role in assessing the genetic membership of the produced bees, demonstrating that the selection and crossbreeding protocols employed effectively preserved the desired characteristics of the subspecies, as previously tested by Danieli et al. (2022). But certainly, in the future, tests such as mitochondrial DNA (mtDNA) or Single Nucleotide Polymorphism (SNP) analysis may be more conclusive if they become economically viable for beekeepers.

Results obtained in 2023 highlighted that starting with high membership values for colonies producing virgin queens and drones enables the development of a progeny closely matching the reference morphotypes in terms of forewings, tergite, and hind legs, underlying to beekeepers the pivotal role of the management of parental lines (*e.g.* isolated breeding stations to avoid genetic drift).

Nevertheless, the results underscored the need for further optimization, particularly to enhance the overwintering survival of F1 colonies. Additionally, queen supersedure events emphasized the importance of refining reintroduction and acceptance procedures, as well as improving the technical

expertise of the operators to ensure greater precision and reliability during operations. Finally, understanding the true hereditability of the investigated morphometric traits and effectively managing drone drift are essential for ensuring the long-term success of crossbreeding initiatives.

In summary, the project delivered promising outcomes for the development of *A. m. ligustica* colonies resilient to climate change. The experience gained, combined with further training and protocol optimization, provides a strong foundation for future advancements, with the potential to significantly contribute to sustainable beekeeping and the conservation of honey bee genetic biodiversity.

6.6 References

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Chapter 7

Precision Beekeeping Systems: State of the Art, Pros and Cons, and Their Application as Tools for Advancing the Beekeeping Sector

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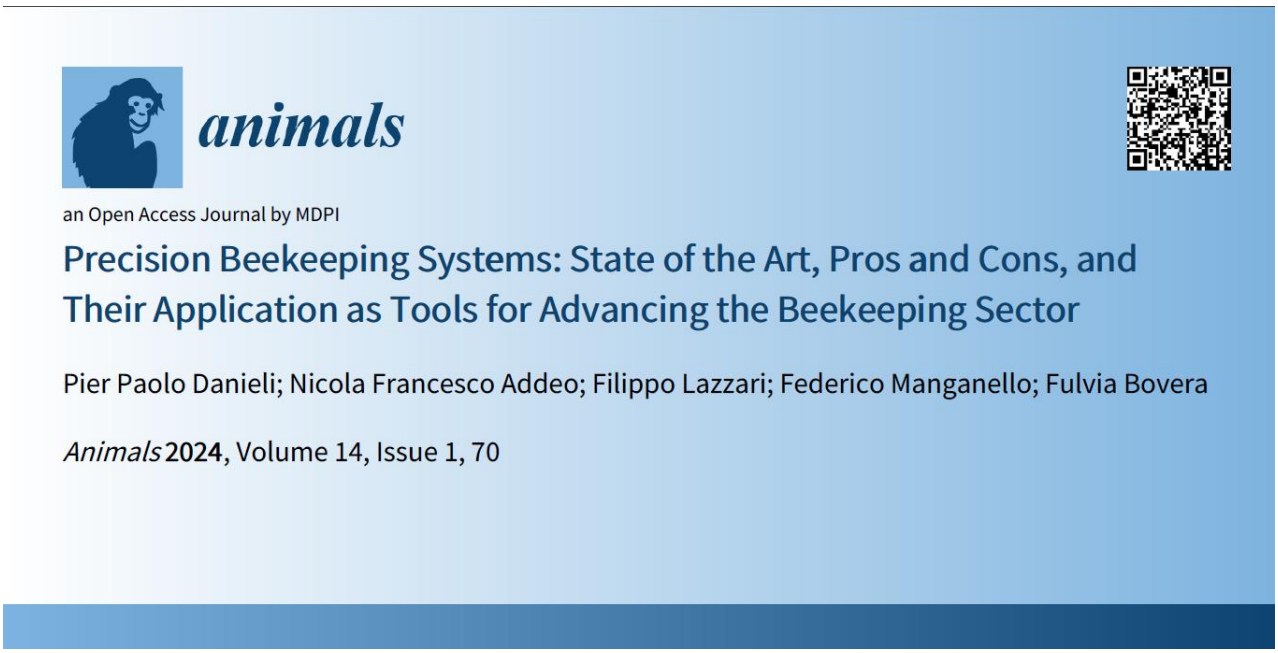

7.1 Simple Summary

This review aims to raise attention to precision technologies applied to the world of beekeeping, the implementation of the precision technological approach by a normal beekeeper, the reliability of the data analysis, the state of the art, and the pros and cons found to date in the apiary sector.

7.2 Abstract

The present review aims to summarize the more recent scientific literature and updated state of the art on the research effort spent in adapting hardware–software tools to understand the true needs of honeybee colonies as a prerequisite for any sustainable management practice. A SWOT (Strengths, Weaknesses, Opportunities, and Threats) analysis was also performed with the aim of identifying the key factors that could support or impair the diffusion of precision beekeeping (PB) systems. Honeybee husbandry, or beekeeping, is starting to approach precision livestock farming (PLF), as has already happened in other animal husbandry sectors. A transition from the current paradigm of rational beekeeping to that of precision beekeeping (PB) is thus expected. However, due to the peculiarities of this species and the related farming practices, the PB technological systems (PB systems) are still undergoing a development process that, to some extent, limits their large-scale practical application. Several physical–chemical (weight, temperature, humidity, sound, gases) and behavioral traits (flight activity, swarming) of the hive are reviewed in light of the evolution of sensors, communication systems, and data management approaches. These advanced sensors are equipped with a microprocessor that records data and sends it to a remote server for processing. In this way, through a Wireless Sensor Network (WSN) system, the beekeeper, using specific applications on a personal computer, tablet, or smartphone, can have all the above-mentioned parameters under remote control. In general, weight, temperature, and humidity are the main hive traits monitored by commercial sensors. Surprisingly, flight activity sensors are rarely available as an option in modular PB systems marketed via the web. The SWOT analysis highlights that PB systems have promising strength points and represent great opportunities for the development of beekeeping; however, they have some weaknesses, represented especially by the high purchasing costs and the low preparedness of the addressed operators, and imply some possible threats for beekeeping in terms of unrealistic perception of the apiary status if they applied to some hives only and a possible adverse impact on the honeybees' colony itself. Even if more research is expected to take place in the next few years, indubitably, the success of commercial PB systems will be measured in terms of return on

investment, conditioned especially by the benefits (higher yields, better colonies' health) that the beekeeper will appraise as a consequence of their use.

7.3 Keywords

Honeybee; sensors and systems; beekeepers; internet of things; literature review

7.4 Introduction

One of the most important challenges of this and, probably, the next century is to safeguard biodiversity around the world. In this regard, great attention has been focused on insects, whose decline on the local and global scales has been reported [1]. Insects are responsible for a wide range of functional roles [2–5], one of which is pollination; in fact, the pollinators can improve the production of 70% of the globally most important crop species and influence 35% of the global human food supply [6]. Medium- and long-term strategies can be studied to control the decline of wild pollinators [7], but, in the short-term, one of the possible strategies is to increase the number of farmed pollinators, in particular honeybees (*Apis mellifera* L.). In this context, honeybees and beekeepers are two important resources for the Earth: insects for their pivotal role in pollination, and humans for their ability to protect and preserve the health and survival of the honeybees. However, the famed honeybee is also exposed to several risks, such as the use of pesticides for intensive crops [8–11], climate change, and the impact of pathogens (parasites, bacteria, and viruses) responsible for a wide range of illnesses. Beekeepers try to contrast these risks by using appropriate farming techniques, different kinds of chemicals, and/or drugs; however-er, the results are not always satisfactory [12,13], and the health and production of the honeybee are often negatively affected. To obtain better production (and better insect health) from a hive, it is important to have proper management, as it is well known that regular inspections of the colonies can improve honey production and thus the remuneration of the farming activity. However, it is important to underline that, in some circumstances, manual visits to the hive can have negative impacts on the colony [14]. In addition, very often the beekeepers could not check their hives with regularity, for example, due to climate conditions or low time availability. In this context, modern technology can be a valuable help for beekeepers. The term "Precision Beekeeping" (PB) has been defined for the first time by Zacepins et al. [15] as a strategy for the management of an apiary based on monitoring individual honeybee colonies to minimize resource consumption and maximize productivity. This new approach can be

considered a new evolutionary phase of beekeeping, as indicated by Zogovic et al. [16]: from "traditional beekeeping", through "rational beekeeping", up to "Precision Beekeeping". The PB is organized into three key points: data collection, data processing, and the data output phase [17]. As recently re-ported by Alleri et al. [18], data can be captured by different kinds of sensors for monitoring, for example, weight, temperature, humidity, sounds, etc.

