Corso di dottorato di ricerca in:

"Scienze e Biotecnologie Agrarie"

Ciclo 35°

"An investigation on the multiple stress factors affecting honey bee health"

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Anno (2023)

CONTENTS

SUMMARY

Honey bees (*Apis mellifera* L.) play a central role in ecosystems providing pollination services.

In the last decades, a serious decline of bee colonies has been observed in many countries in the northern hemisphere, often followed by colony losses. This worrying phenomenon is due to the interaction among a number of stress factors, including parasites and pathogens (i.e. *Varroa destructor* and deformed wing virus), agrochemicals, the availability and quality of food resources and environmental conditions.

To understand how different stress agents (both abiotic and biotic) might positively or negatively interact is fundamental to plan possible actions to maintain and restore bee health.

For this purpose, during the Ph.D., several experiments were carried out under laboratory conditions following a "from detail to general" approach, initially focusing on the interaction between two factors and then gradually incorporating other stressors and assessing how they interfere with the system.

Firstly, we investigated the possible interaction between pollen, an essential nutritional resource for bees, and a xenobiotic substance. Specifically, we considered the toxic alkaloid nicotine that can be found both in nectar and pollen of some plant species. This experiment was replicated both early and late in the season to see if seasonality and hence viral infection, can influence the results. Interestingly, the simultaneous administration of pollen and nicotine resulted in a negative effect on bee survival only late in the season, in the presence of high viral load. We also investigated the above-mentioned interaction after inhibiting the proper functioning of the detoxification system allowing bees to deal with harmful secondary metabolites and xenobiotics. If the detoxification system was compromised, a detrimental effect of nicotine was noted also early in the season, supporting the importance of detoxification. Interestingly, pollen seemed to promote detoxification. However, late in the season, the presence of the virus made the system less predictable.

After confirming the positive role of pollen both in virus free and virus infected bees we investigated which pollen component is associated with its beneficial effect. For this purpose, three different types of pollen were administered to the bees. Since the polar fraction of pollen seems to play a key role in this respect, we decided to test one of the major flavonoids in this pollen: quercetin. However, this compound, at the dose tested here, did not significantly increase the survival of caged bees.

Then, we assessed how the alkaloid nicotine interacts with other stress factors that honey bees can be exposed to: a lower than normal hive temperature (32 °C), pollen deprivation and *V. destructor*, the most dangerous ectoparasite of honey bees. To this purpose, a four-factors factorial experiment was carried out. Further than confirming that both Varroa infestation and a low temperature play a negative role under most conditions, the experiment allowed to identify three significant interactions between factors that open up new avenues of investigation.

Finally, a systems biology approach was used to gain insights into various interactions among the factors that may affect honey bee health. Thus, a conceptual model was created and subsequently validated with dedicated laboratory experiments. This model highlighted a critical positive feed-back loop between virus and immunity; as a consequence, the presence of an immune-suppressive virus creates bistability. Hence, the survival of bees in presence of another stressor, such as a pesticide, does not depend only on that stressor’s level but also on the bee's initial condition.

CHAPTER 1 - **General introduction**

1.1. Biology of *Apis mellifera*

The Western honeybee (*A. mellifera* L.) is widely distributed all over the world and provides honey, wax, royal jelly and propolis. However, the importance of this insect is mainly related to its role as a pollinator. In particular honey bees pollinate several crops and are thus essential for agricultural production; for instance, the production of 39 of 57 monoculture crops is enhanced by animal pollinators (Klein *et al.*, 2007).

The honey bee is an eusocial insect living in colonies of tens of thousands of individuals organized in three castes: a fertile queen, thousands of sterile female workers and hundreds of reproductive males called drones. Gender in honey bees is determined by haplodiploidy: fertilized diploid eggs develop into females while unfertilized eggs evolve into males.

The queen mates with the drones only once and can store all the sperm in the spermatheca throughout her life; she lays about 1,500 - 2,000 eggs per day. The other fundamental role of the queen is to maintain the cohesion of the colony by means of pheromones.

Worker bees have atrophied reproductive organs and perform different tasks depending on their age: the first three weeks of life are spent inside the hive, where they engage in tasks such as cells cleaning, brood feeding, wax production, food storage and colony defence; in the following period, worker bees, then foragers, are responsible for collecting the materials needed to sustain the entire colony such as nectar, pollen, propolis, and water (Seeley, 1982; Johnson, 2008). This division of tasks over the lifespan of worker bees is called polyetism (Winston, 1987). The average lifespan of honey bees is about 40 days.

The main role of drones is to mate with a queen although a little role in thermoregulation and circulation of materials in the hive cannot be excluded.

All three castes go through four stages of development: egg, larva, pupa and adult. All life stages, except the adult stage, take place in the hexagonal wax cells forming the combs inside the nest.

During the egg and the larval stage, the cell is open, and it is sealed when the larva spins the cocoon for pupation. The cell remains sealed until the eclosion of the adult bee. The total developmental time from egg to adult is 16 days for queens, 21 for workers and 24 for drones.

During the summer period, the colony is made of 50,000 - 80,000 individuals; as the cold season approaches, brood production slows down for stopping completely during Winter when the queen bee and about 8,000 - 15,000 worker bees survive depleting the honey and pollen resources accumulated beforehand (Winston, 1987).

1.2. Colony losses and stress factors

Extensive losses of honey bee colonies have been reported all over the northern hemisphere in the last decades (Neumann and Carreck, 2010) causing concern for apiculture and the whole agriculture. A parallel decline of wild bee species has been reported (Potts *et al.*, 2010*a*; Koh *et al.*, 2016). Unfortunately, there are still large gaps in knowledge regarding both the extent and the causes of the observed decline of pollinators. In particular, there is an alarming lack of data concerning certain wild pollinator taxa while the absence of data from certain areas of the world is particularly worrying (Goulson *et al*., 2015). Indeed, most of the available data concerns domestic honey bees.

A consistent decline (25%) in colony numbers was observed in central European countries between 1965 and 2005 (Potts *et al.*, 2010*b*) while in North America, the loss of colonies recorded between 1947 and 2005 was about 59% (National Research Council, 2007; vanEngelsdorp *et al.*, 2008). Since the beginning of modern apiculture, the scale of these events in those regions has increased dramatically (vanEngelsdorp and Meixner, 2010; Osterman *et al.*, 2021). On the other hand, the number of colonies increased in Argentina and China (Aizen and Harder, 2009).

All authors nowadays agree that the loss of honey bee colonies is caused by several stress factors interacting with each other; those factors include: parasites and pathogens, but also, forage resource availability, agrochemicals and adverse environmental conditions (Potts *et al.*, 2010*b*; Goulson *et al.*, 2015). For this reason, we can speak of a multifactorial origin of colony losses (Nazzi and Pennacchio, 2014) (Figure 1). In the following subchapters, the major factors affecting honey bee health and potentially implicated in colony losses will be described.

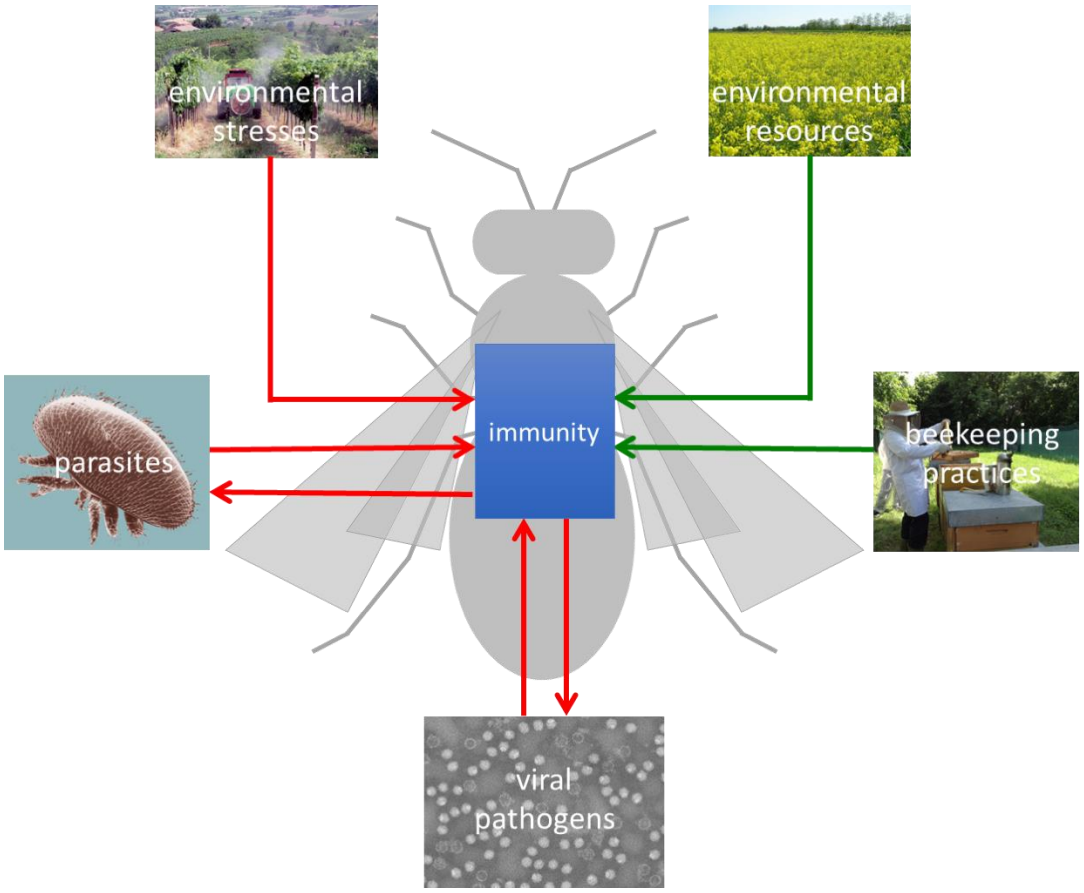

Figure 1. Multiple interactions between honey bees and environmental factors (Nazzi and Pennacchio, 2014).

1.2.1. *Varroa destructor*

Varroa destructor (Anderson & Trueman) is the most important ectoparasite of *A. mellifera*. The Varroa mite shifted from its natural host *A. cerana* to *A. mellifera* in the past century with devastating effects for the beekeeping industry (Rosenkranz, Aumeier and Ziegelmann, 2010).

Varroa lacks a free living stage; its life cycle is strictly synchronized with that of honey bees (Rosenkranz, Aumeier and Ziegelmann, 2010) and can be divided into two distinct parts: the phoretic and the reproductive phase. The first one is spent on the adult bees, while the reproductive phase occurs inside the capped brood cells. After the brood cell is sealed, the Varroa mite starts feeding on the haemolymph of the bee larva, then, 70 h after cell invasion, it lays the first egg, from which a male will develop (Ifantidis, 1983; Rehm and Ritter, 1989; Steiner *et al.*, 1994). Later, at about 30 h intervals, more eggs are laid that will develop into females (Ifantidis, 1983; Rehm and Ritter, 1989). The offspring feed from the same feeding site created by the mother mite (Donzé, Fluri and Imdorf, 1998). The total cycle, from egg to adult, lasts 6 - 7 days for males and 6 - 9 for females (Accorti *et al.*, 1983). This feeding activity underlies all the harmful effects, direct and indirect, of Varroa parasitism (Nazzi and Le Conte, 2016). Upon the emergence of the adult bee from the brood cell, the mother mite and the mature offspring leave the cell with the bee and move onto a nurse bee (phoretic phase) (Le Conte and Arnold, 1987), before entering a brood cell to reproduce again. During this time, the mites can invade other colonies via robbing or drifting bees.

During its entire life a female Varroa may perform two or three reproductive cycles (Nazzi and Le Conte, 2016).

At the individual level, the mite causes water and weight losses (De Jong, De Jong and Gonçalves, 1982; Schatton-Gadelmayer and Engels, 1988; Bowen-Walker and Gunn, 2001; Annoscia, Del Piccolo and Nazzi, 2012) as well as proteins and carbohydrates deprivation (Bowen-Walker and Gunn, 2001). Furthermore, Varroa leads to behavioural modifications (Annoscia *et al.*, 2015; Zanni *et al.*, 2018) and accelerated behavioural maturation (Downey, Higo and Winston, 2000; Zanni *et al.*,

2018; Frizzera *et al.*, 2022). Moreover, Varroa indirectly promotes secondary infections triggered by bacteria and viruses invading the bee through the mite's feeding hole (Boecking and Genersch, 2008; Vanikova *et al.*, 2015); other indirect effects are related to the transmission and replication of pathogens (de Miranda and Genersch, 2010; Nazzi and Le Conte, 2016; Annoscia *et al.*, 2019).

At the colony level, the mite infestation reduces the growth of bee populations (Rosenkranz, Aumeier and Ziegelmann, 2010), such that, beyond a certain threshold, the system can no longer hold and the colony collapses. Indeed, untreated mite infested colonies normally collapse within six months to two years (Le Conte, Ellis and Ritter, 2010).

1.2.2. Deformed wing virus (DWV)

Deformed wing virus (DWV) has become the best-studied honey bee virus (McMenamin and Flenniken, 2018; Grozinger and Flenniken, 2019; Paxton *et al*., 2022). It is a positive single stranded RNA (+ssRNA) picorna-like virus in the family Iflaviridae (de Miranda and Genersch, 2010). DWV negatively impacts honey bee health and it is the main virus associated with the collapse of honey bee colonies infested by *V. destructor* (Sumpter and Martin, 2004; Tentcheva *et al.*, 2004; Ribière, Ball and Aubert, 2008). The symptoms caused by the virus are wing deformities, smaller body size, discoloration in adult bees and reduced lifespan (de Miranda and Genersch, 2010; Grozinger and Flenniken, 2019). There are different routes of infection; the virus can be transmitted vertically (from queen to offspring) or horizontally (from one individual to another individual) mainly through larval food or trophallaxis (Chen, Evans and Feldlaufer, 2006).

This virus normally causes asymptomatic covert infection (de Miranda and Genersch, 2010) which however can turn into devastating overt infections, when the bee's immunocompetence is altered by further stressors (Nazzi *et al*., 2012). In 2012, Nazzi *et al*., provided evidence of the immunosuppressive action of DWV, characterized by the downregulation of the nuclear factor-kappaB (NF-kB) which is implicated in the antiviral response of bees. DWV is now widely distributed

also due to the intimate relationship with the Varroa mite (Wilfert *et al.*, 2016). The mite enables the transition from latent covert infections to devastating overt infections (Nazzi and Pennacchio, 2014), both because it vectors DWV (Bowen-Walker, Martin and Gunn, 1999) and because it activates the virus already infecting the bee. Furthermore, other stressors, like agrochemicals used in agriculture and acaricides used in beekeeping can trigger DWV replication (Di Prisco *et al.*, 2013; Sponsler and Johnson, 2017; Grozinger and Flenniken, 2019).

In 2018, Nazzi and Pennacchio proposed that covert infections by deformed wing virus (DWV) represent a "sword of Damocles" permanently threatening the survival of honey bee colonies and suggested that any factor affecting the honey bee's antiviral defences can turn this pathogen into a killer.

1.2.3. Xenobiotics

There are several xenobiotics in the environment to which bees may be exposed. They can be substances naturally present in nectar and pollen such as alkaloids and flavonoids (Detzel and Wink, 1993; Serra Bonvehí, Soliva Torrentó and Centelles Lorente, 2001; Johnson, 2015), but also residues of agrochemicals used in agriculture or acaricides used within hives to control Varroa infestation (Di Prisco *et al.*, 2013; Grozinger and Flenniken, 2019). Indeed, several chemical substances are used by beekeepers in order to keep Varroa mite populations under control. These synthetic acaricides include the organophosphate coumaphos, the pyrethroids tau-fluvalinate and others (Rosenkranz, Aumeier and Ziegelmann, 2010). Most of the substances are easy to apply and economically convenient. Both coumaphos and tau-fluvalinate are non-polar compounds and therefore tend to accumulate in wax (Murcia-Morales *et al.*, 2022).

Neonicotinoids represent a major class of insecticides (Jeschke and Nauen, 2008). They are nicotine-like compounds with a higher affinity for the nicotinic acetylcholine receptor (nAChR). These compounds are used for the protection of agricultural crops and their residues can be found both in

nectar and pollen (Blacquière *et al*., 2012). Neonicotinoids can affect the flight ability, immunity, and reproduction of bees (Henry *et al.*, 2012; Di Prisco *et al.*, 2013). For this reason, in 2018, based on previous studies (Gross, 2013), three neonicotinoids (Clothianidin, Imidacloprid, and Thiamethoxam) were banned for use in the open field in Europe.

Nicotine is a natural alkaloid well-known for its bitter taste (Gurevitch, Scheiner and Fox, 2006). Pollinators may encounter this alkaloid in plants belonging to the family Solanaceae (Siegmund, Leitner and Pfannhauser, 1999) and *Tilia* species (Naef *et al.*, 2004). Nicotine can be found both in pollen and nectar (Detzel and Wink, 1993). It is a broadly effective defence against herbivores, with a mode of action similar to that of synthetic neonicotinoids (Rand *et al*., 2015). In fact, nicotine mimics the neurotransmitter acetylcholine at the neuromuscular junction, activates the nicotinic acetylcholine receptor, causing twitching, convulsion and death (Tomizawa and Casida, 2003; Steppuhn *et al.*, 2004; Casida and Durkin, 2013).

Only a few insect species such as *Bemisia tabaci* and *Manduca sexta* are known to tolerate nicotine in their diet (Snyder, Walding, and Feyereisen, 1994; Kliot *et al.*, 2014). Nicotine tolerance is linked to cytochrome P450-mediated detoxification (Snyder *et al.*, 1995; Kliot *et al.*, 2014). Honey bees actively detoxify nicotine and detoxification is associated with an increase in energetic investment (Rand *et al.*, 2015).

1.2.4. Sub-optimal temperatures

Insects have limited thermoregulation capacity (Chown and Nicolson, 2004) and are strongly dependent on environmental temperature (Angilletta Jr., 2009). The temperature inside the bee hive is around 32 - 36 °C (Heinrich, 1981) and any temperature deviating from the optimal value triggers either cooling or heating by the bees. Bees can either ventilate moving their wings to cool the air inside the hive or contract their thorax muscles to warm this body part and the surrounding environment (Heinrich, 1993). Furthermore, when the temperature drops below 10 °C the bees form

a cluster whose internal temperature is optimal (Döke, Frazier and Grozinger, 2015). A correlation between winter temperature and colony losses was reported (vanEngelsdorp *et al.*, 2008). Actually, honey bees have to invest heavily in metabolic heat production to regulate the temperature during cold periods (Kronenberg and Heller, 1982; Jones *et al.*, 2004), and this is energetically expensive (Stabentheiner *et al.*, 2003). Furthermore, brood reared at lower temperatures shows morphological deformities and impaired learning, communication and navigational abilities at the adult stage, emphasizing the importance of thermoregulation within the hive (Tautz *et al*., 2003; Jones *et al*., 2005).

1.2.5. Food deprivation

The development and survival of the honey bee colonies are associated with nutrients availability (Brodschneider and Crailsheim, 2010). In fact, the quantity and balance of macro- and micronutrients, as well as secondary metabolites, in the diet of insects, can determine their longevity and ability to respond to environmental pressures, such as xenobiotics (Simpson and Raubenheimer, 2012).

The simplification of agricultural landscapes, the fragmentation, loss, isolation and modification of the landscape threaten arthropod communities worldwide. These modifications can influence the availability of food resources (Montero-Castaño and Vilà, 2012) and can lead to nutritional stress. Nutrition affects a variety of phenomena associated with honey bee biology and development because bees require appropriate floral resources for the sustenance of the colony (Goulson *et al*., 2015). The combination of land use, habitat degradation and the spread of disease contribute to the decline of many pollinator insects (Breeze *et al*., 2014). A balanced and adequate nutrition plays a fundamental role in preserving honey bee health since their environment has been rapidly modified by human presence and activities, and intensive monocultures, loss of natural environments and biodiversity can undermine the bees' nutritional needs (Naug, 2009).

Moreover, in the presence of scarce nutritional resources, the presence of large numbers of honey bees could lead to competition for food resources (Goulson, 2003), negatively affecting wild bees (Iwasaki and Hogendoorn, 2022). This is due to the size of the honey bees colony and the efficient communication within the hive (waggle dance), which is absent in wild species. For instance, Herbertsson *et al.*, (2016) showed that honeybees negatively affected bumblebee densities when landscapes are homogeneous, i.e. flower resources are limited. For this reason, management of bee colonies must be prudent (e.g. preferring small and well-spaced apiaries).

The foragers fly outside the hive to collect food and water for the colony. The diets of bees consist of nectar and pollen. Due to its sugar rich composition, nectar is the major source of energy for bees (Vaudo *et al*., 2015) while pollen is the primary source of protein. The amount of nutrients in nectar and pollen can differ between plant species: 6.3 - 85% for sugar concentration in nectars (Pamminger *et al*., 2019), and 2.5 - 61% and 1 - 20% for protein and lipid contents in pollens, respectively (Roulston and Cane, 2000; Vaudo *et al*., 2020).

Pollen can be mono-floral and poly-floral. In the first case, the abundance of the main taxa is no less than 80% while the latter contains pollen from more plant taxa. (Campos *et al*., 2008). Pollen contains amino acids, carbohydrates, lipids, sterols, vitamins and minerals necessary for normal growth and development of the colony (Stanley and Linskens, 1974; Roulston and Buchmann, 2000; Wright, Nicolson and Shafir, 2018). Workers eat 3.4 - 4.3 mg of pollen per day, with a peak at the age of nurses when they produce larval food in their hypopharyngeal glands (Crailsheim *et al*., 1992). According to some studies, bee pollen also possesses antimicrobial, antioxidant, antifungal, anti-inflammatory, antiviral, immunostimulant and local analgesic properties (Kroyer and Hegedus, 2001; Gercek, Celik and Bayram, 2021; Saisavoey *et al.*, 2021). Indeed, pollen can influence the longevity of bees (Haydak, 1970), affects the tolerance to stress (Naug, 2009), positively influences physiological metabolism (Alaux et al., 2011), immunity (Alaux *et al*., 2010) and the sensitivity to pesticides, as observed for the first time by Wahl and Ulm in 1983. Moreover, pollen intake can

mitigate the deleterious effects of *V. destructor* and the related virus infections, enhancing the lifespan of mite-infested bees under lab conditions (Annoscia *et al*., 2017). A recent study showed how the increase in survival of mite-infested bees is due to the reversing of the faster maturation induced by the parasite at the gene expression level (Frizzera *et al*., 2022). Furthermore, the pollen intake reduces the toxicity of acute doses of pesticides, revealing that pollen quality can influence the ability of bees to metabolize toxic chemicals (Barascou *et al*., 2021).

1.3. Aim of the thesis

The worrying decline of honey bee colonies observed in the recent years stimulated a great deal of research on the stress factors potentially affecting honey bee health. However, although the loss of bee colonies is attributed to the interaction among stress factors, most studies so far analysed the impact of those factors separately. In fact, the number of studies concerning the interaction between two stressors is much lower than that of studies dedicated to the effect of single factors; studies on triple interactions are extremely rare, and nobody so far has considered the possible effect of four factors together (Kaunisto, Ferguson and Sinclair, 2016). Hence the work described in this doctoral dissertation, focusing on the effect of interacting stress factors on honey bee health, the underlaying rules and the implications.

In particular, the aim of this study was to investigate how stress factors and nutrition interact to influence the survival of honey bees. We first concentrated on toxic chemicals and then expanded our view to include other stress factors.

We started studying the interaction between pollen and a toxic compound, trying to answer the following question:

1. How does pollen influence the capacity of bees to sustain an intoxication?

This first study opened further interesting questions:

2. How is this interaction modulated by the seasonal increase in viral infection?
3. What is the very cause of the beneficial effect of pollen on bee survival?
4. How is the interaction between pollen and toxic compounds affected by other concurring stressors?
5. How does the immunosuppressive action of DWV affect the behaviour of this delicate system?

These research questions are addressed in different chapters of this dissertation.

Specifically, following a first introductory chapter (chapter 1 "*General introduction*"), the first two questions are considered in chapter 2 *"An efficient detoxification system, supported by pollen nutrition, is required to contrast mild intoxication under natural conditions*".

Question 3 is analysed in chapter 3 "*The beneficial effect of pollen on virus infected honey bees is related to the polar components*".

In chapter 4*" The effect of a mild intoxication in honey bees is modulated by concurring stress factors*", a fully factorial experiment is described which allowed to investigate how Varroa infestation and a sub optimal temperatures could influence the interaction previously described.

Finally, the last question about the impact of the immunosuppression by DWV was addressed in the article "*A deeper understanding of system interactions can explain contradictory field results on pesticide impact on honey bees*" which constitutes chapter 5 of this thesis.

Following are the general conclusions (chapter 6) and the references cited in this thesis. The appendix includes other scientific works produced during the Ph.D.

CHAPTER 2 - **An efficient detoxification system, supported by pollen nutrition, is required to contrast mild intoxication under natural conditions**

2.1. The interaction between nutrition and toxic compounds

2.1.1. Introduction

Pollen is the main source of proteins and lipids for honey bees and also provides minor nutrients such as minerals, vitamins, phenolic compounds and flavonoids (Campos *et al*., 2008). Pollen has a positive effect on bee longevity (Haydak, 1970) and dietary access to pollen counteracts the accelerated transition to foraging caused by Varroa, influencing the key regulators of that process i.e. Vitellogenin and juvenile hormone (Frizzera *et al.*, 2022). Moreover, pollen can influence the ability of bees to metabolize pesticides (Ardalani *et al.*, 2021; Barascou *et al.*, 2021), the production of some antimicrobial peptides (Alaux *et al.*, 2011) and more in general immune competence (Alaux et al., 2010; DeGrandi-Hoffman *et al*., 2010).

Altogether, the literature underlines the great importance of pollen for the health of honey bees; on the other hand, pollen may also contain toxic compounds such as residues of pesticides and plant's secondary metabolites (Johnson, 2015). Indeed, honey bees are exposed to several xenobiotic substances of both natural and anthropic origin which through foraging are brought back to the colony (Johnson, 2015). For instance, from 9 to 55% of nectars also contain plant-synthesized xenobiotics (Singaravelan *et al*., 2005).

Nicotine is a natural alkaloid found in the pollen and nectar of some plants (Siegmund, Leitner and Pfannhauser, 1999; Naef *et al.*, 2004). In 2015, Singaravelan showed that low concentrations of nicotine elicited a significant feeding preference in honey bees while Detzel and Wink, in 1993, reported a median lethal concentration of nicotine for adult workers of 2.000 ppm. Moreover, nectar nicotine is deterrent at high concentrations, but the workers are more tolerant of this alkaloid when the sugar concentration is higher (Köhler, Pirk and Nicolson, 2012).

Metabolic resistance includes the mechanisms that insects put in place against toxic compounds (Li, Schuler and Berenbaum, 2007; Rand *et al.*, 2015). Indeed, metabolic detoxification is a major mechanism accounting for insect resistance to xenobiotics, including insecticides. Three major insect enzyme systems are: cytochrome P450 monooxygenases (P450s), carboxylesterases (COEs) and glutathione S-transferases (GSTs) (Johnson *et al.*, 2006).

In general, xenobiotic detoxification involves the conversion of lipid-soluble substances to water-soluble, excretable metabolites. It consists of three phases: a first phase, called functionalization, where the superfamily P450s is mainly involved, a second phase of conjugation operated by GSTs and a third phase of transport for excretion (Berenbaum and Johnson, 2015).

The honey bee genome includes only a small number of genes linked to detoxification as compared to other insects (Claudianos *et al.*, 2006). For instance, honey bees count only 46 P450 genes, compared to 85 P450 genes in *D. melanogaster* (Claudianos *et al.*, 2006). This lower number of detoxification genes could limit the capacity of honey bees to metabolize multiple toxins simultaneously and lead to greater sensitivity to pesticides (Johnson, Pollock and Berenbaum, 2009; Johnson *et al.*, 2012). Four genes belonging to the CYP6 family of cytochrome P450s metabolize quercetin (Mao *et al.*, 2009) and are upregulated by honey, pollen and propolis (Johnson *et al.*, 2012). Also, nicotine is detoxicated by honey bee's metabolism. Nicotine is oxidised to less toxic metabolites, cotinine and cotinine N-oxide, by phase I detoxification enzymes, most likely by constitutively expressed CYP6 or CYP9 enzymes (Rand *et al.*, 2015). The cytochrome P450s are also involved into the detoxification of tau-fluvalinate and coumaphos in honey bees (Mao, Schuler and Berenbaum, 2011).

The detoxification activity summarized above can be influenced by some compounds. In particular, piperonyl butoxide (PBO) inhibits P450s (Hodgson and Levi, 1999); in fact, P450 inhibitor PBO elevated the toxicity of tau-fluvalinate, coumaphos and other pyrethroids in bees (Iwasa *et al.*, 2004; Johnson *et al.*, 2006) by inhibiting three P450 enzymes belonging to the CYP9Q family (Mao, Schuler and Berenbaum, 2011). Both tau-fluvalinate and coumaphos are frequently used by

beekeepers to control Varroa mite (Rosenkranz, Aumeier and Ziegelmann, 2010). In 2020, Wu *et al*. found that PBO treatment significantly increased the mortality of thiacloprid or fluvalinate treated workers.

In order to study the effect of nicotine on honey bees and how this is modulated by pollen, we carried out an experiment in which bees were fed with pollen, nicotine or the two substances together. In addition, to assess the importance of detoxification, we used piperonyl butoxide to prevent this function in treated honey bees.

2.1.2. Materials and methods

2.1.2.1. Biological material

Newly emerged adult bees were collected randomly from several colonies of the experimental apiary of the Dipartimento di Scienze AgroAlimentari, Ambientali e Animali of the University of Udine (46°04′53.3″ N, 13°12′33.1″ E). Previous studies indicated that honey bees from this area are hybrids between *A. mellifera ligustica* and *A. mellifera carnica* (Comparini and Biasiolo, 1991).

The bee colonies used in the trials were not treated against Varroa. To this purpose, each year, at the end of the experimental period (~ end of October), the surviving colonies of the experimental apiary are used to start new nuclei and treated with oxalic (5 cc per comb); if they survive the winter, they are used to establish new colonies for the following beekeeping season.

Under these conditions, mite infestation, as assessed by checking both adult bees and brood (see Nazzi et al., 2012), is around 5 mites/1000 bees in June, and gradually increases up to 250 mites/1000 bees in September/October.

The limited chemical treatments applied to the bees used in this experiment makes it rather unlikely the possibility that the detoxification system of bees was already under stress in the studied biological material.

2.1.2.2. Experiments on caged bees

At the emergence, a convenient number of honey bees from a sealed brood comb collected the evening preceding the experiment were transferred into plastic cages (185 × 105 × 85 mm) and maintained in a climatic chamber (34.5 °C, 75% R.H., dark). Bees were fed with sugar syrup and water *ad libitum*. Sugar syrup was a solution made of 2.4 mol/L of glucose and fructose (61% and 31%, respectively) (Thom, Gilley and Tautz, 2003).

Sugar syrup was supplied through 20 mL syringes that were daily weighed to record food consumption; the diet was replaced every week. Also, water was dispensed to bees through 20 mL syringes and changed weekly.

Every day the cages were inspected and the dead specimens were counted and removed; the experiment finished at day 45, when honey bees still alive were censored.

2.1.2.3. Substances used in the experiment

Nicotine (Sigma Aldrich, USA) was added to the sugar syrup. A preliminary dose-response experiment with the following doses: 0 ppm, 0.1 ppm, 1 ppm, 10 ppm and 50 ppm, revealed that 50 ppm was the lowest dose causing significant excess mortality as compared to the control.

PBO is a widely used P450 enzyme inhibitor (Johnson *et* al., 2006), and the treatment with both pesticide and PBO reduces the honey bee survival rate (Iwasa *et al*., 2004; Wu *et al*., 2020). In our experiment, PBO was used according to the dose (0.1% in syrup) used by Wu *et* al. (2020) to impair the P450s. PBO is miscible in ethanol (O'Neil, 2006).

A previous study (our data unpublished) showed no difference between the survival of honey bees fed with 8‰ ethanol and untreated bees, therefore we used this amount of ethanol for dissolving piperonyl butoxide.

To prepare a stock solution of 150 mL of diet, we added 150 µL of PBO (Sigma Aldrich, USA) to 1.2 mL of ethanol (8‰ of 150 mL). Then, we added 148.65 mL of syrup and the solution was mixed.

By doing so, we obtained the required concentration of PBO (0.1%) in a sugar syrup containing 8‰ of ethanol.

To assess any possible harmful effect of PBO at the dose selected for this study and the solvent used for the solutions, a preliminary study was carried out using 30 caged honey bees for each experimental group.

Three experimental groups were established:

- 1 control group fed with sugar syrup and water (Control);
- 1 group fed with sugar syrup with 8‰ ethanol and water (Control+EtOH);
- 1 group fed with sugar syrup with 8‰ ethanol and 0.1% PBO (Control+EtOH+PBO).

The experiment did not reveal any significant difference between the survival of control bees and those treated with 8‰ ethanol (Figure 2; CONTROL vs. CONTROL+EtOH, Log-rank test: Chi-Square = 0.1383, d.f. = 1, $P = 0.7099$). Also, we found no significant difference between the survival of control bees and those fed with sugar syrup containing ethanol and PBO (Figure 2; CONTROL vs. CONTROL+EtOH+PBO, Log-rank test: Chi-Square = 0.5998, d.f. = 1, $P = 0.4387$). For this reason, we assumed that neither ethanol nor PBO were harmful to bees at the tested doses and could therefore be used in the experiment.

