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"A multifactorial approach to honey bee health: from multiple causes to consequences and possible actions"

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SUMMARY

In the last decades, large-scale losses of honey bee (*Apis mellifera* L.) colonies have been recorded all over the world. After years of intense investigation, no specific causal agent for the widespread colony losses has been found but rather a multifactorial origin has been proposed for this phenomenon. Biotic and abiotic factors contribute to this situation, but several studies indicate that the ectoparasitic mite *Varroa destructor* and the deformed wing virus (DWV) play an important role. Recent research has shown that DWV infections at low viral levels are asymptomatic because the honey bee immune competence is able to contain virus replication. As soon as any stress factor interferes with this equilibrium, competing for metabolic resources or negatively acting on immunity, uncontrolled viral replication is promoted, resulting in the transition from a benign covert infection to a devastating overt disease. Xenobiotics, abiotic stressors, malnutrition and other factors can further contribute to complicate the situation.

The aim of this thesis was to investigate, at the individual level, how different stress factors and nutrition interact to influence the survival of honey bees. To this aim, we subdivided the study in two phases; in the first one, we assessed how several stress factors as well as pollen influence honey bee health. In a second phase of this study, we investigated how some of these stressors act in combination with the others and in combination with nutrition.

We selected seven factors that are possibly implicated in the multifactorial syndrome related to colony losses: pollen, as a natural supply of amino acids and lipids for the honey bee; hydroxymethylfurfural (HMF), a toxic compound contained in additional sugars syrups; acidity, which normally characterizes the sugar syrups mentioned above; nicotine, which is a toxic alkaloid that bees can encounter in the environment and with a mode of action similar to that of neonicotinoid insecticides; a temperature 2-3 degrees below that normally found within the hive; *V. destructor*, the most dangerous ectoparasite of honey bees and deformed wing virus (DWV), a key pathogen of honey bees.

We confirmed the beneficial effect of pollen nutrition on honey bees as a positive factor on individuals affected by parasites and pathogens. To gain insight into the mechanisms underlying these effects, we interfered with the energetic pathway of mite infested bees using rapamycin, a chemical inhibitor of mTOR, a protein complex that regulates cell growth and other key cellular processes. We observed that rapamycin, just like pollen, decreases DWV load in a manner that could be related to the stimulation of autophagy.

More in general, we noted the important role played by nutrition in the interactions between honey bee and other stressors. We also documented for the first time a kind of physiological anorexia triggered by *V. destructor* infestation, which leads to a reduced energy availability that influences the capacity of honey bees to cope with other stressors.

Moreover, we shed light on the possible detrimental side effects of supplementary nutrition administered to bees by beekeepers with homemade sugar syrups. In fact, acidity and HMF that are normally present in such syrups are toxic for honey bees.

Lastly, with this work we showed how unpredictable it can be the relationship between stress factors. Since an analytic study of all the factors that can affect honey bee health is unimaginable, the attention should be focused on the metabolic process accounting for the observed interactions in order to develop one or several models that could help to predict the outcome of such interactions.

1. INTRODUCTION

1.1. The importance of *Apis mellifera*

Apis mellifera is an Hymenopteran insect belonging to the superfamily Apoidea. This insect, also known as "honey bee" or "western honey bee", is widely distributed all over the world, and lives in colonies formed by tens of thousands of individuals. The colony lives in a nest made of several wax combs built inside a natural cavity or in a hive box provided by beekeepers.

A. mellifera L. is an eusocial insect because of three distinctive characteristic: division of reproductive and non-reproductive castes, overlapping of two or more generations and brood care operated by unfertile individuals. Indeed, inside an *A. mellifera* colony there are both female and male individuals divided in three castes. The first caste includes the queen bee which is the only fertile female of the hive; she lays eggs for the most of the time (e.g. during the Spring a queen bee can lay 1500-2000 eggs per day). Thanks to an organ called spermatheca, she can preserve, for the entire life, the spermatozoa collected during her first and only mate event with drones (i.e. the male honey bees). The other fundamental role of this individual is to maintain the cohesion of the colony by means of pheromones. A queen can live an average of 3-4 years.

The second caste is represented by the worker honey bees. This group represents the non-fertile female caste whose reproductive organs are atrophic because of a pheromone produced by the queen. These bees live an average of 40 days during which they undertake different tasks depending on age; the firsts three weeks of the adult life are normally spent inside the hive taking care of the brood and operating other nest activities; instead, in the lasts weeks of their life, the workers fly outside the hive as foragers to collect nectar and pollen.

Drones bees are male individuals that are observed mostly during Spring and Summer; their principal role is mating with the queen.

Under temperate climates, when the income of nutrients is sufficient, a situation normally occurring in Spring and Summer, a honey bee colony contains one queen bee, a few thousand drone bees and tens of thousands of worker bees, including adult worker bees (20,000 – 60,000) and immature worker bees (10,000 – 30,000) at different developmental stages (egg, larvae and pupae).

The brood is reared in wax cells produced and shaped by the worker bees. During the egg and the larval stage, the cell is open, while, when the larva spins the cocoon for pupation the cell is sealed. The cell remains sealed until the eclosion of the adult bee. The development of the individuals (from egg to adult) depends on the caste lasting 16 days for queens, 21 and 24 days for workers and drones, respectively.

During Autumn, 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).

Because of its products (honey, wax and propolis) *A. mellifera* has a long history of domestication. This fact promoted international transport resulting in a cosmopolitan distribution including all continents except Antarctica and other oceanic islands (Hung et al., 2018). The importance of this insect is mainly related to its pollination role; indeed, the western honey bee provides an important pollination services for a wide number of agricultural crops (Calderone, 2012) and ranks as the most frequent species of crop pollinators (Garibaldi et al., 2014). More precisely 39 of 57 monoculture crops are pollinated by honey bees (Klein et al., 2007). This results in an estimated 35% of food for human consumption dependent by the honey bees activity; both directly (e.g. pollination of fruit and vegetables) and indirectly (e.g. pollination of fodder cops). This accounts for an estimated 14.5 billion Euro value for the pollination service carried out by honey bees in Europe, while, in the world, the economic impact of honey bees is estimated around 153 billion Euro in 2005 (Moritz et al., 2010).

The real importance of honey bees, however, is related to the maintenance of ecosystems' biodiversity. Indeed, *A. mellifera* is thought to be the most important pollinator species in natural ecosystems with an average proportion of floral visit that is more than double than that contributed

by all bumblebee (Apidae: *Bombus*) species (Kearns and Inouye, 1997; Russo, 2016; Geslin et al., 2017; Hung et al., 2018).

In light of the dramatic decline of wild pollinator insects, both in abundance and diversity (Potts et al., 2010a; Cameron et al., 2011), the importance of honey bees is becoming increasingly important.

1.2. Colony losses

During the winter of 2006 – 2007, in the United States, important losses of managed honey bees were reported and the losses continued through the winter 2007 – 2008 (vanEngelsdrop et al., 2007). The losses, that were attributed to a syndrome called "Colony Collapse Disorder" (CCD), triggered a generalized worry for the health of bees contributing to the spread of large interest in bees which, however, is often related to a simplistic view of the real problems affecting the honey bee.

Indeed, it is indisputable that in the last decade, managed bee colonies have experienced a severe crisis with extensive colony losses (20-30% per year) reported all over the northern hemisphere (Le Conte et al., 2010; vanEngelsdrop et al., 2010; Neumann and Carreck, 2010). Moreover, historic records show that apiculture has been in decline in both Europe and United States as testified by a generally decreasing trend in the number of managed honey bee colonies (Ellis et al., 2010; Potts et al., 2010b).

However, extensive losses are not unusual in the history of beekeeping (vanEngelsdrop and Meixner, 2009).

Reports of bee diseases are in Aristotele's "*The History of Animals*" or in the ancient writings by Pliny and Virgil (Nazzi and Pennacchio, 2014). In modern time, the first recorded losses date back to 1869 followed by 18 further events until today (Underwood and vanEngelsdrop, 2007).

An interesting example comes from a little island close to the seashore of England. In 1906 on the Isle of Wight (England), beekeepers noticed a considerable number of bees crawling outside their hives, unable to fly. Subsequently this condition and the related colony losses, were ascribed to a malady called "Isle of Wight Disease" (Bailey and Ball, 1991; Bailey, 2002). This "disease" then

spread throughout Britain, but the response of the scientific community was not the most appropriate. Indeed, eager to quickly find the responsible for this disease, scientists jumped to conclusions that the tracheal mite *Acarapis woodi* (Rennie et al., 1921) should be blamed for this emergency (Neumann and Carreck, 2010). Seventy years later, Bailey and Ball (1991) and Bailey (2002) concluded that the disease had been due to a more complex combination of stress factors (Neumann and Carreck, 2010).

The history of the Isle of Wight Disease can help to clarify the problem of today colony losses. At the beginning these losses were ascribed to CCD, notwithstanding that the term was specially coined for a defined set of symptoms (vanEngelsdrop et al., 2009) and not just for colony losses. However, nowadays it is largely accepted that the losses of honey bee colonies can be related to many causes and CCD is just one of them (vanEngelsdrop et al., 2010); more importantly, it was highlighted that all factors threatening honey bee colonies can interact with each other (Nazzi and Pennacchio, 2014). Indeed, colony losses have a multifactorial origin with parasites and pathogens playing an active role, reinforced by abiotic stress factors such as pollution, increased mono-agriculture landscapes, climate change and deterioration of natural environments (Le Conte and Navajas, 2008; Goulson et al., 2015; Di Prisco et al., 2016).

1.3. Multifactorial stress affecting honey bee health

As in the case of the Isle of Wight disease, the quest for the cause of recent colony losses started with the attempt to identify a possible single causal agent (Nazzi and Pennacchio, 2014). In fact, similarly to 100 years ago, most early studies attempted to correlate colony losses to a vast range of stress factors (e.g. viruses, pesticides, parasites, fungal pathogens) identifying one or another as the main causal agents (Ratnieks and Carreck, 2010). However, subsequent studies revealed that these factors, while certainly involved, were not the principal responsible for the losses but were part of a complex multifactorial syndrome (Ratnieks and Carreck, 2010). Thus, the theory of a single detrimental

stressor turned into a new paradigm that the decline of honey bees colonies can be induced by a variety of stress factors (abiotic and biotic), often showing synergistic interactions.

Notwithstanding the complexity of this intricate network of interactions between stress factors (Lafferty, 2010), two biotic agents seem to characterize the system: the deformed wing virus (DWV), and the parasitic mite *Varroa destructor* (de Miranda and Genersch, 2010; Rosenkranz et al. 2010; Nazzi and Le Conte, 2016).

The first is an endemic pathogen, that develop asymptomatic covert infections (de Miranda and Genersch, 2010) and this peculiar ability to entail limited acute effects on honey bee colonies has favoured its spread in virtually all honey bee colonies (Nazzi and Pennacchio, 2018). However, covert infections of the virus are not due to a low pathogenicity of the virus but rather to the fact that the honey bee's antiviral barriers are able to contain viral infection (Nazzi et al., 2012). Thus, immunity plays an essential role in maintaining under control viral infection; this implies that any further stressor altering the immunocompetence of the host, and in particular the antiviral function, can cause a transition from benign DWV covert infections to devastating outbreaks of the pathogen (Nazzi et al., 2012; Nazzi and Pennacchio 2014; Di Prisco et al., 2013; Nazzi and Pennacchio 2018). This scenario has been corroborated by a study which proved that DWV exerts an immunosuppressive action, characterized by the downregulation of the nuclear factor-kappaB (NF-kB) (Nazzi et al., 2012) which plays a fundamental role in immunity (Silverman and Maniatis, 2001; Hayden and Ghosh, 2008) and is implicated in the antiviral response of bees (Nazzi et al., 2012). This last evidence accounts for the positive feed-back explaining the impressive dynamics of viral infection as soon as any further stress factor influencing the expression of this crucial gene comes into play (Fig. 1). In particular, this is what normally occurs in case of a severe mite infestation (Nazzi et al., 2012).

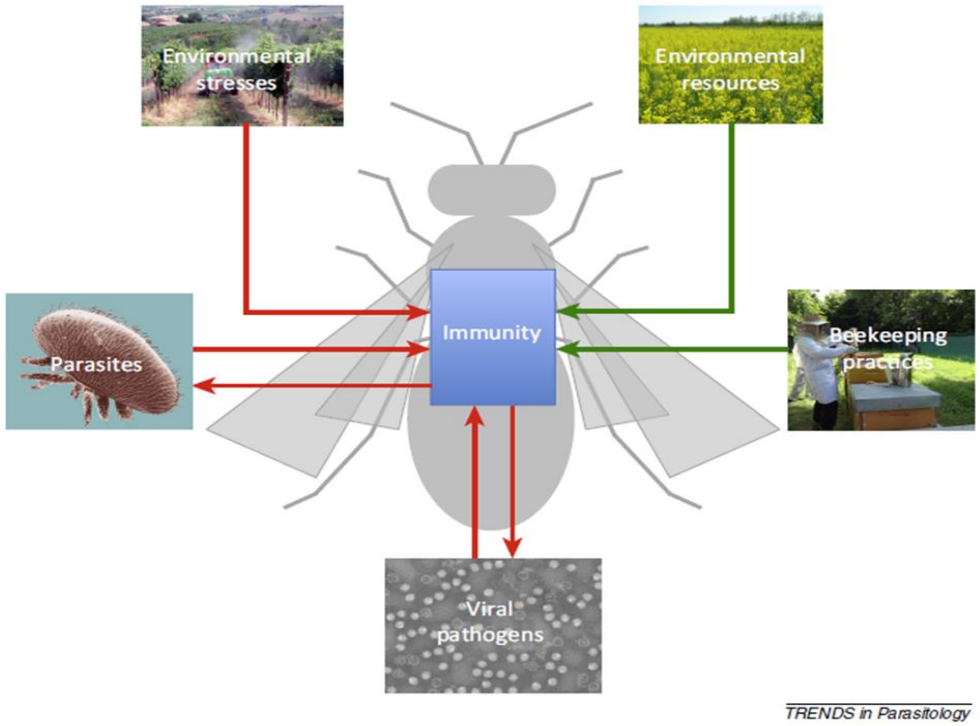

Figure 1. Multiple interactions between honey bees and environmental factors from Nazzi and Pennacchio 2014.

1.4. Stressors

Honey bees are threatened by several stress factors that contribute to their decline. Following the major factors affecting honey bees survival are described.

1.4.1. *Varroa destructor*

Varroa destructor is an ectoparasitic mite originally confined to the Eastern honey bee *Apis cerana*; however, during the first half part of the last century, this parasite shifted to the western honey bee: *A. mellifera*. Therefore, the mite represents a "new" parasite for the western honey bee and a balanced host – parasite relationship is lacking, making *V. destructor* a major threat for apiculture (Rosenkranz et al., 2010).

The mite presents a distinct sexual dimorphism (Ifantidis, 1983) with grey and pear-shaped males and red-brown flat ellipsoidal females (Rosenkranz et al., 2010).

The life cycle of the parasite is closely synchronized with that of the host and lacks a free-living stage; it includes a phoretic phase that is spent on adult bees and a reproductive phase within the sealed brood cells.

The reproductive phase occurs exclusively into the capped brood cells. This phase begins with the adult female reaching a cell with a 5th instar bee larva; there, approximately 5 h after the cell capping, the mite starts sucking haemolymph from a hole made in the larva's body. About 70 h after cell invasion, the mite lays an unfertilized egg which develops into a male (Infantidis, 1983; Rehm and Ritter, 1989; Steiner et al., 1994). Subsequently fertilized female eggs are laid at 30 h intervals (Rehm and Ritter, 1989; Infantidis, 1990). Forty-eight hours after oviposition, protonymphs emerge and start feeding from a communal feeding site on the ventral side of the bee pupa (Donzè et al., 1998). Protonymphs then moult into deutonymphs and eventually into adults. The total cycle, from egg to adult, lasts 6 – 7 days for males and 6 – 9 for females (Accorti et al., 1983).