The sensors can be placed inside or outside the hive; in this way, the hive becomes "smart". In this way, the data is checked in real-time through specific applications on different devices (personal computers, tablets, and smartphones) and processed thanks to algorithms. Beekeepers can thus consult and download the data obtained from their archives. The data processing is important to define if a condition measured at a specific time falls or is not within a "normal" range. In addition, the processing data system can produce signal alarms if some parameter is out of the "normal" standard. From a practical perspective, PB is not only the application of intelligent technologies to hive bee farming but also an in-depth knowledge of the various genetical, nutritional, physiological, and physio-pathological aspects linked to the world of bees. Precision beekeeping is at the beekeeper's service, but the beekeeper must be able to properly read the indications that the digital system provides him, including the alarm signals. Technology is a very important aid, but it is not infallible; therefore, it can support the beekeeper but not replace him, be-cause obviously, it is up to him to make the final decision on any corrective measures to be taken in the presence of anomalous situations. All in all, it seems clear that talking about a multifaceted topic such as precision beekeeping is not easy. The purpose of this review is to illustrate the most recent developments in technologies applied to bee production and how they can be linked to the genetic, physiological, ethological, nutritional, and pathological aspects to arrive at rational management of the apiary for the protection of animals and profitability of productions. A SWOT (Strengths, Weaknesses, Opportunities, and Threats) analysis was also performed with the aim of identifying the key factors that could support or impair the diffusion of precision beekeeping systems.

7.5 Data Collection and Storage: Sensors and Systems

In PB, data collection is the first and probably one of the most important steps. It is typically performed using sensors integrated into beehives and connected to the main processing system [19]. A sensor (or node) is a device sensitive to a specific physical–chemical cue and, if solicited, can generate an electrical signal [20]. These advanced sensors are equipped with a microprocessor

powered by batteries; the microprocessor records the data and, thanks to a Wireless Sensor Network (WSN) system [21], sends it to a remote server for processing [18]. Sensors, microprocessor, battery, and remote server are part of the "system" that is completed by the device of the beekeeper (personal computer, smartphone, or tablet) in which a specific application shows the data to the user on demand and sends signals of alarm if a parameter, for example, the temperature, the weight, the relative humidity, the buzz sounds, the gases, as well as the honeybee activity and the location of the hive, are outside the normal range [18,22]. Thus, the type of sensor (and thus its accuracy) is very important. It is possible to use very simple and low-cost systems. The problem is whether these systems can achieve useful control over the hive. For example, Romanov [23] used an on-site bee colony approach to evaluate the hive temperature, in which a small digital sensor is placed inside the hive and connected to an external dis-play where the temperature is reported. This very simple system is not able to send the da-ta via the web, and the temperature can be observed remotely only by placing a camera near the digital display. This system may seem functional, but the main problem is that the data cannot be stored [19], and it is possible to only do on-site or on-time observation without an alarm signal available for beekeepers. The progress of these technologies al-lows for different and more flexible systems to record, store, and process data. For example, the data recorded by sensors can be transmitted by wired or wireless connection to a PC on the apiary [15,24]. This kind of solution allows for more information to be given to the beekeeper, but to record and send the data, the PC must be turned on (energy expenditure). Another possibility is double-data sending. The sensors recorded the data and transmitted them to the apiary PC, which is connected to a remote service or a cloud device [25]. In this case, the local PC is only an intermediary, and the data storage and processing are from the remote service. The remote service will send the data to the beekeeping devices. When the apiary's PC is turned off, it is possible to store the data on the remote server, but they cannot be processed. Another approach relies on micro-controller platforms to collect and send the data to the remote controller. The micro-controller platforms need less energy and thus are more stable than a PC, but if the system is turned off, the data processing is interrupted, and no data or alarms are sent to the beekeeper's devices. However, more modern technologies are producing sensors able to connect to the web using 4G or 5G connections. In this way, recorded data is sent directly to the remote server for storage and processing, and only the interruption of the network or the breakage of the sensors can interfere with the monitoring of the hives. All this is possible where the apiary is reached by a 4G or 5G connection (even 3G in some sensors). Though networks are by now fairly spread in rural areas, it is possible that some areas are not completely covered by the

service. In addition, with this kind of approach, each sensor point needs a battery as a source of energy and a SIM for the connection to the network. It means that each hive beekeeper must provide these resources.

7.5.1 Weight

The weight of a beehive fluctuates according to the season (the lowest during winter, the highest during the productive period) and can be a valuable predictor of honey pro-duction and, in general, of the activity of the hive. The weight is probably the easiest parameter to measure by using, in general, non-invasive sensors. The different systems available on the market use a scale consisting of load cells, which rely on mechanics and resistive theory, integrated with amplifier modules that are linked to the microcontroller [26]. The load cells are made of a material that moves back when pressure (weight) is ap-plied. One used technology is a “resistive strain gauge” in the load cell, which makes it possible to measure the resistance (Ohm) of the cell [27]. An important aspect when work-ing with load cells is the temperature and humidity around the cell, which will make the value change for the same load, resulting in different measurement values [27]. The load cells can be converted into dedicated electronic circuits (Analog to Digital Conversion, ADC). The ADC circuits are standardized, and one interesting characteristic of ADC converters is the resolution, defining how detailed values the ADC will provide as output. For accurate, reliable measurements and ease of use, the weight sensor or weighing scale is placed under a hive, where each hive can comprise multiple supers/chambers. A single honey chamber can weigh up to 30 kg, which means that a hive with three honey cham-bers and a brood chamber can weigh up to 120 kg. The weighing scale needs to have an appropriate range of measurements and must be sensitive enough to detect daily changes in the weight of the hive with a resolution of a few grams (in general, less than 100 g) [28]. The choice of hive type also plays a role in this variation. For instance, beekeepers may opt for Dadant hives, which are characterized by their built-in base, greater capacity, and, albeit heavier weight. Alternatively, they may choose Langstroth hives, which offer reduced weight and enhanced versatility but necessitate more meticulous oversight from the beekeeper [29,30]. The weighing scale is often the only major component sitting outside the hive. It can be conFigured to operate independently by having its own power source, microcontroller, and communication system, or it can be conFigured to draw power from the monitoring system within the hive and relay detector data back to the monitoring system via wired connections. Drawing power from the internal system and communicating data using wired connections is cost-effective, but the external wiring of such a setup hinders hive inspections and

transportation. On the other hand, a stand-alone system provides a lot of ease of use but equipping each hive with such a scale can be prohibitively expensive. As a special case, Sakanovic and Kevric [31] proposed an innovative system able to record the weight of each single frame inside the hive: a connected frame holder inserted between the roof and the base of a beehive at the top of the frames. The authors stated that the system is not so invasive and does not affect the behaviour of the honeybees. That could have very interesting applications not only from a scientific point of view but also for the beekeepers because it would be possible to know the increase in brood or forages.