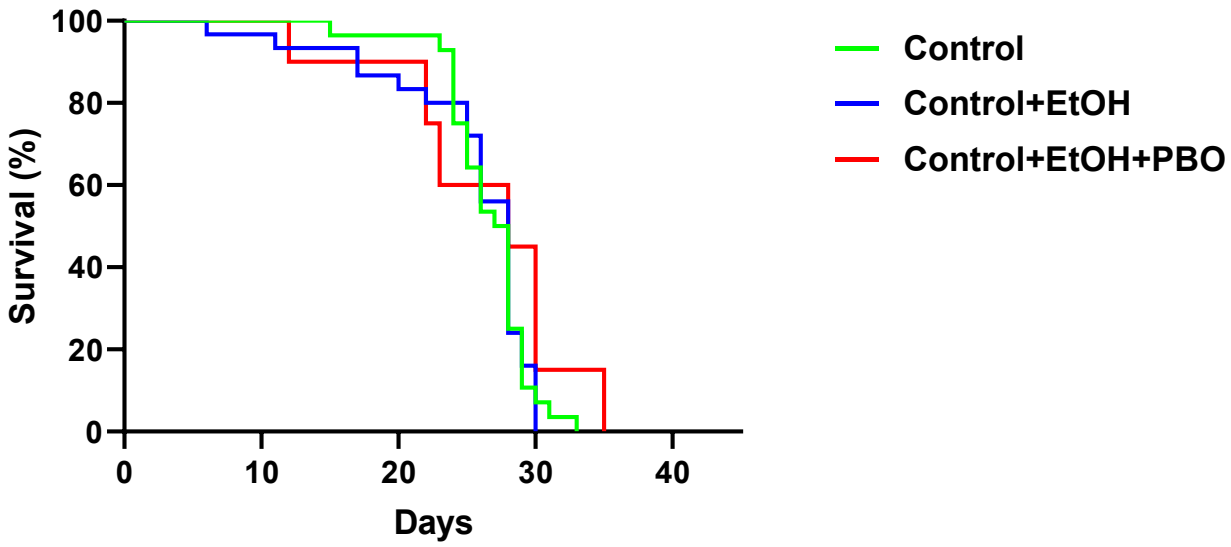

Figure 2. Survival of bees in a preliminary study to assess the possible toxicity of ethanol and PBO.

2.1.2.4. Experimental plan

Four different sugary diets were administered to caged bees:

- sugar syrup with 8‰ ethanol (two cages with about 25 bees per cage received this diet at each replication);

- sugar syrup with 8‰ ethanol and 50 ppm of nicotine (for this purpose, 10 µL of pure nicotine were added to 200 g of the sugar solution (two cages with about 25 bees per cage received this diet at each replication);

- sugar syrup with 8‰ ethanol and 150 µL of piperonyl butoxide (two cages with about 25 bees per cage received this diet at each replication);

- sugar syrup with 8‰ ethanol and 50 ppm of nicotine and 150 µL of piperonyl butoxide (two cages with about 25 bees per cage received this diet at each replication).

Half of the bees used in the experiment received pollen as well as sugar. The pollen used in this experiment was obtained from False indigo (*Amorpha fructicosa L.)* and it was delivered in a Petri dish placed on the floor of the cages.

A mono-floral pollen was chosen here to improve the replicability of the experiment, which is more difficult to achieve with a non-homogeneous pollen mixture. Indeed, a multi-floral pollen mix would have simulated a more general situation; on the other hand, this would have led to greater experimental variability that is difficult to control and replicate.

Overall, there were eight experimental groups each represented by one cage of bees (summarized in Table 1):

- 1 control group fed with sugar syrup, pollen and water (control);
- 1 group fed with sugar syrup and water (pollen deprivation);
- 1 group fed with sugar syrup, pollen, nicotine and water (nicotine);
- 1 group fed with sugar syrup, nicotine and water (nicotine+pollen deprivation);
- 1 group fed with sugar syrup, PBO and water (pollen deprivation+PBO);
- 1 group fed with sugar syrup, pollen, PBO and water (PBO);
- 1 group fed with sugar syrup, nicotine, PBO and water (nicotine+pollen deprivation+PBO);
- 1 group food with sugar syrup, pollen, nicotine, PBO and water (nicotine+PBO).

Experimental group	**Pollen**	**Nicotine**	**PBO**
Control	✓		
Pollen deprivation			
Nicotine	✓	✓	
Nicotine+pollen deprivation		✓	
Pollen deprivation+PBO			✓
PBO	✓		✓
Nicotine+pollen deprivation+PBO		✓	✓
Nicotine+PBO	✓	✓	✓

Table 1. Experimental groups considered in this experiment.

About 100 bees per group were used (25 for each replication).

The experiment was replicated four times early in the season (May - June) when viral infection is low and the contribution of this further stressor can be regarded as negligible.

2.1.2.5. Assessment of viral infection level

qRT-PCR analysis of viral infection was carried out as follows:

Ten newly emerged bees for each replication were sampled in liquid nitrogen and transferred in a −80 °C refrigerator at each replication. After defrosting of samples (2 for each replication) in RNA later, the gut of each honey bee was eliminated to avoid the congestion of the mini spin columns. The bodies of sampled bees were manually homogenized using a pestle, mortar and liquid nitrogen. RNA extractions were performed with Rneasy® Plus Mini Kit (Qiagen) and the provided protocol. The amount of RNA in each sample was quantified with a NanoDrop® spectrophotometer (ThermoFisher™, USA). cDNA was synthetized starting from 500 ng of RNA following the manufacturer specifications (PROMEGA, Italy). Additional negative control samples containing no RT enzyme were included. 10 ng of cDNA from each sample were analysed using Master mix SYBR™ green (AppliedBiosystems™, US) according to the manufacturer specifications, on a BioRad CFX96 Touch™ Real time PCR Detector. All samples were run in triplicate. The thermal cycling profiles was: one cycle at 95 °C for 10 minutes, 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute, and one cycle at 68 °C for 7 minutes.

We considered as positive all samples with a C_t value lower than 30. DWV Forward (GGTAAGCGATGGTTGTTTG) and DWV Reverse (CCGTGAATATAGTGTGAGG) were the primers used (Mondet *et al*., 2014). β-actin was used as a reference gene (Forward: GATTTGTATGCCAACACTGTCCTT; Reverse: TTGCATTCTATCTGCGATTCCA) (Di Prisco *et al.*, 2016).

We also assessed the expression of the following detoxification genes. The qRT-PCR analysis of detoxification genes was carried out according to the same protocol mentioned above, using six 7-

days-old bees and β-actin was used as a reference gene (Forward: GATTTGTATGCCAACACTGTCCTT; Reverse: TTGCATTCTATCTGCGATTCCA) (Di Prisco *et al.*, 2016).

CYP6AS1 (De Smet *et al.*, 2017)	Forward: GCGACCAATGCGAATGAAAC Reverse: TCACGGCATTCCACCATTTC	CYP6AS1 is involved in quercetin metabolization (Mao *et al.*, 2009)
CYP6AS3 (De Smet *et al.*, 2017)	Forward: TCGAAAGGGACGAGGATATG Reverse: AGTCATGGGATGCCTACTGG	CYP6AS3 is involved in quercetin metabolization (Mao *et al.*, 2009); imidacloprid up-regulates this gene (n.s.) (De Smet *et al.*, 2017)
CYP6AS4 (De Smet *et al.*, 2017)	Forward: GGCTGGATTTGAAACGTCAT Reverse: CGCGTGGAATTCTTTCATTT	CYP6AS4 is involved in quercetin metabolization (Mao *et al.*, 2009); imidacloprid up-regulates this gene (n.s.) (De Smet *et al.*, 2017)
CYP6AS10 (Wu *et al.*, 2020)	Forward: TGGCAGTGTATCATTTTACAAAACA Reverse: TGGTATTGGCTTGGGTCCAG	CYP6AS10 is involved in quercetin metabolization (Mao *et al.*, 2009)
CYP9Q3 (Mao, Schuler and Berenbaum, 2011)	Forward: GTTCCGGGAAAATGACTAC Reverse: GGTCAAAATGGTGGTGAC	CYP9Q3 is involved in the detoxification of pesticides (tau-fluvalinate and coumaphos) (Mao, Schuler and Berenbaum, 2011)

2.1.2.6. qRT-PCR raw data

Relative viral load and gene expression were analysed according to the Pfaffl Method (Pfaffl, 2001; Bustin *et al.*, 2009). The Pfaffl Method was used to calculate relative gene expression and viral load data while accounting for differences in primer efficiencies. Primer efficiency was calculated according to the formula $E=10^{(-1/slope-1)*100}$. In order to assess the efficiency of the used primer, a five-step 10-fold dilution series was made from cDNA. All dilutions were run in triplicate.

2.1.2.7. Statistical analysis

All statistical analysis were performed with GraphPad Prism©.

Log-rank test was used for the statistical analysis of bee survival. Multiple comparisons problem was corrected according to Benjamini – Hochberg procedure (Benjamini and Hochberg, 1995), setting the false discovery rate (Q) at 0.1.

Viral load differences between bees sampled early or late in the season were analysed with the Mann-Whitney test.

2.1.3. Results

The experiment was replicated four times early in the season, when the viral load is normally lower and the influence of this pathogen is reduced to the minimum. The qRT-PCR analysis of a sample of bees used in the experiment confirmed this circumstance, showing that the bees used in the experiment had a significantly lower viral load as compared to bees sampled late in the season (Figure 3A; Early season vs. Late season, Mann-Whitney U test: n1 = 6; n2 = 6; U = 0; P = 0.002). The DWV prevalence was 33% early in the season, reaching 100% late in the season (Figure 3B).

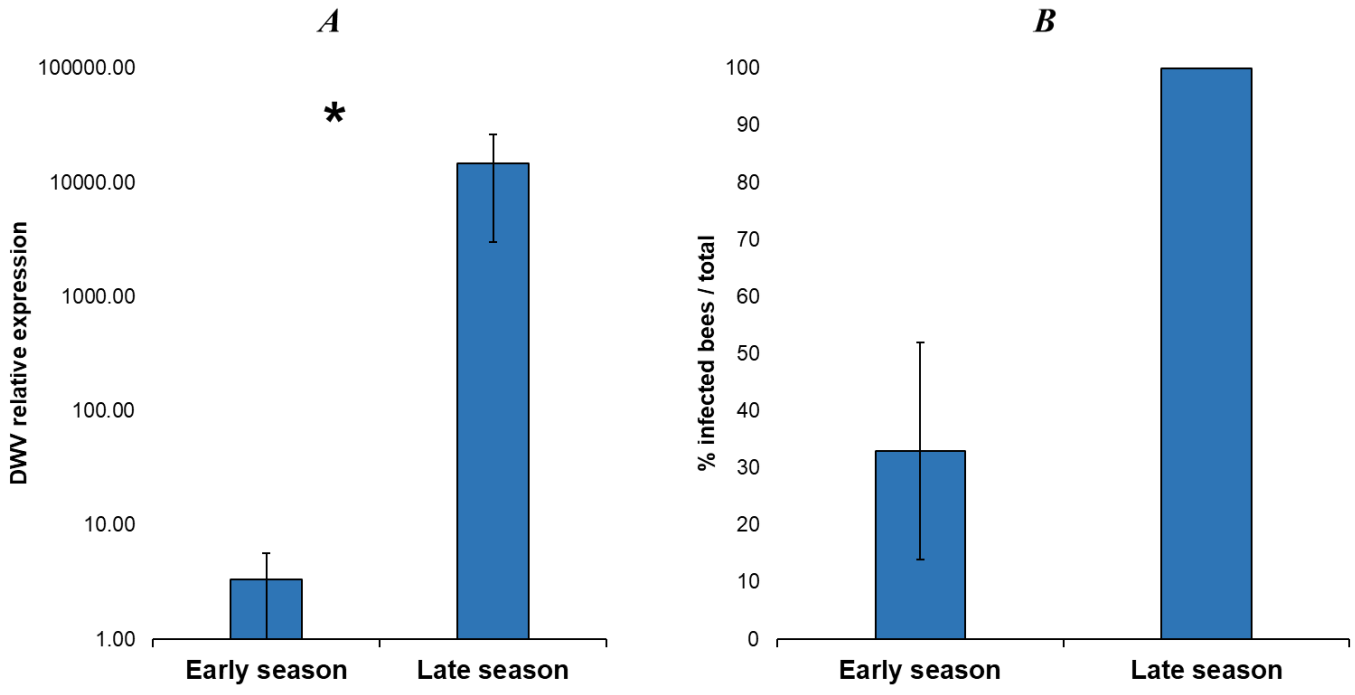

Figure 3. A - DWV relative expression (with standard error) of honey bees sampled early and late in the season. B - DWV prevalence in honey bees sampled early and late in the season.

In order to study the effect of a toxic compound (i.e. nicotine) on honey bees and how this effect is modulated by pollen, we carried out an experiment in which bees were fed with pollen, nicotine or the two substances together; to assess the importance of detoxification, we also used piperonyl butoxide to prevent this function in treated honey bees. For this purpose, we carried out an experiment involved eight experimental groups. To facilitate data interpretation, both the survival curves of bees belonging to all experimental groups (Figure 4) and the median survival of the same bees (Figure 5) are reported. In this experiment, the control group is the group of bees supplied with pollen, since this should be regarded as the standard situation in a well-placed hive for most of the season.

The lack of pollen, hereafter called pollen deprivation (PD), reduced honey bees survival by 28% (Figure 4 and Figure 5; CONTROL vs. POLLEN DEPRIVATION, Log-rank test: Chi-Square = 49.15, d.f. = 1, P = <0.0001; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0143; P<(i/m)Q = 0; significance = confirmed).

Nicotine, at the dose tested here (i.e. 50 ppm), did not cause any significant effect on the lifespan of control bees (Figure 4 and Figure 5; CONTROL vs. NICOTINE, Log-rank test: Chi-Square = 2.459, d.f. = 1, P = 0.1169; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0714; P<(i/m)Q = 0.1169; significance = confirmed). However, the same dose of nicotine appeared to be harmful in nutritionally stressed bees. Indeed, this toxic alkaloid aggravated the negative effect of pollen deprivation mentioned above (Figure 4 and Figure 5; POLLEN DEPRIVATION vs NICOTINE+POLLEN DEPRIVATION, Log-rank test: Chi-Square = 4.578, d.f. = 1, P = 0.0324; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0429; P<(i/m)Q = 0.0324; significance = confirmed).

Nicotine and other possible toxic compound present in pollen must be detoxified, and cytochrome P450s are one of the main systems involved in the detoxification of pesticides and secondary metabolites in plants. To impair detoxification, we used piperonyl butoxide, a P450 inhibitor (Hodgson and Levi, 1999; Wu *et al.*, 2020). Piperonyl butoxide had no significant effect on the survival of control bees (Figure 4 and Figure 5; CONTROL vs. PBO, Log-rank test: Chi-Square =

0.2076, d.f. = 1, *P* = 0.6487; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.1000; *P*<(i/m)Q = 0.6487; significance = confirmed). However, when PBO was added to nicotine a significant impact was noted (Figure 4 and Figure 5; NICOTINE vs. NICOTINE+PBO, Log-rank test: Chi-Square = 5.704, d.f. = 1, *P* = 0.0169; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0286; *P*<(i/m)Q = 0.0169; significance = confirmed). The same was not noted in nutritionally stressed bees (Figure 4 and Figure 5; NICOTINE+POLLEN DEPRIVATION vs NICOTINE+POLLEN DEPRIVATION+PBO, Log-rank test: Chi-Square = 1.326, d.f. = 1, *P* = 0.2496; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0857; *P*<(i/m)Q = 0.2496; significance = confirmed).

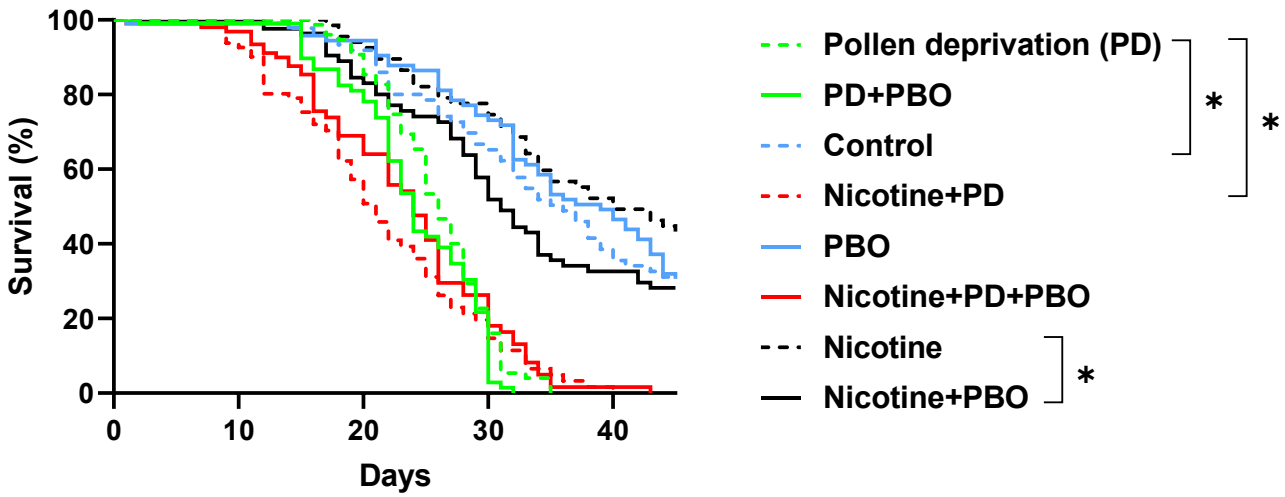

Figure 4. Survival of honey bees fed with pollen, nicotine and PBO. Asterisks mark comparisons that are statistically significant (p<0.05).

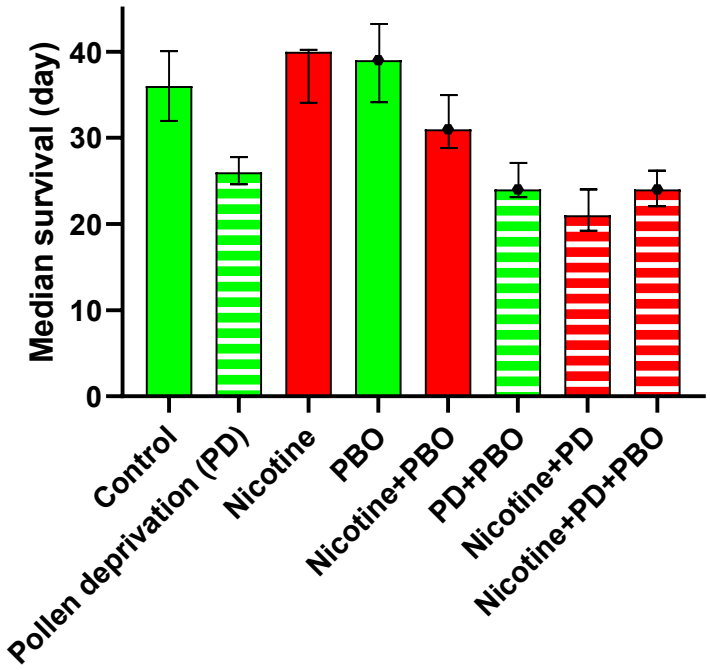

Figure 5. Median survival of honey bees fed with pollen, nicotine and PBO. This experiment was carried out early in the season when the viral load is low. The full bars represent pollen fed bees while the unfilled bars represent bees that did not receive pollen (PD: pollen deprived bees). The red bars show the median survival of nicotine fed bees. A black symbol on the top of the bar is present when PBO was administered to the bees. 95%LCL and 95% UCL are reported.

Out of 5 P450 genes considered in this study (CYP6AS1, CYP6AS3, CYP6AS4, CYP6AS10 and CYP9Q3), one was upregulated by nicotine. Specifically, the presence of nicotine led to significant upregulation of CYP6AS4 (Figure 6; CONTROL vs. NICOTINE, Mann-Whitney U test: n1 = 6; n2 = 6; U = 0; P = 0.008; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.02; P<(i/m)Q = 0.008; significance = confirmed).

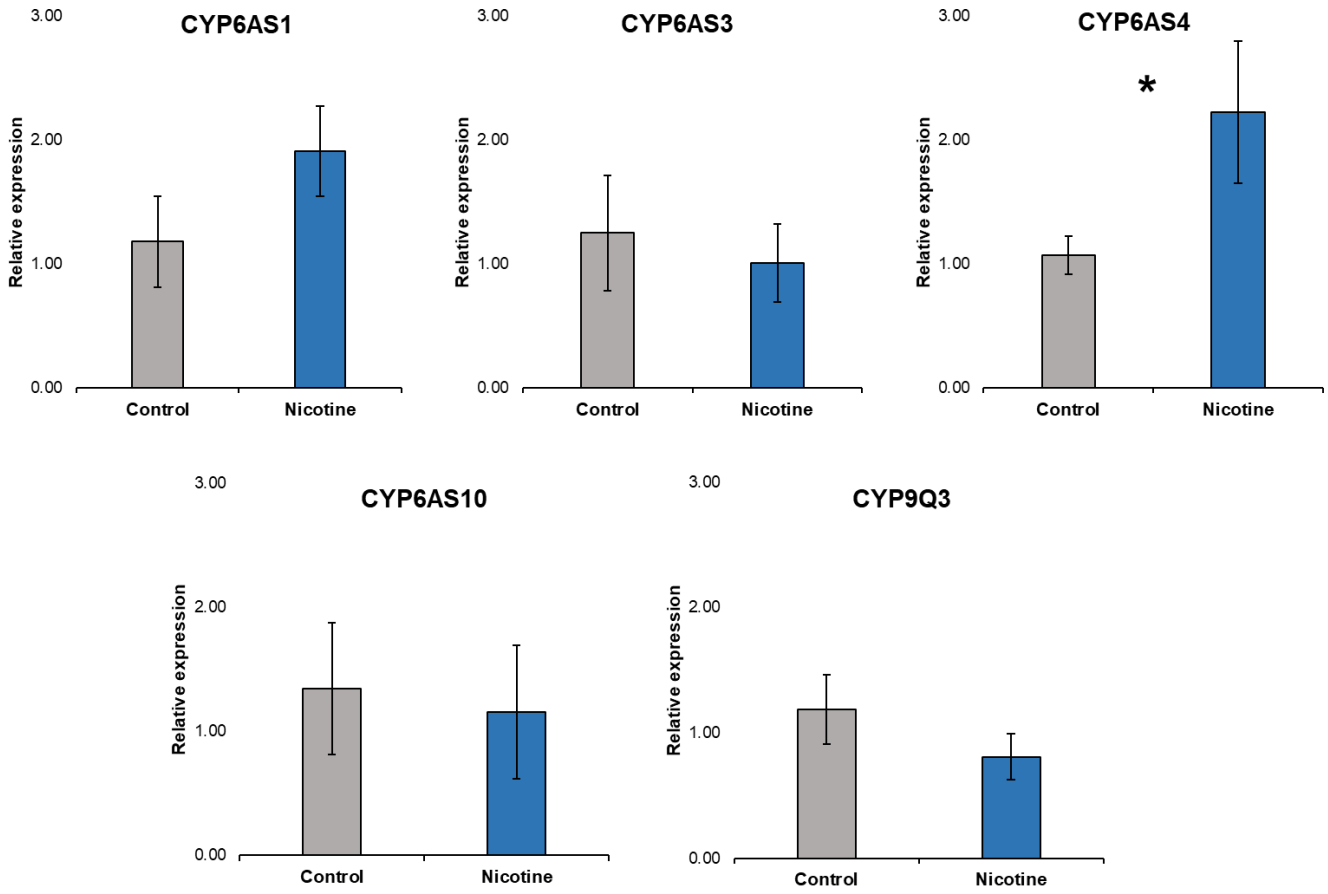

Figure 6. Relative gene expression of the studied detoxification genes. An asterisk marks significant differences ($p < 0.05$).

2.1.4. Discussion

This experiment was carried out early in the season, in May - June, when the prevalence of a common viral pathogen (DWV) is low, both in general (de Miranda and Genersch, 2010) and in the area where the bees were collected from (Nazzi *et al.*, 2012). The low prevalence and viral load were also confirmed by our analysis on a sample of bees used in the experiment (see Figure 3). Therefore, the effects reported here should be regarded as the results of the sole applied stressors and the interference of DWV should be regarded as minimum.

To elucidate the role of pollen in the tolerance of uninfected bees towards a natural toxic compound, we fed bees with pollen, nicotine or both; furthermore, to assess the importance of detoxification we also treated bees with a common inhibitor of this process.

Pollen is an important nutrient for the honey bee and our data confirm that pollen promotes honey bee health, as indicated by the shorter survival of pollen deprived bees which confirms a wealth of previous results (Haydak, 1970; Frizzera *et al.*, 2022). This can be related to the nutritional value of this pabulum supporting the metabolism of bees but could also be related to the role of pollen for the maintenance of some specific functions, such as, for example, immunity and detoxification (Alaux *et al.*, 2010; DeGrandi-Hoffman *et al.*, 2010).

Foraging bees may be exposed to various xenobiotic substances, from both natural and anthropic origin that can be introduced into the hive. Among the several xenobiotic substances present in the environment, nicotine is a toxic alkaloid, which can be found in both nectar and pollen of some plants (Detzel and Wink, 1993; Siegmund, Leitner and Pfannhauser, 1999; Naef *et al.*, 2004). Furthermore, nicotine has a chemical affinity with some insecticides (Rand *et al.*, 2015).

Nicotine, at the low dose used here, appeared to be not harmful to honey bees when plenty of pollen was available.

Toxic substances such as nicotine must be detoxified not to exert their negative effect on bees (Li, Schuler and Berenbaum, 2007; Berenbaum and Johnson, 2015; Rand *et al*., 2015) and the family of cytochrome P450 monooxygenases is one of three major insect detoxification enzyme systems acting on pesticides and secondary metabolites from plants (Johnson *et al.*, 2006). Apparently, in normally fed bees this system acted properly and no negative effects were recorded in bees treated with the toxic compound. Indeed, the gene CYP6AS4 was upregulated in nicotine fed bees compared to the control, suggesting that the contamination with this toxic alkaloid activates detoxification, in presence of pollen.

However, detoxification can be artificially impaired and this is what we did using piperonyl butoxide (Wu *et al.*, 2020), a P450 inhibitor that enhanced the toxicity of pyrethroid insecticides and neonicotinoid insecticides on honey bees (Iwasa *et al.*, 2004; Johnson *et al.*, 2006).

In PBO treated bees, nicotine appeared to be harmful to bees, suggesting that the normal survival recorded before resulted from the activity of an efficient detoxification system; in fact, when detoxification was impaired using the inhibitor, nicotine displayed a significant negative impact on bees. Actually, piperonyl butoxide made bees more vulnerable to an otherwise harmless dose of nicotine.

Interestingly, nicotine, which was not harmful in pollen fed bees, negatively influenced the survival of pollen deprived bees. We can speculate that nicotine may mediate the availability of nutrients, as observed by Bentz and Barbosa in tobacco hornworms (1992), in which dietary nicotine reduces the efficiency of food conversion. However, the negative effect of nicotine in pollen deprived bees can also be interpreted in the context of detoxification; under this point of view, pollen would be important to maintain an effective detoxification. This underlines a further role of pollen which, beside its nutritional value, can support also some specific functions such as detoxification. However, this possibility, supported by the upregulation of CYP genes after the nicotine treatment, should be confirmed by testing gene expression in the other experimental groups.

Moreover, we cannot exclude the possibility of hormesis with nicotine causing a different response depending on the biologically available dose which in turn depends on the honey bees' conditions.

The counterintuitive observation that PBO treated pollen deprived bees exposed to nicotine did not show a shorter survival as compared to pollen deprived bees exposed to nicotine is difficult to explain but may be related to the fact that a simple additive interpretative framework cannot be applied when interactions are too complex such as in this case.

2.1.5. Conclusion

In conclusion, at the beginning of the season, in the presence of low viral loads, pollen deprivation determines a negative impact on honey bee health. Instead, nicotine does not induce a negative effect on the survival of pollen-fed bees due to an efficient detoxification system acting against xenobiotics.

Indeed, when the detoxification system is compromised, i.e. using piperonyl butoxide, the detrimental effect of nicotine can be noted. Interestingly, pollen seems to promote the detoxification system. These considerations can be summarized in the following conceptual model (Figure 7).

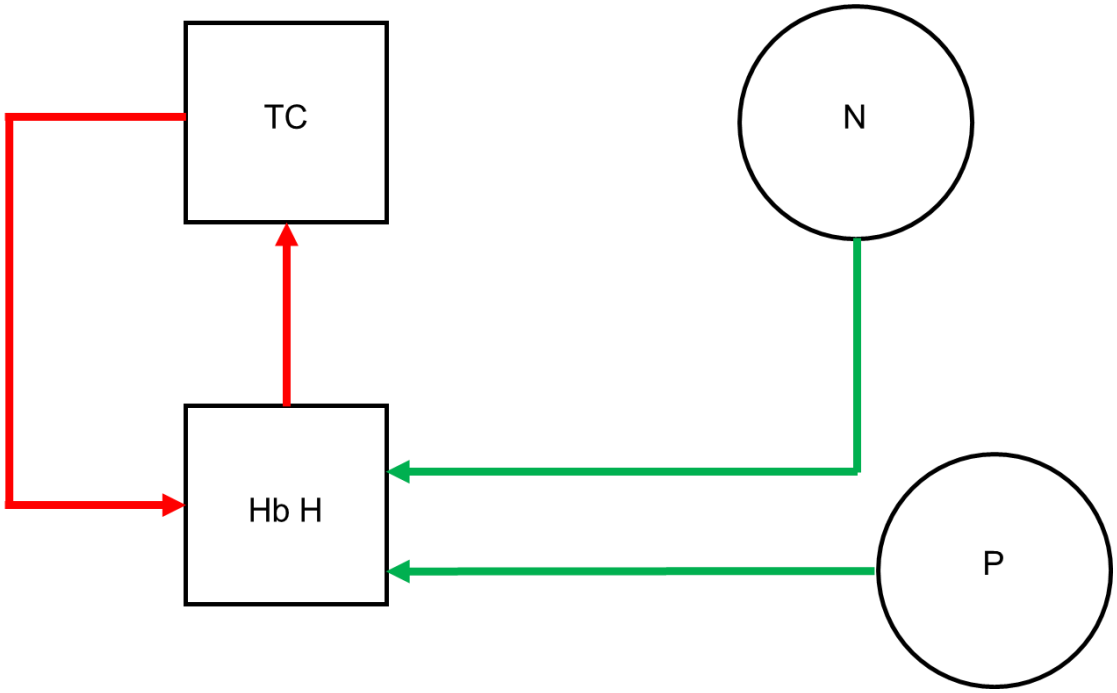

Figure 7. The health of honey bees (Hb H) is positively influenced by pollen (P) and nectar (N) (see the green lines connecting N and P to Hb H). Toxic compounds (TC), such as nicotine, can negatively affect honey bee metabolism and health (see the red line from TC to Hb H) but an efficient detoxification system acting upon TC can reduce this effect (see the red line from Hb H to TC).

2.2. Deformed wing virus influences the interaction between nutrition and toxic compounds

2.2.1. Introduction

In the northern hemisphere most honey bee colony losses occur during the Autumn - Winter period (Amdam *et al*., 2004) when resources are limited and Varroa infestation is high (Martin, 1998). Late in the season, due to high mite infestation, both prevalence and abundance of DWV are the highest and devastating viral outbreak are common (de Miranda and Genersch, 2010; Nazzi *et al.*, 2012).

To study if DWV, and thus seasonality, can influence the results obtained in the previous experiment (see subchapter 2.1.), we replicated the previous experiment four more times late in the season (September - October) when DWV infection is widespread and viral load in infected bees is higher. Again, we studied the survival of bees fed with pollen, nicotine or both; half of the bees were treated with piperonyl butoxide, a P450 inhibitor.

2.2.2. Materials and methods

For the materials and methods see subchapter 2.1.

2.2.3. Results

Quantitative analysis confirmed that late in the season, bees had a higher viral load (Figure 3A; Early season vs. Late season, Mann-Whitney U test: n1 = 6; n2 = 6; U = 0; P = 0.002) and the DWV prevalence rose from 33% to 100% (Figure 3B).

Overall, a reduction in survival was observed when comparing the median survival of control bees from the experiment carried out late in the season with those from the early season experiment (median survival of control early vs median survival of control late, Mann-Whitney U test: n1 = 3; n2 = 3; U = 96; P = 0.05; Figure 8; CONTROL EARLY vs CONTROL LATE, Log-rank test: Chi-Square = 4.433, d.f. = 1, P = 0.0352).