During the host's pupal stage, the mite's offspring feed on a site prepared by the mother mite, sucking haemolymph from the bee (Donzè et al., 1998). This feeding activity is central to all the detrimental effects of the varroa parasitism, both direct and indirect (Nazzi and Le Conte, 2016). The direct effects, linked to haemolymph subtraction, are reduced weight (De Jong et al., 1982; Schatton-Gadelmayer and Engels, 1988; Bowen-Walker and Gunn, 2001; Annoscia et al., 2012), and increased water transpiration (Annoscia et al., 2012) as well as depletion in proteins and carbohydrates (Bowen-Walker and Gunn, 2001), while the 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). In particular, *V. destructor* can vector the following bee viruses: slow paralysis virus (SPV) acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), Cloudy wing virus (CWV) Israeli acute bee virus (IAPV) and the deformed wing virus (DWV) (Allen et al., 1986; Bakonyi et al., 2002; Ongus et al., 2004; Tencheva et al., 2004; Chen et al., 2005; Tencheva et al., 2006). Furtermore, *V. destructor* can promote secondary infections (Vanikova et al., 2015) caused by bacteria and viruses invading the bee through the mite's feeding hole (Boecking and Genersch, 2008; Vanikova et al., 2015). Other effects

related to mite infestation are behavioural modifications (Annoscia et al., 2015; Zanni et al., 2018), accelerated behavioural maturation (Downey et al., 2000; Zanni et al., 2018) and decreased flight performance (Kralj and Fuchs, 2006; Kralj et al., 2007), but a clear distinction between the role of the parasite and the associated DWV virus in causing these effects is difficult.

At colony level, with a moderate mite infestation, the growth of the bee population is reduced (Rosenkranz et al., 2010), while beyond a certain threshold of infestation the colony function is compromised. Indeed, untreated mite infested colonies normally collapse within six months to two years (Le Conte et al., 2010).

Recently one study (Ramsey et al., 2019) suggested that the mite feeds primarily on honey bee fat body tissue and not haemolymph; however the detrimental effects of the feeding activity remains unchanged while more data are necessary to confirm this hypothesis.

1.4.2. Deformed wing virus (DWV)

The deformed wing virus (DWV) is one of the most common viruses of bees (Chen and Siede, 2007) and the main virus associated with the collapse of honey bee colonies infested by *V. destructor* (Ribière et al., 2008; Sumpter and Martin, 2004; Tentcheva et al., 2004). The DWV consists of a 30 nm icosahedral particle containing a single positive strand RNA genome with three structural protein (Bailey and Ball, 1991; Lanzi et al., 2006; Ongus et al., 2004); its genomic organization is typical of iflaviruses, within the picorna like family Iflaviridae (de Miranda and Genersch, 2010). DWV is transmitted between honey bees both horizontally (from individual to individual) and vertically (parent - offspring) (Chen et al., 2006). In presence of overt infection, the virus causes wing deformities, smaller body size, discoloration and reduced lifespan (de Miranda and Genersch, 2010; Grozinger and Flenniken, 2019).

DWV is now widely distributed also due to the intimate relationship with the *Varroa* mite (Wilfert et al., 2016). The virus develops asymptomatic covert infections (de Miranda and Genersch, 2010) related to an effective immune control (Nazzi and Pennacchio, 2014); however, additional stress

factors can influence the outcome of viral infection, and, in particular, both abiotic and biotic factors. As for the biotic stressors, *V. destructor* represents the major agent favouring the transition from common covert infections to devastating overt infections (Nazzi and Pennacchio, 2014). In fact, *V. destructor*, beside vectoring DWV (Ball, 1989; Bowen-Walker et al., 1999), can activate the virus inside the host. Several mechanisms have been proposed to explain the higher viral load observed in mite infested bees (Yang and Cox-Foster, 2005; Gisder et al., 2009; Martin et al., 2012; Annoscia et al., 2019). However, a hypothesis involving virus activation via haemolymph removal and the depletion of a common immune resource (Nazzi et al., 2012; Di Prisco et al., 2016) recently appeared the most supported in a detailed lab study (Annoscia et al., 2019).

As for the abiotic stressors, DWV infection could be triggered by other chemicals acquired through the diet (Vaudo et al., 2015), agrochemicals found in the environment by foragers (Mulin et al., 2010; Sponsler and Johnson, 2017; Di Prisco et al., 2013) and acaricides used by beekeepers inside the hive (Grozinger and Flenniken, 2019).

It is therefore clear that DVW covert infections represent a kind of "sword of Damocles" permanently pending above the bee colony such that any single or a combination of stress factors weakening the antiviral defence barriers maintaining under control those infections and thus supporting the sword, can promote viral replication, thus allowing the sword to fall with dramatic consequences (Nazzi and Pennacchio, 2018).

1.4.3. Temperature

Insects are strongly dependent on environmental temperature (Angilletta, 2009) because of their limited thermoregulation capacity (Chown and Nicolson, 2004). In particular, honey bees are born in a stenothermic environment with larvae completely dependent on the adult bees for heat (Heinrich, 1993). Adult bees instead, are able to generate endothermic heat from muscle contraction (Free and Spencer-Booth, 1960; Heinrich, 1993). In honey bee colonies, adult honey bees maintain the nest temperature within the 32-36 °C range, with an average temperature in the brood area between 34

and 35 °C (Seeley and Heinrich, 1981). This temperature guarantees an optimal larva and pupal development. Indeed, bees raised at sub-optimal temperature are more susceptible to stress (i.e. pesticides) than adults (Medrzycky, 2009). Studies report that the temperature of 32 °C represents the lower limit for both adult and brood since, long periods under this temperature result in malformities as well as neuronal and behavioural insufficiencies (Tautz et al., 2003; Groh et al., 2004; Jones et al., 2005; Becher et al., 2009).

In recent years, a gradual modification of the planet's climatic conditions, provoked by anthropogenic causes, has been recorded; in particular, a general increase in the average global temperature has been reported which has been linked to extreme weather conditions, including both heat waves and cold waves (IPCC, 2012).

1.4.4. Xenobiotics

In addition to parasites, viruses and abnormal environmental temperatures, honey bees are exposed to xenobiotic substances in the environment: phytochemicals acquired through nectar, pollen and propolis (Di Pasquale et al., 2013), agrochemicals, acaricides used for mite control (e.g. the pyrethroid Tau-Fluvalinate and the organophosphate Coumaphos) (National Research Council of the National Academies, 2007). In particular, neonicotinoid insecticides are the subject of intense debate (Blacquière et al., 2012); indeed, based on the scientific evidence regarding the negative effect of these compounds, the European Commission banned three of them (Gross, 2013). Neonicotinoids are nicotine-like compounds used for the protection of agricultural crops and their residues can be found both in nectar and pollen (Blacquière et al., 2012). These compounds act negatively on honey bee immunity (Di Prisco et al., 2013) as well as behavioural traits, such as communication, homing and foraging (Henry et al., 2012).

Nicotine is a highly toxic alkaloid found in nature, primarily in the plant family Solanaceae, where it serves as a defence against herbivores with a mode of actions similar to that of synthetic neonicotinoids (Johnson et al., 2009). Nicotine mimics acetylcholine at the neuromuscular junction,

causing convulsion and death (Tomizawa and Casida., 2003; Steppuhn et al., 2004). Honey bees can naturally acquire nicotine mostly through certain nectars (Detzel and Wink, 1993); this could have diverse effects on honey bee survival, depending on the health status of the colonies (Kohler et al. 2012).

1.4.5. Environmental impoverishment and nutrition

Landscape alteration is the result of the fragmentation, loss, isolation and modification of the landscape; this alteration can affect honey bee and pollinators in general, in several different ways but the most important outcome of these modifications is the change in the availability of food resources (Montero - Castano and Vilà, 2012) that can lead to nutritional stress. In fact, in the last years a number of studies revealed that the widespread decline of many pollinator insects are due to a combination of land use, habitat degradation and the spread of disease (Breeze et al., 2014) and, in particular, a recent study commissioned by European Union, linked the poor variety and quantity of food to increasing honey bee mortality (Donkersley et al., 2014). Thus, it can be assumed that an insufficient availability of food, resulting from environmental impoverishment, can be regarded as a further stress factor affecting honey bees.

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 (Standifer et al., 1977). Honey bees gather these substances by collecting nectar, pollen and water from the natural environment in quantities that can exceed colony demands and store the surplus for periods of dearth and for juvenile stages. Nectar (which is transformed into honey if stored in the colony) is the only source of carbohydrates; it provides energy for metabolic processes associated with the innate humoral and cellular immune reactions and can also provide secondary plant metabolites (e.g. nicotine) that can work together with the immune system reducing microbial or pathogen pressure because of their antimicrobial properties (Erler et al., 2014). Pollen instead, provides proteins and

nutrients required for physiological processes such as growth and immunity (Di Pasquale et al., 2013; Vaudo et al., 2015). In particular pollen can influence longevity, the development of hypopharyngeal glands, (Pernal and Currie, 2000), the production of some antimicrobial peptides (Alaux et al., 2011) and more in general immune competence (DeGrandi-Hoffman et al., 2010; Alaux et al., 2010).

Nutrition is an important determinant of immune response, and the immune system is one of the costliest physiological system in animals (Lochmiller and Deerenberg 2000; Schmid-Hempel 2005). Malnutrition can impair the immune function and increase the susceptibility to disease. In humans, for example, lack of proteins in the diet reduces the concentration of amino acids in plasma and compromises the immune system (Li et al., 2007), while an adequate provision of proteins is required to sustain normal immune-competence. The ectoparasitic mite *V. destructor*, compromises the normal relationship between nutrition and immunity (DeGrandi-Hoffman and Chen, 2015). In fact, workers parasitized during development emerge with lower protein level that can not be restored even with pollen feeding during the adult life (van Dooremalen et al., 2013). Moreover, mite parasitized honey bees pupae have lower emergence weight, lower protein content and elevated free amino acids levels, suggesting that protein synthesis and growth are inhibited by *Varroa* (Aronstein et al., 2012).

In cage condition however, pollen can mitigate the deleterious effects of *V. destructor* and the related virus infections expanding the lifespan of infested bees (Annoscia et al., 2017). It was demonstrated that the apolar fraction of pollen (i.e. lipidic compound) plays a key role in prolonging the lifespan of honey bees (Annoscia et al., 2017) even if it cannot be excluded that other compound may play a role.

The TOR (target of rapamycin) pathway is a major nutrient-sensing pattern that regulates growth and metabolism in response to amino acids, stresses, changes in cellular energy status (Bjedov et al., 2009). It also controls protein translation and ribosome biogenesis, the upregulation of which is required for growth. More recently, the TOR pathway has emerged as an important modulator of ageing (Kaeberlein and Kennedy, 2008).

The principal component of TOR pathway is TOR protein-kinase, which is divided into two different complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 regulates

translation and growth through phosphorylation of two downstream effectors: S6K and 4E-BP. In favourable conditions such us an amino acid-rich diet, phosphorylated S6K promotes cellular and organismal growth while, S6K – deficient animals are smaller and their metabolisms replicate low-calorie diet conditions (Um et al., 2006). In addition, mTROC1 promotes autophagy when the cell or the organism is under starvation (Lum et al., 2005). Autophagy provides the cell with supplementary nutrients, but it also removes damaged cellular components playing an important cytoprotective function.

mTORC1 is activated by insulin and environmental nutrients and naturally repressed by AMP-activated protein kinase (AMPK) a sensor of cellular energy status (Johnson et al., 2013). Thus, there is an interaction between TOR and insulin signaling pathway, although this interaction is complex and the outcome may depend on cell type and on the intensity and duration of the signal (Sarbassov et al., 2006).

The TOR kinase is chemically inhibited by rapamycin, which is a natural macrolide compound isolated from a bacterium from the island of Rapa Nui (Easter Island). Rapamycin is the most specific TOR inhibitor and works by binding the FK506-binding protein EKBP12, which interact with mTORc1 and decreases its activity (Johnson et al., 2013). mTORC2 instead, is not directly affected by rapamycin (Lamming, 2012).

Rapamycin is generally accepted as stimulator of autophagy and inhibition of cap-dependent translation (Ravikumar et al., 2004). The inhibition of TOR activity can increase lifespan and delay the ageing process in yeast (Kaeberlein et al., 2007), worms (Hansen et al., 2008), flies (Kapahi et al., 2004; Luong et al., 2006) and mice (Selman et al., 2009).

In honey bees the TOR pathway and the associated insulin pathway, play a fundamental role in regulation of ageing of individuals (Corona et al., 2007; Münch and Amdam, 2010) and division of labour between worker bees (Wang et al., 2009; Ament et al., 2008), respectively. Moreover, recently it has been demonstrated that the mTOR/insulin pathway responds positively to pollen nutrition but could be inhibited by *Varroa* parasitism (Alaux et al., 2011).

In the northern hemisphere most of the colony losses occur during the Autumn-Winter period (Amdam, 2004) when the resources are naturally limited and *V. destructor* infestation is high (Martin, 1998). Moreover, since in some periods of the year and in some areas, natural resources can be naturally limited and not match the colony's needs, beekeepers normally sustain colonies with additional sources of carbohydrates (Haydak, 1970; Brodschneider and Crailsheim, 2010; Krainer et al., 2016), using homemade inverted sugar syrups, high fructose corn syrup (HFCS) or starch syrup (Jachimowicz and El Sherbiny, 1975; LeBlanc et al., 2009; Brodschneider et al., 2010; Brodschneider and Crailsheim 2010; Krainer et al., 2016). Additional sources of proteins, consisting of pollen supplements or pollen substitutes (Standifer et al., 1977), can also be provided. Carbohydrate rich supplementary food provides an alternative source of energy, increases colony strength, prevents starvation and may reduce wintering losses (Emsen and Dodologlu, 2014). Indeed, a mixture of sucrose and water is commonly used to feed honey bees (Free and Spencer-Booth, 1961; Barker, 1971; Semkiw and Skubida, 2016) especially in the Autumn–Winter period in temperate areas, when honey bees may suffer from low nectar flow and bad weather. However, these syrups may contain hydroxymethylfurfural (HMF) and/or its degradation compounds (i.e. laevulinic acid and formic acid) which are toxic for honey bees. HMF, is an organic compound consisting of a furan ring containing both an aldehyde and an alcohol function, which has been proved to be harmful to adult bees at 150 ppm (Jachimowicz and El Sherbiny, 1975) and 8000 ppm in sugar solution (Krainer et al., 2016), 250 ppm in HFCS syrup (LeBlanc et al., 2009) and 915 ppm in sugar candies (Smodiš Škerl and Gregorc, 2014); while negative effects on larvae were observed at concentrations higher than 750 ppm (Krainer et al., 2016). This compound can be formed both through the Maillard reaction and the thermal and acid-catalyzed degradation of sugars and carbohydrates (Zirbes et al., 2013; Krainer et al., 2016). Moreover, the sugar syrups provided by the beekeepers are also characterized by a marked acidity due to substances added to the mixture to invert sucrose in glucose and fructose (Bailey, 1966); thought to be more digestible by honey bees.

1.5. Aim of the thesis

Honey bees losses are the result of multiple stressors that affect bee health reducing the lifespan of these insects. These stressors are both abiotic and biotic, natural and artificial, with no single one being entirely responsible for the problem. At the same time, nutrients in the diet play a crucial role in the maintenance of homeostatic balance and in developing optimal immune response.

The aim of this study was to investigate, at the individual level, how different stress factors and nutrition interact to influence the survival of honey bees. To this aim, we subdivided the study in two phases; in the first one, we assessed how several stress factors as well as pollen influence honey bee health. In a second phase of this study, we investigated how some of these stressors act in combination with the others and in combination with nutrition.