7.5.2 Temperature

Temperature was one of the first parameters recorded in a hive. Almost a century ago, in 1926, Dunham [32] conducted one of the first experiments by using eight thermocouples placed in different sites of the hive and manually recording the temperature each hour [33]. The temperature is a very important parameter for bee colonies, as its detection can be used to identify different conditions in the hive, such as brood development, the pre-swarming condition, and, in general, the health status of the hive. The temperature of the hive can be monitored by using different systems. Cook et al. [34] equipped the experimental hives with four temperature sensors located in the middle of the frames, from the central one to the outskirts of the hives. In hives with active bee colonies, there was an average gradient of 0.03 °C/cm in the observed temperature range ($p < 0.001$). In hives with no bees, this slope was reduced from 0.025 °C/cm ($p < 0.001$) to 0.005 °C/cm [34]. However, based on a total of 776,872 observations, the authors concluded that the variation in the time to reach the peak temperature at different sensor points was not significant [34]. In general, the most common choice is to place a single temperature sensor in the middle of the hive, where the queen bee is [35]. There are several concerns about the possibility that the physical obstruction of the sensors can affect the activities of the honeybee inside the hive. For this reason, Meikle et al. [36] placed the sensors in the upper part of the hive, and Giammarini et al. [37] chose the inner side of the beehive base rather than directly on the frames. In both cases, the accuracy of the data might be lower than the on-frame alternative [22].

7.5.3 Sounds

Sounds, produced through body vibrations, are one of the methods used by honeybees for on-colony communication [38]. The detection of specific sound signals produced by honeybees can be useful in

assessing physiological or pathological conditions in the hive. The honeybees can produce sound with frequencies ranging from about 0 up to a few thousand hertz [22]. Thus, the first requirement for an acoustic sensor to be included in the hive is to work in this frequency range. Another very important point is to choose the most appropriate acoustic sensor, as the technology supplies different kinds of microphones: electret microphones, a type of electrostatic capacitor-based microphone that eliminates the need for a polarizing power supply by using a permanently charged material, used by Qandour et al. [39] and Anand et al. [40]; and microelectromechanical system (MEMS) microphone, a micro-scale device used, for example, for smartphones, that provide high-fidelity acoustic sensing and is small enough to be included in a tightly integrated electronic product [41,42]. In the past, another system has been studied to measure the vibration in the hive: the laser Doppler vibrometer proposed by Michelsen et al. [38]. The la-ser Doppler vibrometer (LDV) is a tool that measures such vibrations by directing a laser at a surface and comparing the frequency of the returning light to an internal reference beam [43]. This system is very precise and has several applications in different kinds of industries, but it is also too expensive. In fact, no other research is available in the literature on the application of this system for vibration detection in the hive. As for the sensor for temperature, microphones must be placed inside the hive, but in a different position. In the system described by Cecchi et al. [44], the microphone is located in a protective box on the backside of a hive. Robles-Guerrero et al. [45] placed the microphones on the roof of the hive, on the top of the frames. Ferrari et al. [41] sited the microphones inside the hives together with a temperature and humidity sensor to obtain an early prediction of swarming. All the researchers using microphones for sound detection considered it useful to protect the sensors from propolisation through nets, plastic, or metal boxes [39–42,44,45]. Recently, another possibility to evaluate the sounds in the hive has been proposed by using accelerometers, devices that measure the vibration, or acceleration of motion, of a structure. Bencsik et al. [46,47] and Ramsey et al. [48] used accelerometers to evaluate the swarming and the queen activity, respectively. The use of accelerometers has several advantages compared to microphones: accelerometers can stay in a hive for several years, and there are very few problems tied to propolisation; it is possible to detect vibrations with a high precision signal at a low and very low frequency; all considered, probably monitoring vibrations makes more sense than the detection of sounds [49]. Ramsey et al. [48] used two accelerometers placed directly on the frame after creating a cavity at the center of the frame. Those authors indicated that after two seasons of use, no negative effects were detected on the colonies. However, it is important to prevent contact between the bees and the metal components. Aumann et al. [50] stated that vibration sensors, when mounted on the

out-side wall of a beehive, are capable of detecting swarming and robbing activity. However, as recently indicated by Uthoff et al. [51], more work needs to be undertaken to develop a robust classification of sounds and vibrations in the hive, at least for the detection of the queen or of the swarming activity. As an example, during parasitic or other infections in the hive, as well as during warning situations, sounds with a range frequency between 300 and 3600 Hz are produced [52]. At the current state of the research, it is not possible to obtain accurate information on the different behaviours with vibration detection alone.

7.5.4 Images

The collection of images is an interesting and very useful approach. By acquiring videos of pictures, the beekeeper can directly see the honeybees and, in some cases, detect a problem without the necessity of data processing. Optical cameras have, in general, been placed at the entrance of the hive to monitor the honeybee "traffic", including foraging, surveillance, and fan activities. Edwards-Murphy et al. [27] tried to use an infrared camera inside the hive to monitor the activity of the family, but this system had no further applications, probably due to technical problems. To control the honeybee traffic, the external camera must be placed at an adequate distance from the hive entrance. Crawford et al. [53] placed the cameras 27.4 cm from the hive entrance; Yang and Collins [54] placed the camera 30 cm above the hive entrance; and, to permit better vision of the animals, the platform was painted green; and Sledevic [55] placed the cameras 40 cm above the hive. Kulyukin and Mukherjee [42] placed the cameras on top of supers (one or two with a distance cam-era-landing platform of 35 and 60 cm, respectively) and showed no problems with computer vision and the consequent algorithm application. Shimasaki et al. [56,57] placed the cameras in front of the beehives. All those approaches have proven to be effective for monitoring honeybee activity, but their efficacy depends on the resolution of the camera used. As a specific type of camera, thermal cameras can also be used to monitor the temperatures of the honeybees or the hives, with the aim of detecting temperature changes in pre-swarming conditions [58,59]. To date, no thermal camera has been used to count the incoming and outgoing activity at a hive entrance [60] due to its high cost, low resolution, and low frame rate in comparison to optical cameras. Probably, this kind of system can have future development as the technology recently produced low-cost thermal cameras for medical applications [61]. In fact, Williams et al. [60] stated that thermal cameras are a contender for bee counting applications and can be better than optical cameras in poorly lit conditions and without visual aids. Images can also be used for the early detection of honeybee pathology through a visual examination

of the animals. Under laboratory conditions, Elizondo et al. [62] used thermal cameras to study the movement of the mite in the brood. Schurischuster et al. [63] used a visual system to count the mites on adult honeybees. For this purpose, honeybees were forced to enter the hive through narrow tunnels, and artificial light was used to create better conditions for image processing. Braga et al. [64] proposed an interesting grading scale to evaluate the health status of adult honeybees from the morphological aspect of the animals, obtaining 95% accuracy for the health classification of a bee and 82% accuracy in detecting the presence of bees in an image.