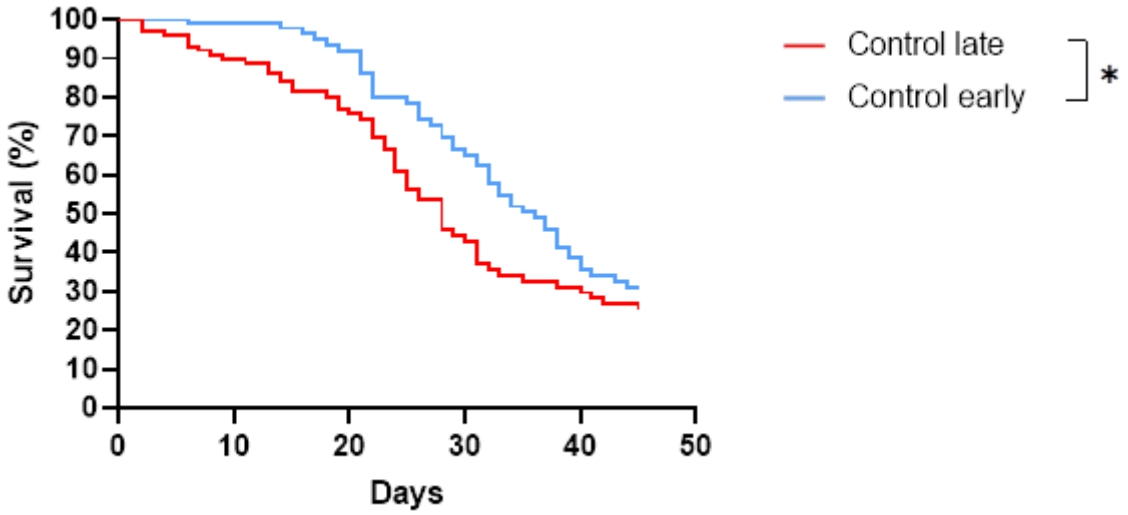

Figure 8. Survival of honey bees belonging to the control group (i.e. fed with pollen) early in the season (in presence of low viral prevalence and abundance) and late in the season (when DWV is widespread and viral load in infected bees is higher). Asterisks mark comparisons that are statistically significant (p<0.05).

Similar to what was observed early in the season, pollen deprivation reduced honey bee lifespan (Figure 9 and Figure 10; CONTROL vs. POLLEN DEPRIVATION, Log-rank test: Chi-Square = 29.08, d.f. = 1, *P* = <0.0001; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0143; *P*<(i/m)Q = 0; significance = confirmed).

Differently from what was observed previously, in presence of higher viral loads, nicotine was harmful even in pollen fed bees, reducing by 32% honey bee survival (Figure 9 and Figure 10; CONTROL vs. NICOTINE, Log-rank test: Chi-Square = 10.64, d.f. = 1, *P* = 0.0011; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0286; *P*<(i/m)Q = 0.0011; significance = confirmed). A negative, but not significant, effect of nicotine was also observed in pollen deprived bees, similar to what was noted before in uninfected bees (Figure 9 and Figure 10; POLLEN DEPRIVATION vs. NICOTINE+POLLEN DEPRIVATION, Log-rank test: Chi-Square = 2.681, d.f. = 1, *P* = 0.1016; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.1000; *P*<(i/m)Q = 0.1016; significance = confirmed).

Again, piperonyl butoxide did not affect the survival of treated bees (Figure 9 and Figure 10; CONTROL vs. PBO, Log-rank test: Chi-Square = 3.210, d.f. = 1, P = 0.0732; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0857; P<(i/m)Q = 0.0732; significance = confirmed) but contrary to the expectations, PBO reduced the effect of nicotine in pollen fed infected bees rather than aggravating it, as already observed in uninfected bees (Figure 9 and Figure 10; NICOTINE vs. NICOTINE+PBO, Log-rank test: Chi-Square = 3.847, d.f. = 1, P = 0.0498; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0714; P<(i/m)Q = 0.0498; significance = confirmed).

Instead, PBO aggravated the impact of nicotine in pollen deprived bees (Figure 9 and Figure 10; NICOTINE+POLLEN DEPRIVATION vs NICOTINE+POLLEN DEPRIVATION+PBO, Log-rank test: Chi-Square = 7.510, d.f. = 1, P = 0.0061; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0571; P<(i/m)Q = 0.0061; significance = confirmed).

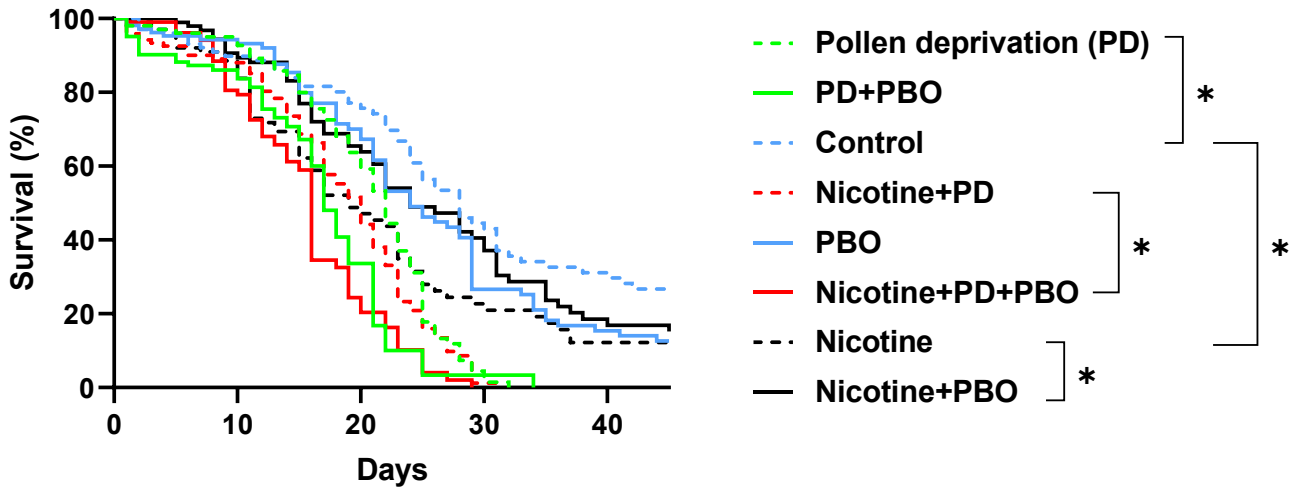

Figure 9. Survival of honey bees fed with pollen, nicotine and PBO. This experiment was carried out late in the season when DWV is widespread and viral loads are higher. Asterisks mark comparisons that are statistically significant ($p<0.05$).

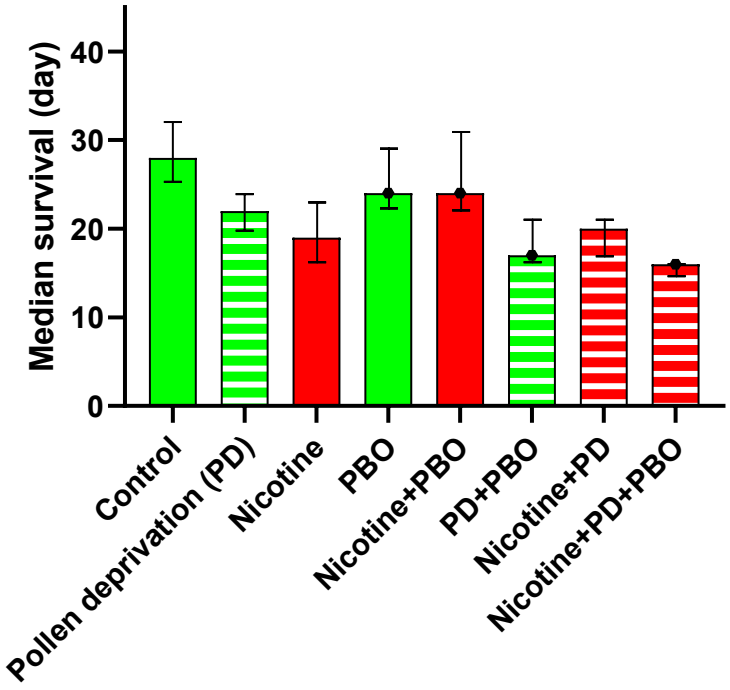

Figure 10. Median survival of viral infected bees fed with pollen, nicotine and PBO. This experiment was carried out late in the season when DWV is widespread and viral loads are higher. The full bars represent pollen fed bees while the unfilled bars represent bees that did not receive pollen. The red bars show the median survival of nicotine fed bees. A black symbol on the top of bars is present when PBO was administered to the bees. 95%LCL and 95% UCL are reported.

2.2.4. Discussion

Deformed wing virus is a key pathogen of honey bees, normally causing asymptomatic covert infections (de Miranda and Genersch, 2010) likely because the pathogen is kept under control by an efficient immunity. When the bee's immunocompetence is compromised by additional stressors, a sudden transition from covert infections to devastating outbreaks of the pathogen can be observed (Nazzi *et al.*, 2012).

For this reason, we decided to investigate if and how deformed wing virus can interfere with the interaction between pollen and the toxic compound nicotine. To do that, the same experiment described in subchapter 2.1. was replicated later in the season when viral infection is widespread and viral load in infected bees is higher.

In general, median survivals lower than those observed at the beginning of the season were recorded, confirming the notable impact of the virus on the honey bee's lifespan.

Pollen deprivation reduced honey bee survival by 21% further highlighting the importance of pollen for honey bee survival previously demonstrated (Haydak, 1970; Frizzera *et al.*, 2022). Furthermore, together with the results of the experiment carried our early in the season, when viral load is lower, our results show that the effect of pollen deprivation does not change according to viral infection.

Interestingly, late in the season, nicotine has a detrimental effect on honey bee health also in pollen fed bees. This result can be explained with the reduced capacity of viral infected bees to deal with the additional stressor here represented by xenobiotics.

The other observed results are in general more difficult to interpret. In particular, the effect of PBO on virus infected bees followed a surprising pattern with no negative effect on pollen fed nicotine treated bees and an apparently clear effect on nicotine detoxification in pollen deprived bees. These unexpected results may result from some other important circumstances. In particular, we could think that pollen may also contain some toxic compounds that needs to be detoxified (Detzel and Wink, 1993) putting an extra-burden on the detoxification system of bees which may already be under stress in presence of the virus. Also, we could speculate about a possible antiviral capacity of nicotine. In support of this hypothesis, in addition to the antiviral effects of nicotine on hepatitis C virus (Yamashina *et al.*, 2008), some authors have shown that secondary metabolites such as alkaloids can reduce the most prevalent parasite of bumblebees, *Crithidia bombi* (Manson, Otterstatter and Thomson, 2010; Richardson *et al.*, 2015) and infected bumblebees use the alkaloid nicotine from nectar to slow the progression of the infection (Baracchi, Brown and Chittka, 2015). Moreover, in 2012, Köhler, Pirk and Nicolson, showed that weak workers (presumably infected with a viral disease transmitted by Varroa or weakened from excessive feeding by the mites during early honeybee development) survived less on sugar-only diets as compared to nicotine enriched diets. Eventually, we may think that the very presence of the virus may make the system much less predictable than

expected on the ground of the simple relationship here studied; this subject will be further discussed in chapter 5.

2.2.5. Conclusion

In conclusion, late in the season, in presence of high viral loads, honey bees are debilitated by viral infection and the median survival of bees tend to be lower than that observed at the beginning of the season. As expected, pollen deprivation causes a negative impact on honey bee health. Interestingly, the effect of nicotine seems to change according to the season and thus viral infection. Indeed, a detrimental effect of 50 ppm nicotine was observed when the toxic compound was administered to viral infected pollen fed bees and a smaller but not significant effect was noted also on pollen deprived bees.

Detoxification was confirmed as an important component of the reaction to chemical stressors in pollen deprived bees but its role in viral infected pollen fed bees proved to be difficult to explain. This and other unexpected results could be interpreted by admitting that nicotine may also have a limited positive impact on honey bees and pollen itself may contain compounds that need to be detoxified.

These further considerations can be incorporated in the following conceptual model derived from Figure 7 (Figure 11).

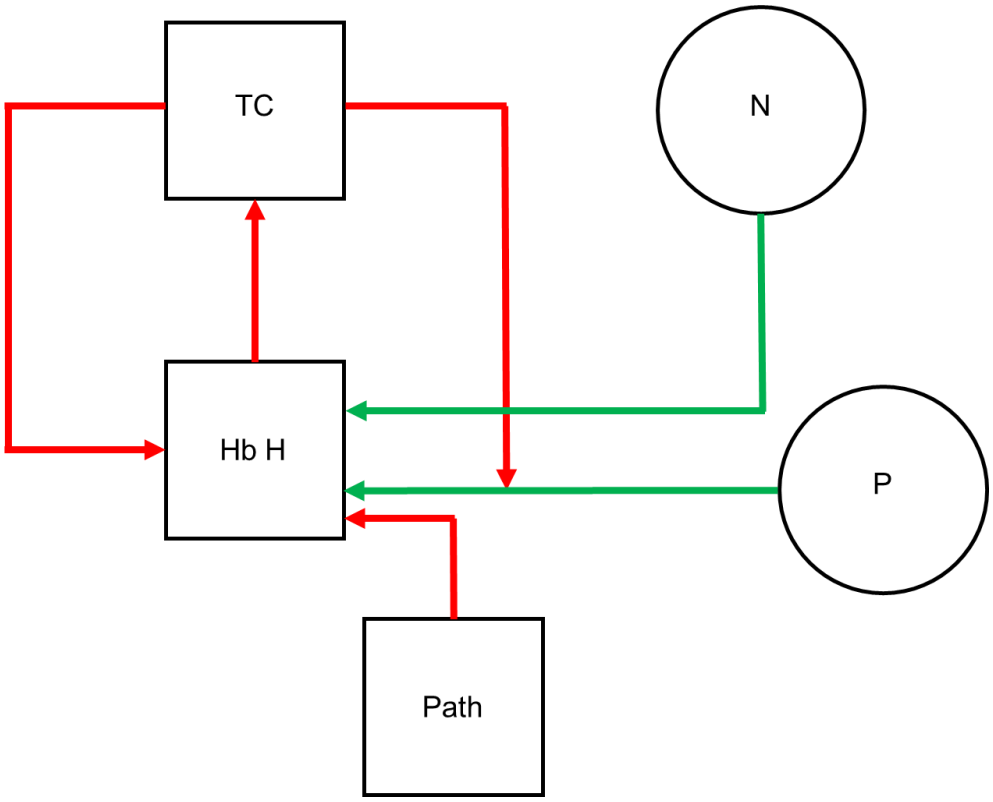

Figure 11. A conceptual model of the interactions based on the results obtained in this study and those reported before (Figure 7). In this figure the negative contribution of deformed wing virus (Path) was added as well as an arrow denoting the possibility that pollen may contain toxic chemical exerting a negative effect on honey bee health.

CHAPTER 3 - **The beneficial effect of pollen on virus infected honey bees is related to the polar components**

3.1. Introduction

Pollen represents an important food source for honey bees and is composed by dozens of compounds belonging to several classes including: amino acids, lipids, and proteins (Roulston and Cane, 2000; Di Pasquale *et al.*, 2013; Vaudo *et al.*, 2015). The polar fraction of pollen contains amino acids, polyphenols and vitamins (Campos *et al.*, 2008). Specifically, quercetin and kaempferol are ubiquitously present in pollen and together can make up 2 - 4 % of the pollen dry weight (Wiermann and Vieth, 1983), being the most abundant flavonoids in the bee diet (Rzepecka-Stojko *et al.*, 2015). Quercetin also occurs in nectar and propolis (Mao *et al.*, 2009). The composition of pollen varies according to the botanical and geographical origin, and different studies report different values for quercetin content (Serra Bonvehí, Soliva Torrentó and Centelles Lorente, 2001; Kaškonienė *et al.*, 2015; Almeida *et al.*, 2017). Quercetin is metabolized by three enzymes in the CYP6AS subfamily and two enzymes in the CYP9Q subfamily (Mao *et al.*, 2009; Mao, Schuler and Berenbaum, 2011). The intake of quercetin reduces the concentration of imidacloprid in honey bees (Ardalani *et al.*, 2021).

Pollen can influence the capacity of bees to tolerate various stress factors (DeGrandi-Hoffman *et al.*, 2010; Annoscia *et al.*, 2017; Frizzera *et al.*, 2022). Furthermore, pollen influences bee longevity (Haydak, 1970) and is involved in the production of some antimicrobial peptides (Alaux *et al.*, 2011); more in general, pollen is involved in immune competence as a whole (Alaux *et al.*, 2010; DeGrandi-Hoffman *et al.*, 2010). Moreover, pollen quality can influence the ability of bees to metabolize pesticides (Barascou *et al*., 2021).

The positive role of pollen was confirmed in this study (see chapter 2), where pollen deprived bees showed a shorter survival as compared to bees having free access to pollen. In particular, we noted that in presence of a virus infection, pollen fed bees survived longer than pollen deprived bees.

Although, the positive role of pollen for honey bees has been underlined by several studies, so far to our knowledge, only one attempt to determine which of the many components of pollen are involved in the observed function has been carried out. In particular, in 2017 Annoscia *et al.*, using a bioassay-assisted fractionation, showed that the lipid fraction can account, at least partly, for the beneficial effects of pollen on mite infested bees but, based on the results, a possible function of the polar fraction was not excluded.

In this study we wanted first to test the effect of pollen on virus infected bees and then shed light on the components responsible for the observed biological effect. For the purpose, we supplied three different kinds of pollen: whole pollen, pollen deprived of the polar components or the apolar components, to bees infected or not with DWV. To better control for the effect of viral infection we carried out the experiments early in the season, when DWV prevalence is low, and artificially infected bees with a known amount of the pathogen. After assessing the importance of the polar fraction of pollen we tested the effect of one of the main components of it.

3.2. Materials and methods

3.2.1. Biological material

Newly emerged adult bees were collected randomly from several colonies of the experimental apiary of the Dipartimento di Scienze AgroAlimentari, Ambientali e Animali of the University of Udine (46°04′53.3″ N, 13°12′33.1″ E). Previous studies indicated that honey bees from this area are hybrid between *A. mellifera ligustica* and *A. mellifera carnica* (Comparini and Biasiolo, 1991).

The bee colonies used in the trials were not treated against Varroa. To this purpose, each year, at the end of the experimental period (~ end of October), the surviving colonies of the experimental apiary are used to start new nuclei and treated with oxalic (5 cc per comb); if they survive the winter, they are used to establish new colonies for the following beekeeping season.

Under these conditions, mite infestation, as assessed by checking both adult bees and brood (see Nazzi et al., 2012), is around 5 mites/1000 bees in June, and gradually increases up to 250 mites/1000 bees in September/October.

The limited chemical treatments applied to the bees used in this experiment makes it rather unlikely the possibility that the detoxification system of bees was already under stress in the studied biological material.

3.2.2. Experiments on caged bees

For the experiment, we used caged bees. At the emergence, a convenient number of honey bees from a sealed brood comb collected the evening preceding the experiment were transferred into plastic cages (185 × 105 × 85 mm) and maintained in a climatic chamber (34.5°C, 75% R.H., dark).

During the experiment, honey bees were fed with sugar candy (Apifonda®) *ad libitum* and water. Sugar candy was dispensed in a Petri dish (Ø = 3.5 cm) and placed on the floor of the cages. Petri dishes were completely covered with laboratory film (Parafilm®), to prevent the exsiccation of the candy, except for a little cut on the top, to ensure bee feeding.

For the artificial virus infections, the bees were starved by removing the sugar candy for one hour; then, bees were individually fed with 5 µL of sugar solution containing or not 10,000 viral copies DWV.

Deformed wing virus particles were isolated and purified by ultracentrifugation from four symptomatic bees collected from the apiary of the University of Udine, following the protocol described by de Miranda *et al.* (2013). The DWV in the extract was quantified according to the protocol described by Di Prisco *et al.* (2016) and the presence of additional viruses besides DWV was ruled out through a molecular analysis carried out elsewhere (Di Prisco, personal communication). The virus material (DWV) was maintained at 4°C.

Every day the cages were inspected and the dead specimens counted and removed; the experiment ended at day 45, when honey bees that were still alive were censored.

3.2.3. Substances used in the experiment

Quercetin was obtained from Sigma Aldrich (USA) and dissolved in the sugar syrup.

3.2.4. Experimental plan

3.2.4.1. Experiment 1 - Pollen fractionation

In this experiment, three different kinds of pollen were administered to the bees: pollen deprived of the apolar fraction by means of dichloromethane extraction, pollen deprived of the polar fraction by means of water extraction and whole pollen. Pollen extraction protocols are reported below. Pollen was delivered in an open Petri dish (Ø = 3.5 cm) placed on the floor of the cages. The pollen used in this experiment was obtained from False indigo (*Amorpha fructicosa L.)*. For more information regarding the choice of the pollen used in the experiment, see subchapter "2.1.2.4. Experimental plan".

The following six experimental groups, summarized in Table 2, were established (one cage with 25 bees per experimental group):

- 1 control group fed with sugar candy and water (control);

- 1 group fed with sugar candy, whole pollen and water (pollen);

- 1 group infected with 10,000 DWV viral copies and fed with sugar candy, and water (DWV);

- 1 group infected with 10,000 DWV viral copies and fed with sugar candy, whole pollen and water (DWV+pollen);

- 1 group infected with 10,000 DWV viral copies and fed with sugar candy, pollen deprived of apolar fraction, and water (DWV+pollen- apolar fraction);

- 1 group infected with 10,000 DWV viral copies and fed with sugar candy, pollen without the polar fraction, and water (DWV+pollen- polar fraction).

Experimental group	Pollen	DWV	Pollen - apolar fraction	Pollen- polar fraction
Control				
Pollen	✓			
DWV		✓		
DWV+pollen	✓	✓		
DWV+pollen- apolar fraction		✓	✓	
DWV+pollen- polar fraction		✓		✓

Table 2. Experimental group present in this experiment

The experiment was replicated three times in May - June.

3.2.4.2. Experiment 2 - Quercetin

In this experiment bees infected or not with DWV were fed either pollen, its aqueous extract and quercetin.

The following experimental groups (Table 3) were established (one cage with 25 bees per group):

- 1 group of bees fed with sugar syrup and water (control);

- 1 group of bees fed with sugar syrup with quercetin (0.56 mg of quercetin were added to 10 mL of sugar syrup according to Almeida *et al.*, 2017) and water (control+quercetin);

- 1 group infected with 10,000 DWV viral copies and fed with sugar syrup and water (DWV);

- 1 group infected with 10,000 DWV viral copies and fed with sugar syrup, pollen and water (DWV+pollen);

- 1 group infected with 10,000 DWV viral copies and fed with sugar syrup with quercetin and water (DWV+quercetin);

- 1 group fed infected with 10,000 DWV viral copies and with sugar syrup with the aqueous extract of pollen (500 µL of aqueous phase were added to 9.5 mL of the sugar solution) and water (DWV+aqueous extract).

Experimental group	Pollen	Quercetin	DWV	Aqueous extract
Control				
Quercetin		✓		
DWV			✓	
DWV+pollen	✓		✓	
DWV+quercetin		✓	✓	
DWV+aqueous extract			✓	✓

Table 3. Experimental group present in this experiment

Sugar syrup was a solution made of 2.4 mol/L of glucose and fructose (61% and 31%, respectively) (Thom, Gilley and Tautz, 2003). Sugar syrup and water were dispensed through two different syringes (20 mL) that were refilled every week.

The experiment was replicated three times early in the season (May - June) and bees were taken from a different colony in each replication.

3.2.5. Pollen extraction

The lipids extraction was conducted following Annoscia *et al*., 2017.

Briefly, the pollen deprived of lipids was obtained as described below. An aliquot of 10 g of lyophilized pollen was extracted with 100 mL of dichloromethane by sonication for 15 minutes at room temperature. After decantation of the solvent, the residue was re-extracted with 50 mL of dichloromethane under the same conditions. The pooled extracts were filtered on a Bückner filter and the solvent was removed placing the extracted pollen in the stove at 40 °C. Then, the extracted pollen was stored at 4 °C.

The pollen deprived of the polar fraction was obtained as described below. An aliquot of 10 g of lyophilized pollen was extracted with 100 mL of demineralized water by sonication for 15 minutes at room temperature. The extract was filtered on a Bückner filter, and the residue was re-extracted with 50 mL of demineralized water under the same conditions, and subsequently filtered again on a

Bückner filter. Then, the extracted pollen was placed in the stove at 40 °C and then stored at 4 °C. The aqueous phase obtained during the extraction was stored at 4 °C.

3.2.6. Statistical analysis

All statistical analysis were performed with GraphPad Prism©.

A log-rank test was used for the statistical analysis of bee survival. Multiple comparisons problem was corrected according to Benjamini – Hochberg procedure (Benjamini and Hochberg, 1995), setting the false discovery rate (Q) at 0.1.

3.3. Results

In a first experiment the effect on virus infected bees of pollen and pollen deprived of its two main components was assessed in a cage experiment.

No significant difference was found between the survival of control bees and those artificially infected with DWV (Figure 12; CONTROL vs. DWV, Log-rank test: Chi-Square = 1.192, d.f. = 1, P = 0.275; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.1; P<(i/m)Q = 0.275; significance = confirmed).

All kinds of pollen significantly increased the bee lifespan (Figure 12; CONTROL vs. POLLEN, Log-rank test: Chi-Square = 49.61, d.f. = 1, P = <0.0001; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0143; P<(i/m)Q = 0; significance = confirmed; DWV vs. DWV + POLLEN, Log-rank test: Chi-Square = 51.74, d.f. = 1, P = <0.0001; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0143; P<(i/m)Q = 0; significance = confirmed; DWV vs. DWV + POLLEN-APOLAR FRACTION, Log-rank test: Chi-Square = 40.97, d.f. = 1, P = <0.0001; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0143; P<(i/m)Q = 0; significance = confirmed; DWV vs. DWV + POLLEN-POLAR FRACTION, Log-rank test: Chi-Square = 9.841, d.f. = 1, P = 0.0017; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0714 P<(i/m)Q = 0.0017; significance = confirmed).

Bees fed with pollen deprived of the apolar fraction (i.e. lipids), survived less than those fed with whole pollen (Figure 12; DWV + POLLEN vs. DWV + POLLEN-APOLAR FRACTION, Log-rank test: Chi-Square = 4.645, d.f. = 1, $P = 0.0311$; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0857; P<(i/m)Q = 0.0311; significance = confirmed); however, the difference seems to be mainly related to a prolonged late survival and the median longevity is similar. A bigger effect was noted when the survival of bees fed with pollen deprived of the polar fraction was compared to that of bees fed with whole pollen (Figure 12; DWV + POLLEN vs. DWV + POLLEN-POLAR FRACTION, Log-rank test: Chi-Square = 32.11, d.f. = 1, $P = <0.0001$; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0143; P<(i/m)Q = 0; significance = confirmed).

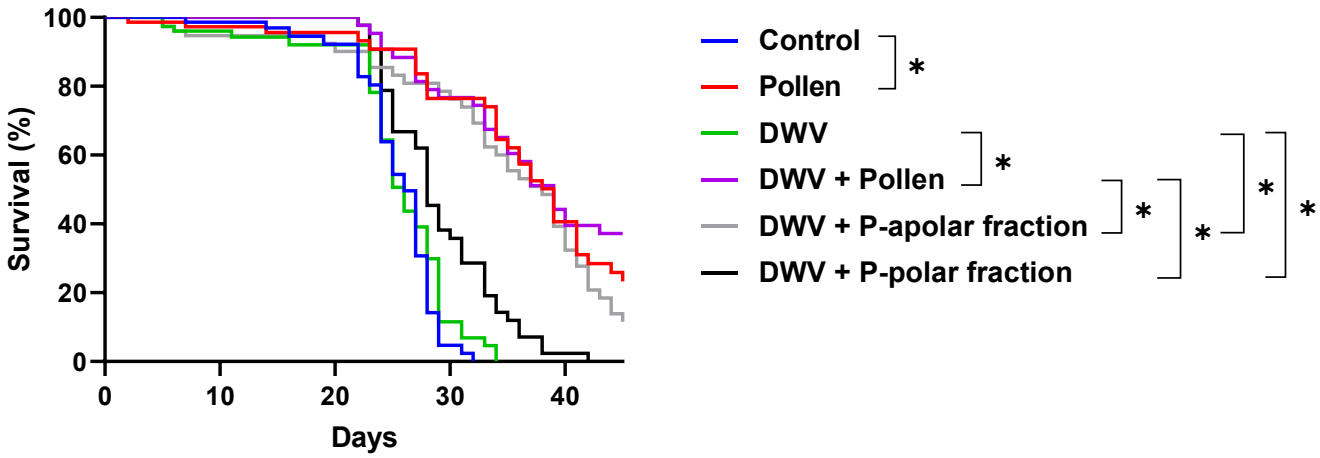

Figure 12. Survival of honey bees fed with different kinds of pollen. Asterisks mark comparisons that are statistically significant ($p<0.05$).

In a second experiment, we tested with a different method the bioactivity of the polar fraction of pollen and of quercetin: one of the major components of that fraction.

Again, no significant difference was found between the lifespan of control bees and DWV infected bees (Figure 13; CONTROL vs. DWV, Log-rank test: Chi-Square = 0.1002, d.f. = 1, $P = 0.7516$;

Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.1; *P*<(i/m)Q = 0.7516; significance = confirmed).

As predicted, pollen significantly increased bee survival (Figure 13; DWV vs. DWV + POLLEN, Log-rank test: Chi-Square = 29.62, d.f. = 1, *P* = <0.0001; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0167; *P*<(i/m)Q = 0; significance = confirmed). Also, the aqueous extract of pollen had a significant positive effect on the lifespan of tested bees (Figure 13; DWV vs. DWV + AQUEOUS EXTRACT, Log-rank test: Chi-Square = 7.906, d.f. = 1, *P* = 0.0049; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.05; *P*<(i/m)Q = 0.0049; significance = confirmed); however, there was a significant difference between the survival of bees fed pollen and those fed with the aqueous extract of it (Figure 13; DWV + AQUEOUS EXTRACT vs. DWV + POLLEN, Log-rank test: Chi-Square = 10.24, d.f. = 1, *P* = 0.0014; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0333; *P*<(i/m)Q = 0.0014; significance = confirmed). Quercetin did not exert any positive effect at the dose tested here (Figure 13; CONTROL vs. CONTROL + QUERCETIN, Log-rank test: Chi-Square = 2.044, d.f. = 1, *P* = 0.1528; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0667; *P*<(i/m)Q = 0.1528; significance = confirmed DWV vs. DWV + QUERCETIN, Log-rank test: Chi-Square = 0.2652, d.f. = 1, *P* = 0.6066; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0833; *P*<(i/m)Q = 0.6066; significance = confirmed).

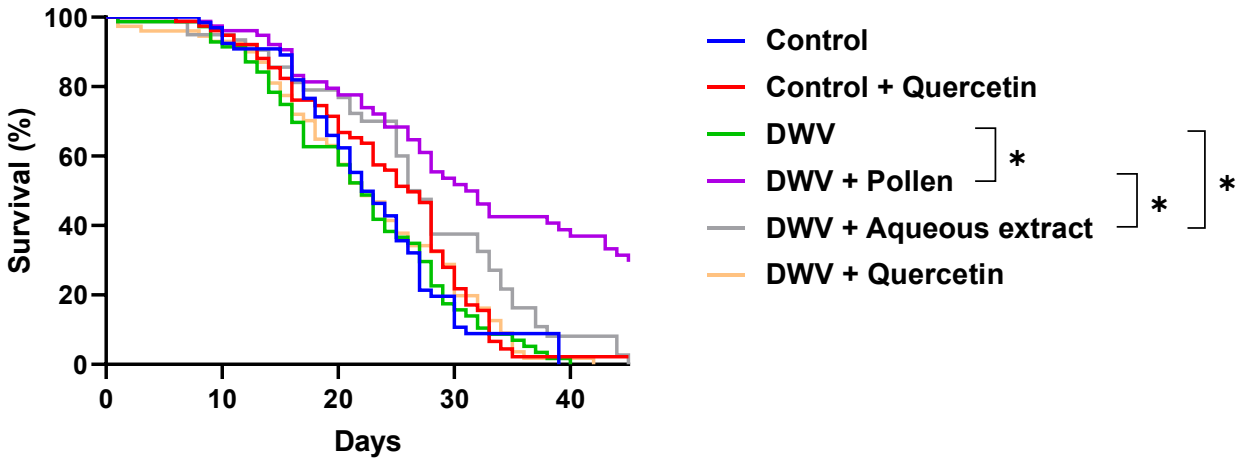

Figure 13. Survival of honey bees fed with quercetin, pollen and the aqueous extract of pollen. Asterisks mark comparisons that are statistically significant ($p<0.05$).

3.4. Discussion

Pollen is important for honey physiology and survival. Here we tested its effect on virus infected bees, then we investigated which of the pollen components accounts for this positive effect. To this aim, we carried out a dedicated experiment early in the season when the virus is rare and compared the effect of whole pollen and pollen deprived of its polar or apolar fraction on bees artificially infected with DWV.

Contrary to our expectations, we observed no difference in the survival between bees infected or not with DWV. This probably depends on the low dosage of DWV (10,000 viral copies) and, moreover, on the selected route of infection (i.e. oral), which is known to trigger lower replication as compared, for example, to direct injection (de Miranda *et al.*, 2013). Furthermore, the instability of DWV particles maintained in isolation (de Miranda *et al.*, 2013; Škubník *et al.*, 2017; Thaduri *et al.*, 2019) may have contributed (i.e. structural instability implies that the virus does not preserve its viability and persistence for a long time outside its host). Nevertheless, since the main purpose of this experiment was to investigate the pollen component responsible for its biological activity, the main focus here was the comparison between virus infected bees receiving or not different kinds of pollen.