The factors that were individually studied in the first phase of this work. were:

- Pollen: the only source of amino acids and lipids for honey bees.
- Hydroxymethylfurfural (HMF): a toxic compound contained in additional sugars syrups produced by beekeepers to sustain colonies in some periods of the year.
- Acidity: that normally characterizes the sugar syrups as reported above.
- Nicotine: a toxic alkaloid that bees can encounter in the environment and was used here as a model compound for its mode of action similar to that of neonicotinoid insecticides (du Rand et al., 2015).
- Temperature: a temperature of 32 °C, 2-3 degrees below that normally found in the hive, was selected as an example of abiotic stress factor in view of the recurrent alarms related to the impact climate change on bee health.
- *V. destructor*: the most dangerous ectoparasite of honey bees.
- Deformed wing virus (DWV): a key pathogen of honey bees.

The second phase of the work, involving the study of multiple interactions between the above cited factors, included the following analyses.

- Study of the interaction between dietary pollen and DWV infection; for this purpose, honey bees infected orally with different doses of DWV, were fed with two different diets, one composed by sugar only and the other made of sugar and pollen.
- Study of the interaction between dietary pollen and *V. destructor*; for this purpose, honey bees infested or not by *Varroa* mite were fed with sugar only or sugar and pollen. An experimental study aiming at clarifying the positive effect of pollen through chemical inhibition and subsequent expression analyses was also carried out.
- Study the interaction between cold stress and *V. destructor;* to this aim we maintained mite infested individuals at 32 °C and compared their survival with that of bees exposed to a single stressor at a time.
- Study of the interaction among nicotine, temperature, *V. destructor* and dietary pollen; to this aim a fully factorial experiment was carried out and the results analysed by means of Cox proportional hazards regression.
- Study of the interaction between hydroxymethylfurfural and acidity and hydroxymethylfurfural and *V. destructor*; for this purpose, we exposed honey bees to HMF and/or to acidified food and to *V. destructor*.

In all cases, caged bees were studied and the response variable was the survival.

Additional relative genes expressions analyses were carried out to complement the survival studies and thus to better understand the effects of stressor on individual honey bees.

Table 1 summarizes the stressors and the interactions that were considered in this study.

Table 1. Interaction of stressors considered in this study. Blue colour refers to sugar only diet, yellow colour to sugar and pollen diet. Blank boxes refer to non-studied interaction.

	Nicotine		Varroa destructor		Temperature		Rapamycin		HMF		Acidity		DWV	
Nicotine														
Varroa destructor														
Temperature														
Rapamycin														
HMF														
Acidity														
DWV														

2. MATERIALS AND METHODS

In order to understand if and how potential stressors, belonging to different categories, and nutrition can impact on honey bee survival, several experiments were carried out under lab conditions. In this chapter the reader will find the experiments that were carried out during this study. Each paragraph refers to a single experiment (except 2.1.).

2.1. Honey bees, *Varroa* mites and methodology used in this study

Experiments were carried out between May 2017 and October 2019. Newly emerged adult bees and mites were collected randomly (unless otherwise specified) 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 local honey bee colonies are hybrids between *Apis mellifera ligustica* Spinola and *Apis mellifera carnica* Pollmann (Comparini and Biasiolo, 1991).

If not otherwise specified, every experiment consisted of three replicates carried out at different times, while the number of bees refers to the total number of individuals used in the experiments.

2.2. Effect of pollen nutrition on survival and diet consumption of deformed wing virus-infected honey bees

Pollen, along with nectar, is the natural food for *Apis mellifera*. Specifically, pollen represents the only source of amino acids, lipids, and protein for honey bees. It has been demonstrated that pollen feeding in some cases reduce virus infection (DeGrandi – Hoffman et al., 2010; Annoscia et al., 2017). To gain insight the effects of pollen nutrition on honey bee survival challenged by DWV, we design a dose-response experiment where honey bees infected with an increasing number of viral copies were feed with a sugar diet or with a sugar diet complemented with pollen.

At the beginning of the beekeeping season (June), when the DWV load in the area is low inside the hives (Nazzi et al., 2012), newly emerged bees were collected from one colony. The bees were then divided in 4 groups, transferred into plastic cages (185 x 105 x 85 mm) in a climatic chamber (34.5 °C, 75% R.H., dark) and fed with water and a sugar solution. After two hours, the bees were starved by removing the sugar solution for one hour; then, bees were individually fed with 5 µL of sugar solution containing different concentrations of DWV: 0, 100, 1000, 10000 viral copies. Subsequentially each group were split in two sub-groups (8 cages in total): one group (4 cages) fed with sugar candy (Apifonda®) and water, while the other group (4 cages) fed with sugar candy (Apifonda®), crude pollen and water. The final situation was composed by eight cages:

- 1 control group fed with sugar and water (control).
- 1 control group fed with sugar, pollen and water (control P+).
- 1 group infected with 100 DWV viral copies and fed with sugar and water (100 vc).
- 1 group infected with 100 DWV viral copies and fed with sugar, pollen and water (100 vc P+).
- 1 group infected with 1000 DWV viral copies and fed with sugar and water (1000 vc).
- 1 group infected with 1000 DWV viral copies and fed with sugar, pollen and water (1000 vc P+).
- 1 group infected with 10000 DWV viral copies and fed with sugar and water (10000 vc).
- 1 group infected with 10000 DWV viral copies and fed with sugar, pollen and water (10000 vc P+).

Sugar were dispensed with the use of small plastic button (Ø = 1.5 cm) filled with fresh sugar candy every 2 days and placed on the floor of the cages. Plastic buttons were completely covered with laboratory film (Parafilm®), to prevent the exsiccation of the candy, except a little cut on the top, to ensure the bee feeding.

Survival and diet consumption were recorded daily.

Sugar consumption was analysed from day 4 to day 20, because of the high variability in sugar intake before day 4, and the too low number of bees after day 20. Indeed, a low number of honey bee per cage (< 5 individual), influence negatively the quality of data. Pollen consumption analysis instead, were calculated from day 4 to 12, since after this day the consumption drastically dropped below the sensitivity of our weighing method.

From 118 to 124 bees per group were used. Log rank test was used for statistical analysis.

For a better understanding of the results, the comparison between control curves ("control" and "control P+") was extrapolated and plotted individually in the paragraph 3.1.1. ("Effect of pollen on adult honey bees under normal conditions").

2.3. Effects of different doses of hydroxymethylfurfural (HMF) on honey bee survival

Beekeepers normally sustain colonies with homemade sugar syrups that could contain hydroxymethylfurfural (HMF), which is harmful to honey bees nevertheless its toxicity in syrups is still debated and complex. To assess the possible negative effects of HMF in homemade wintering food, we investigated the survival of uninfested honey bees fed with HMF at doses similar to those developed in previous prepared homemade syrups (see 6.3. "Attachment 3" for more information) and also compatible with those reported in literature. To this aim, the day before the experiment, several combs containing emerging bees were randomly collected from the apiary and stored overnight in a climatic chamber (34.5 °C, 75% R.H., dark). The day after, newly emerged honey bees were transferred into plastic cages (185 x 105 x 85 mm) and maintained under the same controlled conditions. Bees were divided in two groups. The first one was fed with a sucrose solution (sucrose – water, 2:1 ratio) ("control" in figures) and the second one with a sucrose solution containing 85 ppm of HMF ("85 ppm HMF" in figure). The dose of 85 ppm is equal to the concentration of HMF produced in previous homemade syrups (see 6.3. "Attachment 3" for more information).

The experiment was replicated three times. From 62 up to 80 honey bees per experimental group were used.

Since the concentration of HMF that can be observed in homemade syrups depends on both acidity and boiling time of the homemade sugar syrups (see 6.3. "Attachment 3" for more information), we tested two additional HMF doses corresponding respectively to 400 ("400 ppm HMF" in figure) and 10000 ppm ("10000 ppm HMF" in figure). To this aim, we followed the same protocol reported above to manage honey bee experimental group.

2.4. Effects of acidity on honey bee survival

Since homemade sugar syrup are acidified to invert sucrose, we decided to investigate the possible side effects of a low pH diet on honey bee survival. To this aim, honey bees were collected using the same protocol reported for the previous experiments; the individuals were divided in three groups: a control group fed with water and sugar solution (sucrose : water, 2:1 ratio) (control), a group fed with the same sugar solution acidified with lemon (lemon), and a third group fed with the same sugar solution acidified with HCl (HCl). The three different diets were provided *ad libitum* to bees, together with water. The experiment was replicated three times using three different cages per experimental group (each replicate corresponded to one cage).

2.5. Effect of different concentration of nicotine on honey bee lifespan

Nicotine is not only a xenobiotic that honey bees could find naturally in the environment, but also a compound that likely interact with the same metabolic pathways of neonicotinoids – like compounds, that are used for agricultural crops protection. The first step of our nicotine study was to find a possible sublethal dose to be used in following experiments. To this aim, we investigated the survival of honey bees fed with different concentrations of this natural xenobiotics, as reported in literature. For this purpose, during the early season (May - June) sealed brood combs from different colonies of

the apiary were collected and stored overnight in a climatic chamber (34.5 °C, 75% R.H., dark). The day after, newly emerged bees were transferred into plastic cages (185 x 105 x 85 mm) and fed *ad libitum* with water and different diets, which consisted in a sugar solution (glucose 61%, fructose 39%; Thom et al., 2003) added with 0, 0.1, 1, 10, 50 ppm of Nicotine (Sigma Aldrich, USA), respectively labelled as: "0 ppm", "0.1 ppm", "1 ppm" and "50 ppm" in figures and maintained in a climatic chamber (34.5 °C, 75% R.H., dark). The different diets were dispensed through 20 mL syringes that were daily weighed to record the food consumption. A total of 100 bees per group were used.

2.6. Effects of cold stress on *V. destructor* parasited honey bees

Varroa destructor is a well-known parasite for *Apis mellifera* and a lot of studies had already highlighted the magnitude of the detrimental effects caused to the host. On the other hand, honey bees are affected also by other stressors contemporary to mite infestation. In the northern hemisphere, most colony losses occur during the autumn – winter period when temperature decrease according to latitude and continentality. Thus, cold stress could be a supplementary factor that, along with *Varroa* infestation, could affects colony survival. To investigate the combined effect of low temperature and *V. destructor* infestation, we treated honey bees with both stressors.

For this purpose, we collected mites and last instar bee larvae from brood cells capped in the preceding 15 h obtained as follows: in the evening of the day preceding the experiment the capped brood cells of a comb were marked and the following morning the comb was transferred to the lab and unmarked cells, that had been capped overnight, were manually unsealed. The comb was then placed in an incubator at 35 °C, 75% R.H., where larvae, either infested or not, spontaneously emerged. Last instar bee larvae were transferred into gelatin capsules (Agar Scientific ltd., 6.5 mm diameter) with no mites or one mite, and maintained at 35 °C, 75% R.H. for 12 days (Nazzi and Milani, 1994). Upon eclosion, newly emerged adult bees were separated from the infesting mite and transferred into four plastic cages (185 × 105 × 85 mm) with water and sugar candy (Apifonda®) *ad libitum*, provided as

described above (paragraph 2.2). In this experiment, sugar consumption was analysed from day 4 to day 23, using the Kruskal – Wallis extension Scheirer-Ray-Hare (SHR) statistical method.

One cage, composed by uninfested bees, was maintained in a climatic chamber at 34.5 °C, 75% R.H., in dark condition (labelled as "control" in figures), while another cage, also composed by uninfested bees, was maintained in a climatic chamber at 32 °C, 75% R.H. Even the mite infested bees were divided into two cages, one maintained at standard temperature (34.5 °C, 75% R.H, dark) and the other one kept in cold stress condition (32 °C, 75% R.H, dark).

Survival and diet consumption were recorded daily. A total of 155 bees per group were used.

For a better understanding of the results, the comparison between control curve and cold stress and control curve and *V. destructor* ("control vs. 32° C" and "control vs. V+" respectively) were extrapolated and plotted individually in the paragraph 3.1.5. ("Temperature") and 3.1.6. ("*Varroa destructor*").

To gain insight of the detrimental effects of these two stressors, we further analysed, by quantitative real time PCR (qRT-PCR), the relative expression of some fundamental genes involved in the stress response in organisms, as described below.

Apidaecin (forward: 5'- TTTTGCCTTAGCAATTCTTGTTG - 3'; reverse: 5'- GAAGGTCGAGTAGGCGGATCT - 3') is a gene encoding the antimicrobial peptide involved in specific responses to bacterial challenge (Evans et al., 2006) and thus fundamental for secondary infections vectored by *Varroa destructor*. A similar role has Defensin (forward: 5'- CATGGCTAATGCCGGAGAGG - 3'; reverse: 5'- CTGCACCAGCTTGAAGAGC - 3'), an antimicrobial peptide with an active role against a broad spectrum of Gram-negative bacteria and fungi (Evans et al., 2006). Heat shock protein (Hsp90) (forward: 5'- TTTTGCCTTAGCAATTCTTGTTG - 3'; reverse: 5'- GAAGGTCGAGTAGGCGGATCT - 3') is a protein responding to the proteostatic disruption in the cytoplasm due to high temperature (McKinsty et al., 2017). To maintain a constant temperature in the hive, honey bees can heat themselves up to 49 °C, exposing routinely to significant proteostatic stressors. Temperature slightly lower than normal

(i.e. 32 °C) could bring honey bees to thermoregulate more, leading to higher proteostatic stress than normality.

Vitellogenin (Vg) (forward: 5'-TTGACCAAGACAAGCGGAACT-3'; reverse: 5'-AAGGTTCGAATTAACGATGAA-3') was selected as a generic marker of stress (Dolezal et al., 2016; Smart et al., 2016; Zanni et al., 2017).

Because of its role in the insulin signalling/mTor pathway, and thus in food intake, we studied the Insulin Receptor Substrate 1 (IRS-1) (forward: 5'- TTTGCAGTCGTTGCTGGTA - 3'; reverse: 5'-TAGCGGTAGTGGCACAGTTG - 3') (Mutti et al., 2011). Finally, we investigated the relative virus load of deformed wing virus (DWV) (forward: 5'- GCGCTTAGTGGAGGAAATGAA - 3'; reverse: 5'- GCACCTACGCGATGTAAATCTG - 3') (Mondet et al., 2014), since it represents a constant pathogenic threat for honey bees (Nazzi and Pennacchio, 2018). Actin (forward: 5'-GATTTGTATGCCAACACTGTCCTT - 3'; reverse: 5'- TTGCATTCTATCTGCGATTCCA - 3') (Di Prisco et al., 2016) was used as housekeeping gene. RNA extractions were performed on 12 bees per experimental group with Rneasy® Plus Mini Kit (Quiagen) homogenisation the whole body of the honey bee using a mortar. cDNA was synthetized with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega) and the real-time PCR analysis were performed using SYBR® green dye (Ambion®), according to the manufacturer specifications, on an ABI prism® 7900 sequence detector (Applied Biosystems™, United States). Relative gene expression data were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

The Kruskal – Wallis extension Scheirer-Ray-Hare (SHR) was used to study statistical differences.

2.7. *V. destructor* effects on honey bee thermoregulation

Since we noted a decreasing in sugar intake by honey bees during *Varroa* infestation, and since body temperature affects the rate of energy expenditure and vice – versa, we investigated the body temperature of uninfested and mite infested honey bees. For this purpose, using the same protocol

described above (Nazzi and Milani, 1994), we artificially infested honey bee larvae. Upon eclosion, newly emerged adult bees were separated from the infesting mite and transferred into plastic cages (185 × 105 × 85 mm). Uninfested and infested honey bees were maintained in a climatic chamber at 34.5 °C, 75% R.H., dark.

Starting from day 4, three honey bees collected randomly from the two groups (infested and uninfested) were photographed with an infrared thermographic camera (brand: FLIR; model: i5; thermal resolutions = ± 0.1 °C) with emissivity settled at 0.97 according with (Stabentheiner et al., 2010). The photos were taken for four consecutive days. Three technical replicates (i.e. three pictures) were taken for each photo. Images were analysed with FLIR Tools® software and temperature data were collected, considering the higher degree Celsius reached by the thorax of the honey bee.