7.5.5 Gases

The air inside the hive is a complex mixture of many different volatile compounds re-leased by the honeybees (e.g., pheromones, other chemicals released to repel pests and predators, metabolites, etc.), within the hive (from honey, nectar, larvae, beeswax, pollen, and propolis, or materials out of which hives are constructed), and external sources (from vehicles, farms, industries, and households in the vicinity of hives) [65]. Each hive has an individual gas profile. Considering that the other gas producers are, in general, constant, changes in gas composition inside the hive are tied to changes in gas emissions by honeybee adults or larvae. The simplest gas to detect is carbon dioxide, whose increasing concentrations can indicate, for example, an increase in workers' population in the hive and, over a certain limit, can indicate an inappropriate environment for honeybees [66]. Metal oxide semiconductors (MOx) are gas sensors whose small size allows their placement in the hive [67]. One family of these MOx sensors responds to give an estimate for CO2. These sensors have a low power consumption [68], but the cost is still high, so a practical application in beekeeping is difficult at this stage. In addition, MOx sensors can detect a wide range of gases, so a specific calibration is mandatory to understand what kind of gas the sensor is measuring at a specific time [69,70]. Another solution for CO2 detection in the hive is the use of nondispersive infrared (NDIR) sensors. The basic principle is that an infrared (IR) source, closely matched to the absorption frequency of CO2, shines down a sample tube containing air. The difference between the amount of light radiated by the IR source and the amount of IR detected is directly proportional to the number of CO2 molecules in the air sample in the tube [70,71]. Unlike MOx sensors, this is highly selective for CO2 and can be calibrated to directly measure the concentration of CO2 in ppm [67]. How-ever, to date, the availability of this kind of sensor is limited because the industry has focused its production on MOx sensors.

7.5.6 Humidity

The measurement of humidity is readily available in low-cost, small capacitive sensors that provide a high level of accuracy both in analog and digital formats [72]. Digital devices usually include temperature measurements in the same package and reduce measurement errors by undertaking Analog to Digital Conversion on the sensor chip rather than introducing possible noise in the measurements [71,73]. Easily deployable breakout boards start for as little as EUR 8, with the raw chips costing even less. Many manufacturers produce these with inter–integrated circuit (i2c) interfaces, each with different addresses, allowing some spatial variation over a hive to be monitored with relatively simple and inexpensive hardware [67].

7.6 Internet of Bees and Data Management

In an article published in the online journal Make Magazine, the term "Internet of Bees" (IoB) is reported to indicate the application of IoT to beekeeping. The data collected with the different kinds of sensors applied to the hive must be stored in a system (from a local PC to a cloud). As more data are collected, the more precise will be any sort of prediction of hive behavior or activity. From this point of view, a great role plays in the possibility of creating archives of big data and the possibility of sharing the data collected from different apiaries. Different datasets can be available in the libraries of the companies producing PB systems, where data is protected from other web users. The working process of data can be described by using the OODA loop as described by Brehmer [74] and At-wood [75]: Observe, Orient, Decide, Act. Also, in traditional and rational beekeeping, the same loop is used, but with manual or semi/automated inspection instead of using electronics, like IoB devices. All IoB devices need internet connectivity. There are multiple techniques used, including Wi-Fi, Bluetooth, Mobile Internet (5G, 4G, or 3G), Fibre Broad-band, etc. The development of embedded electronics like Arduino® and Raspberry PiTM has created new opportunities to have a low-cost, standardized device to use as an IoT device. The processing phase of bee colony data is typically limited to basic statistical analysis [76] to determine such bee colony states as queenlessness, bloodlessness, pre-swarming, swarming, and after-swarming. The data output phase includes methods to provide processed data—information—to the end user in the form of a graphical or tabular representation. As recently underlined by [77], the use of wireless network technologies in PB has some limitations, represented by data imperfections, granularity, or inconsistency, but also technical problems such as internet or mobile network coverage. To reduce mistakes that can generate false alarms, it is possible

to use multi-sensors with additional data sources. However, this solution poses another challenge: processing data from different sources as a singular unit. This kind of advanced approach is defined as data fusion. Data fusion is the process of integrating multiple data sources to produce more consistent, accurate, and useful information than that provided by any individual data source. In the OODA approach, orienting and deciding acts are possible with the application of specific mathematical models (algorithms) able to analyze data and predict possible evolutions or consequences of a specific situation. Machine Learning (ML) integrates statistics and computer science to build algorithms that are more efficient when they are subject to relevant data rather than being given specific instructions [78]. Different mathematical models can be used to process the data, and the precision of the output depends on the quantity and quality of the data as well as the accuracy of the prediction model. Brini et al. [79] used different tree methods: Random Forest [80], Extreme Gradient Boosting (XGB) [81], and a regression tree [82] to analyze data on hive weight and concluded that the first two methods outperformed linear models when predicting the hive weight variation. Andrijevi et al. [83] applied mathematical models based on recurrent neural networks to the data obtained from a bee counter at the hive entrance and showed a very high accuracy of the prediction. Pham et al. [84] tested different algorithms to process data on the foraging behaviour of honeybees and observed that all were highly competitive in terms of learning accuracy and speed. Dimitrios et al. [85] tested three different classification algorithms: the k-Nearest Neighbors algorithm (k-NN) and Support Vector Machine (SVM), and a newly proposed by the authors, U-Net Convolutional Neural Network (CNN), developed for biomedical image segmentation. The results show that k-NN and SVM, which are already used for bee sound analysis processes, provide the most accurate results for late and early detection of swarming, respectively. Focusing on early detection, which can alleviate or prevent the event, our experiment showed that SVM is the most appropriate method, while k-NN fails to detect it accurately. For early detection of swarming events, U-Net CNN per-forms almost as well as SVM and has the potential to perform even better with frequency-targeted data input and model parameter fine-tuning. The authors set, as future work, the extensive evaluation of the proposed U-Net CNN algorithm fine-tuning towards swarming events and the extension of their experiments to other deep learning algorithms.

7.7 Study Cases and Market Options

In the last decade, PB system research and development transformed beehive management, enhancing bee health and apicultural efficiency. These breakthroughs have laid the groundwork for the contemporary commercial products we have today. Smart PB systems that meet the needs of beekeepers to remotely check their hives/apiaries have been developed within the paradigm of PB. Some case studies can provide an overview of re-al-world examples that highlight the practical applications of cutting-edge technologies and beekeeping practices.

7.7.1 Study Cases

Monitoring of internal hive temperature and humidity was the focus of the investigation conducted by Rodriguez et al. [86], who constructed their PB system, known as myBee, based on the system architecture outlined by Kviesis and Zacepins [19]. This system underwent testing on ten beehives within a farm environment, and the internal temperature data collected within the hives were validated using an infrared camera system. The study was aimed at showing that the use of a PB system may have many advantages, such as the need not to handle each beehive to identify the colony's conditions and to process the data to obtain reports and statistics, thus detecting any unfavorable conditions in the beehive in real-time. Shaw et al. [87] monitored the internal temperature of bee hives by using a thermal camera mounted in front of the hive. The images had a radiometric un-certainty of 0.5 W m−2 sr−1 when the camera was allowed to stabilize for at least 20 min be-fore recording. In those experimental conditions, the radiance of images was highly correlated ($r2 = 0.62$) with the normalized manual counting (e.g., storage frames, brood frames). As a result, by applying a simple data correction model, it is possible to accurately estimate the demography of a colony without making manual inspections, which turns out to be very promising in the winter when it is not always possible to visit the colonies. To better distinguish the adult bees from the brood masses, Meikle et al. [36] proposed an approach based on continuous weight and temperature monitoring. To develop a predictive model, they systematically recorded data on hive weights and surface densities of frames with adult bees, capped/uncapped honey, and capped broods. Mass data obtained on adult bees and brood from hive inspections were regressed on the amplitudes of sine curves that fit the detrended temperature data. Weight data amplitudes were significantly correlated with adult bee populations ($r2 = 0.82$, $p < 0.0001$) during nectar flows, and temperature amplitudes were found to be inversely correlated with the log-transformed data of brood weight ($r2 = 0.65$, $p < 0.01$). Remote knowledge of daily honey