As previously observed, honey bee survival was enhanced by pollen supply (Alaux *et al.*, 2010; Di Pasquale *et al.*, 2013; Frizzera *et al.*, 2022). Interestingly, feeding the bees with pollen deprived of both fractions (polar or apolar) resulted in a significantly reduced survival compared to bees fed with whole pollen, indicating the importance of both components. However, the absence of the apolar fraction, mainly composed of lipids, resulted in a limited albeit significant impact on bees survival, whereas, the absence of the polar fraction, including flavonoids, amino acids and vitamins, led to a

greater reduction in survival (28%), supporting the importance of this component and its key role for the beneficial effect of pollen.

The polar fraction of pollen contains several flavonoids including: rutin, quercetin and kaempferol Flavonoids are bioactive substances with antiviral, antibacterial, anti-oxidant properties (Duan *et al.*, 2019). Here we concentrated on quercetin, one of the most common flavonoids that are present in pollen.

In a second experiment, we further confirmed the positive effect of pollen and its polar fraction with a different approach (i.e. by addition of the aqueous extract rather than subtracting the polar fraction). Similar to the previous experiment we could not show the negative impact of DWV likely because of the selected dose and mode of infection.

At a dose similar to that expected in pollen (Almeida *et al.*, 2017), quercetin did not significantly increase the survival of caged bees. This could indicate that another component is more important (e.g. kaempferol (Rzepecka-Stojko *et al.*, 2015)) or that the whole mixture is needed to exert its positive effect.

3.5. Conclusion

Pollen appeared to be beneficial for virus infected bees and its positive effect depends both on the polar and apolar fraction in that the removal of any of the two components led to reduced survival compared to bees fed whole pollen. However, the lack of the polar fraction resulted in a greater effect on survival, indicating the importance of its components for honey bee health. Flavonoids are important components of the polar fraction and quercetin is one of the most abundant. However, at the dose tested here, quercetin did not increase survival and thus does not seem to be responsible of the observed beneficial activity of pollen. More experiments are needed to determine the identity of the polar compound/s accounting for the bioactivity of pollen; nevertheless, this experiment may lay the foundation for further dedicated studies on this remarkable topic.

CHAPTER 4 - **The effect of a mild intoxication in honey bees is modulated by concurring stress factors**

4.1. Introduction

Honey bees are exposed to a considerable variety of stress factors, of both biotic and abiotic nature (Potts *et al.*, 2010*a*; Goulson *et al.*, 2015); such factors interact with each other influencing honey bee health in ways that are not always predictable on the ground of the effect of single factors in isolation (Grassl *et al.*, 2018). Nevertheless, stress factors are usually tested individually or at most in pairs for easier experimental design. For instance, Kaunisto, Ferguson and Sinclair, (2016) showed that multiple stressor studies on insects are still relatively rare, in particular, they found only 133 full-factorial studies, fewer than ten studies included three stressor combinations, and none included more than three stressors.

In chapter 2 of this thesis, we reported how pollen and the xenobiotic nicotine affect bee health according to the season and thus viral load. Here we asked how the impact of nicotine (and pollen deprivation) on bee health can be modulated by other concurring stress factors.

For the selection of the further stressors to be tested in our fully factorial experiment, we tried to cover the most important classes of factors that are deemed responsible of both bee decline and insect rarefaction (Nazzi and Pennacchio, 2014; Wagner *et al.*, 2021). Recent studies on the factors implicated in bee decline (as well as insect decline) support the notion that four major classes are responsible: agrochemicals, lack of nutrients resulting from landscape deterioration, parasites and pathogens and adverse environmental conditions (Goulson *et al.*, 2015). Therefore, further than nicotine and pollen deprivation, we considered the most common ectoparasite of honey bees *V. destructor* and a temperature lower by two degrees to the normal internal hive temperature (Heinrich, 1981;Rosenkranz, Aumeier and Ziegelmann, 2010; Stabentheiner, Kovac and Brodschneider, 2010; Nazzi and Le Conte, 2016).

Varroa destructor is the most important ectoparasite of *A. mellifera*; its feeding activity causes several harmful effects on honey bees (Nazzi and Le Conte, 2016), debilitating the bee (De Jong, De Jong and Gonçalves, 1982; Annoscia *et al.*, 2019), promoting secondary infections, vectoring pathogens and facilitating their replication (de Miranda and Genersch, 2010; Vanikova *et al.*, 2015; Nazzi and Le Conte, 2016).

Temperature can also influence honey bees health. In fact, in regions with lower average temperatures higher colony losses are reported (vanEngelsdorp *et al.*, 2008; Johannesen *et al.*, 2022). This may be due to the direct effects of ambient temperature, or to the fact that more food is necessary for nest homeostasis when it is colder, leading to starvation in times of nectar shortage (vanEngelsdorp *et al*., 2008). To counteract low temperatures, honey bees can contract their thorax muscles (Heinrich, 1981) after consuming an adequate supply of honey (Rothe and Nachtigall, 1989).

In order to understand how a parasitic challenge and suboptimal environmental conditions interact with nicotine and pollen deprivation, we carried out a fully factorial experiment, involving, in addition to the previously studied factors (pollen and nicotine), an abiotic stressor (i.e. a lower than normal temperature) and a parasite (i.e. *V. destructor)*.

4.2. Materials and methods

4.2.1. Experimental procedure

Newly emerged adult bees were collected randomly from several colonies of the experimental apiary of the Dipartimento di Scienze AgroAlimentari, Ambientali e Animali of the University of Udine (46°04′53.3″ N, 13°12′33.1″ E). Previous studies indicated that honey bees from this area are hybrids between *A. mellifera ligustica* and *A. mellifera carnica* (Comparini and Biasiolo, 1991).

The bee colonies used in the trials were not treated against Varroa. To this purpose, each year, at the end of the experimental period (~ end of October), the surviving colonies of the experimental apiary are used to start new nuclei and treated with oxalic (5 cc per comb); if they survive the winter, they are used to establish new colonies for the following beekeeping season.

Under these conditions, mite infestation, as assessed by checking both adult bees and brood (see Nazzi et al., 2012), is around 5 mites/1000 bees in June, and gradually increases up to 250 mites/1000 bees in September/October.

The limited chemical treatments applied to the bees used in this experiment makes it rather unlikely the possibility that the detoxification system of bees was already under stress in the studied biological material.

For mite infestation, 5th instar bee larvae and mites were obtained from brood cells capped in the preceding 15 hours (Nazzi and Milani, 1994). Fifth instar larvae were transferred into gelatine capsules (Agar Scientific Ltd., 6.5 mm Ø) with one mite (V+, infested bees) or without any mite (V−, uninfested bees) (Figure 14).

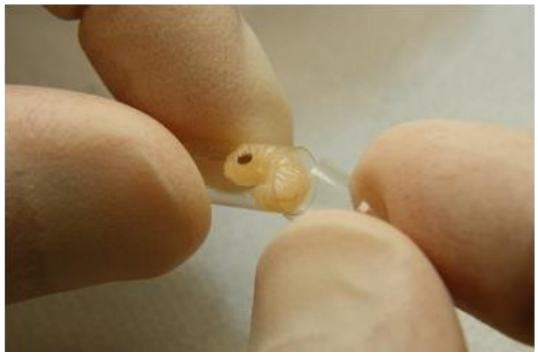

Figure 14. Artificial mite infestation: one V. destructor *female and an L5 bee larva are inserted together in a gelatine capsule.*

The infested and uninfested bees were maintained under controlled conditions (34.5 °C, 75% R.H., dark) for 12 days, until eclosion. Then, the emerging bees, previously separated from the infesting mite (if present), were transferred into 8 plastic cages (185 × 105 × 85 mm) per condition (V+ and V-). Every plastic cage contained about 25 - 30 honey bees. Half of the cages of each group (n=4 V+ and n=4 V-) were put in a different climate chamber. One chamber was set at 34.5 °C, 75% R.H., dark (T-, normal temperature), the other at 32 °C, 75% R.H., dark (T+, suboptimal temperature).

Pollen of *Amorpha fructicosa* (for more information regarding the choice of the pollen used in the experiment, see subchapter "2.1.2.4. Experimental plan") was delivered through an open Petri dish

(Ø = 3.5 cm) placed on the floor of the cages in half of the cages and changed once a week; 50 ppm nicotine were added to the sugar syrup in half of the cages.

Bees were fed with sugar syrup and water *ad libitum*. Sugar syrup was a solution made of 2.4 mol/L of glucose and fructose (61% and 31%, respectively) (Thom, Gilley and Tautz, 2003). Sugar syrup was supplied through 20 mL syringes and the diet was replaced every week.

Water was also dispensed to the bees through 20 mL syringes. Every day the cages were checked to count and remove the dead bees. The experiment was ended at day 45, when honey bees that were still alive were censored.

In total there were sixteen experimental groups (Table 4), as follows:

1. Climate chamber set at 34.5 °C:

- Uninfested honey bees fed with sugar syrup (PD);
- Uninfested honey bees fed with sugar syrup and pollen (control);
- Uninfested honey bees fed with sugar syrup containing 50 ppm of nicotine (PD+N);
- Uninfested honey bees fed with sugar syrup containing 50 ppm of nicotine and pollen (N);
- Infested honey bees fed with sugar syrup (V+PD);
- Infested honey bees fed with sugar syrup and pollen (V);
- Infested honey bees fed with sugar syrup containing 50 ppm of nicotine (V+PD+N);
- Infested honey bees fed with sugar syrup containing 50 ppm of nicotine and pollen (V+N).

2. Climate chamber set at 32 °C:

- Uninfested honey bees fed with sugar syrup (PD+T);
- Uninfested honey bees fed with sugar syrup and pollen (T);
- Uninfested honey bees fed with sugar syrup containing 50 ppm of nicotine (PD+N+T);
- Uninfested honey bees fed with sugar syrup containing 50 ppm of nicotine and pollen (N+T);

- Infested honey bees fed with sugar syrup (V+PD+T);

- Infested honey bees fed with sugar syrup and pollen (V+T);

- Infested honey bees fed with sugar syrup containing 50 ppm of nicotine (V+PD+N+T);

- Infested honey bees fed with sugar syrup containing 50 ppm of nicotine and pollen (V+N+T).

Experimental group	Pollen	Nicotine	Varroa	Pollen deprivation	Temperature
PD				✓	
Control	✓				
PD+N		✓		✓	
N	✓	✓			
V+PD			✓	✓	
V	✓		✓		
V+PD+N		✓	✓	✓	
V+N	✓	✓	✓		
PD+T				✓	✓
T	✓				✓
PD+N+T		✓		✓	✓
N+T	✓	✓			✓
V+PD+T			✓	✓	✓
V+T	✓		✓		✓
V+PD+N+T		✓	✓	✓	✓
V+N+T	✓	✓	✓		✓

Table 4. Experimental group present in this experiment

The experiment was replicated three times late in the season (August - September).

4.2.2. Assessment of viral infection level

qRT-PCR analysis of viral infection was carried out as follows:

Eighteen 6-days-old bees were sampled in liquid nitrogen and transferred in a −80 °C refrigerator at each replication. After defrosting of samples in RNA later, the gut of each honey bee was eliminated to avoid the congestion of the mini spin columns. The bodies of sampled bees were manually homogenized using a pestle, mortar and liquid nitrogen. RNA extractions were performed with Rneasy® Plus Mini Kit (Qiagen) and the provided protocol. The amount of RNA in each sample was quantified with a NanoDrop® spectrophotometer (ThermoFisher™, USA). cDNA was synthetized

starting from 500 ng of RNA following the manufacturer specifications (PROMEGA, Italy). Additional negative control samples containing no RT enzyme were included. 10 ng of cDNA from each sample were analysed using Master mix SYBRTM green (AppliedBiosystemsTM, US) according to the manufacturer specifications, on a BioRad CFX96 Touch™ Real time PCR Detector. All samples were run in triplicate. The thermal cycling profiles was: one cycle at 95 °C for 10 minutes, 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute, and one cycle at 68 °C for 7 minutes.

We considered as positive all samples with a C_t value lower than 30. DWV Forward (GGTAAGCGATGGTTGTTTG) and DWV Reverse (CCGTGAATATAGTGTGAGG) (Mondet *et al.*, 2014) were the primers used. β-actin was used as reference gene (Forward: GATTTGTATGCCAACACTGTCCTT; Reverse: TTGCATTCTATCTGCGATTCCA(Di Prisco *et al.*, 2016).

4.2.3. Graphical representation of data

A fully factorial experiment with four factors and 16 experimental groups, generates a large amount of data whose interpretation can be difficult; to facilitate this task we adopted the graphical representation described below.

We placed the control group in the centre of the four axes of a Cartesian space with the axes x, y, z, t representing the direction of change of the four stressors tested in the multifactorial experiment (Figure 15).

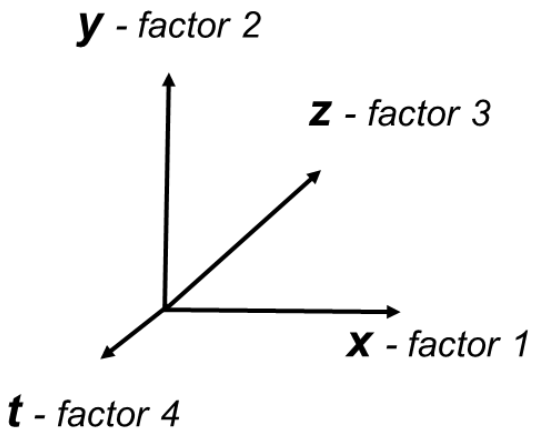

Figure 15. Graphical representation of the four stressors in a Cartesian space.

Then we created the "hypercube" depicted in Figure 16. This model consists of two cubes; at the corners of the cubes the median survival of each experimental group, that is based on the pooled data from the three replications, is reported.

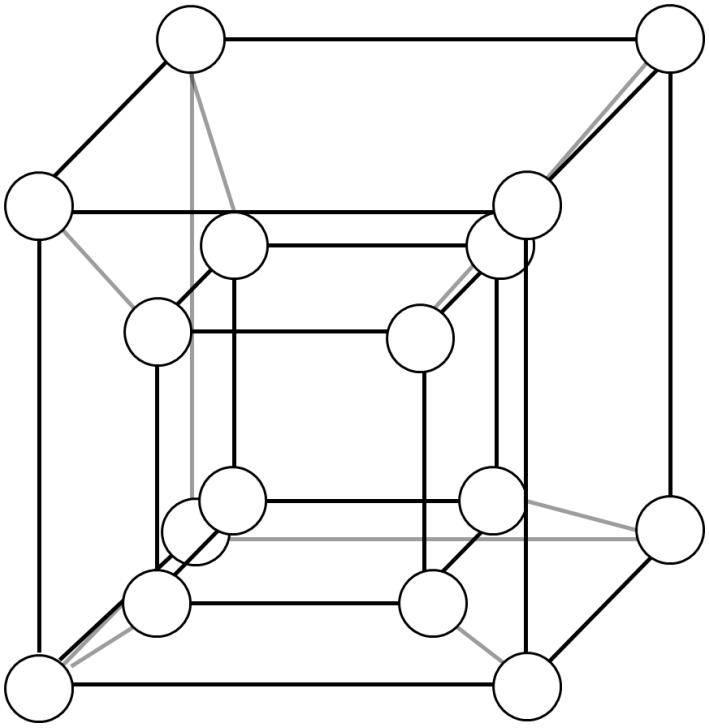

Figure 16. The hypercube used to synthesize the results of the factorial experiment.

In this way, starting from the bottom left vertex of the internal cube, representing the median survival of control bees, and moving along the edges or the diagonals of the hypercube the effect of each factor or their binary, ternary and quaternary combinations on bee survival can be recognized.

4.2.4. Statistical analysis

We first tested the effect of each single factor as compared to the control; this was done by means of a log-rank test on bee survival data using the pooled data from the three replicates. The problem of multiple comparisons was solved according to Benjamini and Hochberg (1995), setting the false discovery rate (Q) to 0.1. These statistical analyses were performed with GraphPad Prism©.

To test the effect of each factor in combination with all the others as well as all the interactions we carried out an analysis of variance which was performed with Minitab 16®, after the data normalisation with logarithm.

4.3. Results

This experiment allowed to study the effect of four stress factors alone and in combination with each other on the bee survival under controlled laboratory conditions (Figure 17).

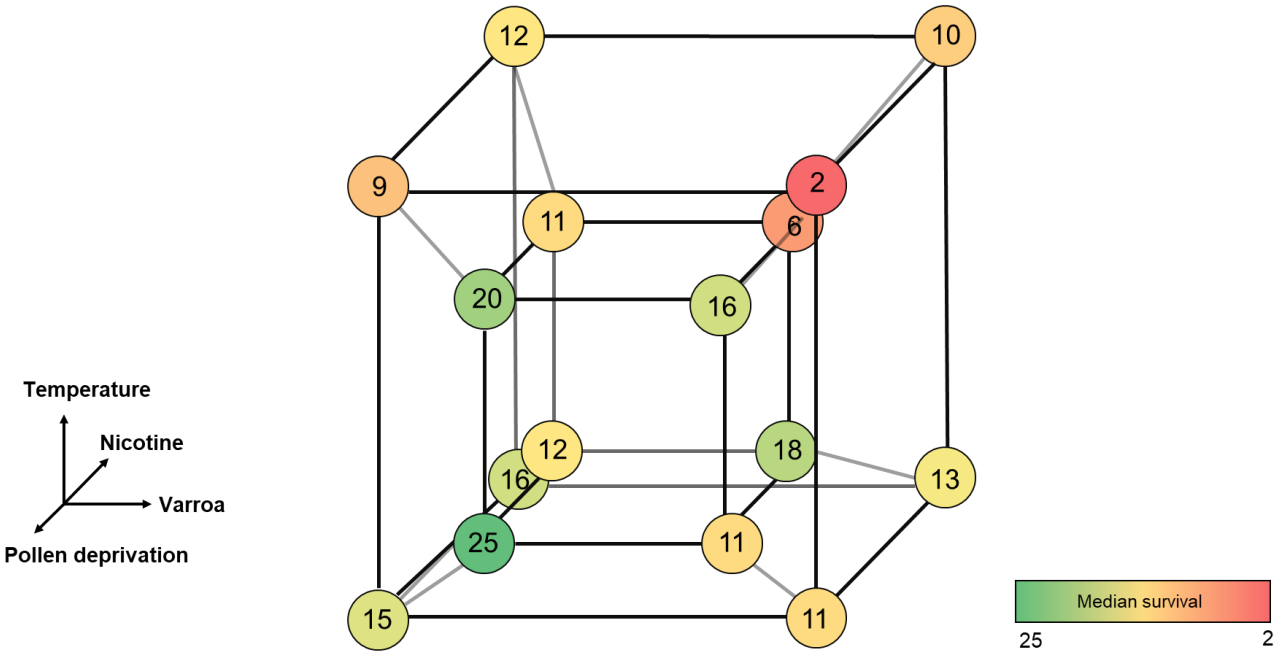

Figure 17. Graphical representation of the results of a four factors factorial experiment, testing the effect of the following stressors: Varroa infestation, intoxication with nicotine, pollen deprivation and a low temperature. The median survival of the honey bees belonging to each experimental group is represented on the vertices of the hypercube and highlighted with a colour indicating the deviation from the control group, represented in green at the bottom left corner of the inner cube. To see the effect of a tested factor, alone and in combination with the others, one should move along the respective edge.

Only 3 out of 18 samples, collected 5 days after the emergence, showed a C_t value lower than 30, indicating that the prevalence of the virus when the experiment was conducted was high (83%).

We first examined the effect of each single stressor on bee health; this is done by comparing the control group with each single stress treated group.

Mite infestation significantly reduced the survival of honey bees (median survival of mite infested bees = 11.0, median survival of uninfested bees = 25.0; Log-rank test: Chi-square = 18.69, d.f. = 1, P = <0.0001; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0250; P<(i/m)Q = 0; significance = confirmed). Instead, an environmental temperature lower by only 2 °C with respect to the internal hive temperature didn't significantly reduce the survival of honey bees (median survival of control bees = 25.0, median survival of bees exposed to a low temperature = 20.0; Log-rank test: Chi-square = 1.733, d.f. = 1, P = 0.1880; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.1000; P<(i/m)Q

= 0; significance = confirmed). In contrast, pollen deprivation significantly reduced the survival of bees by 40% (median survival of control bees = 25.0, median survival of nutritionally stressed bees = 15.0; Log-rank test: Chi-square = 15.49, d.f. = 1, P = <0.0001; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0250; P<(i/m)Q = 0; significance = confirmed). Finally, nicotine with pollen significantly reduced the survival of bees (median survival of control bees = 25.0, median survival of bees fed with a contaminated diet = 12.0; Log-rank test: Chi-Square = 11.06, d.f. = 1, P = 0.0009; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.0750; P<(i/m)Q = 0; significance = confirmed).

Next, we considered the effect of each stressor when applied together with any of the other factors. In this way we wanted to assess if any stressor is harmful under all or most circumstances. For this purpose, we applied an analysis of variance, after the data normalisation with logarithm. Graphically this is done by comparing the two halves of the hypercube, obtained by cutting the cube with a plane perpendicular to the axis along which the factor of interest varies. If a difference between the two half cubes is noted, we can assume a generally negative effect of that stressor, because shorter survival is observed in bees exposed to that stressor both in presence and not of three other stressors of different quality. A significant effect associated to that factor in the analysis of variance can corroborate this visual impression.

Varroa effect: this is noted by dividing the hypercube in half, with the vertical plane α as reported in Figure 18. A decrease by 27% is observed when moving from left to right, relative to the plane α; the average median survival of uninfested bees, in the left half cube, being 15.0 as compared to the average median survival of mite infested bees in the right half cube which was 10.9 (Figure 18 and Table 5; V+ vs. V-, F = 36.51, P = 0.000).

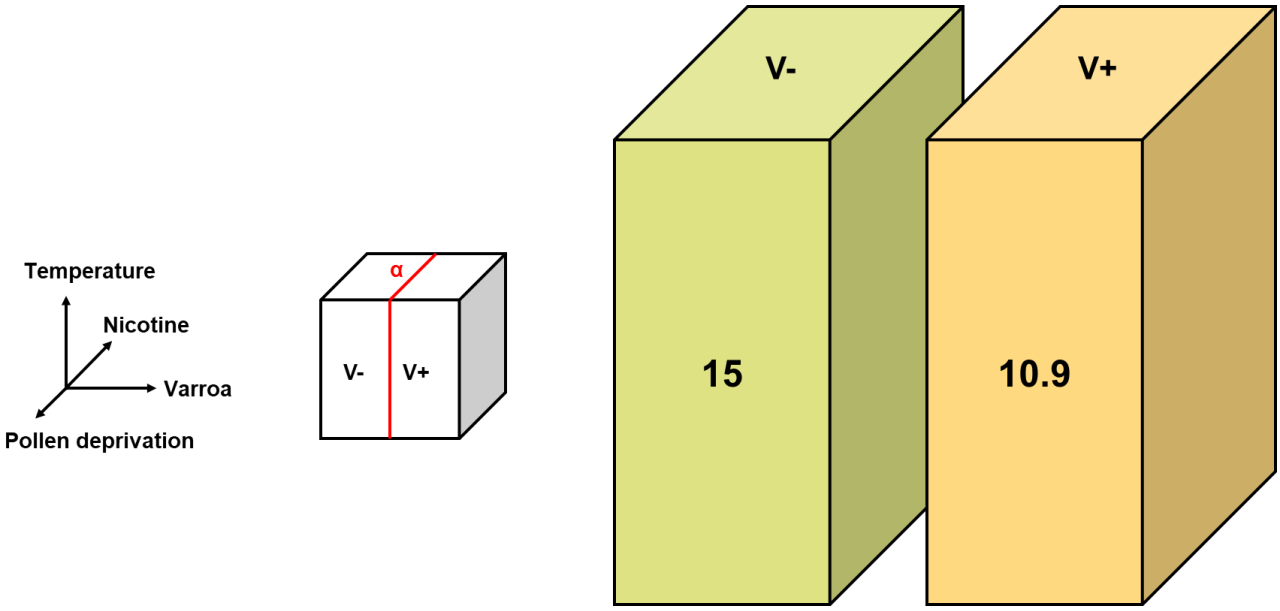

Figure 18. Varroa effect. The two half cubes obtained dividing the hypercube with plane α include all the experimental groups infested (V+) or not infested (V-) by the mite, respectively.

Low temperature effect: this is noted by dividing the hypercube with the horizontal plane β (Figure 19). A 28% decrease in bee survival is noted comparing the bottom half cube, including bees maintained at the optimal temperature, with the upper one, including the experimental groups exposed to the lower temperature; the average median survival of bees exposed to normal temperature (34.5 °C) was 15.1 while the average median survival of bees exposed to the lower temperature (32 °C) was 10.8 (Figure 19 and Table 5; T+ vs. T-, F = 28.38, $P = 0.000$).

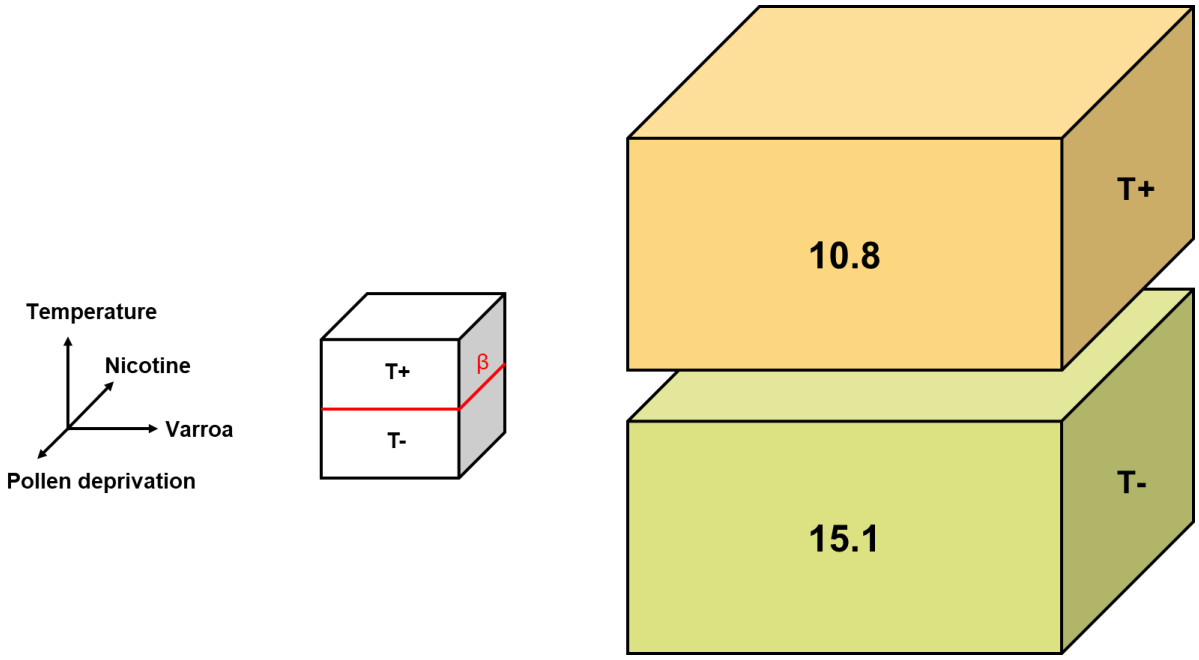

Figure 19. Low temperature effect. The two half cubes obtained dividing the hypercube with plane β include all the experimental groups exposed to low temperature (T+) or not (T-), respectively.

Pollen deprivation effect: this is noted by comparing the internal cube with the external one (Figure 20). A 23% decrease in bee survival is noted comparing the internal cube, including pollen fed bees, with the outer one, including the groups of bees that did not receive pollen; the average median survival of pollen fed bees was 14.9 while the average median survival of bees that did not receive pollen was 11.4; however, the effect of pollen deprivation was not statistically significant (Figure 20 and Table 5; PD+ vs. PD-, F = 0.14, $P = 0.713$).

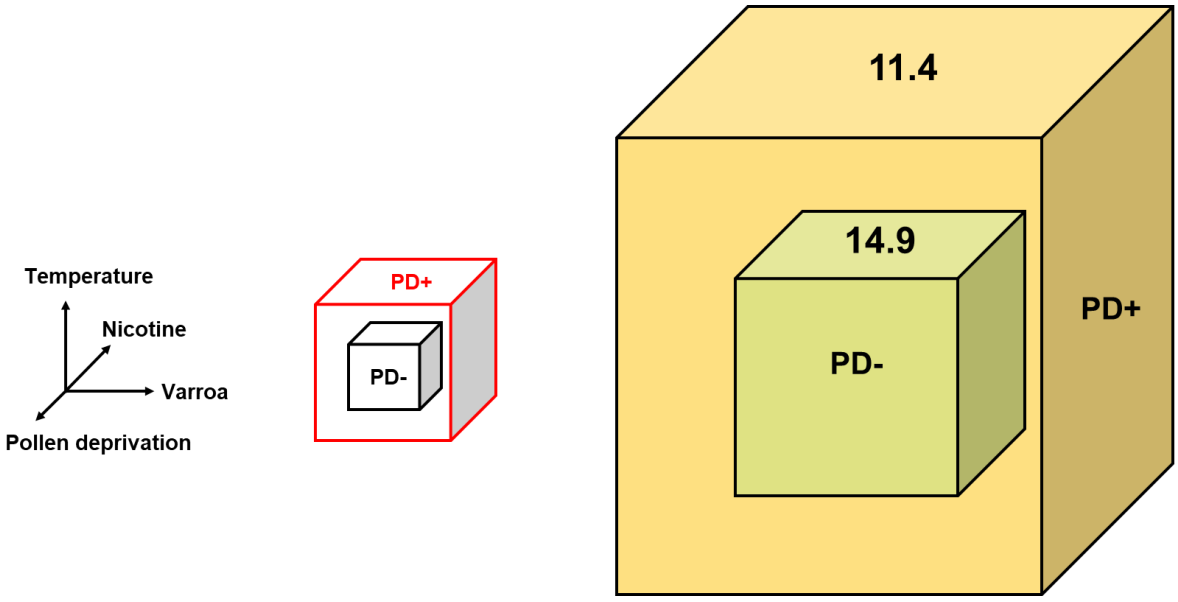

Figure 20. Pollen deprivation effect. The inner cube includes all the experimental group of pollen fed bees (PD-) while the outer cube includes all the experimental group of bees that did not receive pollen (PD-).

Nicotine effect: this is noted by dividing the hypercube in half, with the vertical plane γ as reported in Figure 21. A decrease by 10% is observed comparing the front half cube, including bees fed with an untreated diet, with the back one, including the groups of nicotine fed bees. The average median survival of bees fed with an untreated diet, in the front half cube, was 13.6; the average median survival of nicotine fed bees in the back half cube was 12.3 but the effect of nicotine was not statistically significant (Figure 21 and Table 5; N+ vs. N-, F = 0.15, $P = 0.702$).

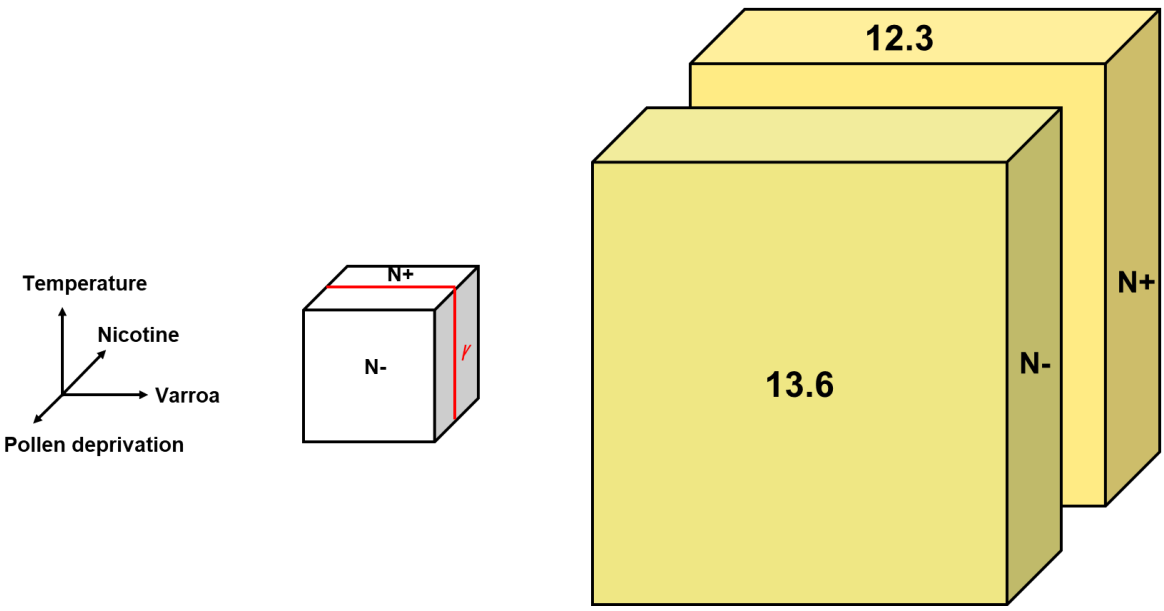

Figure 21. Nicotine effect. The two half cubes obtained dividing the hypercube with plane γ include all the experimental groups of bees fed with nicotine (N+) or not (N-), respectively.