Following, since we noted a decrease on thermoregulatory capacity of the mite parasitised bees, we investigated indirectly the status of flight muscles. Indeed, these muscles are involved in honey bee thermoregulation because of their tetanic contraction that lead to a non-shivering thermogenesis.

To investigate this issue, we artificially infested or not honey bee larvae as described before. The two groups of bees (infested or not) were maintained in an environmental chamber at 34.5 °C, 75% R.H., dark, for 12 days. Upon eclosion, newly emerged honey bee were weighed and then head, thorax, and abdomen were dissected and separately weighed. Data were analysed with Mann-Whitney test to investigate differences in thorax weight between infested and uninfested bees.

2.8. Effect of pollen nutrition on mite infested bees and study of the possible causes of the observed beneficial effect

Pollen nutrition has a beneficial effect on lifespan of *V. destructor* parasited bees (Annoscia et al., 2017). To further investigate this effect, we tested the hypothesis that pollen can influence the energetic pathways of the honey bees, compensating nutrients that are lost because of the mite's feeding activity (e.g. glycogen, trehalose and proteins (Glinski and Jarosz, 1984; Zóltowska et al., 2007).

The experimental plan envisaged the feeding of honey bees with pollen and rapamycin, to verify if the beneficial effects of pollen were lost. Rapamycin indeed, inhibit the mTOR pathway, which is central in the energetic metabolisms of the organisms.

However, before using rapamycin we carried out preliminary experiments aiming at assessing the best solvent to dilute the compound to the required concentration.

The first preliminary experiment was set up to study the best solvent for rapamycin (Glentham life sciences). Since the solubility of the chemical inhibitor in water is only 2.6 µg/mL, we tested if ethanol (Sigma Aldrich, USA) and dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA), whose rapamycin solubility are >90 mg/mL and >250 mg/mL respectively (source: www.sigmaaldrich.com), could represent a health issue for honey bees. To investigate this problem, newly emerged honey bee were collected into plastic cages (185 x 105 x 85 mm) and divided in three groups: the control group (control), fed *ad libitum* with water and sugar solution (glucose 61%, fructose 39%; Thom et al., 2003); the "8‰ ethanol" group, fed *ad libitum* with water and sugar solution containing 8‰ ethanol (Sigma Aldrich, USA); the "5‰ DMSO" group, fed *ad libitum* with water and sugar solution containing 5‰ DMSO (Sigma Aldrich, USA). Concentrations of 8‰ and 5‰ were used because they allowed the total solubilization of rapamycin at doses that we would have been used in the next steps of our study. Survival and diet consumption were recorded daily. The experiment was replicated two times.

The second step in the use of rapamycin as inhibitor, was to investigate the right concentration of this drug in food. Indeed, rapamycin could have deleterious, beneficial or no effects on organism health, depending on the working dose. Our idea was to use a hypothetical maximum dose with no negative effect on honeybee survival. After preliminary bibliography investigation, the choice fall down on four possible concentrations: 0.5 µM, 5 µM, 50 µM, 500 µM. Rapamycin were administered via oral feeding by sugar solutions. After the results of the previous experiment, ethanol at 8‰ was used as solvent for rapamycin since no effect were highlighted in survival and in sugar intake of treated bees.

Rapamycin (Glentham life science, UK) was thus first solubilized in ethanol 8‰ (Sigma Aldrich, USA), and then diluted in sugar solution (glucose 61%, fructose 39%; Thom et al., 2003) to obtain a concentration of 500 µM ("500 µM" in figures). Subsequently, this solution was diluted 10, 100, 10000 times with sugar solution to reach: 50 µM ("50 µM" in figures), 5 µM ("5 µM" in figures) and 0.5 µM ("0.5 µM" in figures). These food solutions were respectively administered to honey bees in different experimental group. The control solution ("control" in figures) was created adding 8‰ ethanol (Sigma Aldrich, USA) in sugar solution.

As for previous experiments, uninfested newly emerged honey bees were collected into plastic cages (185 x 105 x 85 mm) and stored in a climatic chamber (34.5 °C, 75% R.H., dark).

Survival and diet consumption were recorded daily. From 74 to 104 bees per group were used.

After the previous experiment, 500 µM was choose as working concentration for our experiment since no negative effects were highlighted both on survival of bees and in daily intake of food.

To study the hypothesis that pollen acts on energetic pathway to promote survival in mite infested bees we used rapamycin, which is an inhibitor of mTOR pathway, together with pollen and *V. destructor*. The experimental plan was composed by six groups divided as follow: the control group ("control"), formed by uninfested bees fed with sugar and water *ad libitum*; a group formed by uninfested bees fed with a sugar solution containing 500 µM of rapamycin (Sigma Aldrich, USA) and water *ad libitum* ("V-R+" in figure); the mite infested group, formed by *Varroa* infested honey bees fed with sugar and water *ad libitum* ("V+" in figure); mite infested bees fed with sugar, pollen and water *ad libitum* ("V+P+" in figure); mite infested bees fed with a sugar solution with 500 µM of rapamycin (Sigma Aldrich, USA) and water *ad libitum* ("V+R+" in figure); and finally, the last group formed by mite infested bees fed with a sugar solution with 500 µM of rapamycin (Sigma Aldrich, USA), pollen and water *ad libitum* ("V+R+P+" in figures). To obtain mite infested bees or uninfested bees, we artificially incapsulated larvae as described previously (Nazzi and Milani, 1994). Upon eclosion, newly emerged adult bees were separated from the infesting mite and transferred into plastic cages (185 × 105 × 85 mm), maintained in a climatic chamber (34.5 °C, 75% R.H, dark).

Sugar solution were dispensed with the use of 20 mL syringes inside the cages, while pollen was delivered with the use of an open petri dish placed on the floor of the cages. Survival and diet consumption were recorded daily. A minimum of 65 and a maximum of 85 bees per group were used. Relative gene expression analysis was firstly utilized to assess the biological effect of the rapamycin and to investigate the impact of pollen on the mTOR pathway. Since we had three independent variables (rapamycin, *V. destructor* and pollen) with unequal groups (absence of uninfested bees fed with sugar solution and pollen since this effect was well studied in previous experiments) we used the Mann Whitney test to study defined comparisons. In particular, the studied comparisons were:

- Uninfested honey bees versus *Varroa* infested honey bees.
- *Varroa* infested honey bees versus *Varroa* infested honey bees fed with pollen.
- *Varroa* infested honey bees versus *Varroa* infested honey bees fed with rapamycin.
- *Varroa* infested honey bees fed with pollen versus *Varroa* infested honey bees fed with pollen and rapamycin.
- Uninfested honey bees versus uninfested honey bees fed with rapamycin.

We choose these comparisons to highlight the effectiveness of the independent variables in respond to a single variable factor. Since we did five comparisons per gene, with a total of 25 comparisons, we control the false discovery rate with Benjamini – Hochberg procedure, setting the false discovery rate (Q) to 0.1. This false discovery rate was chosen because of the large number of statistical test and because it excludes potentially false negatives.

To investigate the relative expression of genes, we performed a quantitative real time PCR (qRT-PCR) assessing the relative expression of some key genes involved both in mTOR and insulin signalling pathways (rather than PI3K – Akt signalling pathway). These genes were: Insulin – like peptide 2 (ILP-2) (forward: 5'- TTCCAGAAATGGAGATGGATG -3'; reverse: 5'- TAGGAGCGCAACTCCTCTGT -3'); Insulin Receptor Substrate 1 (IRS-1) (forward: 5'- TTTGCAGTCGTTGCTGGTA - 3'; reverse: 5'- TAGCGGTAGTGGCACAGTTG - 3'); Phosphoinositide 3-kinases (PI3K) (forward: 55'- TGAATTTGGCTTAACTGGAT - 3'; reverse: 5'-

TTTCAACTGCTCGTTCGTAT - 3'); mTOR (forward: 55'- GTTGCAGTCCAGGCTTTTTG- 3'; reverse: 5'- AACAACTGTTGCTGACGGTG- 3'); ATG2 (forward: 5' – GCCACTGGATTCTTCAACAGG – 3'; reverse: 5' – GCGTAGAACCCCTGCTAGAT– 3'). Vitellogenin (Vg) (forward: 5'-TTGACCAAGACAAGCGGAACT-3'; reverse: 5'-AAGGTTCGAATTAACGATGAA-3') was selected as a generic marker of stress (Dolezal et al., 2016; Smart et al., 2016; Zanni et al., 2017). In addition to gene expression we also studied the relative viral load of deformed wing virus (DWV) (forward: 5'- GCGCTTAGTGGAGGAAATGAA - 3'; reverse: 5'- GCACCTACGCGATGTAAATCTG - 3'), because it represents a constant pathogenic threat for honey bees (Nazzi and Pennacchio, 2018) and it is regulated by pollen presence in diet (Annoscia et al., 2017). Elf – s8 (forward: 5'- TGAGTGTCTGCTATGGATTGCAA - 3'; reverse: 5'- TCGCGGCTCGTGGTAAA - 3') and GAPDH were used as housekeeping genes. RNA extractions were performed on 12 bees per experimental group with Rneasy® Plus Mini Kit (Quiagen) homogenization the whole body of the honey bee using a Fast- Prep® (Savant™TermoFisher™, United States) homogenizer. cDNA was synthetized with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega) and the real-time PCR analysis were performed using SYBR® green dye (Ambion®), according to the manufacturer specifications, on an ABI prism® 7900 sequence detector (Applied Biosystems™, United States). Relative gene expression data were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.9. Interactions between nicotine, temperature, *V. destructor* and pollen

Since honey bees are exposed to a considerable variety of stress factors in the environment, both biotic and abiotic, is essential investigate how multiple stressors could interact to influence honey bees health. In fact, stressors are usually tested individually; however, in natural conditions the effect of a tested stressor could be enhanced or repressed by the presence of other factors.

To this aim we selected four factors and we tested them simultaneously in a fully nested experiment. These factors were: *V. destructor*, nicotine (50 ppm in sugar solution), low temperature (32 °C) and the presence of pollen in the diet.

During the months of August-September we collected mature bee larvae and mites from brood cells capped in the preceding 15 hours. Then, we artificially infested or not honey bee larvae with one mite or no mites, respectively (Nazzi and Milani, 1994). Such honey bee larvae were maintained in an environmental chamber at 34.5 °C, 75% R.H., dark, for 12 days and, upon eclosion, newly emerged adult bees were separated from the mite (if present) and divided in eight experimental groups (each composed by two cages) organized as follows:

- Uninfested honey bees fed with sugar solution (glucose 61%, fructose 39%).
- Uninfested honey bees fed with sugar solution and pollen.
- Uninfested honey bees fed with sugar solution containing 50 ppm of nicotine.
- Uninfested honey bees fed with sugar solution containing 50 ppm of nicotine and pollen.
- Infested honey bees fed with sugar solution.
- Infested honey bees fed with sugar solution and pollen.
- Infested honey bees fed with sugar solution containing 50 ppm of nicotine.
- Infested honey bees fed with sugar solution containing 50 ppm of nicotine and pollen.

Each of the two cages belonging to the eight different groups were then stored in two different climatic chambers, with the same relative humidity (75 %) but different temperature 34.5 °C and 32.5 °C, respectively.

Survival and diet consumption were recorded daily. From 53 to 65 bees per group were used.

For a better understanding of the results, we extrapolated the survival curves of caged bees fed with sugar diet and honey bees fed with sugar and pollen and we plotted them individually in the paragraph 3.1.1. (“Effect of pollen on adult honey bees under normal conditions”).

2.9.1. Survival data analysis

The Cox proportional-hazards model (Cox, 1972) was used to analyse the data. The Cox model is commonly used in medical research for investigating the association between the survival time of patients in relation to one or more predictor variables. The main assumption of Cox model is the proportionality of the hazard ratios (HR) between groups. However, in our model the proportionality was rejected because of HR pollen trend (data not showed); therefore, the weighted Cox regression was used. Weighted estimation of Cox regression is a parsimonious alternative which supplies well interpretable average effects also in case of non-proportional hazard (Dunker et al., 2018). The R package "coxphw" was utilized for the purpose.

2.9.2. Model building (R script)

Since theory suggests that Cox regression with more than two interaction factors may be non-predictive, we started our analysis with the pairwise comparison of our four factors and progressively omitted the non-statistically significant interactions, starting with those with the higher p-value. For simplicity we report below the starting and final regression equation:

Starting equation:

```
> res.cox <- coxphw(Surv(time, status) ~ (temperature+varroa+pollen+nicotine)^2, data = mydata2, template =
"AHR")
```

Final equation:

```
> res.cox <- coxphw(Surv(time, status) ~ (temperature+varroa+pollen+nicotine)^2 - varroa:pollen -
temperature:pollen - temperature:nicotine - temperature:varroa, data = mydata2, template = "AHR")
```

2.10. Combination of HMF and acidity on honey bee survival

Since we showed that HMF at a low concentration is not harmful for honey bees while acidity has a negative effect on bee, we decided to study the combinate effects of these stressors together. In order to study the interactive effect of these two potential stressors we compared the survival of newly

emerged honey bees (obtained with the protocol mentioned above; Nazzi and Milani, 1994) fed with an acidified sucrose solution at 2.91 pH containing 85 ppm of HMF ("acid + HMF" in figure), versus honey bees fed with sucrose solution containing HMF but not acid ("control + HMF") and versus a acidified (with HCl) solution at 2.91 pH ("acid") . From 62 up to 80 honey bees per experimental group were used in total.

2.11. Effects of a monosaccharide based diet on honey bees

To verify the need to invert sucrose in homemade syrups and thus the importance of the lemon juice addition in the syrups, we studied the survival of bees fed either with monosaccharide or disaccharide sugars. We therefore reared newly emerged honey bees obtained as above and provided either a water and sucrose solution *ad libitum* ("sucrose" in figure) or a 1:1:1 water, glucose and fructose solution ("glu+fru" in figure). Three replicates with three different cages were made for this experiment (each replicate corresponded to one cage).

2.12. Combination of HMF and *V. destructor*

Homemade sugar syrups, which can hide possible side detrimental effects, are administered to honey bee colonies during the autumn – winter periods. This part of the season however, if often characterized by high *V. destructor* infestation.

To assess the possible negative interaction of HMF with the ectoparasite, we investigated the survival of infested bees fed with 400 ppm of HMF. To do so, we collected mature bee larvae from brood cells capped in the preceding 15 hours and transferred them into gelatin capsules (Agar Scientific Ltd., 6.5 mm Ø) with no mites or one mite that had been collected from recently sealed brood cells (Nazzi and Milani, 1994); bees were maintained in an environmental chamber at 34.5 °C, 75% R.H., dark, for 12 days. Upon eclosion, newly emerged adult bees were separated from the infesting mite and transferred into plastic cages (185 × 105 × 85 mm), maintained in a climatic chamber at 34.5 °C, 75% R.H., dark. Bees were divided in three groups: infested honey bees fed with sugar solution (glucose 61%, fructose

39%) and water (“V+” in figures); infested honey bee fed with sugar solution (glucose 61%, fructose 39%) containing 400 mg/L of HMF and water *ad libitum* (“V+ HMF+”); uninfested honey bees fed with sugar solution (glucose 61%, fructose 39%) containing 400 mg/L of HMF and water *ad libitum* (“V+ HMF-”). The experiment was replicated twice. From 62 up to 80 honey bees per experimental group were used in total.

3. RESULTS

3.1. Effect of single factors on bee health

In order to understand if and how potential stressors, belonging to different categories, and pollen can impact on honey bee survival, several experiments were carried out under lab conditions. These experiments involved exposing to different stressors caged honey bees whose survival was monitored on daily basis. For a better comprehension, results are reported following the sequence "from single to multiple stressors" rather than the consecutiveness of the experiments. Thus, results of an experiment could have been subdivided in more than one paragraph. This, of course did not influenced the validity of the data. The most relevant data are reported below.