production is crucial to estimate with a certain degree of accuracy the start and end of the nectar inflow and the amount of honey that could be retrieved from each hive/apiary, as well as to correlate any production failure to external factors (e.g., undesired weather events). In this respect, Ca-tania and Vallone [35] monitored the main environmental factors, both inside and outside the hive, to assess their influence on the daily honey production. Their platform, founded on Arduino® technology, measured internal and external air temperature, humidity, and the weight of the hive as well. In that trial, honey production (5 Kg in 18 days from the placement of the hives in the fields) was extrapolated by zeroing the hive scale once ap-plied (this approach, however, does not exclude the problem of taking into account the mass of expansion of the colony). The data set acquired in that work allowed for valid decision support for the operator, such as the time of addition of supers or their removal at the end of the import of nectar. To date, there are no PB systems that allow us to estimate exclusively the supers' weight, and therefore there is no way to know directly and accurately the production of honey; this could be a crucial aspect in the development of new PB systems in the future. To gain an overall picture of the behaviour of the hive, it can also be beneficial to acquire information about the flight activity of bees. Using object tracking and Machine Learning, Fruet et al. [88] were able to count and monitor ongoing and outgoing worker bees and drones with an average error of 5% compared to the manual count made by operators on 27 videos captured from the entrance of 24 beehives. This prototype, called ApisFlow, represents a non-invasive, fairly accurate PB system for the honeybees' flight activity.

Danieli et al. [89] tried to assess the ongoing and outgoing flight count accuracy of a prototypal device based on infrared cells' technology by using a manual bee simulation and by manual counting of bees' flight recorded through a camera trap. Both methods al-lowed them to assess that the flight sensor was unreliable, being affected by a high asymmetrical distribution between incoming and outgoing flights that made it impossible to find a simple data-correction model to be implemented at the hive or server level. Fiedler et al. [90], and subsequently Wakjira et al. [91], tried to apply the same PB systems prototype, called SAMS HIVE, to try to find valid solutions to some Ethiopian and Indonesian management problems, mainly due to the great distance between the hives, often located in deep forests, or the aggressive behaviour of local honeybees that make remote monitoring of the hives themselves useful. The initial PB system design [90] featured a horizontal frame incorporating internal and external sensors for monitoring temperature and humidity, positioned beneath the first super, alongside a scale for tracking the total hive weight. Subsequently, this design was enhanced by

integrating the sensor-equipped frame directly in-to the brood chamber [91]. The SAMS HIVE allowed for a decrease in the number of on-site observations of the bee colonies by beekeepers concerning unmonitored hives, reducing the risk for the operator, who can only carry out inspections if necessary. It helps to detect abnormal behaviours of the colonies and could help to prevent death, absconding, and cases of colony collapse disorder (CCD) as well. Implementation of the bee colony monitoring system to support the development of PB in remote areas can have added value for the beekeepers by increasing their honey production and subsistence. To predict swarming events, the monitoring of hive sounds could be a useful tool. As an example, Ferrari et al. [41] analyzed sound characteristics in beehives and their correlation with temperature and humidity changes by studying 270 h of sound recordings in three beehives alongside temperature and humidity data. During increased bee activity, like swarming, sound frequencies changed from about 100–300 Hz to 500–600 Hz, attributed to the bees' vigorous wing flapping, resulting in a temperature drop from 35 °C to 33 °C [41]. In a recent study, Danieli et al. [92] remotely identified three distinct swarming events, validated through as many swarm captures in the field, by monitoring weight loss (1.1–2.1 Kg) using a commercial PB system (Melixa S.r.l., Trento, Italy). As found by others [93], a hive temperature increase (1–2 °C) was detected in the first part of each swarming event. Combining in-hive temperature increases and hive weight decreases can improve the overall reliability of swarm management practices based on the PB system's warnings.

Optimization of technical problems is a focal point in the development of the systems to be adopted in PB. Energy consumption of data acquisition systems, with particular consideration given to installations in remote locations lacking a readily available energy supply, has a pivotal role in the optimization and reliability of PB systems. In this context, Hadjur et al. [68] measured the consumption and thermal performance (working CPU's temperature during the data acquisition and transmission) of a Raspberry PiTM chip with-in a PB system in different environmental conditions (room temperature, hot and cold field test). According to that study, it seems that the working temperature of the system does not significantly affect the energy consumption of the execution of a script. In addition, the current approach to acquiring data, which consists of completely shutting down the system and waking it every hour to perform data collection, seems to be an efficient energy solution because the residual consumption of an inactive Raspberry Pi device has a significant impact on energy consumption [68].

The accuracy of data measurements, especially in relation to the weight and temperature of the beehive, can be critical for the beekeeper in the surveillance of colony well-being and honey production. Danieli et al. [94] showed that some simple data manipulation that can be easily implemented at the data generation (hive/apiary), or storage (cloud) level can enhance the accuracy of the informative potential of two commercial PB systems that, probably due to different sensors' technology, exhibited slight but not-negligible differences. One system exhibited a relative deviation of 35% for weight in-creases (e.g., nectar flow) below 1 kg, but the other one demonstrated relatively substantial deviations of 25% for weights extending up to 6.5 kg [94]. The author addressed this issue by employing a straightforward approach to correlating the recorded data with known weights, thus facilitating the application of corrections to attain precise and highly usable data.

7.7.2 Market Options

Precision beekeeping systems are already commercially available products, and it is feasible to access producers' websites via an online survey to obtain relevant information on offered options. This information can be beneficial not only for those involved in ap-plied research but also, most importantly, for beekeepers. However, market options for such systems often lack comprehensive packages encompassing all the possible sensors with which a PB system could be equipped (Table S7.1). In addition, the pricing is very often not available to a broad range of users, but it is expected to vary considerably for each system depending on how many sensors, which can usually be purchased separately, the beekeeper is interested in. The most common system (equipped with scale, temperature, and humidity sensors) has an average cost of 422 EUR/hive (216–874 EUR/hive), calculated on 16 of 32 PB systems found on the web market. A bit less than 70% of the PB system found were European, about a fifth were American, and a tenth were Asian, mostly from the Middle East and India, while the least represented were Australians (Figure S7.1). No data concerning Chinese or African PB systems can be easily retrieved due to the linguistic difficulty. Unfortunately, probably due to patenting, it was difficult to find detailed information about the types of sensors and technologies used in marketed PB systems. However, in about one case out of ten (90.6%), the sensors most integrated into PB systems were those measuring internal temperature, followed by weight (84.4%) and humidity (81.3%) (Figure 7.1). Overall, these sensors are among the earliest to have been developed and thus feature prominently in nearly all systems. A significant proportion of PB systems also include sound sensors (46.9%), which are frequently combined with internal temperature, weight, and humidity sensors. This four-sensor combination appears to be the most