We next studied the binary interactions between factors. In particular, we focused on binary interactions involving nicotine, which was studied in detail in chapter 2. As for the statistics this is done with ANOVA (Table 5); graphically, these effects can be identified by further dividing the halves of the hypercube obtained as before in quarter cubes highlighting the effect of the second factor on bees exposed or not to the first. The binary interactions are revealed by quarter cubes differing more in presence of the second factor than when this is not present.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	17	9750.4	573.55	6.53	0.000
Blocks	2	1434.6	717.28	8.17	0.000
Linear	4	4091.3	1022.82	11.65	0.000
Varroa	1	2469.1	2469.15	28.13	0.000
Nicotina	1	102.2	102.21	1.16	0.281
Temperatura	1	1200.6	1200.55	13.68	0.000
Pollen deprivation	1	305.9	305.85	3.48	0.062
2-Way Interactions	6	3171.2	528.53	6.02	0.000
Varroa*Nicotina	1	986.5	986.51	11.24	0.001
Varroa*Temperatura	1	59.3	59.29	0.68	0.411
Varroa*Pollen deprivation	1	91.0	91.04	1.04	0.309
Nicotina*Temperatura	1	27.9	27.88	0.32	0.573
Nicotina*Pollen deprivation	1	1780.7	1780.71	20.29	0.000
Temperatura*Pollen deprivation	1	193.8	193.79	2.21	0.138
3-Way Interactions	4	794.6	198.64	2.26	0.061
Varroa*Nicotina*Temperatura	1	20.1	20.12	0.23	0.632
Varroa*Nicotina*Pollen deprivation	1	188.0	188.04	2.14	0.144
Varroa*Temperatura*Pollen deprivation	1	46.5	46.48	0.53	0.467
Nicotina*Temperatura*Pollen deprivation	1	543.8	543.80	6.20	0.013
4-Way Interactions	1	290.0	289.97	3.30	0.070
Varroa*Nicotina*Temperatura*Pollen deprivation	1	290.0	289.97	3.30	0.070
Error	690	60563.7	87.77		
Lack-of-Fit	30	10274.7	342.49	4.49	0.000
Pure Error	660	50289.0	76.20		
Total	707	70314.0			

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	11.791	0.366	32.22	0.000	
Blocks					
1	-1.374	0.481	-2.85	0.004	1.17
2	1.861	0.501	3.71	0.000	1.17
Varroa					
Yes	-1.892	0.357	-5.30	0.000	1.02
Nicotina					
Yes	-0.384	0.356	-1.08	0.281	1.02
Temperatura					
Yes	-1.317	0.356	-3.70	0.000	1.02
Pollen deprivation					
Yes	-0.666	0.357	-1.87	0.062	1.02
Varroa*Nicotina					
Yes Yes	1.193	0.356	3.35	0.001	1.02
Varroa*Temperatura					
Yes Yes	-0.292	0.356	-0.82	0.411	1.02
Varroa*Pollen deprivation					
Yes Yes	-0.362	0.356	-1.02	0.309	1.02
Nicotina*Temperatura					
Yes Yes	-0.201	0.356	-0.56	0.573	1.02
Nicotina*Pollen deprivation					
Yes Yes	1.603	0.356	4.50	0.000	1.02
Temperatura*Pollen deprivation					
Yes Yes	-0.529	0.356	-1.49	0.138	1.02
Varroa*Nicotina*Temperatura					
Yes Yes Yes	-0.170	0.356	-0.48	0.632	1.02
Varroa*Nicotina*Pollen deprivation					
Yes Yes Yes	-0.521	0.356	-1.46	0.144	1.02
Varroa*Temperatura*Pollen deprivation					
Yes Yes Yes	0.259	0.356	0.73	0.467	1.02
Nicotina*Temperatura*Pollen deprivation					
Yes Yes Yes	0.886	0.356	2.49	0.013	1.02
Varroa*Nicotina*Temperatura*Pollen deprivation					
Yes Yes Yes Yes	0.647	0.356	1.82	0.070	1.02

Table 5. Results of the analysis of variance.

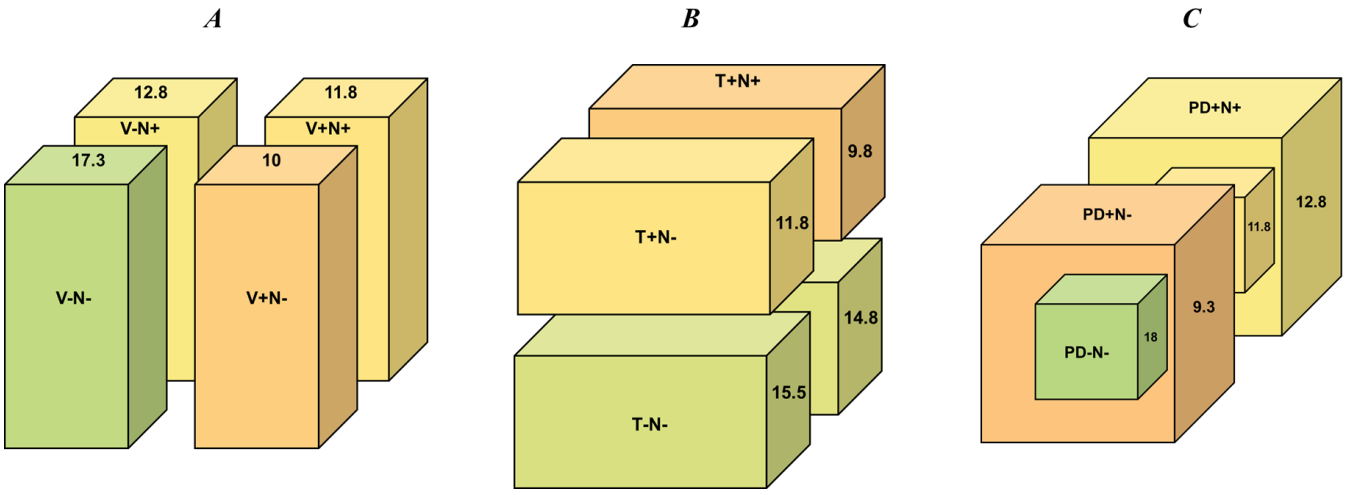

Figure 22. Binary interactions with nicotine of the following factors: Varroa (A), low temperature (B); pollen deprivation (C).

The following significant interactions were revealed by ANOVA: Varroa x nicotine (Table 5; F = 12.57, *P* = 0.000), pollen deprivation x nicotine (Table 5; F = 29.65, *P* = 0.000), temperature x Varroa (Table 5; F = 4.20, *P* = 0.041) and nicotine x temperature x pollen deprivation (Table 5; F = 6.62, *P* = 0.010). Interestingly, while the effect of nicotine was not significant when considered in isolation, a significant interaction with both Varroa and pollen deprivation was noted. In particular, it appeared

that nicotine can reduce the effect of Varroa (as highlighted by the higher similarity between the half cubes in the right hand side of the hypercube in Figure 22A), while nicotine can aggravate that of a low temperature (as highlighted by the higher diversity between the half cubes in the top of the hypercube in Figure 22B).

4.4. Discussion

This experiment allowed to assess the impact of four different stress factors, alone and in combination with each other. These factors were chosen from the following categories: parasites, xenobiotics, lack of an adequate nutritional supply and adverse environmental conditions.

Individually, both Varroa infestation, pollen deprivation and nicotine negatively impacted bee survival.

The negative effect of Varroa infestation is well known (Nazzi and Le Conte, 2016) and largely depends on the pathogenic virus DWV transmitted and facilitated by the mite (Nazzi *et al.*, 2012; Annoscia *et al*., 2019). Moreover, the mite causes water and weight losses (Annoscia, Del Piccolo and Nazzi, 2012), behavioural modifications (Annoscia *et al*., 2015) and accelerated behavioural maturation (Downey, Higo and Winston, 2000; Zanni *et al.*, 2018).

Pollen can influence individual and colony development, affecting the longevity of bees and their immunocompetence (Haydak, 1970; Alaux *et al.*, 2010; DeGrandi-Hoffman *et al.*, 2010). Therefore, a decreased survival in the absence of this important nutrient was expected and confirmed here.

Also, the negative impact of nicotine was expected in this experiment which was carried out late in the season thus matching the condition of the experiment reported in chapter 2 of this thesis. Late in the season, in presence of a higher viral load (in this case a qRT-PCR analysis of viral infection demonstrated a DWV prevalence of 83%), the bees are already debilitated (de Miranda and Genersch, 2010; Grozinger and Flenniken, 2019) and an additional stressors, here represented by the toxic

alkaloid nicotine, that must be detoxified (Rand *et al.*, 2015), may further aggravate bee health conditions.

Surprisingly, the effect of a low temperature did not reach statistical significance. This could be explained by the fact that these bees had pollen at their disposal, a very important nutritional source. Indeed, Frizzera *et al*. (in press) report a significant decrease in survival in no pollen fed bees maintained at 32°C compared to those maintained at the normal temperature of 34°C.

As for the study of the general effect of the four factors studied here, our data indicate that the Varroa mite exerts an effect that is constantly negative, regardless of the number and identity of the other concurring stressors. Similarly, a lower-than-normal temperature decreased survival regardless of the other concurring stressors. This last result is interesting especially considering the lack of a negative effect when low temperature was tested in isolation. This supports once again the multifactorial origin of bee losses. On the other hand, we did not observe a general statistically significant impact of pollen deprivation and the alkaloid nicotine, likely because the effect of these further stressors is largely influenced by the others. The lack of a general effect of pollen deprivation could depend on the fact that pollen and nicotine interact with each other as suggested in chapter 2 of this thesis and demonstrated here by the significance of the interaction pollen deprivation x nicotine. For a more detailed discussion about this subject see chapter 2 of this thesis.

The significant interaction between nicotine and Varroa may be related to an antiviral action of nicotine. Indeed, Varroa mite vectors and triggers DWV infection (Bowen-Walker, Martin and Gunn, 1999; Nazzi *et al.*, 2012) and it was shown that nicotine has an antiviral effects on hepatitis C (Yamashina *et al.*, 2008). In addition, another study conducted by Köhler, Pirk and Nicolson in 2012 reported a lower survival in weak honey bees colonies fed with sugar-only diets as compared to those fed with nicotine enriched diets; in that case, the authors speculated that the weak group was infected with a viral disease transmitted by Varroa or weakened from excessive feeding by the mites during

early honeybee development. Furthermore, other alkaloids were shown to negatively affect *Crithidia bombi*, a bumblebees' parasite (Manson, Otterstatter and Thomson, 2010; Richardson *et al*., 2015).

The significant interaction between Varroa and temperature matches with the correlation between winter temperature and colony losses reported by vanEngelsdorp *et al*., (2008) but will not be discussed in detail here because of the focus on toxic compounds of this dissertation.

Finally, the statistical test identified a significant interaction between nicotine x temperature x pollen deprivation which could also be considered as a side effect of the already discussed interaction nicotine x pollen deprivation.

4.5. Conclusion

The above described fully factorial experiment allowed us to study the effect on bee survival of four stress factors here considered both alone and in combination with each other. One of the main achievements include the identification of some interesting interactions between Varroa mite infestation and the other stressors to which bees are normally exposed. Among these interactions that one between mite infestation and a temperature slightly lower than optimal is particularly interesting in that it may shed light on the observed upsurge of colony losses during autumn and winter months. However, an interesting interaction between mite infestation and the alkaloid nicotine was also noted, suggesting how this substance could mitigate the detrimental effect of Varroa infestation. This is an important piece of information in view of the crucial role played by Varroa and the lack of effective mitigation measures against parasitic infections in honey bees.

Finally, the experiments highlighted a positive interaction between pollen deprivation and nicotine, an interesting result in light of what was discussed in Chapter 2.

In conclusion, an experimental plan such as the one adopted in this study, has proven to be a powerful tool to support the analysis of the multiple stressors affecting honey bee colonies in their environment;

indeed, it allowed us to highlight interactions that could not be assessed from the simple study of individual stressors. Such approach may be adopted in the future to study the effects of more stressors on complex organisms as honey bee colonies and other eusocial insects.

The further data obtained through the factorial experiment reported above can be incorporated into the conceptual model presented in chapter 2 of this thesis (Figure 23).

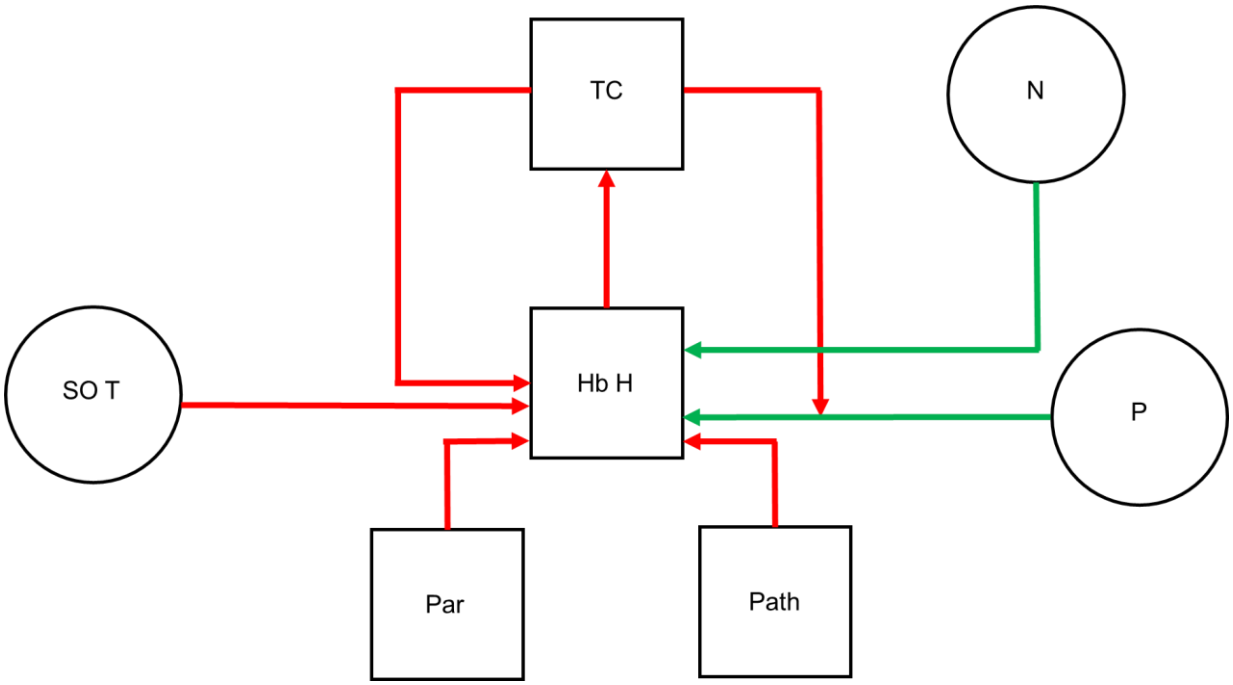

Figure 23. The conceptual model of interactions has improved with the results of the four factors factorial experiment. In this figure the negative contribution of Varroa mite infestation (Par) was added as well as an arrow denoting the negative effect of a sub optimal temperature (SO T) on honey bee health.

CHAPTER 5 - **A deeper understanding of system interactions can explain contradictory field results on pesticide impact on honey bees**

nature communications

Article

https://doi.org/10.1038/s41467-022-33405-7

A deeper understanding of system interactions can explain contradictory field results on pesticide impact on honey bees

Received: 24 February 2022

Accepted: 16 September 2022

Published online: 29 September 2022

Check for updates

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While there is widespread concern regarding the impact of pesticides on honey bees, well-replicated field experiments, to date, have failed to provide clear insights on pesticide effects. Here, we adopt a systems biology approach to gain insights into the web of interactions amongst the factors influencing honey bee health. We put the focus on the properties of the system that depend upon its architecture and not on the strength, often unknown, of each single interaction. Then we test in vivo, on caged honey bees, the predictions derived from this modelling analysis. We show that the impact of toxic compounds on honey bee health can be shaped by the concurrent stressors affecting bees. We demonstrate that the immune-suppressive capacity of the widespread pathogen of bees, deformed wing virus, can introduce a critical positive feed-back loop in the system causing bistability, i.e., two stable equilibria. Therefore, honey bees under similar initial conditions can experience different consequences when exposed to the same stressor, including prolonged survival or premature death. The latter can generate an increased vulnerability of the hive to dwindling and collapse. Our conclusions reconcile contrasting field-testing outcomes and have important implications for the application of field studies to complex systems.

Losses in honey bee colonies have been reported since the beginning of modern apiculture[1], but the scale of these events has increased dramatically[2]. These losses potentially affect pollination services and food sustainability[3] and are therefore a cause for concern. Losses are multifactorial with several interacting stress factors affecting honey bee health leading to potential cascade effects on colony stability[4]. Some of the factors which significantly contribute to colony losses are parasites and pathogens, agrochemicals, forage resource availability, and environmental conditions such as external temperature[5].

Pesticides, and in particular neonicotinoid insecticides, have attracted considerable attention for their potential negative effects on pollinators including honey bees[6]. These compounds have both lethal and sublethal effects on bees, affecting navigation, immunity, and reproduction[7–9]. However, even though the negative effects of neonicotinoid insecticides have been established in the laboratory[6], field testing has resulted in contradictory outcomes (Supplementary Table 1). No detectable negative effects were reported on honey bees maintained near Clothianidin-treated oilseed rape fields in some countries[10–12], whereas in a large-scale experiment spanning three

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European countries both negative and positive effects were noted[13]. The lack of negative results observed in some cases has been attributed to the buffering capacity of honey bee colonies[12,14] but the reason why such buffering capacity could prevent apparent harm under certain conditions, but not others, remains unclear. The variability in the contexts where the studies were carried out, involving both the possible stress factors and the quantity-quality of available nutrition as well as the availability of other foraging resources in turn affecting the exposure to the pesticide applied to the focal crop, certainly plays a role. However, this plausible explanation lacks the necessary robustness in cases where the absence of evidence has often been regarded as evidence of absence. In fact, after several high-profile well-replicated experiments, the regulation across countries and regions with otherwise similar situations appears different in each. For example, in Europe, the neonicotinoids Clothianidin, Imidacloprid, and Thiamethoxam have been banned in open fields since 2018. While Canada banned the use of neonicotinoids on bee attractive crops in 2019 but still allows other uses including seed treatment and in the US a review on the same chemicals is still in progress. The situation is related to several factors, these include a different interpretation of the precautionary principle, economics, and politics. However, the consistency of scientific evidence provided to support such decisions may have played a role.

To gain a mechanistic understanding of the processes underlying the contrasting field results regarding pesticide harm to honey bees, we adopted a systems biology approach. Based on theoretical and computational parameter-free methodologies[15,16] we assessed the structural properties of the biological system under study (i.e., honey bee health as affected by various factors). These are properties that exclusively rely on the architecture of the system and are independent of the strength, which is often unknown, of each interaction (i.e., any relationship between two components of the system). Structural approaches can provide qualitative insight into complex webs of interactions, even in the absence of knowledge about parameter values, and unravel the synergistic net effect of multiple stressors on bee health. Through these methods we showed, first in theory and then in vivo, how the impact of toxic compounds on honey bee health and colony stability can be shaped by the concurrent stressors affecting bees, eventually leading to multiple outcomes depending on initial conditions.

Results

A conceptual model of honey bee health

The conceptual model of stressors and drivers potentially affecting honey bee health was built from available data (Fig. 1; Supplementary Table 2). This model describes the health of honey bees as influenced by multiple stress factors and effects. These include: (a) ectoparasites such as the mite *Varroa destructor*[17], (b) viral pathogens like the deformed wing virus (DWV)[18], (c) toxic compounds[19], among which neonicotinoid insecticides appear to play a pre-eminent role[6], and adverse environmental factors, in particular (d) sub-optimal thermal conditions[20]. Sugars from nectar (e) and pollen (f) are used by bees as a source of energy and proteins and promote honey bee health[21]. Both nectar and pollen can however be contaminated with toxic compounds (g, h)[22,23]. Honey bees invoke a number of mechanisms to combat stress factors; in particular, an immune response is normally activated to counter parasites (i)[24] and pathogens (j)[25], and a detoxification system (k) can reduce the concentration of toxic compounds[26]. Honey bees can increase sugar feeding to counteract low temperatures (l)[27]. However, this increased feeding may then expose bees to higher contamination with toxic compounds. Some of the factors themselves can influence honey bee homeostatic responses; DWV in particular can impair the immune response (m)[28], which can likewise be reduced by some toxic compounds (n)[7]. Mite-infested honey bees may consume less sugar (o)[29]. We also cannot discount that lower temperatures may have a potentially negative effect on parasites (p)[30].

Many more stressors, including more than twenty viruses, a plethora of toxic substances, several parasites, and a countless combination of environmental factors may influence bee survival[4]. However, as far as our analysis is concerned, the proposed representation of the system already captures all the relevant qualitative interactions, irrespective of the specific identity of the stressors involved and the quantitative details. For example, we included just one toxic compound, even though many pesticides can impact honey bees at the same time[31] and can interact with one another, as in the case of fungicides increasing the toxicity of insecticides[32–34]. Our model may thus be seen as an oversimplification of the system under study. This would be the case if our objective were to derive a descriptive model aiming

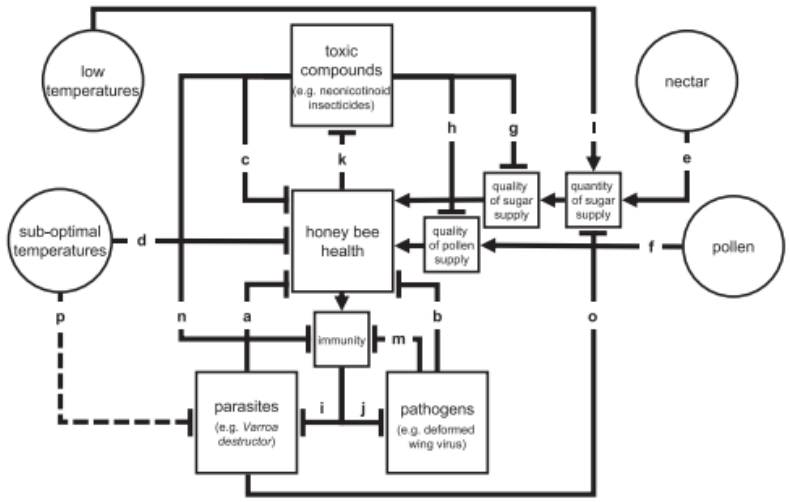

Fig. 1 | The health of honey bees as influenced by multiple factors and their effects. In the conceptual model of bee health bar-headed lines denote negative effects while arrow-headed lines indicate positive ones. See text and Supplementary Table 2 for explanation of lettered effects.

at quantifying bee health at any given time, in the presence of a defined level of certain stressors. However, for the structural analysis of the network of qualitative interactions we carried out, the case of one toxic compound exerting a negative effect, or that of more toxic compounds interacting with one another to exert an even bigger negative effect on honey bees, are equivalent because the sign of the effect is the same. Similarly, flowering resources can impact the way pesticide use affects bees either by deterring them from treated plants or by altering their pesticide tolerance[35–38]. However, such effects would correspond to a lower or higher impact of toxic compounds which have already been incorporated into the model. Thus, in both cases, the outcome of the analysis is not affected by this modeling choice.

According to our conceptual model, the dynamic interplay between honey bee health and the surrounding environment can be described by the following system of ordinary differential equations.

$$\begin{aligned}\tau_{HB}\dot{x}_{HB} = &-\delta_{HB}x_{HB} + g_{TC}(x_{TC}) + g_{VA}(x_{VA}) + g_{VI}(x_{VI}) \\ &+ \bar{f}_{S,C}(u_S, u_C, x_{TC}, x_{VA}) + \bar{f}_P(u_P, x_{TC}) + \underline{f}_{HB}(u_T)\end{aligned} \quad (1)$$

$$\tau_{TC}\dot{x}_{TC} = -\delta_{TC}x_{TC} + g_{HB}(x_{HB}) \quad (2)$$

$$\tau_{VA}\dot{x}_{VA} = -\delta_{VA}x_{VA} + h_{VA}(x_{HB}, x_{TC}, \varepsilon x_{VI}) + \underline{f}_{VA}(u_T) \quad (3)$$

$$\tau_{VI}\dot{x}_{VI} = -\delta_{VI}x_{VI} + h_{VI}(x_{HB}, x_{TC}, \varepsilon x_{VI}) \quad (4)$$

These equations mathematically represent the interactions among the key components (variables) in our conceptual model: honey bee health (x_{HB}), the stress due toxic chemicals (x_{TC}), the stress due to parasites (x_{VA}), and the stress due to pathogens (x_{VI}). The system includes the effects of the external inputs: sugar u_S, pollen u_P, absolute deviation from desired temperature u_T and sub-optimal temperature u_C. The coefficients τ denote the time constants, δ denote the "self-control" of each key-player. All inputs (and possible parameters, e.g., ε) are non-negative. All variables and inputs exert their influence on the variation of the other variables (denoted by a dot on the variable's name) by means of different functions (i.e., $g(x)$, $\bar{f}(x)$, $\underline{f}(x)$, $h(x)$). Functions can be decreasing, in case of negative effects (e.g., function $g_{TC}(x_{TC})$ in Eq. (1), representing arrow c in Fig. 1, indicates that the more toxic compounds x_{TC}, the lower honey bee health x_{HB}). Functions can also be increasing/decreasing according to the variable or input considered (e.g., function $\bar{f}_P(u_P, x_{TC})$ in Eq. (1), representing arrow f in Fig. 1; the function is increasing with respect to u_P, because the more pollen the higher honey bee health, but is decreasing with respect to x_{TC}, because toxic compounds can contaminate the pollen and thus cause a negative effect on honey bee health (see arrow h in Fig. 1)). A detailed description of the various functions, together with a summary of the biological effects they account for and a reference to the conceptual model in Fig. 1, is reported in Supplementary Table 3.

Equation (1) shows that honey bee health (x_{HB}) is self-regulated by internal physiological mechanisms described by δ_{HB}. Also, honey bee health can be negatively influenced by toxic compounds (x_{TC}), parasites (x_{VA}), and pathogens (x_{VI}), according to various mechanisms described by different monotonically decreasing functions (i.e., g_{TC}, g_{VA}, g_{VI}), denoted with the common symbol g because each factor exerts a negative effect on honey bee health. Similarly, honey bee health is affected by other factors (e.g., nutrition, represented by the external inputs u_S and u_P; sub-optimal temperatures u_T and low temperatures u_C), whose influence can be modified by other stress factors (e.g., toxic compounds that can contaminate foodstuff). These interactions are represented by functions that are increasing in the case of favorable influences and decreasing in the case of adverse effects.

Structural analysis of the bee health model

The structure of the system under study (i.e., honey bee health as affected by various factors) was analyzed using the concept of community matrix[39]. The community matrix, whose elements represent the effects of each factor onto every other and itself at equilibrium, formally encapsulates the interactions among the components of an ecological system and corresponds to the Jacobian matrix of the system of growth equations, together with their respective signs. Since the signs of the partial derivatives for the various functions are as described above, if we assume that the negative term $\delta_{VI}x_{VI}$ is dominant with respect to the positive effect from h_{VI}, then the Jacobian matrix of the system has the following parameter-independent sign pattern, where the term in position (i,j) represents the parameter-independent sign (positive, negative, or zero) of the direct effect that key player j has on key player i.

$$sign(J) = \begin{bmatrix} - & - & - & - \\ - & - & 0 & 0 \\ - & + & - & + \\ - & + & 0 & - \end{bmatrix}$$

If the model is reformulated by using as a first state variable the opposite of x_{HB} (viz. an indicator of bee unhealthiness), the community matrix becomes Metzler (i.e., all off-diagonal entries are non-negative); hence, the system is monotone[40]. Monotonicity consists in the remarkable feature of preserving the ordering of solutions with respect to initial data. When this is the case, despite the possible intricacies, some important features of the system dynamics can be inferred based on purely qualitative or relatively basic quantitative knowledge of the system characteristics[41,42] as we will show below.

We then described the effect of an external input applied to the system variables on the steady-state variation of each of the others. If a persistent input is applied to the system, the steady-state variation of a variable may have the same sign as the applied input, or the opposite sign, or may be zero in the case of perfect adaptation. Structural influence means that the sign of the variation does not depend on the value of the system parameters. In this case, the steady-state interactions can be represented by the following structural influence matrix, where the term in position (i,j) represents the parameter-independent sign (positive, negative, or zero) of the variation of the steady state of key player i ensuing from the application of a constant input affecting key player j; this can be seen as the net effect of j on i, including both direct and indirect effects. HB, TC, VA, VI are honey bee health, toxic compounds, parasites, viruses, respectively.

Influence of	HB	TC	VA	VI
on HB	+	−	−	−
on TC	−	+	+	+
on VA	−	+	+	+
on VI	−	+	+	+

Unlike the sign matrix above, which includes only the direct effect of each component on the others, this matrix reports net effects, including both direct and indirect effects of a stressor on the others[16]. The structural influence matrix thus shows that any new stressor applied to the system has a net negative effect on bee health. Thus, a toxic compound, such as for example a neonicotinoid insecticide, can only have a negative effect on honey bee health when applied to individual bees, regardless of the presence of parasites and pathogens. Hence, the lack of a detectable effect reported in some cases could be regarded as a lack of the hypothesized detrimental effect. However, a detailed study of the system equilibria reveals that this conclusion is a consequence of not considering the complexity of the study system (i.e., honey bee health as affected by various factors).

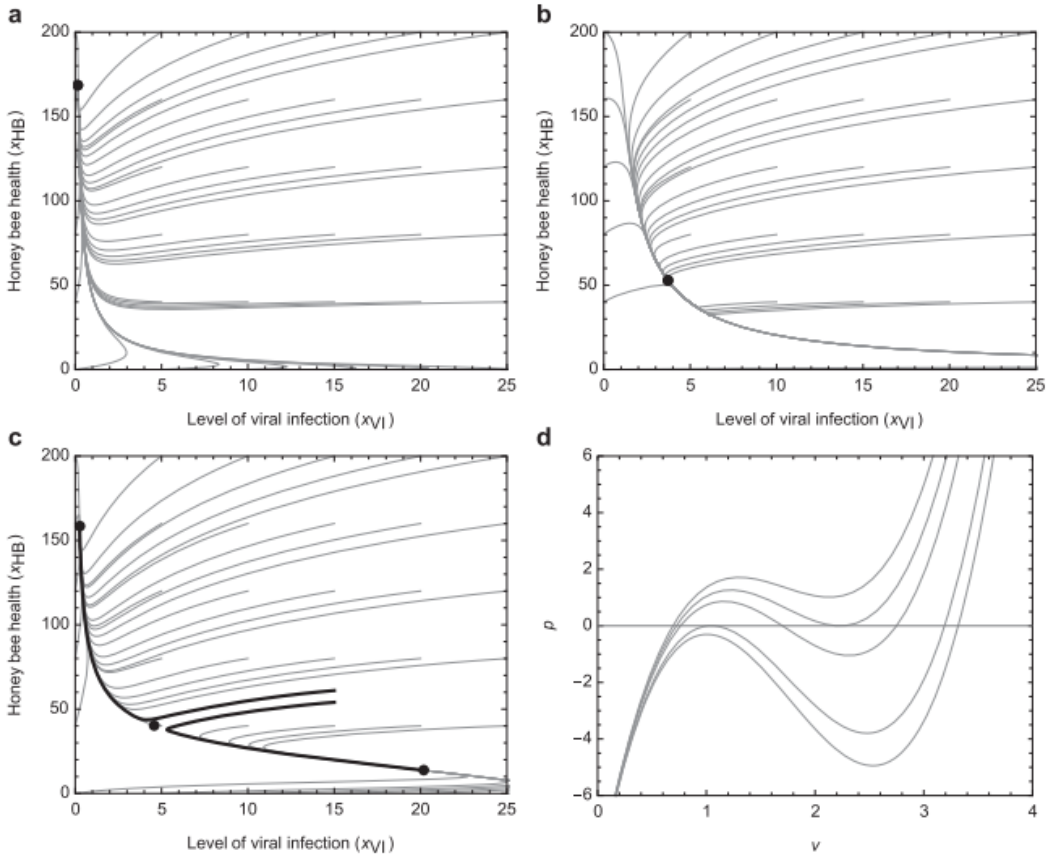

Fig. 2 | The equilibria and some orbits of the full system in the projected phase plane of honey bee health (x_{HB}) and level of viral infection (x_{VI}). Equilibria represent the values of the state variables where they do not change and are indicated with dots, while the orbits are the values that the state variables can assume while approaching the equilibria and are represented with lines. **a** Orbits and the unique equilibrium without immune-suppression, in presence of a low level of parasites. **b** Orbits and the unique equilibrium without immune suppression, in case of a high level of parasites. **c** Orbits and the three equilibria with immune-suppression; two orbits exiting from close initial conditions are marked with thick lines. **d** Equilibria of the subsystem of bee and virus for increasing immune-suppression. p is a function of the level of viral infection v that vanishes at equilibria; top curve: at low immune-suppression there is one equilibrium at high bee health; bottom curve: at high immune-suppression there is one equilibrium at low bee health; intermediate values of immune-suppression can cause three equilibria.