3.1.1. Effect of pollen on adult honey bees under normal conditions

Pollen as well as nectar, is the natural food for *A. mellifera*. In particular, pollen represents the only source of amino acids, lipids, and protein for honey bees. To assess the effect of this substance on adult honey bees survival, we ran two experiments: one early in the active bee season (May) and another later in the season (August - September); during the experiments, caged bees were fed sugar candy and pollen or sugar candy only and their survival assessed through daily observations.

In general, a shorter survival was observed in bees at the end of Summer as compared to bees sampled in Spring (Fig. 2); pollen fed bees seemed to survive longer than bees fed with sugar only. However, in the trial carried out in May, the difference between the survival of pollen fed bees and control bees did not reach significance (Fig. 2A; P- vs. P+, Log-rank test: Chi-Square = 2.451, d.f. = 1, $P = 0.117$), while, in the late season experiment we noted a significant difference in survival between pollen fed

bees and bees fed with sugar only (Fig. 2B; P- vs. P+, Log-rank test: Chi-Square =13.366, d.f. = 1, $P < 0.000$).

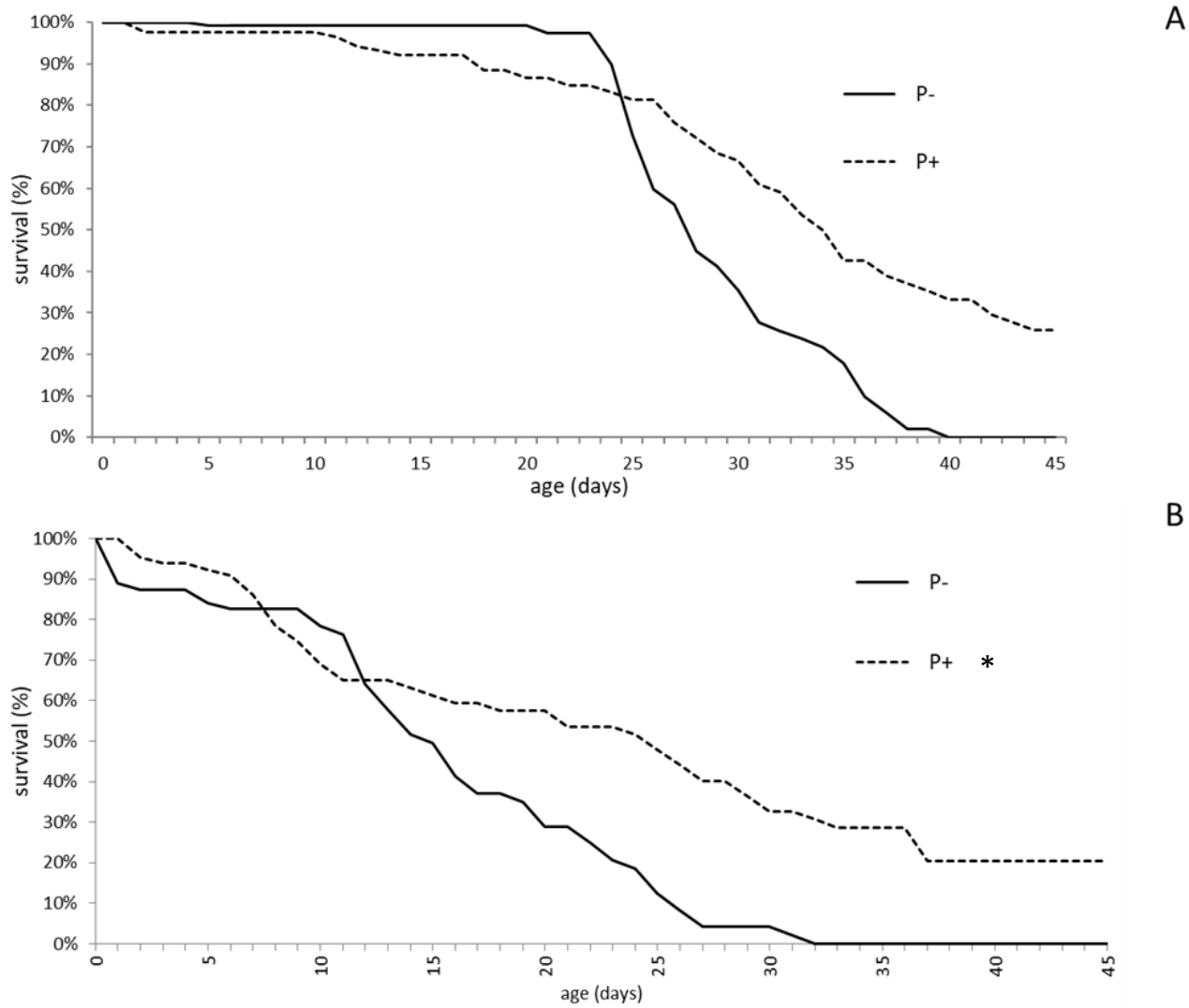

Figure 2. A- Survival of honey bees fed with sugar and with sugar and pollen in early season (May). B- Survival of honey bees fed with sugar and with sugar and pollen in late season (August - September). For a better comprehension, curves of point A coming from Fig.10 while curves of section B coming from data of the paragraph "2.9. Pairwise interactions between nicotine, temperature, V. destructor and pollen"

3.1.2. Hydroxymethylfurfural

Beekeepers normally sustain colonies with homemade sugar syrups that can contain hydroxymethylfurfural (HMF), which is harmful to honey bees. To assess the influence of this toxic compound we fed caged honey bees with a sugar solution (sucrose – water, 2:1 ratio) containing a dose of HMF (85 ± 9.17 ppm) similar to that previously found in homemade syrups and also similar

to that reported in literature. HMF at this low dose had no effect on survival (Fig. 3A; control vs. control + HMF, Log-rank test: Chi-Square = 0.567, d.f. = 1, $P = 0.451$). However, the concentration of HMF that can observed in homemade syrups depends both on acidity and boiling time of these syrups (see appendix 6.3. "Possible side effects of sugar supplementary nutrition on honey bee health" for more information). In particular, at low pH (i.e. 2 pH), HMF can range from 355.3 ppm to 12,005.3 ppm depending on boiling time (0 to 40 minutes). Therefore, we tested the survival of honey bee fed with sugar solutions containing either 400 ppm or 10,000 ppm. 400 ppm of HMF did not affect the survival of honey bees (Fig. 3B; control vs. 400 HMF, Log-rank test: Chi-Square 0.298, d.f. = 1, $P = 0.585$), while 10,000 ppm of HMF appeared to be toxic for bees (Fig. 3C; control vs. 10000 HMF, Log-rank test: Chi-Square = 16.452, d.f. = 1, $P < 0.000$).

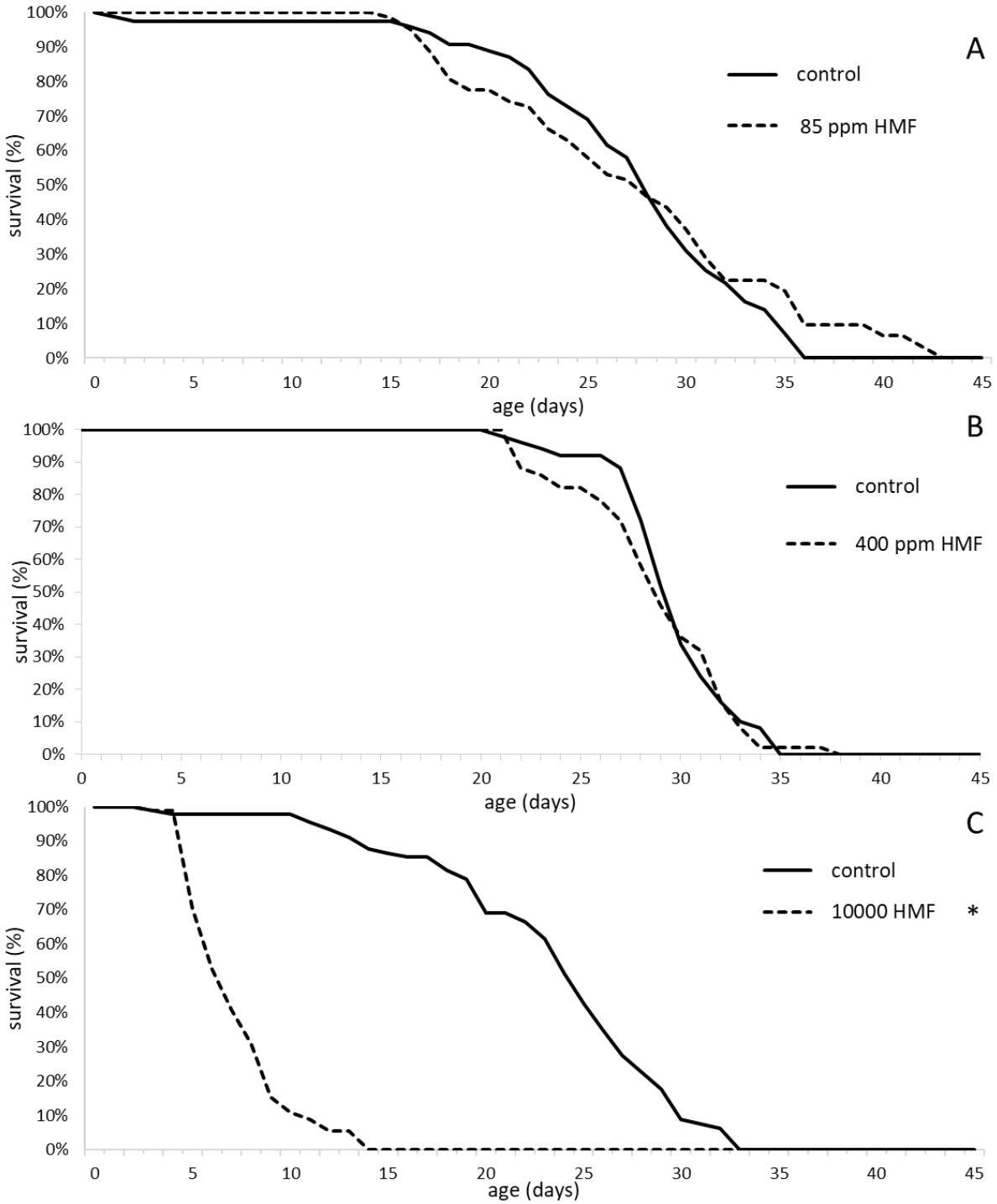

Figure 3. A- Survival of honey bees fed with sugar and sugar with 85 ppm HMF. B- Survival of honey bees fed with sugar and sugar with 400 ppm HMF. C- Survival of honey bees fed with sugar and sugar with 10000 ppm HMF. An asterisk was added in the legend to the name of the experimental group if a significant difference ($p < 0.05$) was found between that group and the control.

3.1.3. Acidity

Since homemade sugar syrup are normally acidified to invert sucrose, we investigated the possible side effects of low pH on honey bee survival. Low acidity (pH = 2.91) obtained by adding either HCl or lemon juice (a common ingredient of homemade sugar syrups) negatively affected the survival of honey bees (Fig. 4; control vs. lemon, Log-rank test: Chi-Square = 47.852, d.f. = 1, $P < 0.000$; control vs. HCl, Log-rank test: Chi-Square = 25.059, d.f. = 1, $P < 0.000$; HCl vs. lemon, Log-rank test: Chi-Square = 1.103, d.f. = 1, $P = 0.294$).

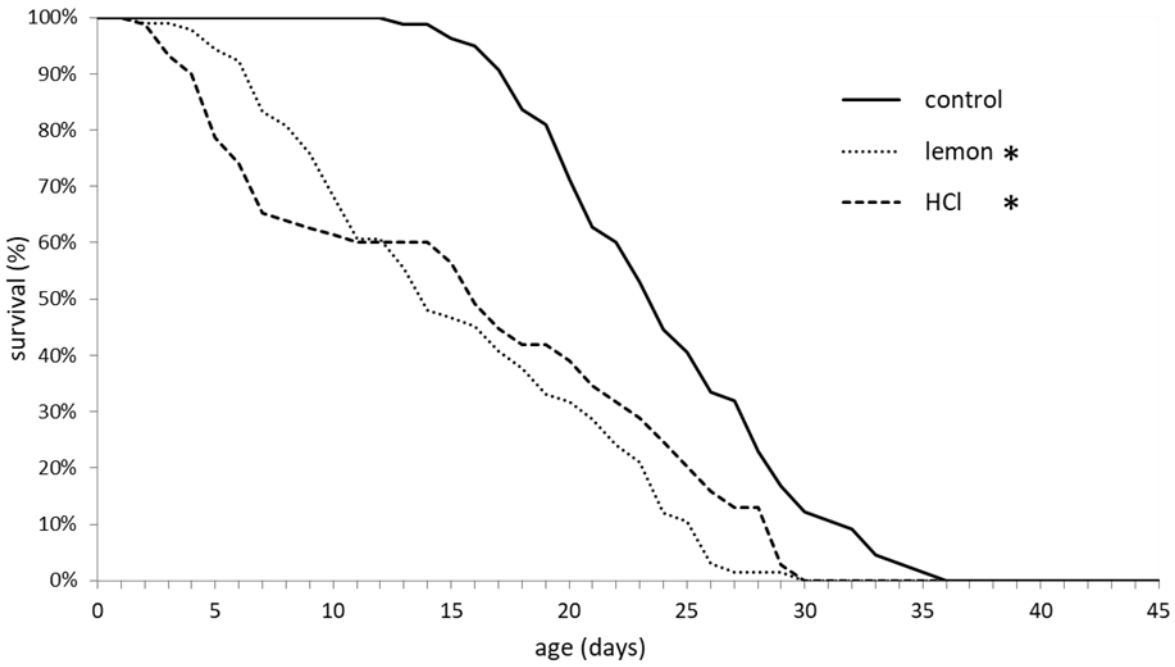

Figure 4. Survival of honey bees fed with sugar syrup acidified or not with different substances (pH = 2.91). An asterisk was added in the legend to the name of the experimental group if a significant difference (p <0.05) was found between that group and the control.

3.1.4. Nicotine

To assess the impact of this natural xenobiotic on bee survival, four doses of the compound were dissolved in sugar solutions and fed to bees as well as a clean control solution.

0.1 and 1 ppm of nicotine slightly increased the lifespan of honey bees (Fig. 5A; 0 ppm vs. 0.1 ppm, Log-rank test: Chi-Square = 11.671, d.f. = 1, $P = 0.001$; 0 ppm vs. 1 ppm, Log-rank test: Chi-Square

= 11.150, d.f. = 1, *P* = 0.001). No effect was observed with 10 ppm of nicotine (Fig. 5A; 0 ppm vs. 10 ppm, Log-rank test: Chi-Square = 19.107, d.f. = 1, *P* = 0.166); instead, 50 ppm of nicotine decreased the survival of treated bees (Fig.5A; 0 ppm vs. 50 ppm, Log-rank test: Chi-Square = 10.408, d.f. = 1, *P* = 0.001).

To exclude the possibility that the negative effects of the highest dose of nicotine was related to a decreased ingestion of sugar solution, and thus, to the effect of starvation, caused by a possible phago-deterrent effect of the compound, we calculated the daily average consumption of sugar solution per experimental group. No differences were found between treated groups and control (Fig. 5B; 0 ppm vs. 0.1 ppm, Mann-Whitney U test: n1 = 20 ; n2 = 20; U = 149; *P* = 0.084; 0 ppm vs. 1 ppm, Mann-Whitney U test: n1 = 20 ; n2 = 20; U = 158; *P* = 0.128; 0 ppm vs. 10 ppm, Mann-Whitney U test: n1 = 20 ; n2 = 20; U = 155; *P* = 0.112; 0 ppm vs. 50 ppm, Mann-Whitney U test: n1 = 20 ; n2 = 20; U = 191; *P* = 0.404).