favored setup for PB systems worldwide. A low percentage (15.6%) of the systems incorporate flight activity recorders. Interestingly, among the PB systems considered (Table S7.1), only one had the capability to provide data separately for the bees entering and leaving the hive. This distinction is noteworthy as, when complemented with weight sensors, it can aid in detecting phenomena such as robbing, swarming, or external perturbative factors, such as the ones that can affect the orientation capability of the foragers [95]. Furthermore, less commonly found sensors in PB systems include cameras (3.1%) to monitor flight activity or theft, and light sensors (3.13%) as theft or accident indicators if the hive is open. The need for remote beehive monitoring and data collection is a crucial resource in scientific research. In Europe, the most (and only) widespread PB system used in published scientific research is the one developed and marketed by Melixa S.r.l. (Trento, Italy). This system is distinguished by its ideal alignment with the requirements of the research community, offering a basic suite of sensors such as temperature, humidity, weight, flight activity, and weather station that collectively facilitate a comprehensive analysis of the dynamics of the hive in the context of each research experiment. Fontana et al. [96] use this PB system to relate flight activity and weight with the health status of colonies exposed to crop-protection pesticides in relation to the presence or absence of contaminants in the main matrices of the hive (wax, pollen, and honey). More recently, the application of the sensor suite of the Melixa PB system (Melixa s.r.l., Trento, Italy) has allowed Bota et al. [97] to find out that the flight activity of the colonies was negatively related to the vocal activity of the bee-eaters (*Merops apiaster* L.), detected by a passive acoustic monitoring system outside the apiary, providing insights about the possible role of PB systems in assessing damages caused by wildlife on apiaries. The same PB system was used by Danieli et al. [92], who detected some swarming events using hive weight decreases combined with in-hive temperature. It is worth noting that none of the marketed PB systems considered here is equipped with real-time, in-app alarm systems for events like swarming (both prevention and response) or robbing. Incorporating such systems could streamline the work of beekeepers significantly.

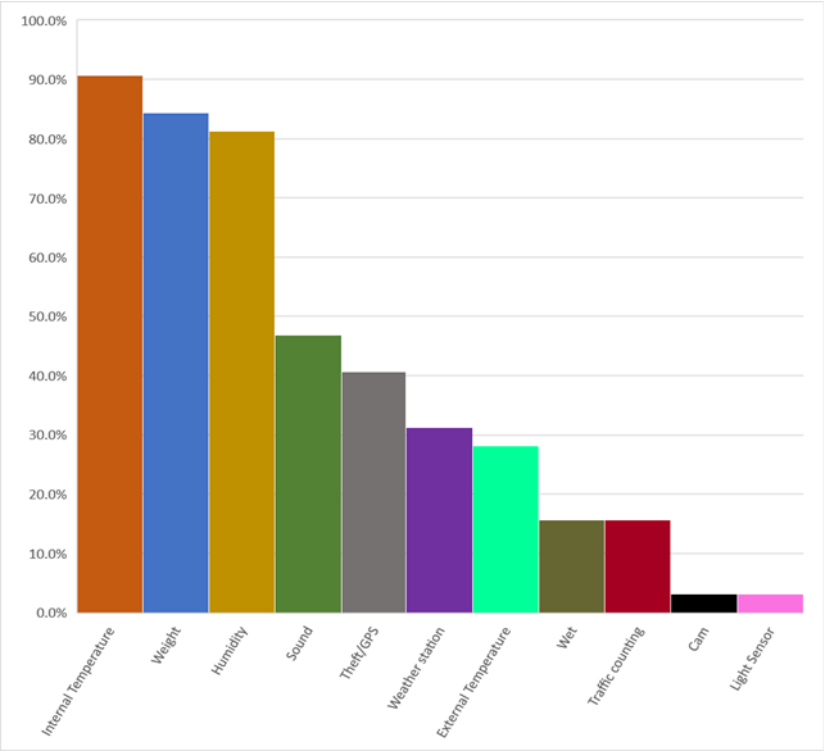

Figure 7.1. Types of sensors fitted on commercial precision beekeeping (PB) systems available on the web market.

7.8 SWOT Analysis on the Application of PB Systems

The pros and cons of using PB systems can be systematically assessed according to a four-box analysis framework approach: strengths, weaknesses, opportunities, and threats (SWOT analysis) [98], as already performed by others [99–101]. As far as the adoption of PB systems in modern apiculture and in apidology research as well, the main factors that can be considered in a SWOT analysis are summarized in Table 7.1.

Table 7.1. SWOT analysis with Internal (Strengths, Weaknesses) and External (Opportunities, Threats) factors on the use of precision beekeeping (PB) systems in beekeeping.

Strengths	Weaknesses
Accurate information Preventing and monitoring troubles and swarming Improving productivity	Cost Energy efficiency Beekeepers' preparedness Sensor accuracy
Opportunities	**Threats**
Management innovations Time-saving Bee knowledge improvement	Technology dependence Bees' health threats

7.8.1 Strengths

The use of advanced sensors and Machine Learning algorithms [41,87,88] provides a wealth of accurate information regarding life inside the beehive [102]. Sensors' data fusion enables timely hive intervention, reducing response time to issues like stress, diseases, and pests and enhancing beekeeping efficiency [18,103]. For example, this means preventing, with a combination of hive temperature or sound data, a possible swarming or being alerted in time, using collected scale data, that swarming has occurred [35,41,51,104,105]. At the same time, many systems are equipped with GPS and alarm systems, which warn the beekeeper in case the hive is stolen [106] and/or subject to accidents such as overturning due to wind, agricultural machining, or wildlife [90,97,107]. In practical applications, PB systems can support the beekeeper by optimizing honey harvesting [108] and by al-lowing product losses to be minimized [35,109].

7.8.2 Weaknesses

Precision beekeeping systems' pricing is one of the main critical points that hinders the large-scale use of this type of equipment in modern apiculture [22]. These systems have a high cost, with components and sensors to be purchased separately, and they can raise the price of a complete system significantly. In addition to the purchase price, additional costs have also to be added for systems' maintenance, troubleshooting by technical assistance services, and related shipment costs, which are difficult to calculate a priori [19]. In addition, it is important to note the difficulty in mass producing these systems on a global scale due to the current low customer demand, differences in hive construction, and the differences in honeybee species (e.g., *A. mellifera* vs. *A. cerana*) and/or subspecies and their behaviors [19,22,110]. It should also be noted that beekeepers hardly find information regarding the return on investment (ROI) of commercial PB systems [91]. In the survey con-ducted on products currently available on the market (see Section 4.2 of this review), only one (BeeSage B.V., Den Haag, The Netherlands) out of thirty-two PB systems found on the web market can give the beekeeper information regarding the ROI, which is estimated in two years for an apiary consisting of 10 hives. The energy autonomy and data connection of these devices are very relevant issues as well [22]. In fact, for the optimal use of the PB devices by beekeepers, it is essential that the system can power itself even in different climatic conditions [27,111] and can provide continuous real-time data to the owner. Though many of the commercial PB systems are equipped with photovoltaic panels, the climate and/or hive location (e.g., near trees, walls, buildings, etc.) cannot

always allow for optimal charging of the batteries. The challenge of batteries, compounded by the potential for weak signals, poses a greater concern for nomadic beekeepers. A remote monitoring system for their hives would be highly advantageous. However, these beekeepers frequently relocate their hives to remote or wooded areas, where both power supply limitations and signal strength can hinder data transmission [25,112,113]. Another critical issue is the beekeeper's preparedness for the proficient use of these technological tools. In fact, beekeepers without basic/advanced training in the use of hi-tech equipment may not always be able to read and evaluate data correctly or take advantage of using software for data manipulation. This issue could affect the major benefits these systems can give to the beekeeping of the future, data understanding, and improved production [19]. In some way, this issue can hamper the wide diffusion of these systems, by which a decrease in selling price can be reasonably expected. Finally, the accuracy of some systems, such as in- and out-flight traffic and sound detection [89,114], remains to be greatly improved before they can be effectively deployed for management purposes.