System equilibria

Although an analytical solution of the differential equations representing our biological system, and thus the calculation of each variable at each time, is not possible, the study of the equilibria of the system can explain its behavior under different conditions.

Equilibria are the simplest solutions of the dynamical system representing honey bee health as affected by stressors and drivers and represent the value of the state variables (e.g., x_{HB}, representing honey bee health) where they do not change, or, in other words, the possible destiny of a variable provided it is allowed to (and can) settle to a constant value. Therefore, the study of system equilibria can discriminate whether honey bee health, represented by Eq. (1), can settle to a high, satisfactory level, or is bound to deteriorate to a lower, dangerous level, when insects are exposed to a certain set of stressors. The equilibria and the orbits (i.e., the values that the state variables can assume while approaching equilibria) are represented in graphs with black dots and lines, respectively (Fig. 2a–c).

To provide a visual description of our results, we specified the form of each function and assumed a set of values for the model parameters (Supplementary Methods; Supplementary Table 4); then we plotted the orbits and the equilibria on the projected phase planes. In this way we could graphically describe the trajectory of each variable with respect to others; in particular, we could see how honey bee health reacts to increasing pressure of viruses, parasites or toxic compounds and the end point of this process. Please note that our arbitrary selection of parameters (which are highly uncertain) does not influence the general qualitative conclusions of this study.

To investigate stability in the presence of different stressors, we considered two alternative cases: (1) a pathogen that cannot influence the immune response of honey bees, and (2) a viral pathogen that can affect honey bee immune system, as in the case of DWV[28].

In the first case, after appropriate mathematical treatment (Supplementary Methods), we found that the system admits a unique positive equilibrium, which is globally asymptotically stable in the positive orthant, whereby the position of the equilibrium on the honey bee axis depends on the intensity of the stressors or their combination (Supplementary Results; Fig. 2a, b). In particular, in presence of a pathogen that cannot impair immunity, honey bee health is high when

the level of parasites (or any other stressor) is low (Fig. 2a), and vice versa (Fig. 2b). In other words, it appears that, in presence of a stable input of the stressors included in our model, honey bee health reaches a well-defined level which depends on the level of the stressors. If either the level of parasite or pathogen pressure or pesticide contamination or both is too high, this equilibrium can be unbearable for the individual bee, resulting in death. In any case, the result can be predicted with a good degree of confidence based on the initial conditions; in fact, global stability makes the result independent of the initial conditions, as highlighted by the orbits in Fig. 2a, b that are converging to the same equilibrium point (represented by the dots in the figures) from different initial conditions (represented by any point on the lines in the figures).

We then considered the presence of a pathogen with the capacity to affect the immune response of honey bees. In this case, a convenient mathematical treatment relying also on bifurcation theory[43] (Supplementary Methods) reveals a completely different scenario: the system can now admit three equilibria, one of which is unstable, and hence bistability arises (Fig. 2c). A dynamical system is bistable when it has got two stable equilibria. This is a common feature of many biological systems and allows to interpret several phenomena from the level of molecules to ecosystems (see for example, refs. 44–47). With a convenient metaphor, a monostable system (i.e., a system with a single stable equilibrium, like the one described above) can be assimilated to a landscape with a single valley such that a ball will inevitably end at the bottom of that valley. Instead, a bistable system can be represented with two valleys separated by a hill, such that a ball sitting on the top of the hill (i.e., in the unstable equilibrium) can either fall into one or another valley, depending on any small initial perturbation.

Bistability is related to the presence of positive feed-back loops in the system that can amplify small differences in the initial conditions[48]. In this case, the addition of a pathogen that is capable of interfering with the immune response corresponds to the introduction of a critical positive feed-back loop into the system (formed by arrows "m" and "j" between "immunity" and "deformed wing virus" in Fig. 1). Indeed, the higher the viral load, the stronger the suppression of the immune system, and the lower the efficiency of the latter to contain the virus, which can then actively replicate leading to higher viral loads. In mathematical terms, this can be seen from the equations of the system: functions h, which convey the effect of the virus, are increasing with respect to x_{VI} (the state variable associated with the virus). When the parameter ε, associated with the immune-suppressing potential of the virus, is large enough, the presence of function h_{VI} in the equation describing the time evolution of x_{VI} yields the ability of the virus to increase its effect. Thus, the presence of an immune-suppressing virus creating a positive feed-back loop is necessary for the system to exhibit the described bistability property.

In practice, under reasonable and biologically meaningful conditions, if the immune suppression capacity is absent or low, a unique stable equilibrium exists in the range of high bee health (Fig. 2d). For higher immune-suppression (i.e., larger values of the crucial parameter ε in Eqs. (3) and (4)) a fold bifurcation[43] creates two additional equilibria (Fig. 2d). Of the resulting three equilibria, two are stable and are located in the high and low bee health regions, respectively. Increasing ε further moves the intermediate unstable equilibrium towards the high bee health stable one, until they collapse and disappear through a second fold bifurcation, leaving just one stable equilibrium in the low bee health region, when the immune suppression capacity is too large (Fig. 2d).

In conclusion, the introduction of a pathogen capable of interfering with the honey bee's immune system generates an unstable intermediate 'watershed' equilibrium, which explains why, in the presence of slightly different initial conditions, vastly different outcomes can be possible (see thick curves in Fig. 2c). Under more descriptive terms, if a stressor is above a certain level, there is only one equilibrium at low bee health, meaning for example that if a toxic compound is present at a harmful concentration, bee survival will be significantly lower, and a negative effect will be noted; instead, if the same stressor is below that dangerous level, one equilibrium at high bee health is certainly possible; meaning that, if the toxic compound is present at a low concentration, bee survival may not be significantly different from normal and a negative effect may not be noted. Interestingly, our analysis revealed that, in the presence of an immune-suppressing virus, bistability can occur so that, for the same intermediate level of one stressor, one can have either low bee health or high bee health depending on the similar, but not identical, initial conditions and therefore the results may become unpredictable. In other words, in the presence of an intermediate amount of insecticide, a virus-infected bee can either die prematurely or survive much longer, depending on its initial, intrinsic individual situation.

Validation of the bee health model

To experimentally test the predictions of our mathematical analysis showing bistability, we used data from several survival experiments, carried out using the same standardized method, over 6 years.

In this case, to test our theoretical predictions we used the longevity of caged bees as an estimator of their health condition, assuming that high honey bee health implies normal survival and low bee health is related to shorter longevity. Furthermore, to determine the effects of an immune-suppressing pathogen on honey bee health we used the seasonality of a common virus, DWV, which, in the area where the study was carried out, is rare in Spring and widespread at the end of the season when high viral loads are normally reached in infected bees[28]. For this reason, bees sampled early in the season can be considered virtually virus free whereas bees sampled late in the season can be considered as virus infected.

We hypothesized that, in the presence of an immune-suppressing pathogen, besides the expected reduction in median survival, the predicted bistability should result in bees at high bee health dying later in life and bees at low bee health dying earlier in life, with a consequent increase in the variability of longevity data.

We first tested the effect that the addition of an immune-suppressing virus has on the survival of caged honey bees. To this aim we compared the survival of bees maintained under the same conditions and sampled either early in the season and late in the season; subsequent qRT-PCR analyses confirmed that virus infection was rare in the first and common in the latter (Table 1; Supplementary Figure 1). Virus-free honey bees from early year populations had a characteristic survival curve with limited mortality during the first three weeks of life, followed by another two weeks of increased mortality with a distribution of lifespans centered around 23 days of age; in fact, 50% of those bees died between 21 and 24 days of age (Fig. 3a; Table 1). Instead, virus-infected honey bees from late-year populations had a shorter median survival and moreover a much broader distribution of lifespans, with a significant number of bees dying at a young age and others surviving much longer (Fig. 3a; Table 1). As a result, the interquartile range of longevities, here used as a measure of the dispersion of data, was 6 in early year bees and 10 in late year populations (Table 1), indicating a higher variability of longevity data in the presence of an immune-suppressing virus.

In a second experiment, virus-free honey bees were artificially fed virus particles or not and the tests were repeated, confirming the results reported above (Fig. 3b; Table 1). In particular, we found that control bees had a median longevity of 18 days and an interquartile range of 5, whereas virus-treated bees had a shorter median longevity (i.e., 10) as a result of a large number of bees dying in the first days, as underlined by a much larger dispersion of longevity data (interquartile range = 12). This further supports the view that the presence of an immune-suppressing virus can create vastly different outcomes depending on the slightly different initial conditions of single bees exposed to otherwise identical situations.

https://doi.org/10.1038/s41467-022-33405-7

Table 1 | DWV infection and survival of the honey bees used in the lab experiments

Treatment	Early										Late									
	Control					Treated					Control					Treated				
	DWV prev.	nv	Median survival	IQR	ns	DWV prev.	nv	Median survival	IQR	ns	DWV prev.	nv	Median survival	IQR	ns	DWV prev.	nv	Median survival	IQR	ns
None	0.09	11	23.0	6.0	107						0.70	63	21.0	10.0	542					
Virus	0.38	8	18.0	5.0	37	0.75	8	10.0	12.0	38										
Nicotine	0.00	3	28.0	5.0	37	0.00	3	25.0	7.0	37	0.83	12	14.0	11.5	51	0.83	12	14.0	8.0	55
Low temperature	0.33	3	23.0	3.0	61	0.33	3	19.5	4.0	54	0.87	31	18.0	12.0	201	0.88	34	17.0	16.0	217

DWV prevalence (proportion of infected bees in a sample of nv bees), median survival (days), interquartile range of the distribution of longevities (IQR), and sample size (ns), are reported.

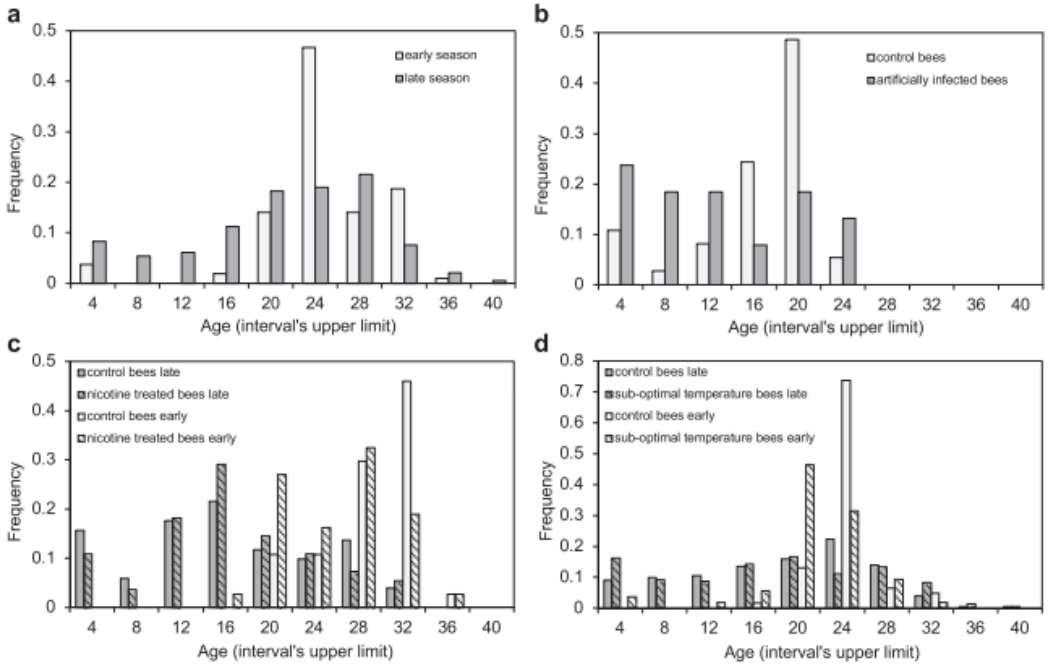

Fig. 3 | Distribution of individual lifespans of honey bees under different conditions. a Early in the season when the prevalence of an immune-suppressing virus is low (white bars) and later when all bees are virus infected (gray bars). **b** Treated or not (gray and white bars, respectively) with a virus administered to mature larvae through the diet. **c** When exposed to a toxic compound, when the prevalence of an immune-suppressing virus is low (white bars with diagonal pattern) or when the virus is widespread (gray bars with diagonal pattern); the corresponding distribution of honey bees sampled early or late in the season and not exposed to the toxic compound as a control (white and gray bars, respectively). **d** As (**c**) but exposed to a sub-optimal temperature in place of a toxin. Source data are provided as a Source data file.

In summary, by carrying out two different comparisons of uninfected versus virus-infected bees (one diachronic, with naturally virus-infected bees sampled at two different times, and one synchronic, by treating or not with the virus some uninfected bees at the same time), we noted that uninfected bees show mortality concentrated after three weeks of life, as expected given the shape of the survival curve of control caged bees previously observed under the same conditions[49]. In contrast, the mortality of virus-infected bees is not concentrated late in life but can also occur at a young age, resulting in a marked variability of longevities. Thus, as predicted by our model analysis, the probability of dying either soon or late does not only depend on the treatment but rather on the slightly different intrinsic conditions of bees. These were not under our control but dictated the bee's final destiny.

To investigate how the presence of an immune-suppressing virus could alter the response of honey bees to different stressors, we carried out two more experiments, whereby we studied the survival of honey bees exposed to 50 ppm of nicotine, here used as an example of a toxic compound, or to the sub-optimal temperature of 32 °C, as compared to the normal in-hive temperature of 34.5 °C[20].

When the virus was not present, both stressors caused a decreased lifespan, showing a distribution of lifespans shifted towards shorter ages (Fig. 3c, d; Table 1). However, in presence of a virus, both in the case of a toxic compound and a low temperature, a much broader survival distribution was generated, consistent with the bistability hypothesis (Fig. 3c, d; Table 1). Accordingly, the interquartile range of longevities increased from values from 3 to 7 in early year populations to values from 8 to 16 in late year populations (Table 1),

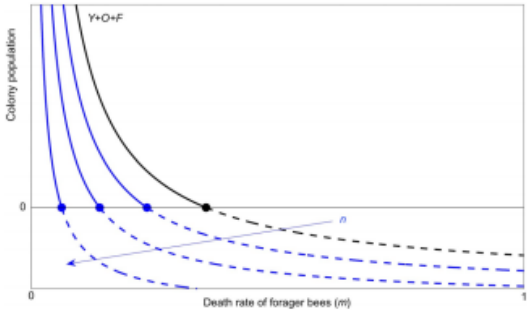

Fig. 4 | Dependence of the colony population at equilibrium on the death rate m of forager bees for varying death rate n of juveniles hive bees. For a forager death rate m exceeding a critical value (black dot) the only stable equilibrium is zero, corresponding to colony failure. The premature death of hive bees (denoted by increasing values of n, represented by the blue curves) moves that critical value left, meaning that colony failure can occur for lower foragers' death rates. Black line: $n = 0$, blue: $n \in (0, 1)$, dots: $m(n)$. The parameter values are $L = 2000$, $w = 27{,}000$, $\alpha = 0.25$, and $\sigma = 0.75$ as in a previously published report[50].

highlighting a higher variability of longevity data, both in case of a toxic compound and a low temperature.

Overall, these results show that the presence of a pathogen capable of interfering with immune control creates a situation whereby the survival of honey bees is not solely determined by external stressors. Rather, it is greatly influenced by some minimal variations in the starting conditions, leading either to an imbalanced condition and premature death (lower thick orbit in Fig. 2c), or coping with the stress much longer (upper thick orbit in Fig. 2c).

From individual health to colony stability

It is important to note that any stress impacting the health of individual honey bees, thus significantly reducing their survival, could be propagated at colony level, eventually leading to colony collapse. However, whilst a mild negative effect could be buffered by the bee colony, a deviation from a favorable initial condition could result in rapid deterioration.

It has previously been shown that the lowered survival of forager bees can disrupt the colony equilibrium, resulting in colony collapse[50]. In particular, it was shown that mortality of forager bees exceeding a certain threshold (i.e., $m = 0.355$ in Fig. 4) could lead to colony failure despite some compensation mechanisms (e.g., a premature transition to foraging by nurse bees to replace dead foragers). To understand how the effects observed here in individual bees can influence colony stability, we used the same model after appropriate modifications. We found that the premature death of bees, at a younger age, as we report above, can be more detrimental than the already demonstrated reduced lifespan of foragers, moving the critical value of mortality to the left (Fig. 4). This mortality limits both the development of brood and the replacement of dying forager bees, adding to the effect described by other studies, and making collapse even more probable.

Discussion

It is widely acknowledged that agricultural systems function as complex systems and agrochemicals are an important component within these systems. In particular, widely used neonicotinoid insecticides are regarded as significant threats to honey bees and the pollination service they provide, benefitting crop production and biodiversity[51]. This concern is based on a large and consistent body of evidence that was largely built under laboratory conditions[6]. Studies carried out under field conditions have not provided similarly convincing data[10–13] (Supplementary Table 1), generating uncertainty about the real risk posed by some substances under more realistic settings. This, in turn, contributed to different regulatory approaches towards the same products under different conditions or countries[52].

Indeed, descriptive models could help draw more or less accurate predictions regardless of the inevitable variability of contexts[53] and thus support risk assessment and the consequent decisions[54]. Unfortunately, the lack of exact quantitative knowledge of the many parameters influencing bee health at individual and colony levels still pose a serious challenge to this approach. On the other hand, theoretical and computational tools are now available to assess the parameter-independent, structural properties of biological systems[15,16]. In fact, our systems biology approach allowed us to uncover some structural properties of the system under study (i.e., honey bee health as affected by various factors), reaching important conclusions that are based on unequivocal mathematical arguments.

We demonstrated in theory, and also confirmed in practice, that the already reported capacity of a widespread virus to impair the immune defenses of honey bees[28] can generate bistability. This implies that honey bees under similar initial conditions can have markedly different destinies when exposed to the same stressor. Our study of the possible consequences of this phenomenon at the colony level indicates that it increases the vulnerability of the colony to dwindling and collapse.

It is important to underline that only the immune-suppressing pathogen can cause the bistability and the described dynamics, because of its capacity to attack the bee defense system, thus exacerbating the pathogen's effect[28]. To our knowledge, no other stress factor can impair the system keeping that stressor under control and thus be implicated in similar dynamics. In some cases, an effect of pesticides on the detoxification system of honey bees has been reported[55]. This is normally expressed as an upregulation of some genes after exposure to pesticides[56–58], likely indicating the activation of a pathway in response to intoxication. This does not necessarily suggest the capacity of that pesticide to impair detoxification but can be regarded as evidence of a well-functioning homeostatic system that reacts to intoxication through a physiological mechanism aimed at reducing the concentration of the toxic chemical. On the other hand, several studies showed that fungicides can increase the toxicity of insecticides[32–34] suggesting impaired detoxification that could be tested with further mechanistic studies. Based on our analysis we can hypothesize that a pesticide exhibiting an anti-detoxification activity could cause system behavior like that reported here for the pathogenic virus DWV. At present this possibility is purely speculative, but it may have important implications for honey bee survival and should therefore be considered with great attention.

Our data allows a retrospective evaluation of published studies that may explain the contrasting results reported. Based on our conclusions we hypothesize that, in the presence of a low prevalence of the immune-suppressing virus, the negative effect of pesticides at field-realistic concentrations can be buffered by the colony's homeostatic response as previously proposed[12,14], provided that other stressor effects are limited. In contrast, when the immune-suppressive virus or the vector mite is present, negative effects are more likely to be observed because of the bistability we demonstrated. This in turn may cause some bees to experience premature mortality which cannot be effectively buffered by the homeostatic response mechanisms of the colony. This concurs with the observation that, in studies that showed no adverse neonicotinoid effects[10–12], DWV prevalence and/or mite infestations were low. Whereas, in the study reporting a country-specific effect of neonicotinoids[13], mite prevalence was low where positive effects were found (i.e., Germany) and high where effects were clearly negative (i.e., United Kingdom). Based on our results we suggest that the relative scarcity of the immune-suppressing virus can account for dynamics characterized by a single stable equilibrium at

satisfactory honey bee health. Under these conditions, it is likely that the buffering capacity of the bee colony can prevent collapse, despite a chemical reducing the bees' lifespan. If the immune-suppressing virus reaches a sufficient prevalence, the ensuing bistability accounts for results that can be either normal, when initial conditions are favorable, or dramatic in all other cases. This does not hold for neonicotinoid insecticides only, but also for parasites and pathogens, not least because *V. destructor* has allowed DWV to spread worldwide[59]. Of course, other factors, such as variable pesticide exposure under different conditions may be implicated in the variability of results about neonicotinoid effects under field-realistic conditions. However, in our opinion, such possible alternative causes would hardly result in a situation where the same chemical can cause either positive or negative effects in a comparative study carried out in a standardized manner[13].

In science we often rely on empirical data to base our predictions of future effects. This approach works well for limited and easily controlled systems, but it is not adequate for complex systems such as agroecosystems. Here, with honey bees, we show how even a small part of such a system can generate complex yet predictable emergent properties that can explain hitherto hard-to-reconcile observations.

Overall, this study demonstrates that considering relationships between components, rather than focusing on the individual, context-dependent, expression of a system state, leads to a much deeper understanding and is a better basis for real-world decisions. In fact, the bee system described here is a good example of the kind of feedbacks found in ecology and biology and is not unique. In cases like this, empirical observations of a single system state in space and time are important but have poor predictive power compared to the system analysis presented here.

Here, we demonstrate that although the complexity of the system representing honey bee health as affected by multiple factors can appear intractable, it may be better to deal with that complexity rather than to factor it away. This thinking suggests more critical evaluation of empirical studies and should help to clarify the debate on pesticides and honey bees. Today's regulatory risk assessment for pesticides relies on a single substance, single-use approach[54], but a new multi-stressor approach is proposed[60]. In parallel, discussions about the protection goals for bees in European environmental risk-assessment seem almost entirely based on empirical observation of variability, and not on mechanistic understanding[61]. Our results could inform regulatory efforts by contributing to re-design honey bee risk assessment and achieve a more homogenous regulatory response to scientific evidence.

Methods

The bee health model

The conceptual model of the interactions of various stressors with honey bee health is described by the following system of ordinary differential equations (ODEs)

$$\begin{aligned}\tau_{HB}\dot{x}_{HB} = -\delta_{HB}x_{HB} + g_{TC}(x_{TC}) + g_{VA}(x_{VA}) + g_{VI}(x_{VI}) \\ + \bar{f}_{S,C}(u_S, u_C, x_{TC}, x_{VA}) + \bar{f}_{P}(u_P, x_{TC}) + \underline{f}_{HB}(u_T)\end{aligned} \quad (1)$$

$$\tau_{TC}\dot{x}_{TC} = -\delta_{TC}x_{TC} + g_{HB}(x_{HB}) \quad (2)$$

$$\tau_{VA}\dot{x}_{VA} = -\delta_{VA}x_{VA} + h_{VA}(x_{HB}, x_{TC}, \varepsilon x_{VI}) + \underline{f}_{VA}(u_T) \quad (3)$$

$$\tau_{VI}\dot{x}_{VI} = -\delta_{VI}x_{VI} + h_{VI}(x_{HB}, x_{TC}, \varepsilon x_{VI}) \quad (4)$$

for the state variables x_{HB} representing honey bee health, x_{TC} the stress due to toxic compounds (e.g., neonicotinoid insecticides), x_{VA} the stress due to parasites (e.g., *V. destructor*) and x_{VI} the stress due to pathogens (e.g., DWV). The system includes the effects of external inputs as sugar u_S, pollen u_P, absolute deviation from desired temperature u_T and sub-optimal temperature u_C. All the inputs and possible parameters are non-negative; the coefficients τ denote the time constants; the coefficients δ denote the self-regulation parameters; ε in the last two equations allows to account for pathogens that can ($\varepsilon > 0$) or cannot ($\varepsilon = 0$) impair the immune system (through link m in Fig. 1). We assume that the functions g are smooth, bounded, positive, convex and decreasing to 0; the functions $\bar{f}$ are smooth, bounded, non-negative, concave and increasing with respect to (w.r.t.) u arguments (vanishing only when the first u argument vanishes) while convex and decreasing to 0 w.r.t. x arguments; the functions $\underline{f}$ are smooth, bounded, non-positive and decreasing (vanishing only when $u = 0$); the functions h are smooth, bounded, positive, convex and decreasing to 0 w.r.t. the first argument while concave and increasing w.r.t. all the other arguments. For a detailed description of the various functions, together with a summary of the biological effects they account for and a reference to the conceptual model in Fig. 1, see Supplementary Table 3.

Structural analysis of the bee health model

We describe here the structural considerations and computations that yield the structural influence matrix for the honey bee health system.

The structural influence matrix M is defined as follows. M is a symbolic matrix with entries M_{ij} chosen among: +,−,0,?, according to the criteria described below. Consider an equilibrium point $\bar{x}$ and a constant perturbation u applied on the j-th system variable (small enough not to compromise the stability of the equilibrium). The equilibrium value will be modified as $\bar{x} + \delta\bar{x}$. Consider the sign of the perturbation of the i-th variable, $\delta\bar{x}_i$. Then $M_{ij} = +$ if $\delta\bar{x}_i$ always has the same sign as u; $M_{ij} = -$ if $\delta\bar{x}_i$ always has the opposite sign as u; $M_{ij} = 0$ if always $\delta\bar{x}_i = 0$; regardless of the system parameters. Conversely, if the sign does depend on the system parameters, we set $M_{ij} = ?$.

In this section we prove that the influence matrix of the honey bee health system is structurally determined, i.e., there are no "?"' entries in M.

We start with the following proposition.

Proposition 1 Assume that a matrix J is Hurwitz stable (i.e., all its eigenvalues have negative real part) and has the sign pattern

$$sign(J) = \begin{bmatrix} - & - & - & - \\ - & - & 0 & 0 \\ - & + & - & + \\ - & + & 0 & - \end{bmatrix}$$

Then, the sign pattern of adj(−J), the adjoint of −J, is

$$sign(adj(-J)) = \begin{bmatrix} + & - & - & - \\ - & + & + & + \\ - & + & + & + \\ - & + & + & + \end{bmatrix}$$

Proof To prove the statement, we just change the sign of the first variable, hence we change sign to the first row and column of matrix J. The resulting matrix M is such that

$$sign(M) = \begin{bmatrix} - & + & + & + \\ + & - & 0 & 0 \\ + & + & - & + \\ + & + & 0 & - \end{bmatrix}$$

We observe that M is a Metzler matrix, namely, all its off-diagonal entries are non-negative. Moreover, the matrix is Hurwitz stable. Then, we can proceed as in the proof of *Proposition 4* in a previous report[16]. Given a Metzler matrix that is Hurwitz stable, its inverse has non-

positive entries; hence, the inverse of $-M$ has non-negative entries: $(-M)^{-1} \geq 0$ elementwise. Moreover, we observe that M is an irreducible matrix, i.e., there is no variable permutation that brings the matrix in a block (either upper or lower) triangular form. This implies that the inverse of $-M$ has strictly positive entries: $(-M)^{-1} > 0$ elementwise. Also, stability implies that the determinant of $-M$ is positive: $\det(-M) > 0$. Then, $adj(-M) = (-M)^{-1}\det(-M) > 0$, hence the adjoint of $-M$ is also positive elementwise. To consider again the original sign of the variables, we change sign to the first row and column of $adj(-M)$, and we get the signature above for $adj(-J)$.

The next step is the characterization of the structural influence matrix, which corresponds to the sign pattern of the adjoint of the negative Jacobian matrix in *Proposition 1*.

To this aim, we first consider the linearized system and write it in a matrix-vector form

$$\dot{x}(t) = Jx(t) + e_j u$$

where $\dot{x}(t)$ is the time derivative of the four-dimensional vector $x(t)$ and e_k, $k = 1,2,3,4$, is an input vector, constant in time, with a single non-zero component, the k-th, equal to 1, while the scalar $u > 0$ is the magnitude of the input. We wish to assess the i-th component of $x(t)$, $x_i(t) = e_i^T x(t)$. If J is Hurwitz, as assumed, the steady-state value of variable $x_i(t)$ due to the input perturbation e_k applied to the equation of variable $x_k(t)$ is achieved for

$$0 = J\bar{x} + e_k u,$$

namely

$$x_i = -e_i^T J^{-1} e_k u,$$

which implies that the sign of the steady-state value $\bar{x}_i$ of variable x_i due to a persistent positive input acting on the k-th equation has the same sign as $(-J^{-1})_{ik}$, the (i,k) entry of matrix $(-J)^{-1}$. Since we assume Hurwitz stability, we have that $\det(-J)$ is positive, hence the sign pattern of the inverse $(-J)^{-1}$ corresponds to the sign pattern of the adjoint, $adj(-J)$. In fact, $adj(-J) = (-J)^{-1}\det(-J)$.

We next consider the nonlinear system under investigation, which we write in the form

$$\dot{x}(t) = f(x(t))$$

and without restriction we assume that the zero vector is an equilibrium point: $0 = f(0)$. This condition can be always achieved, without loss of generality, by a translation of coordinates. We also consider a stable equilibrium: we assume that the linearized system at the equilibrium is asymptotically stable, namely its Jacobian J, which has the sign pattern considered in *Proposition 1* above, is Hurwitz. We also assume that a constant input perturbation of magnitude u is applied to the system, affecting the k-th equation, i.e.,

$$\dot{x}(t) = f(x(t)) + e_k u,$$

and that the perturbation is small enough to keep the state in the domain of attraction of the considered equilibrium. Due to this perturbation, a new steady state $\bar{x}(u)$ is reached that satisfies the condition

$$0 = f(\bar{x}(u)) + e_k u$$

To determine the sign of the new equilibrium components $\bar{x}(u)$, we consider this new equilibrium vector as a function of u in a small interval $[0, x_{MAX}]$. Adopting the implicit function theorem yields

$$\frac{d}{dx}\bar{x}(u) = -J(u)^{-1} e_k u,$$

where we have denoted by $J(u)$ the Jacobian matrix computed at the perturbed equilibrium $\bar{x}(u)$. Hence, for u small enough, the sign of the derivatives of the entries of the new, perturbed equilibrium are, structurally, the same as those in the k-th column of matrix $-J^{-1}$. Since, by construction, $x(0) = 0$, this is also the sign of the elements of vector $\bar{x}(u)$, for u in the interval $[0, x_{MAX}]$.

We have therefore proved that the original nonlinear system describing honey bee health admits the following structural influence matrix:

$$\begin{bmatrix} + & - & - & - \\ - & + & + & + \\ - & + & + & + \\ - & + & + & + \end{bmatrix}$$

System equilibria

The results concerning the system equilibria were obtained through a standard analytical treatment of the nonlinear equations describing the equilibrium conditions of the system of differential Eqs. (1), (2), (3), (4). A detailed description of methods is reported in Supplementary Methods.

Laboratory experiments using honey bees

To confirm the bistability of the system representing honey bee health as affected by multiple stressors, we used data from several survival experiments, carried out in a laboratory environment according to the same standardized method, over a 6-year period (Source data file).

All experiments involved *Apis mellifera* worker bees, sampled at the larval stage or before eclosion, from the hives of the experimental apiary of the University of Udine (46°04′54.2″N, 13°12′34.2″E). Previous studies indicated that the local bee population consists of hybrids between *A. mellifera ligustica* and *A.m. carnica*[62,63]. Ethical approval was not required for this study.

We considered experiments on the effect of the following stressors: infection with 1000 DWV genome copies administered through the diet before pupation, feeding with a 50 ppm nicotine in a sugar solution at the adult stage, exposition to a sub-optimal temperature of 32 °C at the adult stage. All experiments were replicated 3 to 13 times, using, in total, the number of bees reported in Table 1.

For the artificial infection with DWV, we collected with soft forceps individual L4 larvae from the brood cells of several combs. Groups of 20–30 of such larvae were placed in Petri dishes with an artificial diet made of 50% royal jelly, 37% distilled water, 6% glucose, 6% fructose, and 1% yeast. 25 DWV copies per mg of diet were added or not to the diet according to the experimental group (note that a bee larva at this stage consumes about 40 mg of larval food per day, thus the viral infection per bee was 1000 viral copies). After 24 h larvae were transferred onto a piece of filter paper to remove the residues of the diet and then into a clean Petri dish, where they were maintained until eclosion. At the day of emergence, bees were transferred to plastic cages in a thermostatic cabinet, where they were kept until death. The DWV extract was prepared according to previously described protocols[64] and quantified according to standard methods.

For the treatment with nicotine, 10 μL of pure nicotine were added to 200 g of the sugar solution used for the feeding of the caged bees, to reach the concentration of 50 ppm.

Finally, to expose bees to a 32 °C temperature, the plastic cages with the adult bees were kept in a thermostatic cabinet whose temperature was set accordingly.

To monitor the survival of the adult bees treated as above, they were maintained from eclosion until death in plastic cages in a dark incubator at 34.5 °C (or 32 °C, according to the experiment), 75% R.H.; two syringes were used to supply a sugar solution made of 2.4 mol/L of glucose and fructose (61% and 31%, respectively) and water, respectively; dead bees were counted daily.

All the results of these experiments are reported in Source data file.