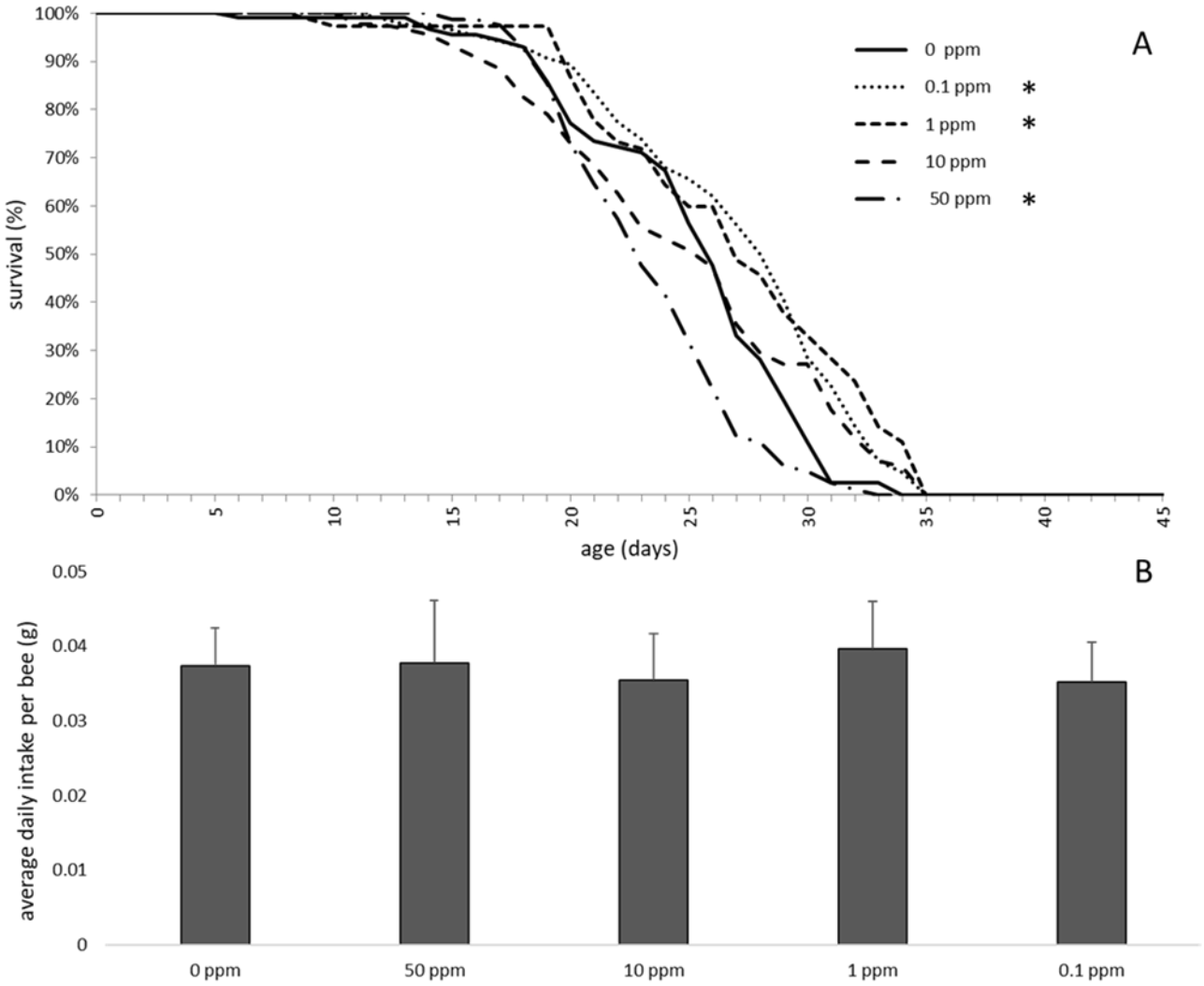

Figure 5. A- Survival of honey bees fed with sugar syrups containing different concentration of nicotine. B- Average daily consumption per bee of sugar syrups containing different concentration of nicotine. An asterisk was added in the legend to the name of the experimental group if a significant difference ($p < 0.05$) was found between that group and the control.

3.1.5. Temperature

To study the possible negative effects of cold stress on survival, honey bees were maintained at 34.5 °C, which is regarded as the normal temperature within the hive, and at 32 °C.

No differences in the shape of survival curves or in the median survival time were observed between the two groups of bees maintained at different temperature (Fig. 6A; control vs. 32 °C, Log-rank test: Chi-Square = 0.900, d.f. = 1, $P = 0.343$). However, a higher average daily intake of sugars was noted

in bees kept at 32 °C (stress temperature) versus bees reared at 34.5 °C (control temperature) (Fig. 6B; control vs. 32 °C, Mann-Whitney U test: n1 = 14 ; n2 = 14; U = 58; *P* = 0.033).

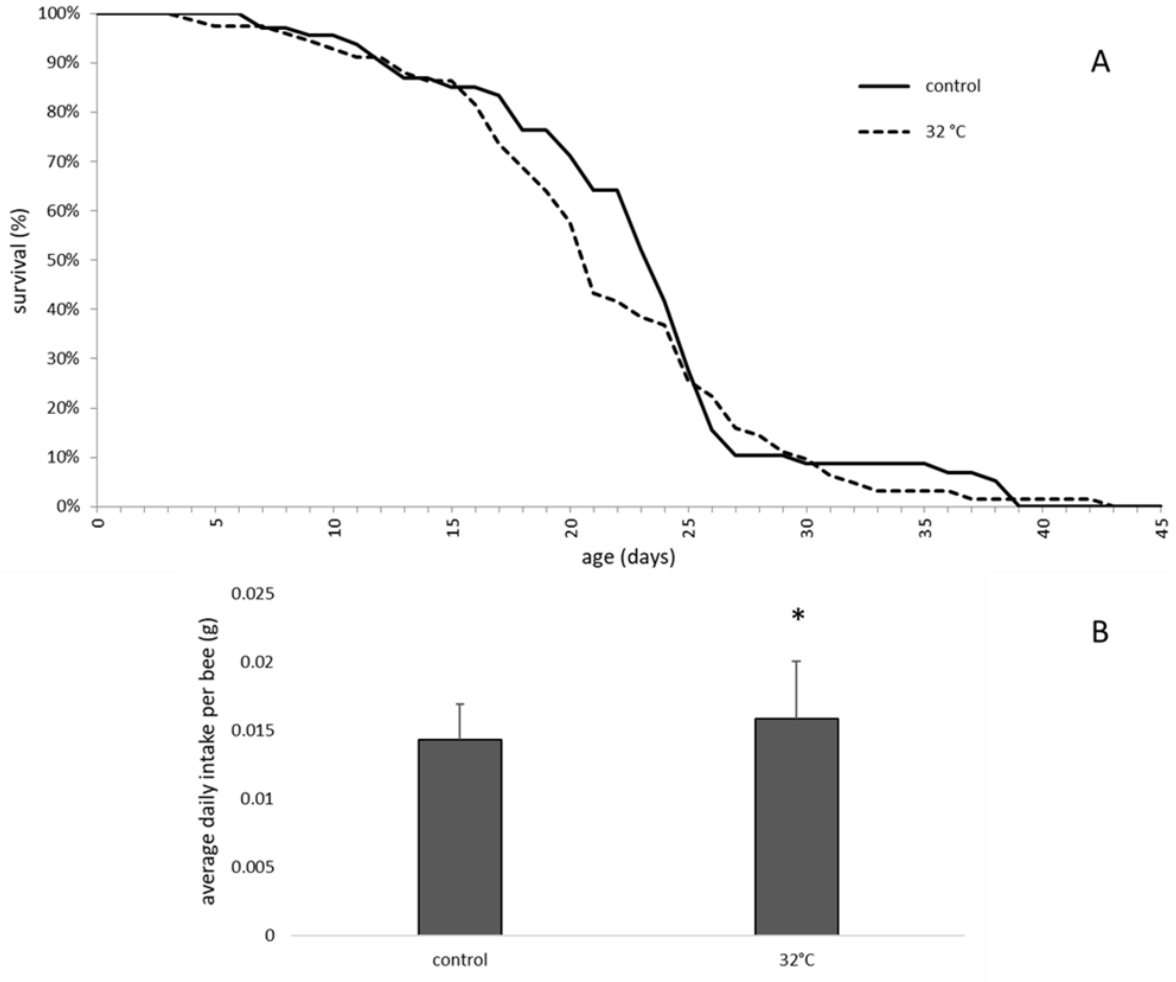

Figure 6. A-Survival of honey bees reared at control temperature (34.5 °C) and at 32°C. B. Average daily consumption per bee of sugar candy at control temperature and under cold-stress conditions.
An asterisk marks values significantly differing from control (p <0.05). For a better comprehension, the curves (A) are extrapolated from Fig.19.

3.1.6. *Varroa destructor*

The effect of Varroa parasitism on honey bee health has been extensively studied. However, to set a standard baseline for the following experiments regarding the multiple interactions among different stressors, an experiment was carried out, whereby the survival of adult bees exposed or not to the parasite during the pupal stage was studied. As expected, bees artificially infested with *V. destructor* showed a reduced lifespan as compared to un-infested bees (Fig. 7A; control vs. V+, Log-rank test:

Chi-Square = 9.6361, d.f. = 1, P = 0.002). Interestingly, mite infestation decreased the daily consumption of sugar in honey bees (Fig. 7B; control vs. V+, Mann-Whitney U test: n1 = 19 ; n2 = 19; U = 56; $P < 0.000$).

Since body temperature affects the rate of energy expenditure and vice – versa, sugar intake should influence honey bee body temperature. To investigate this aspect, we analysed the body temperature of healthy and mite infested honey bees with an infrared thermographic camera. Results showed that mite infested bees have a lower body temperature (Fig. 8; control vs. varroa, Mann-Whitney U test: n1 = 12 ; n2 = 12; U = 19; P = 0.001).

To get insight into why mite infested honey bees are less able to thermoregulate, we weighted honey bees thoraxes, which contains no more than the indirect flight muscles, to assess whether mite infested bees have underdeveloped muscles as a side effect of *V. destructor* parasitization. Data show that there is no difference in thorax weight between uninfested and mite infested honey bees (Fig. 9; control vs. varroa, Mann-Whitney U test: n1 = 10 ; n2 = 10; U = 50; P = 0.500); this is even more convincing in view of the normally reduced weight of mite infested bees.

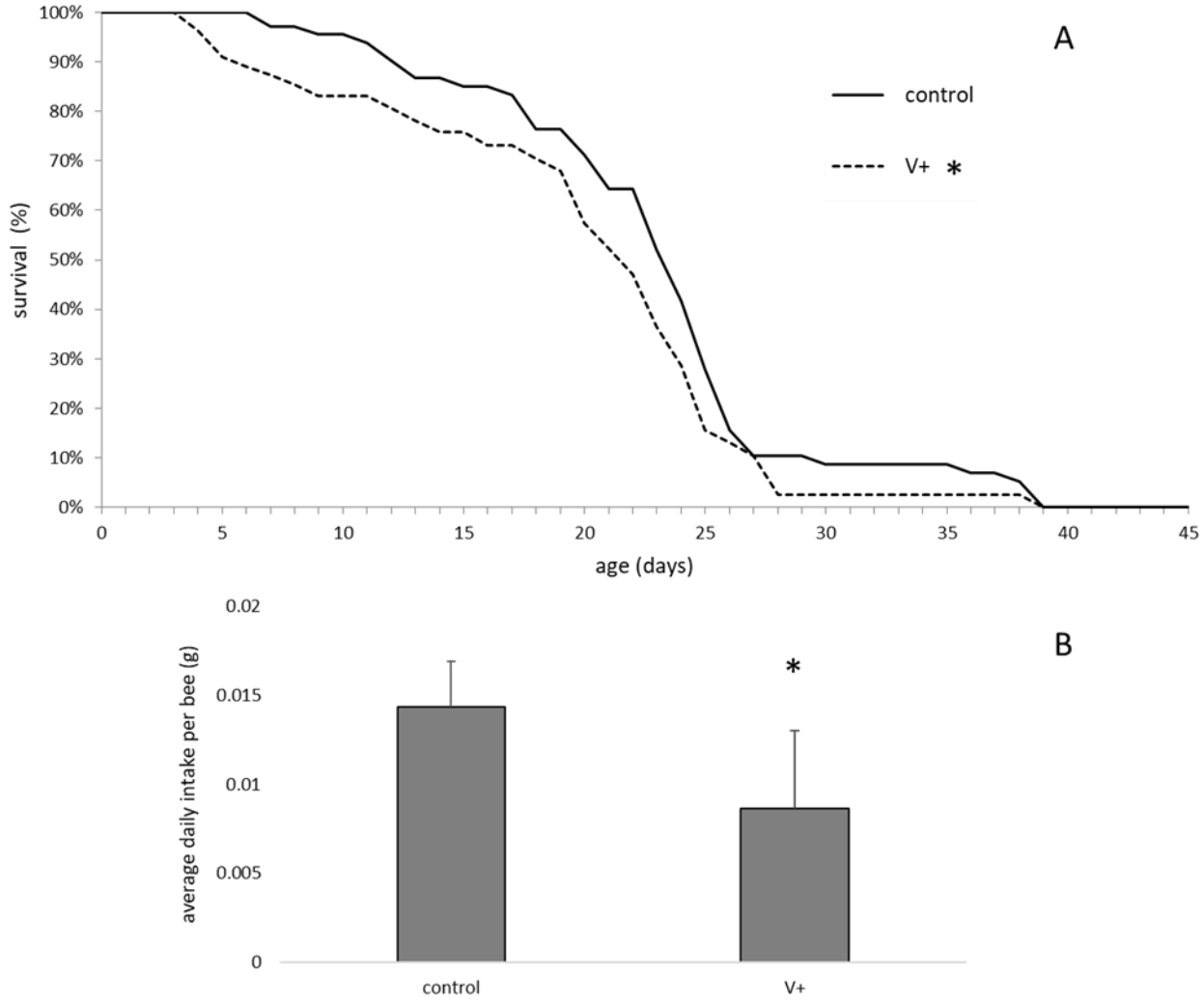

Figure 7. A- Survival of uninfested (control) and mite infested (V+) honey bee. B- Average daily consumption per bee of sugar solution in control and parasitized honey bee. Asterisk refers to statistical significance ($p < 0.05$) compared to control. For a better comprehension, the curves (A) are extrapolated from Fig.19.

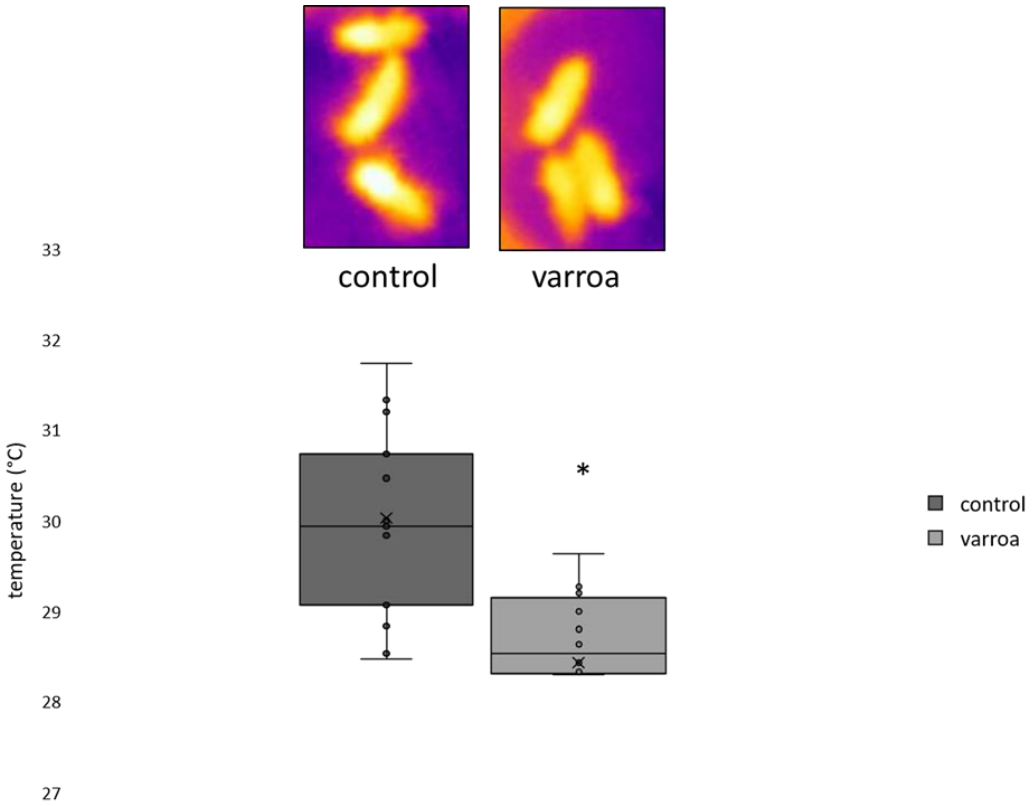

Figure 8. Average body temperature of health and mite infested honey bees. Asterisk refers to statistical significance (p <0.05) compared to control.