7.8.3 Opportunities

Hive sensors generate valuable continuous data that is impossible to obtain otherwise, offering a unique opportunity for research on honeybee colony behavior and their environment [17,19,36]. From a sustainability standpoint, a careful reading of the data sent to the beekeeper can be of help for shrewd hive management (according to the precision beekeeping paradigm itself), allowing fewer on-field beekeepers' interventions, reducing unnecessary hive monitoring trips, and more judicious use of treatments as well [18,108]. The combination of WSN, modern technologies of the Internet of Things (IoT), and a user (in general, the beekeeper) can represent the most suitable way to support or substitute the manual visits to the apiary. The mass diffusion of these systems could also lead to further technological innovations in the beekeeping field. At the same time, a greater diffusion of this type of product could likely lead to a reduction in prices, boosting a greater diffusion of them, as has happened in the economies of scale of other technological products in re-cent decades [115].

7.8.4 Threats

To date, not all the available PB systems on the market offer the accuracy needed to correctly manage apiaries remotely. In principle, it could be a mistake to rely entirely on technology while neglecting the direct field experience that beekeepers have [114], as, all in all, they still play a crucial role in the

proper management of an apiary. Considering also that, due to the high cost, it is economically unfeasible for a beekeeper to place more than one/two systems per apiary (overall, less than 10% of hives could be effectively monitored), reducing thus the real value of PB systems that can be well capitalized if all, at least, or a great part of the beehives of an apiary are equipped with them. As a possible result, the beekeepers can obtain an unreal perception of the more general conditions (production, colonies' health status, etc.) of their apiaries. It also remains to be thoroughly evaluated to determine what impact these systems have on beehive life. Some studies have shown that hives exposed to low-frequency electromagnetic fields may be more affected by stress symptoms [116–119]. In Henry et al. [76], a group of three hives was subjected to a 2.4 GHz Wi-Fi signal (the most common Wi-Fi signal) for 30 days. In that experience, in comparison to a control group of three hives, an increase in temperature and humidity inside the hives was observed during the trial period, even though such differences were not statistically supported. In Odemer and Odemer [120], queen bee larvae in two hives were exposed to radiation from a common GSM device (which is often used by precision beekeeping systems to send data) during all life stages, evaluating hatching and mating success. The hatching ratio was reduced in the radiation-affected colonies, but no interference in mating was shown. However, few studies [120–124] have set out to assess the impacts of these waves and the electromagnetic radiation on beehives, although the topic needs in-depth study.

7.9 Mid-Term Perspectives and Final Remarks

Indubitably, the development of PB systems will move traditional beekeeping into the mainstream of the Precision Livestock Farming (PLV) paradigm, moving from operative farmers' choices based almost entirely on their own experience to decisions driven by quantitative data [125]. In this respect, the aims and goals of PB are similar to those that animated the development of PLF in other livestock sectors, such as, for example, real-time measuring of milk production in dairy herds [126] or body weight increase in broiler husbandry [127,128] to be relevant tools by which to plan and implement precision management strategies to improve productivity and maintain high animal welfare standards as well [129]. However, beekeeping can be regarded as a particular husbandry activity mainly due to the peculiarity of the farmed animal: the honeybee, a not fully domesticated species [130]. In some respects, beekeeping and sheep farming share similar pasture-based management with more limitations for the beekeeper than the sheep farmer in supporting the animals under inclement weather conditions or other unfavorable situations (e.g., seasonality). On the other hand, pasture-based animal

production systems show several constraints that limit adopting PLF solutions [129], as the beekeeping shows: the distances from the farmer center, the uneven access to the communication web, and, very often, the unavailability of electricity supply grids to power electronic devices. Roughly in line with the potentiality of the commercial solutions available to date, the state of the art described in this review allows for an overview of the first key point of PB [17]: data collection. However, even from that point of view, more R&D is required and certainly is expected to take place in the next few years on the real-time monitoring of some relevant hive activities and behavioral features such as in–out flight trafficking, in-hive air composition (i.e., CO2 concentration), or colony sound/vibrations. Looking at the real development and implementation of PB, as for other measurable quantities of a hive (weight, humidity, temperature), gathering data is of utmost importance but, per se, has few if any practical utility if not adequately supported by data processing, but also by data fusion as well, that can effectively support beekeepers in gaining integrated insights more easily and more deeply than the ones they routinely obtain by visiting apiaries and hives. In this respect, further improvements will be deeply rooted in the experiential background of skilled beekeepers (mainly the professional ones) and, wherever possible, in the experience gained in other fields of PLF. In some circumstances, due to time or weather conditions, the skilled beekeeper can obtain a general overview of the status of a colony by knocking on the side of the hive and hearing the bees' sound response. In this respect, upgrades of commercial PB systems should encompass the possibility for the beekeeper to check at a distance the status of his/her hives by stimulating them through any type of knocking-type cue and evaluating the related outcomes (i.e., sound/vibration reply) directly or mediate through any sort of decision support system [25]. At the same time, Machine Learning is widely studied in PLF applications for cattle [125] or poultry production [131], but to date, there are few re-ports in the PB-related literature [84,85] and very few, if any, commercial solutions. To be a very practical and effective option for beekeepers, PB has to aim for tangible improvements not only in apiary productivity, a very relevant issue, especially for practitioner beekeepers, but also in beehive welfare, which includes indubitably the proper management of honeybee pests and parasites [132]. From this specific standpoint, the main R&D pipelines should address some relevant scientific questions and practical issues such as the relationships among food source availability, bee food quality, climate conditions, other environmental stressors, bees' genetics, and the outbreak of hive pathologies (i.e., varroasis, nosemiasis, and virosis), all of which are topics of outstanding relevance to safeguarding honeybees as pollinating insects, for crops and wild flora as well, but also as farm animals.

7.10 Supplementary Materials

Table S7.1. Sensors' type equipped on the survey's considered PBS.

ID	System	Weight	Internal Temperature	External Temperature	Wet	Humidity	Sound	Traffic Counting	Weather Station	Theft/GPS	Cam	Light
1		✓							✓	✓		
2		✓	✓			✓	✓			✓		
3		✓	✓		✓			✓	✓	✓	✓	
4		✓	✓			✓		✓ **	✓			
5		✓	✓			✓	✓			✓		✓
6			✓			✓	✓					
7		✓	✓			✓		✓	✓			
8		✓	✓			✓	✓					
9		✓								✓		
10		✓	✓		✓		✓					
11		✓	✓			✓				✓		
12		✓	✓			✓	✓		✓	✓		
13		✓	✓	✓		✓				✓		
14		✓	✓	✓		✓	✓					
15		✓ *	✓			✓	✓		✓			

16	✓	✓			✓	✓					
17	✓	✓			✓			✓	✓		
18	✓	✓	✓		✓	✓			✓		
19	✓	✓			✓		✓				
20	✓	✓	✓		✓	✓					
21	✓	✓			✓	✓			✓		
22		✓	✓		✓	✓					
23		✓	✓		✓	✓		✓	✓		
24	✓	✓	✓		✓		✓				
25	✓	✓	✓	✓	✓			✓	✓		
26	✓	✓		✓	✓			✓			
27	✓	✓	✓	✓	✓				✓		
28	✓	✓			✓						
29	✓										
30	✓	✓			✓						
31									✓		
32		✓			✓	✓					
Total	27	28	9	5	26	15	5	10	13	1	1

* Multiple intra-frame sensors; ** it can discriminate number of incoming and outgoing bees.

Table S7.2. Commercial precision beekeeping systems found in the web search.