All experiments were carried out during the summer months, from June to September for 6 consecutive years. Previous data indicated that, in this region, virus prevalence increases along the active season starting from very low levels in spring and reaching 100% of virus-infected honey bees by the end of the summer; virus abundance in infected honey bees follows a similar trend[28]. For this reason, it can be assumed that bees sampled early in the season are either uninfected or they bear only a very low viral infection level, whereas bees sampled later in the season are likely to be virus-infected, bearing moderate to high viral infections. To confirm this assumption and identify a method for filtering our data according to viral infection, we assessed viral infection in a sample of bees from the untreated control group of each experiment, by means of qRT-PCR. According to standard practice, we assumed that Ct values below 30 are indicative of an effective viral infection, whereas Ct above that threshold are more likely in virus negative bees. As expected, we found that virus prevalence increases from June to September (Supplementary Figure 1a), in such a way that up to mid July only the minority of bees can be considered as viral infected (Supplementary Figure 1b). Therefore, we classified as "early" all the samples collected up to mid July and assumed that viral infection in those samples was low; on the other hand, samples collected from mid July till September were classified as "late" and we assumed that viral infection in those samples was high.

qRT-PCR analysis of viral infection was carried out as follows. At the beginning of every experiment (i.e., at day 0), two to five bees for each replication were sampled in liquid nitrogen and transferred in a −80 °C refrigerator. After defrosting of samples in RNA later, the gut of each honey bee was eliminated to avoid the clogging of the mini spin column used after. The whole body of sampled bees was homogenized using a TissueLyser (Qiagen®, Germany). Total RNA was extracted from each bee according to the procedure provided with the RNeasy Plus mini kit (Qiagen®, Germany). The amount of RNA in each sample was quantified with a NanoDrop® spectrophotomer (ThermoFisher™, USA). cDNA was synthetized starting from 500 ng of RNA following the manufacturer specifications (PROMEGA, Italy). Additional negative control samples containing no RT enzyme were included. DWV presence was verified by qRT-PCR considering as positive all samples with a Ct value lower than 30. The following primers were adopted: DWV (F: GGTAAGCGATGGTTGTTTG, R: CCGTGAATATAGTGTGAGG[65]). 10 ng of cDNA from each sample were analyzed using SYBR®green dye (Ambion®) according to the manufacturer specifications, on a BioRad CFX96 Touch™ Real time PCR Detector. Primer efficiency was calculated according to the formula $E = 10^{(-1/\text{slope}-1)*100}$. The following thermal cycling profiles were adopted: one cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, and one cycle at 68 °C for 7 min.

Individual survival and colony stability

To investigate how the death rate of forager bees affects colony growth, a compartment model of honey bee colony population dynamics was proposed[50]. This model showed that death rates over a critical threshold led to colony failure. Here we modified this model to include premature death of bees at younger age, as predicted by our model of individual bee health in the presence of an immunosuppressive virus. We show that the critical threshold found in the previously published model[50] becomes a decreasing function of the death rate of the younger individuals, so that premature death (and, in turn, immune-suppression) favors colony collapse.

In more details, we first summarize the results of the previously published model[50] where two populations F (forager) and H (hive) of bees are considered and where conditions are provided on the mortality m of F under which the whole population collapses: namely, mathematically stated, the system admits the zero equilibrium only. Here we extend the model partitioning H in two categories, Y (younger hive bees) and O (older hive bees), as

$$H = Y + O$$

introducing an early mortality factor n for the young population, showing how such a factor worsens the collapsing condition.

The previously published model[50] concerns the interaction between hive bees H and forager bees F and is described by the ODEs

$$\dot{H} = L\frac{H+F}{w+H+F} - H\left(\alpha - \sigma\frac{F}{H+F}\right)$$

$$\dot{F} = H\left(\alpha - \sigma\frac{F}{H+F}\right) - mF.$$

Above, L is the queen's eggs laying rate, w is the rate at which L is reached as the total population $H+F$ gets large, α is the maximum rate at which hive bees become forager bees in the absence of the latter, σ measures the reduction of recruitment of hive bees in the presence of forager bees and, finally, m is the death rate of forager bees (while the death rate of hive bees is assumed to be negligible).

We first summarize the main results in terms of a threshold value for m in view of colony collapse, as our further analysis will follow a similar approach. All the parameters are assumed to be positive.

The search for the equilibria of the above ODEs leads to the unique nontrivial equilibrium (beyond the trivial one)

$$\bar{H} = \frac{L}{mJ} - \frac{w}{1+J}$$

$$\bar{F} = J\bar{H}$$

for

$$J = J(m) := \frac{\alpha - \sigma - m + \sqrt{(\alpha-\sigma-m)^2 + 4m\alpha}}{2m}.$$

Note that J is alway positive (and, moreover, it is independent of L and w). It follows that $\bar{F}$ and $\bar{H}$ have the same sign, so that the existence of the nontrivial equilibrium is equivalent to $\bar{F} + \bar{H} > 0$. It is not difficult to recover that

$$\bar{F} + \bar{H} = \frac{w}{m}\left(l\frac{1+J}{J} - m\right)$$

where $l := L/w$ is introduced for brevity. Then if $\alpha \le l$ we get

$$\bar{F} + \bar{H} = \frac{w}{m}\left(l\frac{1+J}{J} - m\right) \ge \frac{w}{m}\left(\alpha\frac{1+J}{J} - m\right) = \frac{w}{m}(\sigma + mJ) > 0,$$

with the last equality following from

$$\alpha - \sigma\frac{J}{1+J} - mJ = 0,$$

which in turn comes from annihilating the right-hand side of the second ODE and from using $J = \bar{F}/\bar{H}$ while searching for equilibria. We conclude that, independently of m, the colony never collapses if the recruitment rate α of forager bees is sufficiently low.

Hence, we assume $\alpha > l$. Observe that

$$\bar{F} + \bar{H} \Longleftrightarrow l > J(m - l)$$

guarantees existence whenever m is sufficiently small, viz. $m \le l$. Assume then $m > l$, so that the above condition reads

$$J < \frac{l}{m - l}$$

leading to the threshold condition

$$m < \bar{m} := \frac{l}{2}\frac{\alpha + \sigma + \sqrt{(\alpha - \sigma)^2 + 4\sigma l}}{\alpha - l}$$

by using the definition of J, see Eq. (2) the previously published model[50].

A standard stability analysis shows that, assuming $\alpha, m > l$, the nontrivial equilibrium is (globally) asymptotically stable whenever it exists (positive), i.e., whenever $m < \bar{m}$. Otherwise, the only (globally) attracting equilibrium is the trivial one, corresponding to colony collapse (see Fig. 5 for the previously published model[50] or Fig. 4 for $n = 0$). In the mathematical jargon, the disappearance of the positive equilibrium, for m exceeding $\bar{m}$, is referred to as a transcritical bifurcation[43].

Now, in view of the outcome of the analysis of our model of individual bee health, we introduce a mortality term for the younger bees. As forager bees are recruited from adult hive bees, we divide the class of hive bees H in younger Y and older O, assuming that the former die at a rate n, while the death rate of the latter remains negligible according to the previously published model[50]. Obviously, $H = Y + O$. The original ODEs are consequently modified as

$$\dot{Y} = L\frac{H + F}{w + H + F} - Y$$

$$\dot{O} = (1 - n)Y - H\left(\alpha - \sigma\frac{F}{H + F}\right)$$

$$\dot{F} = H\left(\alpha - \sigma\frac{F}{H + F}\right) - mF.$$

Note that the sum of the first two equations above gives

$$\dot{H} = L\frac{H + F}{w + H + F} - H\left(\alpha - \sigma\frac{F}{H + F}\right) - nY.$$

The new negative mortality term for younger hive bees, $-nY$, models the fact that only the younger hive bees die prematurely while the rest of the dynamics is unchanged with respect to the original model.

The search for equilibria soon gives

$$\bar{Y} = L\frac{\bar{H} + \bar{F}}{w + \bar{H} + \bar{F}}$$

from the first ODE above, so that the remaining two equilibrium conditions lead to

$$\bar{H} = \frac{L_n}{mJ} - \frac{w}{1 + J}$$

$$\bar{F} = J\bar{H}$$

for the same J originally defined and $L_n := L(1 - n)$ (note that $n \in (0,1)$, and the case $n = 0$ brings us back to the original model). From this point on the analysis is the same as that previously summarized for the original model, but for replacing L with L_n and l with $l := l(1 - n)$. Consequently, by assuming $\alpha, m > l_n$ (which is less restrictive when $n > 0$), the threshold condition $m < \bar{m}$ becomes

$$m < \bar{m}(n) := \frac{l_n}{2}\frac{\alpha + \sigma + \sqrt{(\alpha - \sigma)^2 + 4\sigma l_n}}{\alpha - l_n},$$

which clearly returns the original threshold condition when $n = 0$. Since

$$\frac{d\bar{m}}{dn}(n) < 0$$

as it can be immediately verified, it follows that the critical value for m, $\bar{m}(n)$, beyond which the colony system admits only the zero equilibrium, i.e., the transcritical bifurcation value, decreases with n (Fig. 4). We thus conclude that colony collapse is favored by the premature death of younger hive bees, possibly caused by a virus impairing the immune system as shown by the analysis of our model of individual bee health.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data generated in this study are provided in the Source data file. Source data are provided with this paper.

Code availability

Figure 2 and Supplementary Figs. 2 and 3 were produced with custom codes developed with the software Mathematica (version 11.3.0.0 run on Mac OS X 10.11.6 MacBook Pro late 2013); Fig. 4 was produced with custom codes developed with the software MATLAB (version R2019a run on Mac OS X 11.6.1 MacBook Pro 2020). All the codes are freely available[66], also at: http://cdlab.uniud.it/software under the heading "BeeStability".

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Acknowledgements

European Union's Horizon 2020 research and innovation program, under grant agreement No. 773921 (PoshBee). C.J.T. was also funded by the European Union's Horizon 2020 research and innovation program, grant agreement No. 817622 (B-GOOD).

Author contributions

Conceptualization: D.B., G.G., C.J.T., F.B., and F.N. Methodology: D.B., G.G., D.A., F.B., and F.N. Investigation: D.B., D.F., G.G., E.S., V.Z., D.A., F.B., F.N. Visualization: D.B., D.A. Supervision: F.B. and F.N. Writing—original draft: D.B., G.G., D.A., C.J.T., F.B., and F.N. Writing—review & editing: D.B., D.F., G.G., E.S., V.Z., D.A., C.J.T., F.B., and F.N.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-33405-7.

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Peer review information *Nature Communications* thanks Dimitry Wintermantel and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

CHAPTER 6 - **General conclusions**

Honey bees play a vital role in ecosystems and contribute to crop production.

In the last decades, a worrying decline of honey bee colonies has been observed in many countries. Losses are caused by the interaction among several stress factors, including parasites and pathogens (i.e. Varroa mite and deformed wing virus), agrochemicals, the availability-quality of food resources and environmental conditions. In this context, toxic chemicals, both of natural and anthropic origin play an important role.

The aim of this thesis was to investigate how different stress factors, and in particular toxic chemicals interact with nutrition to influence honey bee survival.

The approach adopted here involved starting from one single stressor, the toxic alkaloid nicotine, and gradually increasing the complexity of the system by progressively adding more and more factors to the initial framework. With this aim, several experiments were carried out under laboratory conditions in which we investigated the biological effects of the stressors under study by integrating different techniques (e.g. chemical, molecular and mathematical) to achieve the most comprehensive understanding of the complex systems under consideration.

In sum, the studies described in this thesis showed that the effect of nicotine, a toxic alkaloid that can be found both in nectar and pollen of some plant species, depends not only on its own harmfulness but also on the response of bees, and, in particular, on detoxification that is supported by an adequate nutrition. This was confirmed by noting that, when the prevalence of DWV was low, the impairment of the detoxification system through piperonyl butoxide, led to a detrimental effect of nicotine.

The delicate balance between toxic chemicals and bee defences, however, can be impaired when further stressors are added, because of the positive and negative interactions arising from the stressors' combination. In particular, it was demonstrated that DWV can alter the outcome of the interaction between nicotine and nutrition. Furthermore, it appeared that nicotine can interact with

other factors, such as Varroa infestation, mitigating its effect. A very intricate network of interaction is therefore in place which, however, can be adequately dissected using a suitable systems biology approach. In this way, it was demonstrated that the system representing honey bee health includes a dangerous positive feed-back loop generating bistability; this explains the sudden transition from satisfactory bee health to critical conditions that is often observed in honey bees.

To gain insight into the importance of pollen and the negative impact of pollen deprivation on honey bee health, experiments were carried out to study which pollen component is responsible for its biological activity. To this purpose, three different kinds of pollen were administered to bees; the lack of the polar fraction of pollen resulted in a substantial decrease in survival (-28% instead of -2.5% observed in absence of the apolar fraction), underscoring the importance of this component and its role in determining the positive effects of pollen for honeybees.

In this thesis work, for the sake of repeatability, a single kind of pollen (from *A. fructicosa*) rather than a mixture of pollens was used for the experiments. One could argue that this could have affected the general relevance of our results; however, honey bees can collect pollen from hundred of different plants, according to season, availability, weather, etcetera and the ideal mixture does not simply exist.

In any case, only further experiments with other pollens or, even better, the identification of the active principles responsible for its biological activity, down to the molecular level, will definitely confirm our findings.

In this thesis, to assess the role of DWV in shaping the studied interactions, bees collected 'early' and 'late' in the season were compared in view of the well know seasonality of viral infection such that early in the season DWV prevalence and infection level are low while late in the season both 100% prevalence and high viral loads are normally recorded under the local conditions. This was necessary in view of the impossibility to rely upon completely uninfected bees such as those that can be found on a few remote islands. Actually, infected bees could be obtained also by artificially infecting virus free bees collected early in the season but this treatment may induces many more differences in the

biological material because of the perturbation related to the infection method (e.g. intrahemocaelic injection). The used approach could therefore be regarded as a simplification since the difference between early and late season bees is not limited to DWV levels. For example, short-living summer bees and long-living winter bees contain different concentrations of carbohydrates, amino acids, choline-containing compounds, and other unknown compound (Lee *et al.*, 2022). However, it was assumed that those further differences are minor when compared to that related to viral infection, in view of the dramatic implications for survival that were documented.

In conclusion, I believe that the results obtained here may set the stage for further investigations aiming at better characterizing the observed interactions. Moreover, I'm confident that the approach adopted in this study may represent a useful template for similar studies dealing with similarly complex biological systems.

CHAPTER 7 - **References**

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CHAPTER 8 - **Appendix: other works published during the Ph.D. course**

Attachment 1

Frizzera D, Ray AM, Seffin E, Zanni V, Annoscia D, Grozinger CM and Nazzi F (2022) The Beneficial Effect of Pollen on Varroa Infested Bees Depends on Its Influence on Behavioral Maturation Genes. Front. Insect Sci. 2:864238. doi: 10.3389/finsc.2022.864238

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ORIGINAL RESEARCH
published: 27 April 2022
doi: 10.3389/finsc.2022.864238

The Beneficial Effect of Pollen on Varroa Infested Bees Depends on Its Influence on Behavioral Maturation Genes

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Edited by:
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Reviewed by:
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Julius Maximilian University of Würzburg, Germany
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University of Puerto Rico, Puerto Rico
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Specialty section:
This article was submitted to Insect Health and Pathology, a section of the journal Frontiers in Insect Science

Received: 28 January 2022
Accepted: 04 April 2022
Published: 27 April 2022

Citation:
Frizzera D, Ray AM, Seffin E, Zanni V, Annoscia D, Grozinger CM and Nazzi F (2022) The Beneficial Effect of Pollen on Varroa Infested Bees Depends on Its Influence on Behavioral Maturation Genes. Front. Insect Sci. 2:864238. doi: 10.3389/finsc.2022.864238

Honey bees collect nectar and pollen to fulfill their nutritional demands. In particular, pollen can influence longevity, the development of hypopharyngeal glands, and immune-competence of bees. Pollen can also mitigate the deleterious effects caused by the parasitic mite *Varroa destructor* and related deformed wing virus (DWV) infections. It has been shown that *V. destructor* accelerates the physiological and behavioral maturation of honey bees by influencing the interaction between two core physiological factors, Vitellogenin and juvenile hormone. In this study, we test the hypothesis that the beneficial effects of pollen on Varroa-infested bees are related to the hormonal control underpinning behavioral maturation. By analyzing the expression of genes associated to behavioral maturation in pollen-fed mite-infested bees, we show that treatment with pollen increases the lifespan of mite-infested bees by reversing the faster maturation induced by the parasite at the gene expression level. As expected, from the different immune-competence of nurse and forager bees, the lifespan extension triggered by pollen is also correlated with a positive influence of antimicrobial peptide gene expression and DWV load, further reinforcing the beneficial effect of pollen. This study lay the groundwork for future analyses of the underlying evolutionary processes and applications to improve bee health.

Keywords: honey bee, nutrition, pollen, juvenile hormone, behavioral maturation, Vitellogenin

INTRODUCTION

Honey bees use carbohydrates to obtain energy, proteins for growth and development, lipids for energy reserves, whereas minerals, vitamins and water are needed for optimal survival (1). Honey bees gather these substances by collecting nectar, pollen and water from the environment in quantities that can exceed colony demands. The surplus is stored for periods of dearth and for feeding juvenile stages (2). Nectar is the only source of carbohydrates; it provides energy for metabolic processes but it is also associated with the innate humoral and cellular immune reactions. Nectar can also provide secondary plant metabolites complementing the immune system reducing microbial or pathogen pressure due to their antimicrobial properties (3). Pollen provides proteins, lipids, amino acids, sterols and vitamins required for physiological processes such as growth

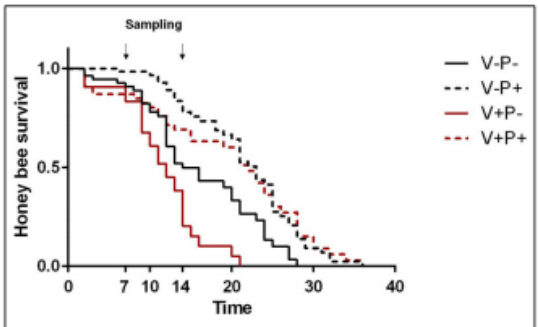

FIGURE 1 | Survival of uninfested (V–) and mite-infested (V+) honey bees fed with pollen (P+) or not (P–). Varroa decreased the survival of honey bees [V–P– vs. V+P-, Log Rank (Chi Square = 10.59, d.f. = 1, $P = 0.001$)]. Pollen increased the survival of both uninfested [V–P– vs. V–P+, Log Rank (Chi Square = 13.31, d.f. = 1, $P < 0.001$)] and mite-infested honey bees [V+P- vs. V+P+, Log Rank (Chi Square = 22.77, d.f. = 1, $P < 0.001$)]. The survival of Varroa-infested bees fed with pollen was similar to that of pollen-fed uninfested bees [V+P+ vs. V–P+, Log Rank (Chi Square = 0.096, d.f. = 1, $P = 0.757$)]. Arrows indicate the timing of sampling for gene expression analysis at day 7 and 14.

and immunity (4–6). Indeed, these nutrients make pollen nutrition one of the most important factors influencing bee longevity (7) and a key factor boosting honey bee tolerance against pesticides, pathogens and viruses (8–11). Pollen nutrition also positively affects the development of hypopharyngeal glands (12), the production of antimicrobial peptides (AMPs) (13), the expression of longevity genes (13), and generally increases immune competence (11, 14).

The mite *Varroa destructor* is the most important ecto-parasite of the western honey bee (15). During the reproductive phase, inside the capped brood cells, the mite feeds on the haemolymph obtained from a hole pierced in the pupal abdomen (16). Recent work suggests that Varroa mites also consume materials from the fat body while they are feeding from the pupating bee (17) but further research is needed to determine the extent to which the nutritional needs of the mite are met by the residual fat body vs. the hemolymph. This feeding activity is central to all the detrimental effects caused by the parasite (15), although it is difficult to distinguish between the direct effects of mite parasitization and the indirect ones related to the viruses vectored and facilitated by the mite. In particular, Varroa can transmit and promote the replication of deformed wing virus (DWV) (18, 19), which, due to its ubiquitous distribution (20), represents a constant threat to the survival of honey bee colonies (21).

Varroa can also compromise the normal relationship between nutrition and immunity (22). Indeed, mite parasitized bees have a lower weight at the emergence, lower protein content and elevated free amino acids levels, suggesting that protein synthesis and growth are disrupted by Varroa (23). Varroa also influences the food intake of adult honey bees parasitized at the pupal stage (i.e., parasite induced anorexia), likely due to an interaction with the insulin pathway (24). Varroa can additionally modulate the honey bee's age-dependent behavioral maturation. Worker bees start out as hive bees nursing larvae and subsequently switch to foraging and this transition represents a turning point in the honey bee's life, involving drastic changes in behavior, physiology and immunity, with foragers showing reduced immune-competence as compared to nurses [for a review, see (25)]. The mite accelerates the physiological maturation of honey bees by influencing the relationship between two core physiological factors, Vitellogenin and juvenile hormone (JH) (26–28). Vitellogenin is a yolk precursor protein that is also involved in immunity (29, 30). The protein, encoded by *vg* gene, is particularly abundant in the haemolymph of nurse honey bees (31–33). JH is a hormone with high titers in forager bees and a low concentration in nurses (34, 35). Juvenile hormone esterases (JHEs), play a major role in JH metabolism. In particular, JH esterase, encoded by *jhe*, degrades JH during larval and pupal development and can therefore be used as marker of JH titer (35).

Nurse bees are thus characterized by a high concentration of Vitellogenin and a low JH titer, while foragers show low levels of the protein and high hormone concentration. These two compounds are involved in a double negative feedback loop that regulates forager transition (36, 37). Moreover, the timing of the switch determines the overall lifespan of the worker (25) such that the transition to foraging can be interpreted as the starting point of a count-down to death.

In a previous study, we showed that pollen intake can mitigate the deleterious effects of *V. destructor* and the related virus infections enhancing the lifespan of mite-infested bees under lab conditions (38). In the article we listed a number of possible mechanisms accounting for the observed beneficial effects of pollen on diseased bees, including: increasing the supply of energetic compounds complementary to sugars (i.e., lipids), reinforcing the cuticle and thus preventing water loss, improving defense against pathogens facilitated by Varroa; influencing the hormonal regulation of the honey bee's homeostasis. This latter potential mechanism is particularly interesting for the possible implications for polyethism which is regulated by hormones.

Here we test the hypothesis that pollen can prolong mite-infested bees' lifespan by inverting the accelerated behavioral maturation caused by the parasite. Since Vitellogenin and JH are the key regulators of behavioral maturation in bees (36, 39), we predicted that pollen stimulates the expression of *vg* and *jhe* in mite-infested bees (prediction 1). Moreover, we predicted that the delay of the transition to foraging caused by pollen should stimulate immunity (prediction 2) because the transition to foraging is associated with a reduced immune-competence (29, 40, 41). To this aim, we used *Apidaecin-1* and *Defensin-1* as indicators of immune system activation. Furthermore, given the reduced immune-competence associated with the transition to foraging, we predicted that the abundance of DWV is affected by age and pollen feeding (prediction 3).

To test our hypothesis and the related predictions, we artificially infested honey bee larvae at the pupal stage and fed the eclosing bees with a diet complemented with or without pollen. Then, after confirming the beneficial effect of pollen on the lifespan of mite-infested honey bees, we studied the expression of key genes involved in behavioral maturation and immunity

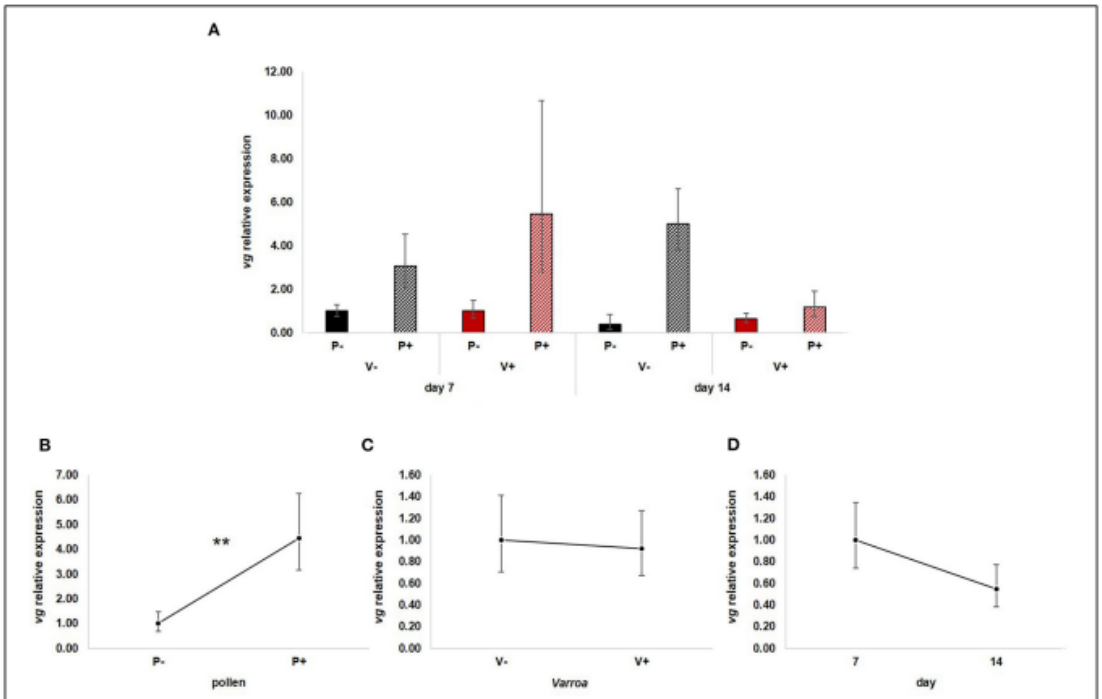

FIGURE 2 | (A) *vg* relative expression in the experimental groups. **(B)** Pollen significantly up-regulated *vg* expression [GLM, pollen ($F = 10.71$, d.f. = 1, $P = 0.003$)]. **(C)** Varroa did not have a significant effect on *vg* expression [GLM, Varroa ($F = 0.01$, d.f. = 1, $P = 0.941$)]. **(D)** *vg* expression decreased with time, but the effect was not significant [GLM, Time ($F = 1.73$, d.f. = 1, $P = 0.197$)]. $^{**}P < 0.01$.

to assess how they are affected by parasitization and how this influence is shaped by the pollen acquired through the diet.

MATERIALS AND METHODS

Effect of Pollen on Varroa-Infested Honey Bees

To confirm that pollen can mitigate the negative effect of mite parasitization and assess the expression of a panel of key genes involved in behavioral maturation and immunity, we reared honey bee larvae inside artificial cells in presence of a Varroa mite or not according to Nazzi and Milani (42). To this aim, we transferred 5th instar larvae into gelatine capsules (6.5 mm Ø; Agar Scientific Ltd.) with one (V+) or no (V−) mites and maintained them in an incubator at 34.5°C, 75% relative humidity (R.H.), dark, for 12 days. At the emergence, Varroa-infested bees (that were separated from the mite) and control bees were transferred into plastic cages (185 × 105 × 85 mm), under standardized environmental conditions (34.5°C, 75% R.H., dark) and fed under two different diet regimes: a sugar diet complemented with pollen (P+) and a sugar diet (P−), supplied *ad libitum*. Sugar was provided as a solution (61% glucose, 39% fructose) with a 20 mL syringe, whereas multifloral pollen (previously maintained at −20°C) was offered to bees in an open petri dish placed on the floor of the cages. Sugar solution and pollen were renewed every 7 days.

In total, we set up four experimental groups (from 54 to 62 bees per group): uninfested bees fed with sugar only (V−P−), uninfested bees fed with sugar and pollen (V−P+), mite-infested bees fed with sugar (V+P−) and mite-infested bees fed with sugar and pollen (V+P+).

Dead bees were counted and removed daily. The experiment was replicated three times.

Gene Expression

Bees to be used for the molecular analyses were sampled on day 7 and 14 and flash frozen in liquid nitrogen. In those bees, we studied the expression of the following genes: (1) *vg* (**Supplementary Table 1**), which encodes for Vitellogenin; (2) *jhe* (**Supplementary Table 1**); (3) *Apidaecin-1* and 4. *Defensin-1* (**Supplementary Table 1**). We also tested the abundance of DWV (**Supplementary Table 1**). According to Corona et al. (43) *vg* expression varies across tissues, being the highest in the abdomen where fat body is concentrated; however, the time-dependent

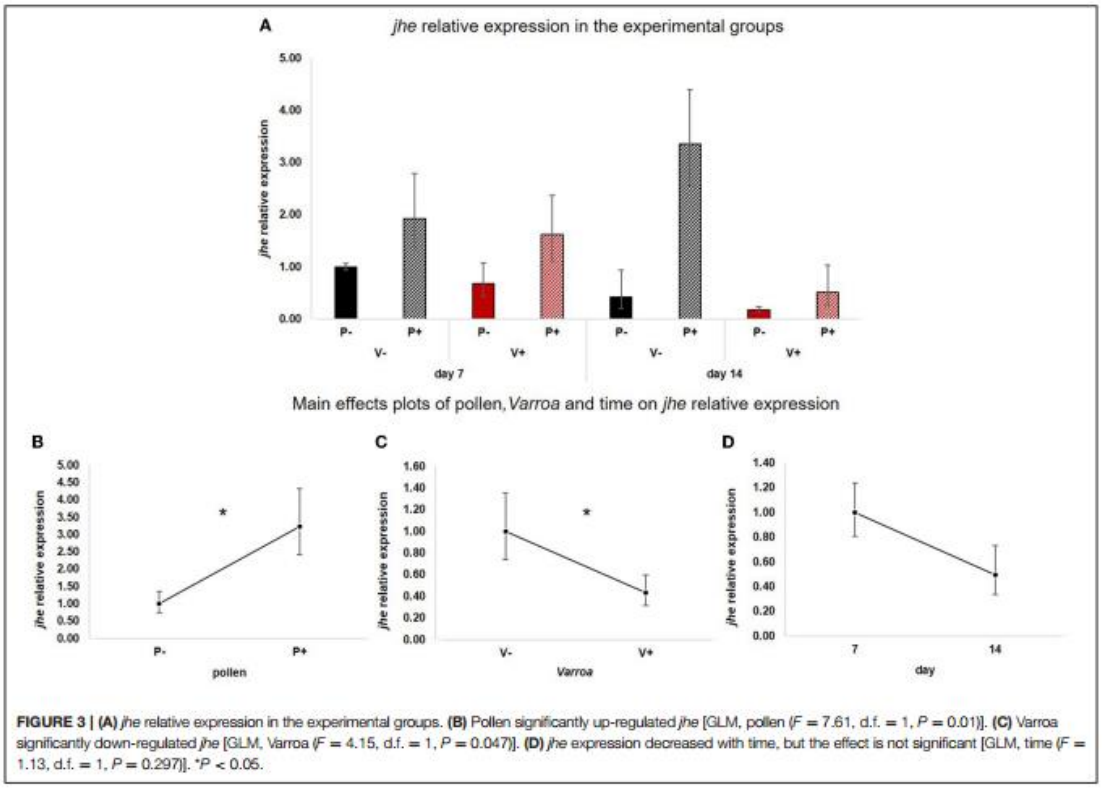

FIGURE 3 | (A) *jhe* relative expression in the experimental groups. **(B)** Pollen significantly up-regulated *jhe* [GLM, pollen ($F = 7.61$, d.f. = 1, $P = 0.01$)]. **(C)** Varroa significantly down-regulated *jhe* [GLM, Varroa ($F = 4.15$, d.f. = 1, $P = 0.047$)]. **(D)** *jhe* expression decreased with time, but the effect is not significant [GLM, time ($F = 1.13$, d.f. = 1, $P = 0.297$)]. *$P < 0.05$.

pattern of expression is the same in different body parts. For this reason, in this study, which was dedicated to the possible influence of diet and mite infestation on worker bees' behavioral maturation, we investigated gene expression using the whole body of honey bees.

Sampled bees were homogenized using mortar and pestle in liquid nitrogen. Total RNA was extracted from each bee according to the protocol provided with the RNeasy Plus mini kit (Qiagen®, Germany). The amount of RNA in each sample was quantified with a NanoDrop® spectrophotometer (ThermoFisher™, US). cDNA was synthesized from 500 ng of RNA per sample, following the manufacturer specifications (PROMEGA, Italy). Additional negative control samples containing no RT enzyme were included. Ten nanogram of cDNA from each sample were analyzed using SYBR® Green dye (Ambion®) according to the manufacturer specifications, on a BioRad CFX96 Touch™ Real time PCR Detector. All samples were run in duplicate; when technical replicates differed by more than 0.5 Ct, the analysis was repeated, in duplicate, in another plate. The following thermal cycling profiles were adopted: one cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min, and one cycle at 68°C for 7 min. Given the high number of samples to be analyzed, an inter-plate calibrator (i.e., a control sample that was run in every analyzed plate) was used. Relative viral load and gene expression were analyzed with the $2^{-\Delta\Delta Ct}$ method (44) using b-actin and GAPDH as housekeeping genes (**Supplementary Table 1**); those genes were selected on the ground of literature data and a preliminary study aiming at comparing the response of some candidate housekeeping genes. Primers' efficiency was between 95 and 99%. Log Normalized values were analyzed using GLM by means of Minitab 16. In total, five bees per treatment and per sampling point were analyzed. All data and the details of the statistical analyses are reported in **Supplementary Data Sheet 1**.