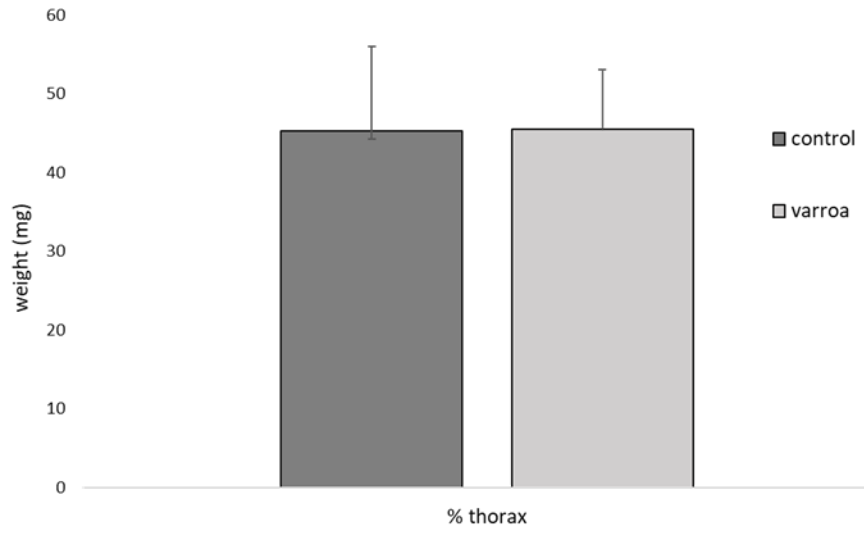

Figure 9. Average thoraxes weight of uninfested (control) and infested (varroa) honey bees.

3.1.7. Deformed wing virus

In order to study the net effect of the virus on honey bee lifespan, while excluding the interference of the vector *V. destructor* and/or the immune challenge related to a possible intrabdominal injection,

we orally administered various doses of deformed wing virus (DWV) to newly emerged honey bees that were fed with sugar only.

Honey bees treated at emergence with 100 viral copies showed no statistical differences in survival from the control group (Fig. 10A; control vs. 100 vc , Log-rank test: Chi-Square = 1.224, d.f. = 1, *P* = 0.269), while bees fed with 1,000 and 10,000 viral copies showed a shorter lifespan (Fig. 10A; control vs. 1000 vc , Log-rank test: Chi-Square = 6.160, d.f. = 1, *P* = 0.013; control vs. 10000 vc, Log-rank test: Chi-Square = 3.896, d.f. = 1, *P* = 0.005).

No differences in daily intake of sugar was observed among bees belonging to the different experimental groups (Fig. 10B; control vs. 100 vc, Mann-Whitney U test: n1 = 17 ; n2 = 17; U = 111; *P* = 0.385; control vs. 1000 vc, Mann-Whitney U test: n1 = 17 ; n2 = 17; U = 136; *P* = 0.124; control vs. 10000 vc, Mann-Whitney U test: n1 = 17 ; n2 = 17; U = 133; *P* = 0.139).

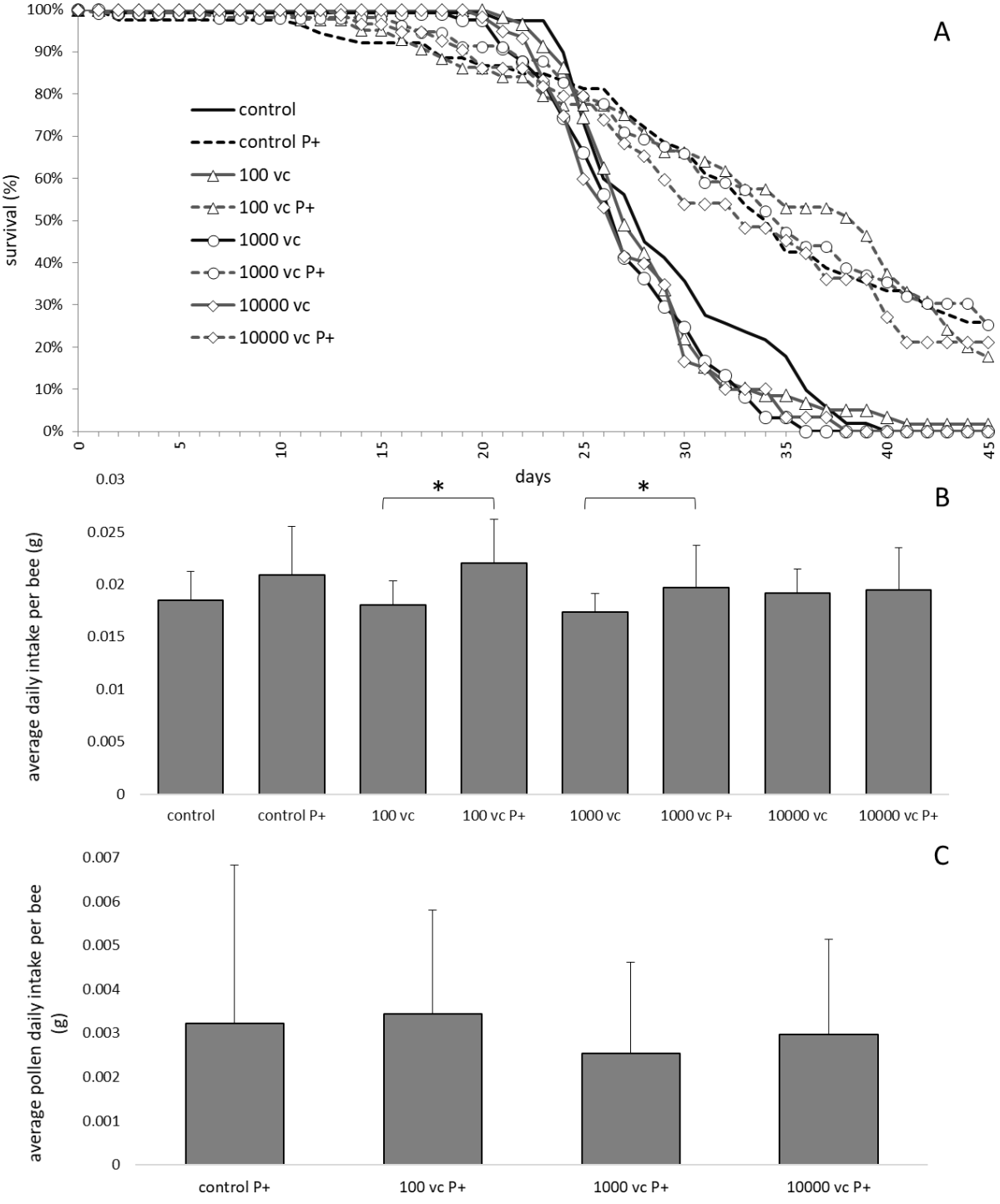

Figure 10. A- Survival of honey bees infected with different viral copies and fed with different diets (pollen presence, pollen absence). B- Average sugar consumption of bees with different diets (pollen presence, pollen absence). Asterisks marks significant differences (p <0.05). C- Average pollen consumption of bees.

3.2. Effects of multiple factors on bee health and interactions with nutrition

In the previous paragraphs we reported the net effect of different stressors on honey bee health. However, under natural conditions, honey bee colonies face a number of different stress factors. In order to understand how these stressors interact with each other and their impact on honey bee survival also in combination with nutrition, several experiments were carried out under lab conditions.

First, we investigated the effects of pollen on virus infected honey bees at different infection levels. Then, we studied the effect of pollen on *Varroa* parasitized bees and tried to assess if this positive effect depends on the contribution of pollen to the energetic balance of bees.

Following, we built a fully four factorial experiment to get insight into the interplay between nicotine, temperature, *V. destructor* and pollen.

Lastly, we tested the combined action of HMF and acidity because these two stressors are often combined in the supplementary nutrition provided by beekeepers to bees in some periods of nectar shortage.

3.2.1. Effect of pollen on virus infected honey bees

To investigate the effect of pollen on the lifespan of virus infected honey bees, we fed bees infected with different viral copies (0, 100, 1,000, 10,000 viral copies) with sugar, water and pollen.

No statistical differences among groups of pollen fed bees were observed (Fig. 10A; control P+ vs. 100 vc P+ , Log-rank test: Chi-Square = 3.784, d.f. = 1, $P = 0.052$; control P+ vs. 1000 vc P+ , Log-rank test: Chi-Square = 0.832, d.f. = 1, $P = 0.362$; control P+ vs. 10000 vc P+ , Log-rank test: Chi-Square = 0.006, d.f. = 1, $P = 0.939$). No differences in both sugar (Fig.10B; control P+ vs. 100 vc P+, Mann-Whitney U test: n1 = 17 ; n2 = 17; U = 121; $P = 0.209$; control P+ vs. 1000 vc P+, Mann-Whitney U test: n1 = 17 ; n2 = 17; U = 128; $P = 0.285$; control P+ vs. 10000 vc P+, Mann-Whitney

U test: n1 = 17 ; n2 = 17; U = 118; *P* = 0.181) and pollen daily intake (Fig. 10C; control P+ vs. 100 vc P+, Mann-Whitney U test: n1 = 9 ; n2 = 9; U = 51; *P* = 0.113; control P+ vs. 1000 vc P+, Mann-Whitney U test: n1 = 9 ; n2 = 9; U = 70; *P* = 0.454; control P+ vs. 10000 vc P+, Mann Whitney: n1 = 9 ; n2 = 9; U = 64; *P* = 0.322) were noted in this experiment.

No significant differences were found in the lifespan of bees belonging to the two control groups (i.e. infected bees fed with pollen or not) (Fig. 10A; control vs. control P+, Log-rank test: Chi-Square = 2.451, d.f. = 1, *P* = 0.117). Instead, pollen significantly increased the survival of virus infected bees at all concentration tested (Fig.10A; 100 vc vs. 100 vc P+, Log-rank test: Chi-Square = 15.554, d.f. = 1, *P* < 0.000; 1000 vc vs. 1000 vc P+, Log-rank test: Chi-Square = 14.745, d.f. = 1, *P* < 0.000; 10000 vc vs. 10000 vc P+, Log-rank test: Chi-Square = 7.758, d.f. = 1, *P* = 0.005).

We also compared sugar consumption in bees fed with pollen or not. We noted higher sugar consumption in bees inoculated with 100 and 1000 viral copies fed with pollen with respect to those treated with the same number of viral copies but without pollen (Fig. 10B; 100 vs. 100 vc P+, Mann-Whitney U test: n1 = 17 ; n2 = 17; U = 57; *P* = 0.001; 1000 vs. 1000 vc P+, Mann-Whitney U test: n1 = 17 ; n2 = 17; U = 87; *P* = 0.024). No differences were noted between the control group and that treated with 10,000 viral copies (Fig. 10B; control vs. control P+, Mann-Whitney U test: n1 = 17 ; n2 = 17; U = 100; *P* = 0.063; 10000 vs. 10000 vc P+, Mann-Whitney U test: n1 = 17 ; n2 = 17; U = 142; *P* = 0.466).

3.2.2. Effect of pollen on mite infested bees and study of the possible causes of the observed beneficial effect

To investigate the effect of pollen on the lifespan of parasitized bees, we fed bees infested by the mite at the pupal stage with pollen or not and assessed their survival.

As expected mite infested bees lived shorter than uninfested ones (Fig. 11A; control vs. V+ , Log-rank test: Chi-Square = 11.381, d.f. = 1, *P* = 0.001) but mite infested bees fed with sugar and pollen,

survived significantly longer than mite infested bees fed with sugar only (Fig. 11A; V+ vs. V+P+ , Log-rank test: Chi-Square = 12.101, d.f. = 1, $P = 0.001$).

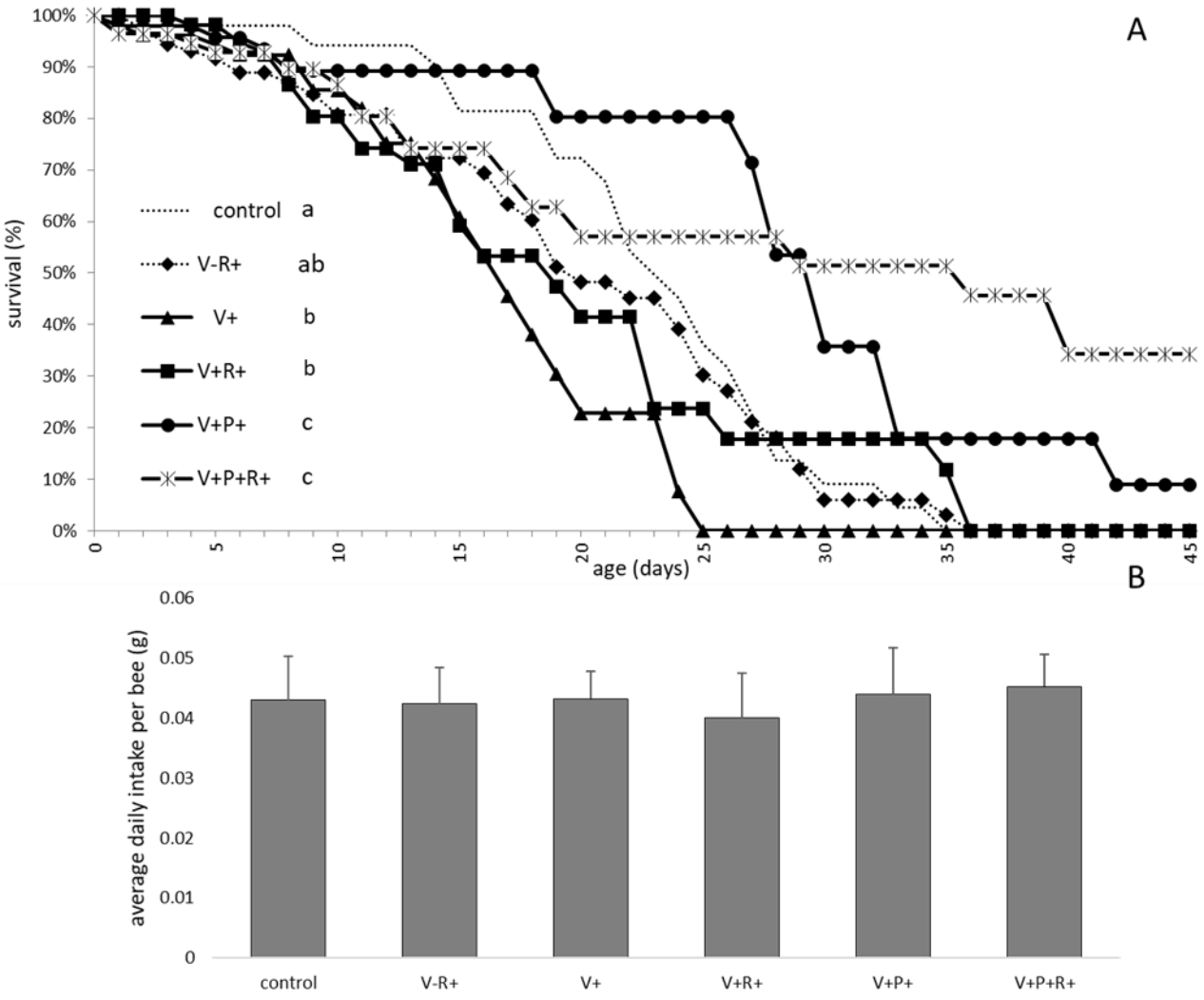

Figure 11. A- Survival of honey bees treated with different combination of Varroa*, pollen and rapamycin. Curves that share the same letter are not significantly different. B- Average daily consumption per bee of sugar solution in treated honey bees.*

To gain insight into how pollen can mitigate the detrimental effects of varroa parasitism on honey bees previously demonstrated, we tested if the observed beneficial effects depends on the positive contributions of pollen to the energetic metabolism of bees. To this aim, we altered the functioning of the energetic pathways of pollen fed bees, by means of a chemical inhibitor of a crucial player of metabolism and checked if the positive effect of pollen was conserved.