PB System/Firm	Website	Country
BeeGuard	www.beeguard.it	ITA
3Bee	www.3bee.com	ITA
Melixa S.r.l.	www.melixa.it	ITA
Bee Hive Monitoring	www.beehivemonitoring.com	SVK
ApisProtect	www.apisprotect.com	IRL
arnia	www.arnia.co	GBR
BuzzBox	www.osbeehives.com	USA
HiveGenie	www.hivegenie.com	USA
Forsage	www.forsage.net	CZE
Antifurtoarnia	www.antifurtoarnia.it	ITA
Beenalytics	www.beenalytics.com	RUS
Arniaperfetta	www.arniaperfetta.it	ITA
Beehold	-	SRB
Sms scale	www.smsvaga.com	SRB
Beep	www.beep.nl	NLD
Bee and me	-	AUT
Solution bee	www.solutionbee.com	USA
Iobee	www.io-bee.eu	FRA
Gobuzzr	www.gobuzzr.com	IND
Beemate	www.beemate.buzz	AUS
Beesage	www.beesage.co	NLD
Intelligent hives	www.intelligenthives.eu	POL
Hyper hyve	www.hyperhyve.com	USA
Beewise	www.beewise.ag	ISR
HiveGenie	https://www.hivegenie.com	USA
Xlogbee	www.xlogbeescale.com	HRV
wolfwaagen	www.wolf-waagen.de	DEU
Savebees	www.save-bees.com	GRC
Optibee	www.optibee.fr	FRA

Opnehivescale	www.openhivescale.org	FRA
Beewatch	www.beewatch.de	DEU
nectar	www.nectar.buzz	CAN
Apisprotect	https://apisprotect.com	IRL

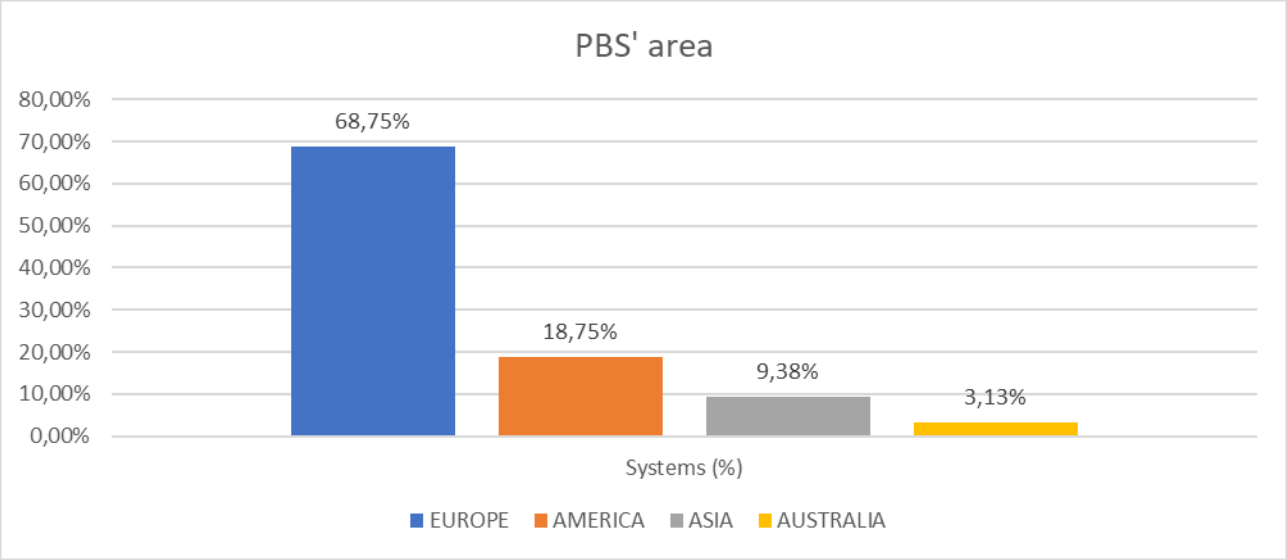

Figure S7.1. Percentage of systems analyzed in the survey in different areas of the world.

7.11 Author Contributions

Conceptualization: P.P.D. and F.B.; literature search: N.F.A., F.M., and F.L.; drafting of sections: 1–3 F.B. and N.F.A.; 4–6 P.P.D., F.M., and F.L.; reviewing and finalization of manuscript: F.B. and P.P.D. All authors have read and agreed to the published version of the manuscript.

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Chapter 8

General conclusions

This study explored the potential of various strategies to address the specific challenges affecting beekeeping in Central Italy, with a particular focus on *Apis mellifera ligustica*. The integrated approach suggested, based on innovative and green methodologies, highlighted how environmental and pathological stressors, such as climate change, abnormal beekeeping seasons with overlapping blooms, and the lack of floral resources at critical moments, are threatening colony resilience, significantly impacting production and sector sustainability.

One crucial aspect is nutritional management, which is particularly important in Central Italy due to the scarcity of floral resources during key periods of the beekeeping season. In this context, the research conducted on supplemental protein nutrition has shown promising potential in improving the physiological condition of honeybees. Specifically, the administration of pollen-based diets with a high biodiversity index led to increased nutrient accumulation (proteins and lipids) in different body parts, enhanced development of the hypopharyngeal glands, slight ovarian activation, and high survival rates in controlled environment tests. These conditions allowed young bees to better challenging *Varimorpha* spp., a nearly ubiquitous microsporidian pathogen in Italy, as demonstrated by the reduction in spore loads at the end of the trials following pollen diet supplementation. The environmental and economic sustainability of this approach is evident: the pollen administered to bees during periods of scarcity is the same one collected by colonies during peak bloom periods, providing a simple and cost-free strategy for beekeepers.

Another major challenge addressed in this study is winter colony losses and the ongoing battle against *Varroa destructor*, a mite that continues to pose a significant threat to honeybee survival. The proposed mineral supplementation approach, using iron (Fe^{2+}) in the form of ferrous fumarate chelated with vinasse and administered as nutrient through sugar syrup, showed some positive effects, such as good tolerance by bees. However, due to its low acaricidal efficacy and limited impact on overwintering success in field trials, the results were inconsistent, suggesting that dose optimization and combination with organic treatments, such as oxalic acid, might be necessary to enhance its

effectiveness. An Integrated Pest Management (IPM) strategy could provide an effective solution, combining multiple practices that remain green, cost-effective, and easy to implement for beekeepers.

Parallelly, genetic improvement of *Apis mellifera ligustica* through controlled breeding demonstrated significant potential, both in preserving this endemic subspecies and in enhancing colony resilience to climatic variations while maintaining phenotypic traits consistent with breed standards. Instrumental insemination and morphometric analyses proved to be sustainable approaches of interest to beekeepers, who could either perform these techniques independently using basic equipment and free analysis software or rely on specialized services. However, further studies are needed to optimize protocols and assess their long-term effectiveness.

An additional innovation in this study is the introduction of precision beekeeping tools, enabling real-time hive monitoring through advanced sensors (*e.g.* temperature, weight, flight activity, sound). These technologies allow beekeepers to optimize colony management, reducing losses and enhancing productivity. Despite their potential, challenges remain regarding the costs of large-scale implementation.

A key perspective of this research is that integrating the tested strategies can help address the multifaceted challenges of modern beekeeping. The synergy between nutrition, genetics, and monitoring technologies would lead to a more resilient and sustainable apicultural system, capable of adapting to evolving environmental and market conditions.

It is essential to emphasize that all strategies proposed in this study were developed with a strong focus on environmental sustainability, in agreement with D.M. 1061/2021 (Action IV.5) “PhD Programs on Green Topics”. The primary goal is to provide beekeepers with practical and effective solutions that are both environmentally friendly and economically viable.

In conclusion, the combination of green nutritional strategies, genetic improvement, and advanced monitoring technologies demonstrates a viable approach to addressing the main challenges of beekeeping in Central Italy. The adoption of these solutions, characterized by environmental sustainability, low cost, and ease of application, can significantly contribute to improving colony health and productivity, providing beekeepers with concrete tools to face future challenges in the sector.

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