RESULTS

Effect of Pollen and Varroa on Honey bee Survival

As expected, under lab conditions, Varroa significantly negatively impacted honey bee survival [**Figure 1**; V−P− vs. V+P−, Log Rank (Chi Square = 10.59, d.f. = 1, $P = 0.001$)] while pollen positively influenced the lifespan of healthy honey bees [**Figure 1**; V−P− vs. V−P+, Log Rank (Chi Square = 13.31, d.f. = 1, $P < 0.001$)]. Also, pollen significantly increased the lifespan of Varroa-infested honey bees [**Figure 1**; V+P− vs. V+P+, Log Rank (Chi Square = 22.77, d.f. = 1, $P < 0.001$)] such that the survival curve of parasitized bees closely resembled that of

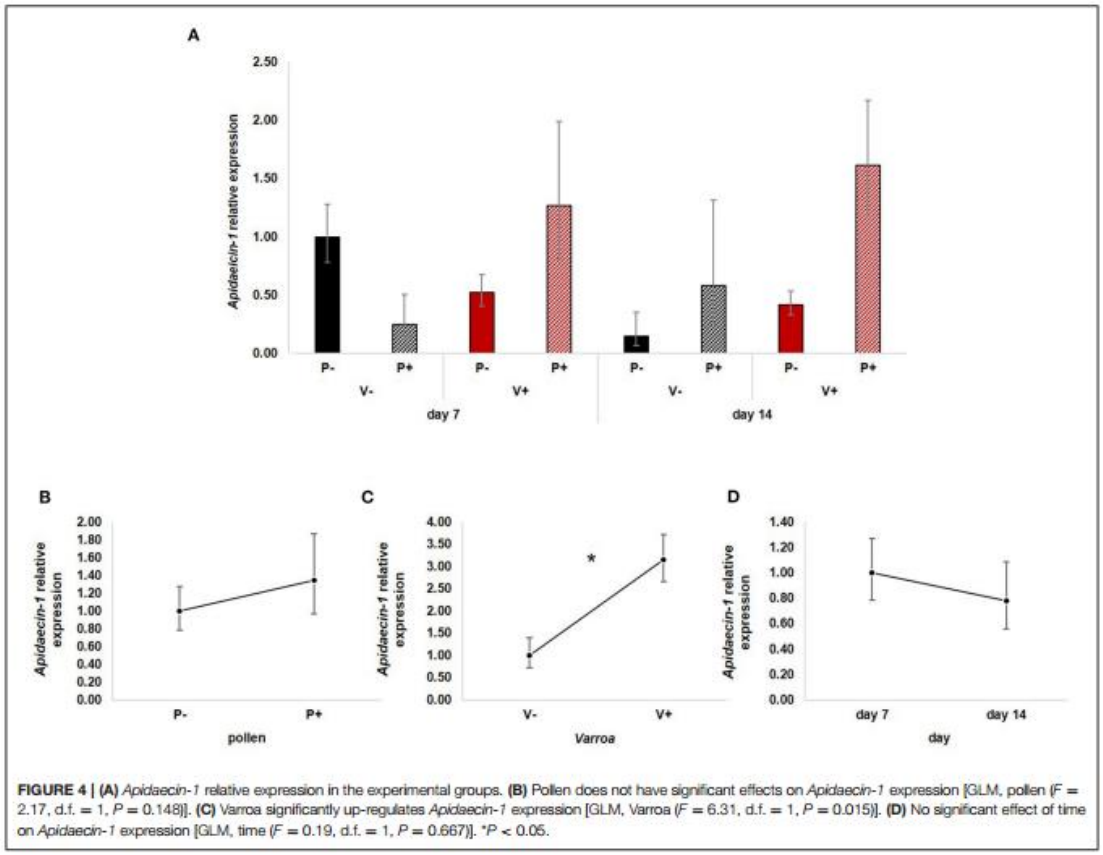

FIGURE 4 | (A) *Apidaecin-1* relative expression in the experimental groups. **(B)** Pollen does not have significant effects on *Apidaecin-1* expression [GLM, pollen (F = 2.17, d.f. = 1, P = 0.148)]. **(C)** Varroa significantly up-regulates *Apidaecin-1* expression [GLM, Varroa (F = 6.31, d.f. = 1, P = 0.015)]. **(D)** No significant effect of time on *Apidaecin-1* expression [GLM, time (F = 0.19, d.f. = 1, P = 0.667)]. *P < 0.05.

uninfested bees [**Figure 1**; V+P+ vs. V−P+, Log Rank (Chi Square = 0.096, d.f. = 1, P = 0.757)].

Effect of Pollen and Varroa on Genes Involved in Behavioral Maturation

Vitellogenin and juvenile hormone play a fundamental role in lifespan and behavioral maturation. The protein is high in nurses and low in foragers, while JH follows an opposite pattern. Therefore, we studied the expression of *vg*, the gene encoding Vitellogenin, and *jhe*, which encodes JH esterase, in relation to pollen diet, Varroa infestation and sampling time. Since *vg* expression is related to Vitellogenin synthesis while *jhe* expression is involved in JH degradation, *vg* and *jhe* are expected to be both high in nurses and low in foragers.

GLM analysis showed that *vg* expression (**Figure 2A**) is significantly up-regulated by pollen [**Figure 2B**; GLM, pollen (F = 10.71, d.f. = 1, P = 0.003)] but not by Varroa [**Figure 2C**; GLM, Varroa (F = 0.01, d.f. = 1, P = 0.941)] and time [**Figure 2D**; GLM, Time (F = 1.73, d.f. = 1, P = 0.197)]. No significant interactions among the three factors were noted (**Supplementary Figures 1A–C**).

Likewise, *jhe* (**Figure 3A**) was positively influenced by pollen [**Figure 3B**; GLM, pollen (F = 7.61, d.f. = 1, P = 0.01)] but was also significantly down-regulated by Varroa [**Figure 3C**; GLM, Varroa (F = 4.15, d.f. = 1, P = 0.047)]. Time did not significantly affect the expression of this gene [**Figure 3D**; GLM, Time (F = 1.13, d.f. = 1, P = 0.297)]. No significant interactions between pollen, Varroa and time were noted (**Supplementary Figures 2A–C**).

Effects of Pollen and Varroa on Antimicrobial Peptides

The transition to foraging is associated with a reduced immune-competence, and antimicrobial peptides are key immune effectors. Therefore, we tested if this further indicator of aging is affected by mite infestation and assessed how pollen feeding shapes this interaction.

Apidaecin-1 expression (**Figure 4A**) was not statistically influenced by pollen [**Figure 4B**; GLM, pollen (F = 2.17, d.f. = 1,

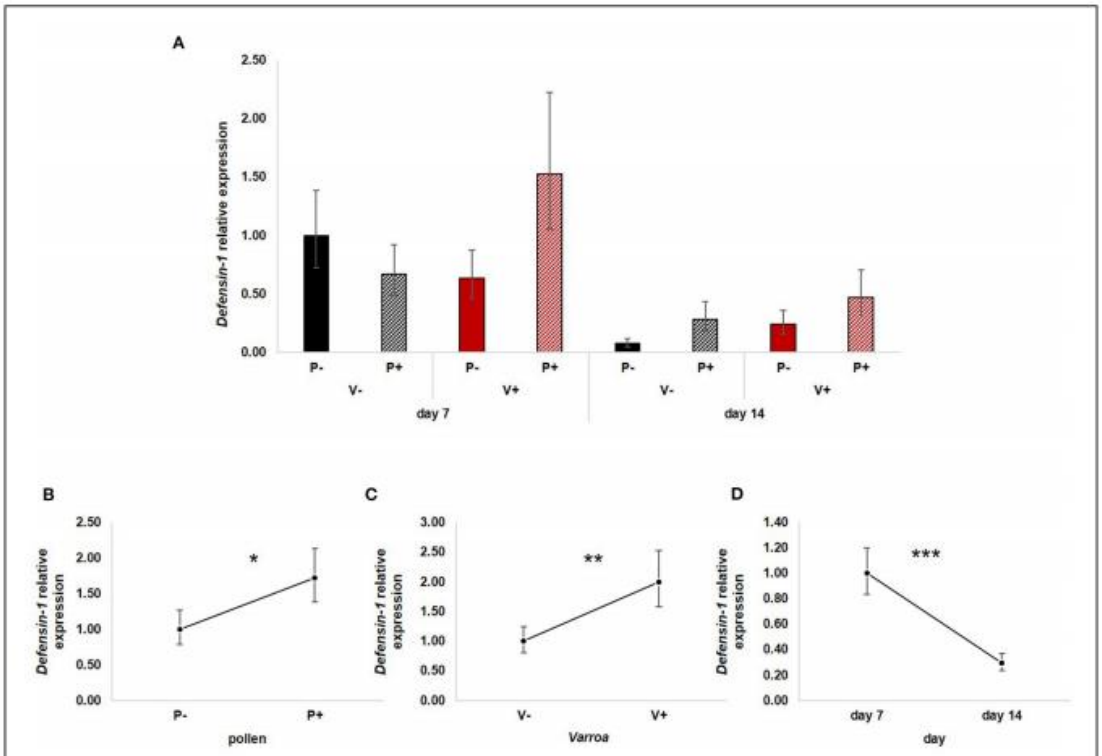

FIGURE 5 | (A) *Defensin-1* relative expression in the experimental groups. **(B)** Pollen significantly up-regulated *Defensin-1* expression [GLM, pollen ($F = 4.22$, d.f. = 1, $P = 0.045$)]. **(C)** Varroa significantly increased *Defensin-1* expression [GLM, Varroa ($F = 7.48$, d.f. = 1, $P = 0.009$)]. **(D)** *Defensin-1* expression significantly decreased with time [GLM, time ($F = 19.54$, d.f. = 1, $P < 0.001$)]. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

$P = 0.148$)] and time [**Figure 4D**; GLM, time ($F = 0.19$, d.f. = 1, $P = 0.667$)] while mite infestation activated the expression of this AMP [**Figure 4C**; GLM, Varroa ($F = 6.31$, d.f. = 1, $P = 0.015$)]. The interaction between Varroa and pollen was close to significance (**Supplementary Figure 3A**); no other significant interactions were noted (**Supplementary Figures 3B,C**).

All three factors—pollen, Varroa and time—had a significant effect on the expression of *Defensin-1* (**Figure 5A**). In particular, pollen up-regulated *Defensin-1* expression [**Figure 5B**; GLM, pollen ($F = 4.22$, d.f. = 1, $P = 0.045$)] as well as Varroa [**Figure 5C**; GLM, Varroa ($F = 7.48$, d.f. = 1, $P = 0.009$)], while the expression decreased with time [**Figure 5D**; GLM, time ($F = 19.54$, d.f. = 1, $P < 0.001$)]. The interaction between Varroa and pollen was close to significance (**Supplementary Figure 4A**); no other significant interactions were noted (**Supplementary Figures 4B,C**).

Effects of Pollen on Viral Load

DWV is normally present in honey bees at low titers, but replication is activated by several stress factors, including Varroa infestation (19). Considering the decreasing immune-competence of foragers, we tested if the abundance of this ubiquitous pathogen is affected by age and how pollen feeding influences this interaction (**Figure 6A**).

GLM analysis revealed a significant negative effect of pollen on DWV load [**Figure 6B**; GLM, pollen ($F = 8.03$, d.f. = 1, $P = 0.007$)], a predictable increase of virus load modulated by Varroa infestation [**Figure 6C**; GLM, Varroa ($F = 12.49$, d.f. = 1, $P = 0.001$)] and a significant reduction with time [**Figure 6D**; GLM, time ($F = 5.07$, d.f. = 1, $P = 0.029$)]. No significant interactions between pollen, Varroa and time were noted (**Supplementary Figures 5A–C**).

DISCUSSION

Earlier studies demonstrated that dietary pollen has a positive effect on the immunity and lifespan of honey bees, while Varroa parasitization has a negative impact on these two traits. Our study identifies a potential mechanism for these

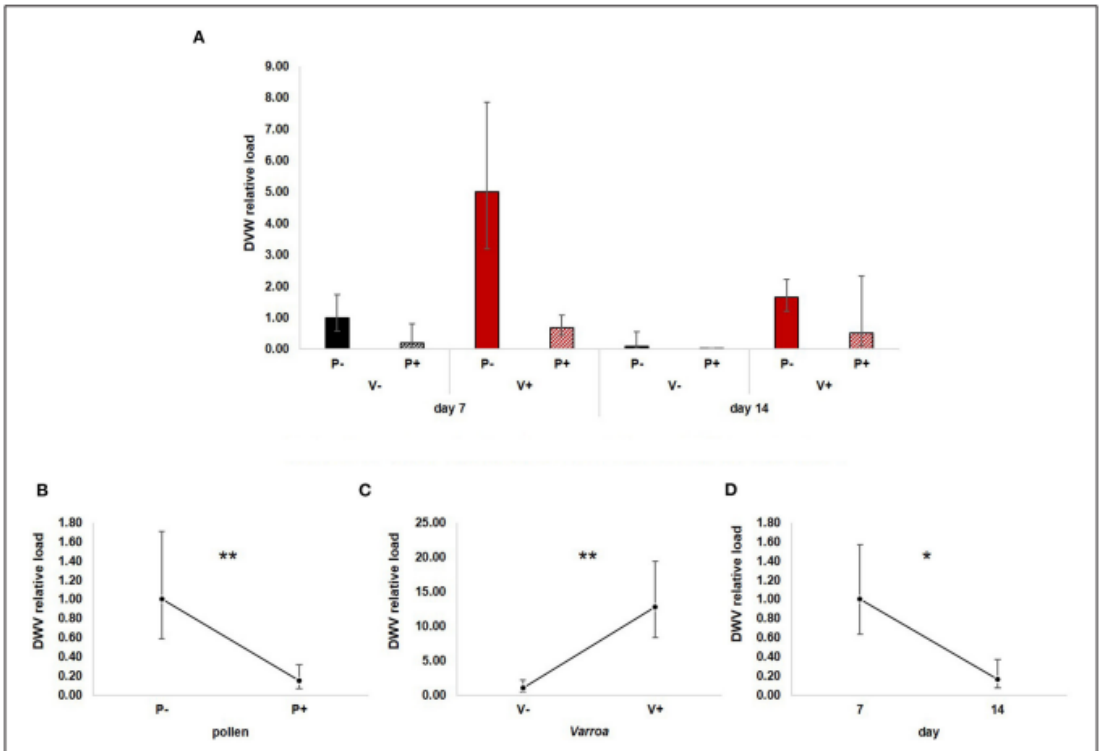

FIGURE 6 | (A) DWV relative load in the experimental groups. **(B)** Pollen diet caused a significant reduction of viral load both in mite-infested and uninfested honey bees [GLM, pollen ($F = 8.03$, d.f. = 1, $P = 0.007$)]. **(C)** Varroa infestation increased virus load [Varroa ($F = 12.94$, d.f. = 1, $P = 0.001$)]. **(D)** DWV load decreased with time [GLM, time ($F = 5.07$, d.f. = 1, $P = 0.029$)]. *$P < 0.05$; **$P < 0.01$.

pleiotropic effects. We demonstrate that pollen consumption alters the expression of two key genes underpinning the juvenile hormone-mediated behavioral maturation process in honey bee workers. Thus, pollen-fed bees are in a physiologically younger state, which is associated with increased immune function and hence increased ability to reduce viral infections. Since Varroa feeding shifts the expression of these key genes to accelerate maturation, parasitization can reduce longevity and immune gene function, leading to higher viral infections. Thus, dietary pollen can mitigate the impact of Varroa parasitization on bee immunity and lifespan, through its influence on these core genes.

In agreement with our previous study, the access to dietary pollen appeared to mitigate the impact of Varroa mite infestation in caged honey bees under laboratory conditions (38). Indeed, Varroa reduced the survival of honey bees, but pollen feeding nearly compensated for that effect, significantly extending the lifespan of mite-infested bees, such that the survival of pollen-fed mite-infested bees was not very different from that of pollen-fed uninfested bees. Indeed, apart from the first few days, when mite-infested bees fed with pollen survived less than uninfested bees, the survival curves of the two groups overlapped at around day 20 (**Figure 1**). These patterns are consistent with the results obtained for the DWV titers: at eclosion, mite-infested bees bear much higher levels of virus which tend to cause higher mortality in the very first days of adult life, before pollen consumption can exert its beneficial action.

Besides confirming previous results on the effects of pollen on the survival of mite-infested bees, in this work we wanted to test the hypothesis that pollen can prolong the lifespan of those bees by mitigating the accelerated behavioral maturation caused by the parasite. In particular, given the role played by Vitellogenin and JH on bees' behavioral maturation (36, 39), we first predicted that pollen stimulates the expression of *vg* and *jhe* in mite-infested bees. The role of pollen, influencing behavioral maturation via its effects on *vg* and *jhe*, was confirmed here. Both *vg* and *jhe* were up-regulated in pollen-fed bees (**Figures 2B**, **3B**). The effect of pollen on Vitellogenin is consistent with results from previous studies (45). The effect of pollen on *jhe* levels, which is regarded as a marker of JH levels (35), supports the double repressor hypothesis proposed

by Amdam and Omholt (36), where the transition to foraging is regulated by those two signals linked in a mutual negative feedback loop, generating the bistability responsible for the sharp transition between the two stages. Under this model, pollen can extend the lifespan of bees due to its action on these two regulators and the resulting delayed transition to foraging (43, 46). Furthermore, the accelerated behavioral maturation caused by the mite (27, 28, 47) was confirmed here by the down-regulation of *jhe* observed in the case of mite infestation (**Figure 3C**). While our study did not show a significant negative impact of *vg* levels as a result of mite infestation, possibly due to high variation among our samples, previous studies have clearly demonstrated that mite infestation results in a significant reduction of *vg* levels (28, 48, 49). Importantly, our results confirm our first prediction that the increase in the expression of *vg* [previously observed also by Alaux et al. (13)] and *jhe*, observed in mite-infested pollen-fed bees, can counteract the accelerated transition to foraging caused by mite infestation described above. Our results also support our second prediction, that pollen feeding stimulates immune function, as an outcome of delayed maturation. The fact that aging is related to a reduced immune competence is supported by the decreasing trend observed in both AMPs according to bees' age (**Figures 4D**, **5D**). Moreover, the higher expression of both AMPs in pollen-fed bees indicates that indeed pollen feeding results in a delayed behavioral maturation and consequently a nurse-like phenotype at older ages (**Figures 4B**, **5B**). In contrast, the up-regulation of AMPs in the case of mite infestation has already been observed (**Figures 4C**, **5C**) (13, 28, 50, 51), and is likely related to the response to the secondary infections triggered by the mite (52), and the proposed implication of AMPs in antiviral response of bees (19, 53). Lastly, the results obtained here by testing DWV load in bees fed or not with pollen (**Figure 6B**), nicely confirm that, by postponing the transition to foraging and thus enhancing immune-competence, dietary pollen can indirectly contribute to reducing viral infections thus confirming our third prediction. The detrimental effects of *V. destructor* parasitism on DWV load have been extensively studied, and our results confirm previous data (13, 19, 38, 54, 55). Interestingly, there was a significant effect of time on DWV (**Figure 6D**). However, this result is mostly affected by the decreasing of virus load in sugar-fed bees from day 7 to day 14 (**Supplementary Figure 5C**). However, on day 14, more than 50% of the bees in this treatment group were already dead (**Figure 1**). Thus, the most infected bees in this sample group died early, likely leaving the less infected bees, which were sampled on day 14.

In conclusion, we confirm that pollen has a beneficial effect on bees challenged with Varroa mite. Varroa infestation at the pupal stage influences the nutritional status of the honey bee (23, 24); this compromises the natural maturation by influencing the relationship between two core physiological factors, Vitellogenin and JH (27). This leads to an accelerated transition to foraging and thus an anticipated death since this transition determines the overall lifespan of the bee (25). Instead, dietary access to pollen counteracts the accelerated transition caused by Varroa, influencing the key regulators of that process. As a further positive side effect, the enhanced immune-competence allows a better response to the secondary infections triggered by the mite, resulting in further reinforcement of the already positive effects of pollen on honey bee survival in case of mite infestation.

In our opinion, the lab work described here lays the foundations for further and necessary field-based studies. Our results well explain the effect of pollen on mite-infested individual bees but the complexity of social life could not be incorporated into our experiments. Indeed, the colony is supported by a complex network of interactions and the behavioral maturation of individual honey bees is affected by a number of external factors. In fact, in the colony, the transition to foraging is influenced by both social and environmental factors (56–59) but can also impact the colony's food intake as well as the individual bee mortality and thus colony composition and in turn pollen availability (56, 57). Furthermore, such colony effects can also influence Vitellogenin and JH levels (60). Further studies at colony level are therefore necessary to fully evaluate the effects of pollen on parasitized honey bees in their natural environment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DF: conceptualization, data curation, investigation, and writing original draft. AR and CG: conceptualization. ES and VZ: data curation and investigation. DA: conceptualization, data curation, and investigation. FN: conceptualization, data curation, and writing original draft. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the European Union's Horizon 2020 research and innovation programme, under Grant Agreement No. 773921 (PoshBee) and by the Italian Ministry of University, PRIN 2017 - UNICO (2017954WNT).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2022.864238/full#supplementary-material

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Attachment 2

XXVI Congresso Nazionale Italiano di Entomologia

Torino, 11th June 2021

Detoxification as a key to understand the interaction between pollen and toxic compounds in honey bees

Elisa Seffin, Davide Frizzera, Virginia Zanni, Desiderato Annoscia, Francesco Nazzi

Oral presentation

Honey bees (*Apis mellifera* L.) play a vital role in ecosystems as plant pollinators and are essential for both plant biodiversity and agricultural production; indeed, one third of world crop production relies on animal pollination. Recent research highlighted the role of multiple interactions among several stress factors as a major cause of widespread colony losses threatening those essential ecosystem services.

Pollen is the only source of proteins for honey bees and contains other substances that are necessary for normal growth and development. On the other hand, pollen may also contain toxic compounds, or it could be contaminated by widespread insecticides.

Therefore, in many cases, to exploit the beneficial effects of pollen, metabolic detoxification (which is often based on Cytochrome P450 monooxygenases) must be activated with a costly process in terms of energy; for this reason, the interaction between pollen and toxic compounds deserves a close scrutiny.

To gain insight into the possible interactions between pollen and toxic compounds that can be associated to it, we carried out dedicated lab experiments using nicotine as a toxic compound, both because it is common in some nectars and pollens and for its affinity with some insecticides.

To this purpose honey bees were fed with nicotine, pollen or both and treated or not with a common inhibitor of detoxification (i.e. the insecticide synergist piperonyl butoxide (PBO), a P450 inhibitor, enhancing the toxicity of pyrethroid and neonicotinoid insecticides).

The experiment was replicated three times in the early season and in the late season, to evaluate possible differences related to the presence deformed wing virus (DWV): a common pathogen that is rare in Spring and widespread in late Summer.

Preliminary results suggest that nicotine negatively affects bee survival only early in the season, when viral infection is low; under this condition, pollen seems to counteract the negative effect exerted by nicotine. Late in the season, when viral infection is higher, nicotine alone doesn't seem to be similarly harmful whereas the presence of pollen and nicotine together significantly reduce the survival. Finally, PBO appears to be active only in presence of both factors.

Attachment 3

2rd Joint Meeting of Agriculture-oriented PhD Programs UniCT, UniFG, UniUD

14-16 September 2020

How interaction among stress factors might affect honey bees health

PhD student: Elisa Seffin
Supervisor: Francesco Nazzi

Oral presentation

Honey bees (*Apis mellifera* L.) play a vital role in ecosystems as plant pollinators and are essential for the conservation of plant biodiversity and agricultural production; indeed, one third of world crop production relies on animal pollination.

In the last decades a serious decline of bee colonies has been observed in many countries, as a result of extensive colony losses. This worrying phenomenon is due to the interaction among a number of stress factors, including parasites and pathogens (i.e. *Varroa destructor*, deformed wing virus-DWV), agrochemicals, the availability and quality of food resources and environmental conditions.

For this reason, to understand how different stress agents (abiotic and biotic) might positively or negatively interact is fundamental to plan possible actions to maintain and restore bee health.

On the other hand, honey bee survival is enhanced by a convenient supply of pollen, which, however, may also contain toxic compounds.

To further investigate the beneficial effects of pollen, an experiment was carried out to determine how DWV effects are mitigated by the introduction of pollen in the diet. Furthermore, to identify the active component, pollen deprived of lipids or amino acids was administer to bees and the effects compared with those of whole pollen.

To gain insight into the role of possible toxic compounds from pollen, I investigated the effects of pollen in bees whose detoxification system was impaired using the insecticide synergist piperonyl butoxide (PBO), a P450 inhibitor.

Preliminary results suggest that pollen increased survival in virus affected bees and pollen deprived of lipids determined a similar outcome if compared to the whole one. Nicotine negatively affected bee survival; furthermore, pollen in the diet enhanced this negative trend. PBO seems to exacerbate the negative effect of nicotine in presence of pollen.

Attachment 4

3rd Joint Meeting of Agriculture-oriented PhD Programs UniCT, UniFG, UniUD

Giovinazzo (BA), 11 - 15 October 2021

A look at the interaction among stress factors that may affect honeybee health

PhD student: Elisa Seffin
Supervisor: Francesco Nazzi

Oral presentation

Honeybees (*Apis mellifera* L.) are important pollinators, contributing to plant biodiversity and agricultural production.

In the last decades, a worrying decline of bee colonies has been observed in many countries. The interaction among stress factors play a crucial role; in particular, nutrition can affect the capacity of bees to tolerate parasitic infections.

Pollen is the only source of proteins for bees and contains substances that positively affect bees' stress resistance. In previous experiments, we highlighted a beneficial effect of the pollen's polar fraction on bees infected by the deformed wing virus (DWV). I therefore tested if, quercetin, one of the most common pollen's flavonoids, may account for that positive effect. Preliminary results suggest that quercetin is important but doesn't explain all the beneficial effects of pollen.

Moreover, pollen may also contain toxic compounds that must be detoxified and the honeybee's detoxification system (e.g., Cytochrome P450 monooxygenases) certainly plays an essential role. To investigate this aspect, honeybees were fed nicotine, pollen or both and treated or not with a common inhibitor of detoxification (i.e. piperonyl butoxide, a P450 inhibitor). The experiment was replicated early in the season when viral infection is limited or later when DWV is widespread to evaluate possible interactions with viral infection.

According to the preliminary results, the effect of nicotine is heavily affected by viral infection, instead, PBO shows a different trend according to the season.

Finally, to assess how other factors can shape the reaction to nutrition and infection, a multifactorial experiment was carried out in which bees were exposed to four different factors: the neonicotinoid sulfoxaflor, a low temperature and a parasitic infestation either in presence of pollen or not. The experiment suggested an interesting interaction between nutrition and toxic compounds that, again, could be mediated by the detoxification system.

Attachment 5

III Convegno AISSA#under40, Bolzano 14-15 luglio 2022

Facoltà di Scienze e Tecnologie, Libera Università di Bolzano-Bozen

Can the interaction between pollen and toxic compounds affect honey bee survival?

Elisa Seffin, Davide Frizzera, Virginia Zanni, Desiderato Annoscia, Francesco Nazzi

Oral presentation

Honey bees (*Apis mellifera* L.) play a vital role in ecosystems and are essential for both plant biodiversity and agricultural production; indeed, one third of world crop production relies on animal pollination.

Pollen is the only source of proteins for honey bees and contains substances that are necessary for growth and development. However, pollen may also contain toxic compounds resulting from plants metabolism or the contamination with pesticides used in agriculture.

In presence of potentially harmful substances, the metabolic detoxification (based on Cytochrome P450 monooxygenases) is activated. Therefore, both pollen and toxic compounds could engage the same physiological response system resulting in a potential interaction between the two factors.

To gain insight into the possible interactions between pollen and xenobiotics potentially associated to it, we carried out dedicated lab experiments using nicotine as toxic compound, both because it is common in some nectars and pollens and for its affinity with some insecticides.

To this purpose honey bees were fed with nicotine, pollen or both and treated or not with the insecticide synergist piperonyl butoxide (PBO), a P450 inhibitor, normally used to enhance the toxicity of pyrethroid and neonicotinoid insecticides.

Replicated experiments were carried out both early in the season, when the prevalence of a common viral pathogen (DWV) is low, and later in the season when viral infection is widespread.

Preliminary results suggest that nicotine, at the tested dose, negatively affects bee survival only early in the season. Under these conditions, pollen appeared to counteract the negative effect exerted by nicotine.

Instead, late in the season, when viral infection is higher, nicotine alone doesn’t seem to be similarly harmful, whereas the concurrent presence of pollen and nicotine significantly reduces honey bee survival.

Finally, PBO shows a different trend according to the season.

Attachment 6

4rd Joint Meeting of Agriculture-oriented PhD Programs UniCT, UniFG, UniUD

Paluzza (UD), 3 - 7 October 2022

Interactions among stress factors and their effect on honeybee health

Elisa Seffin, Davide Frizzera, Virginia Zanni, Desiderato Annoscia, Francesco Nazzi

Oral presentation

Honeybees (*Apis mellifera* L.) play a vital role in ecosystems' maintenance, providing fundamental pollination services and thus contributing to plant biodiversity and agricultural production.

In the last decades, worrying losses of honeybee colonies have been reported in many countries which are related to the interactive effects of several stress factors. In order to plan effective remedial actions, it is essential to better understand how different stress agents might interact influencing honeybee health.

Since nutrition influences bees' stress tolerance, we focused our attention on pollen, an important proteins' source that plays a key role in bees' life.

Firstly, we considered the possible interaction between pollen and virus infection. We investigated if the detrimental effects of the deformed wing virus –that causes deformity and reduced lifespan– could be mitigated by a pollen-based diet and which components can explain its positive biological activity.

To gain insight into the higher order interactions involving pollen, we carried out a multifactorial experiment in which bees were exposed to three different stress factors: the insecticide sulfoxaflor, a low temperature and a parasitic infestation, either in presence of pollen or not.

Finally, since pollen may also contain toxic compounds that must be detoxified, we carried out another multifactorial experiment involving a plants' secondary metabolite: the toxic alkaloid nicotine.

We found that pollen can mitigate the detrimental effects of a viral infection.

Interestingly, we observed a general positive effect of pollen in presence of the other stress factors mentioned above; in particular, it seems that pollen can exert a beneficial impact on honeybees' survival when bees are exposed to low temperature or parasitic infections.

Moreover, the interaction between pollen and nicotine is heavily affected by viral infection, which increases during the summer.

Attachment 7

European PhD Network "Insect Science" - XIII Annual Meeting

Firenze, 16-18 November 2022

c/o CREA - Centro di Ricerca per la Difesa e la Certificazione, Firenze

Pollen and the toxic compound nicotine on honey bees health: an unexpected interaction

Elisa Seffin, Davide Frizzera, Virginia Zanni, Desiderato Annoscia, Francesco Nazzi

Oral presentation

Since honey bees (*Apis mellifera*) are essential for the conservation of plant biodiversity and agricultural production and are the most important pollinators, we can certainly say that honeybees play a vital role in ecosystems.

As with all the living beings, also honey bee survival is enhanced by a convenient supply of essential nutrients.

In fact, pollen is crucial being the only source of proteins for honey bees. Moreover, it contains all the lipids, vitamins and minerals necessary for normal growth and development of the colony.

However, it may also hold toxic compounds such as plant's secondary metabolites or residues of pesticides used in agriculture.

Due to pollinators may encounter the natural xenobiotic nicotine in both nectar and pollen of some plants present in the fields, and because of its affinity for some insecticides, we investigated the interaction between pollen and this toxic alkaloid.

Furthermore, to study if the season can influence this interaction, the experiment was replicated late in the season, when the prevalence of a common honey bees viral pathogen (DWV) is widespread.

Interestingly, the interaction between pollen and nicotine changes if it is affected by viral infection, which increases during the summer.

Finally, given the need to detoxify these substances and to try to explain this interesting interaction, we decided to impair the detoxification systems, Cytochrome P450 monooxygenases, by using Piperonyl butoxide (PBO).

Curiously, also the use of PBO seems to vary depending on the season, and thus the viral load, considered.

Acknowledgements

This Ph.D. project was financed by European Union's Horizon 2020 research and innovation program, under grant agreement No. 773921 (PoshBee).

At the end of my three-year journey, I am deeply grateful to my supervisor Prof. Francesco Nazzi for his presence, enthusiasm for research and science, his patience and immense knowledge.

I would like to extend my sincere thanks to Prof. Desiderato Annoscia for his insightful advice and suggestions.

I would like to offer my special thanks to my colleague Virginia Zanni for all the help, support and friendship. Also, I wish to show my appreciation to my colleague Davide Frizzera for all the help, support and advice during my Ph.D.

Furthermore, I thank so much Silvia Parenzan, the newest member of "UNIUD Bees and Ticks" group for the long talks and comfort during the writing of this thesis.

Thanks to our beekeeper Mauro D'Agaro and to my colleagues from the other labs, especially to Arianna and Gaia.

A big thank also to my American advisor Prof. Michelle Flenniken and her lab members Naomi Kaku, Bridget Doyle and Rose McDonald for the good science, hospitality and the ability to make me feel at home on the other side of the world.

I thank with all my heart my entire beloved family who have always supported me throughout my university career, Ph.D. experience and my life in general. Moreover, I wish my brother Luca a long and fulfilling scientific career.

Finally, I thank Luca, who has believed in me from the very first moment of this fantastic experience, as well as accompanying and supporting me in life.

Comunque vada, sarà un successo.
Ad maiora semper!