As a preliminary step we tested different solvents for dissolving our inhibitor. The survival of honey bees fed with 8‰ ethanol and 5‰ DMSO was similar to that of bees belonging to the control group (Fig. 12A; control vs. 8‰ ethanol, Log-rank test: Chi-Square = 0.867, d.f. = 1, *P* = 0.352; control vs. 5‰ DMSO, Log-rank test: Chi-Square = 0.301, d.f. = 1, *P* = 0.583). However, sugar consumption was significantly lower in DMSO treated honey bees (Fig. 12B; control vs. 5‰ DMSO, Mann-Whitney U test: n1 = 20 ; n2 = 20; U = 267; *P* = 0.035). For this reason, to avoid any possible interference between food intake and rapamycin treatment, we choose ethanol as a solvent for dissolving this inhibitor.

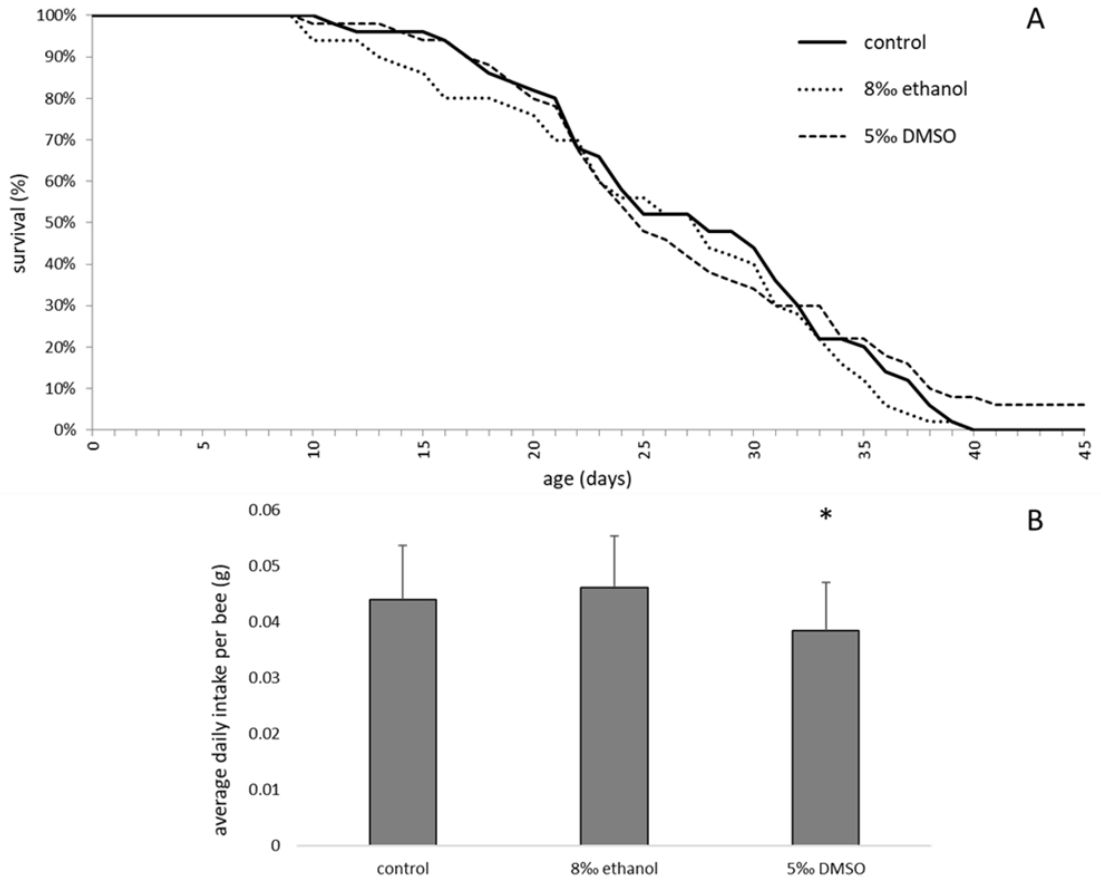

Figure 12. Survival of honey bees treated with different rapamycin solvent. B- Average daily consumption per bee of sugar solution in treated honey bees. Asterisks marks experimental groups that significantly differ from control (p <0.05).

To investigate the effects of rapamycin drug on survival, honey bees were treated with different doses of the compound. Survival was monitored daily. No statistical significant differences in survival were observed (Fig. 13A; control vs. 0.5 µM, Log-rank test: Chi-Square = 2.034, d.f. = 1, *P* = 0.154; control vs. 5 µM, Log-rank test: Chi-Square = 1.288, d.f. = 1, *P* = 0.256; control vs. 500 µM, Log-rank test:

Chi-Square = 3.262, d.f. = 1, *P* = 0.071) apart from 50 µM which had a negative impact on honey bee survival (Fig.13A; control vs. 50 µM, Log-rank test: Chi-Square = 5.170, d.f. = 1, *P* = 0.023).

No significantly differences in sugar intake were found among the treatment (Fig.13B; control vs. 0.5 µM, Mann-Whitney U test: n1 = 18 ; n2 = 18; U = 163; *P* = 0.487; control vs. 5 µM, Mann-Whitney U test: n1 = 18 ; n2 = 18; U = 133; *P* = 0.179; control vs. 50 µM, Mann-Whitney U test: n1 = 18 ; n2 = 18; U = 162; *P* = 0.919; control vs. 500 µM, Mann-Whitney U test: n1 = 18 ; n2 = 18; U = 120; *P* = 0.273). Based on these results, we adopted the dose of 500 µM as the standard one for further experiments.

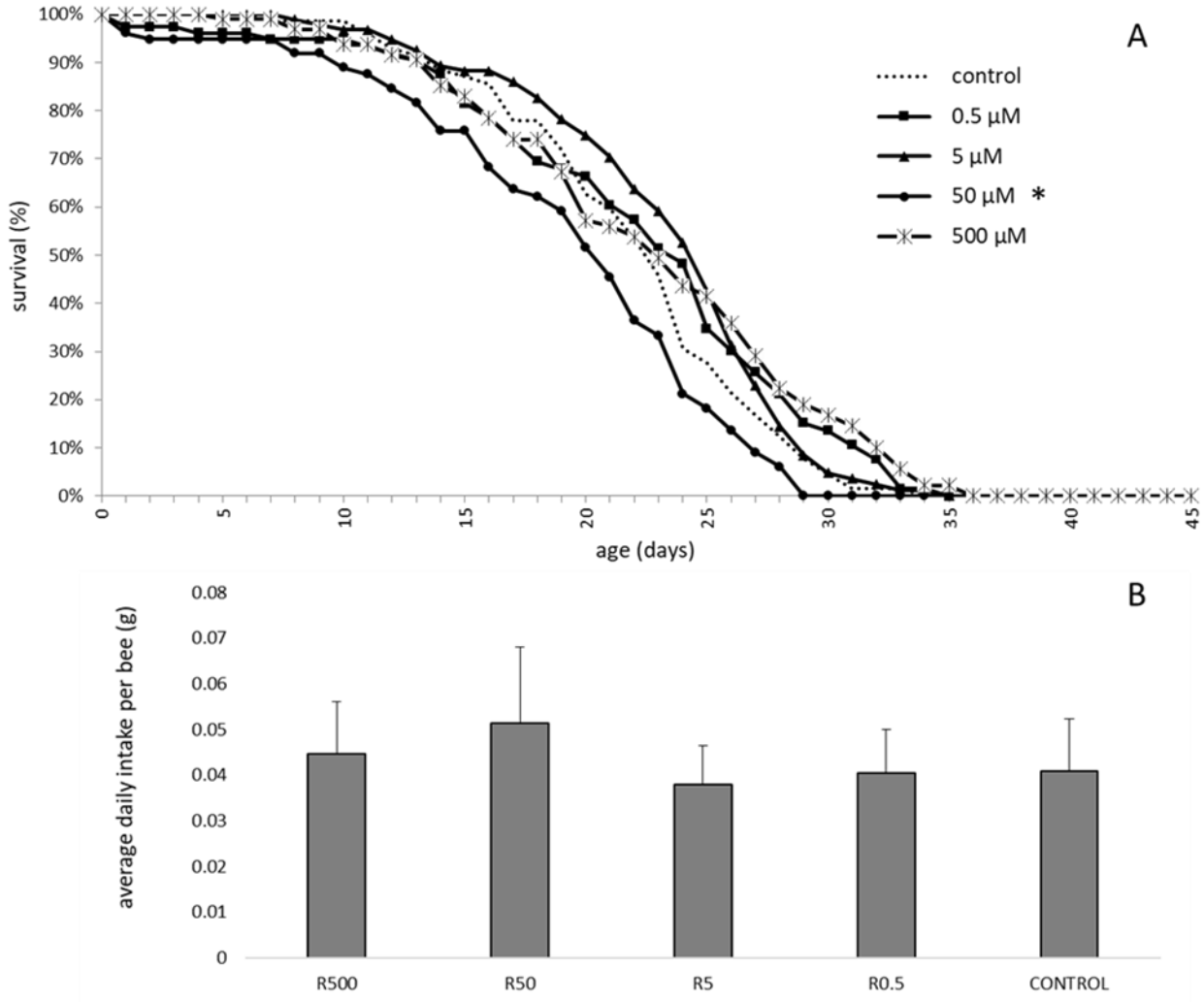

Figure 13. A- Survival of honey bees treated with different concentration of rapamycin. B- Average daily consumption per bee of sugar solution in treated honey bees. Asterisks marks experimental groups that significantly different from control (p <0.05).

To investigate if the beneficial role of pollen in mite infested bees is lost after chemical inhibition of mTOR, a crucial node of the energetic pathways, honey bees were treated with different combinations of varroa, pollen and rapamycin and their survival studied.

Rapamycin had no effect on uninfested honey bees (Fig. 11A; control vs. V-R+ , Log-rank test: Chi-Square = 0.865, d.f. = 1, *P* = 0.352); furthermore, rapamycin did not affect the survival of mite infested bees fed with sugar only (Fig. 11A; V+ vs. V+R+, Log-rank test: Chi-Square = 1.003, d.f. = 1, *P* = 0.317); however, in this case the survival curve of *Varroa* infested bees showed a peculiar extension after day 23 (Fig. 11A); also rapamycin did not influence the survival of mite infested - pollen fed honey bees (Fig. 11A; V+P+ vs. V+R+P+ , Log-rank: Chi-Square = 1,226 d.f. = 1, *P* = 0.68). No differences in sugar consumption were observed among treatment (Fig. 11B; control vs. V-R+, Mann-Whitney U test: n1 = 20 ; n2 = 20; U = 198; *P* = 0.478; control vs. V+, Mann-Whitney U test: n1 = 14 ; n2 = 20; U = 132; *P* = 0.390; V+ vs. V+R+, Mann-Whitney U test: n1 = 14 ; n2 = 20; U = 103; *P* = 0.098; V+ vs. V+P+, Mann-Whitney U test: n1 = 14 ; n2 = 20; U = 120; *P* = 0.242; V+P+ vs. V+P+R+, Mann-Whitney U test: n1 = 20 ; n2 = 20; U = 162; *P* = 0.152).

To further investigate the relationship between pollen and mTOR pathway activation, we performed virus and gene expression analyses on the bees used in the experiments.

Firstly, we investigated the effects of *V. destructor* and pollen in our experimental groups.

As expected mite infestation increased DWV relative load (Fig. 14; control vs. V+, Mann-Whitney U test: n1 = 4; n2 = 7; U = 3; *P* = 0.01882; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.02; P<(i/m)Q = 0.01882; significance = confirmed), while the difference between varroa infested bees fed with pollen or not did not reach statistical significance (Fig. 14; V+ vs. V+P+; Mann-Whitney U test: n1 =7; n2 =3; U = 7; *P* = 0.21252; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.01; P<(i/m)Q = 0.21252; significance = not confirmed).

Varroa and pollen had a significative impact on vitellogenin expression. Indeed, the mite decreased the expression of Vg (Fig. 15; control vs. V+; Mann-Whitney U test: n1 = 12 ; n2 = 9; U = 15; *P* = 0.00279; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.04; P<(i/m)Q = 0.00279; significance

= confirmed), while pollen considerably increased its expression in parasitized bees (Fig. 15; V+ vs. V+ P+; Mann-Whitney U test: n1 = 9 ; n2 = 11; U = 11; *P* = 0.00011; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.02; P<(i/m)Q = 0.00011; significance = confirmed).

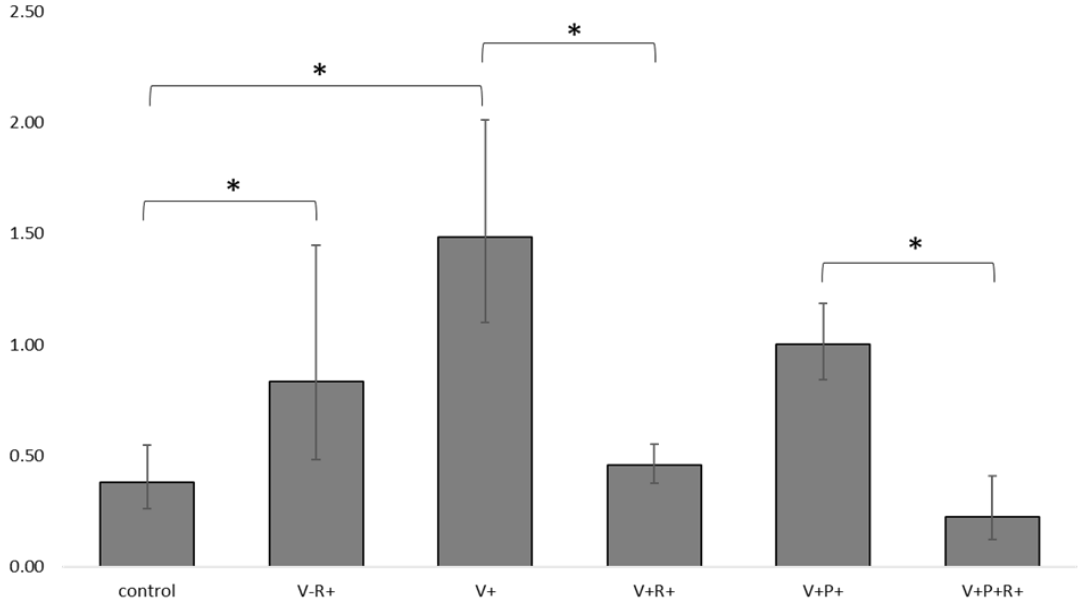

Figure 14. Relative DWV expression in the experimental groups. Asterisks mark comparisons that are statistically significant (p<0.05).

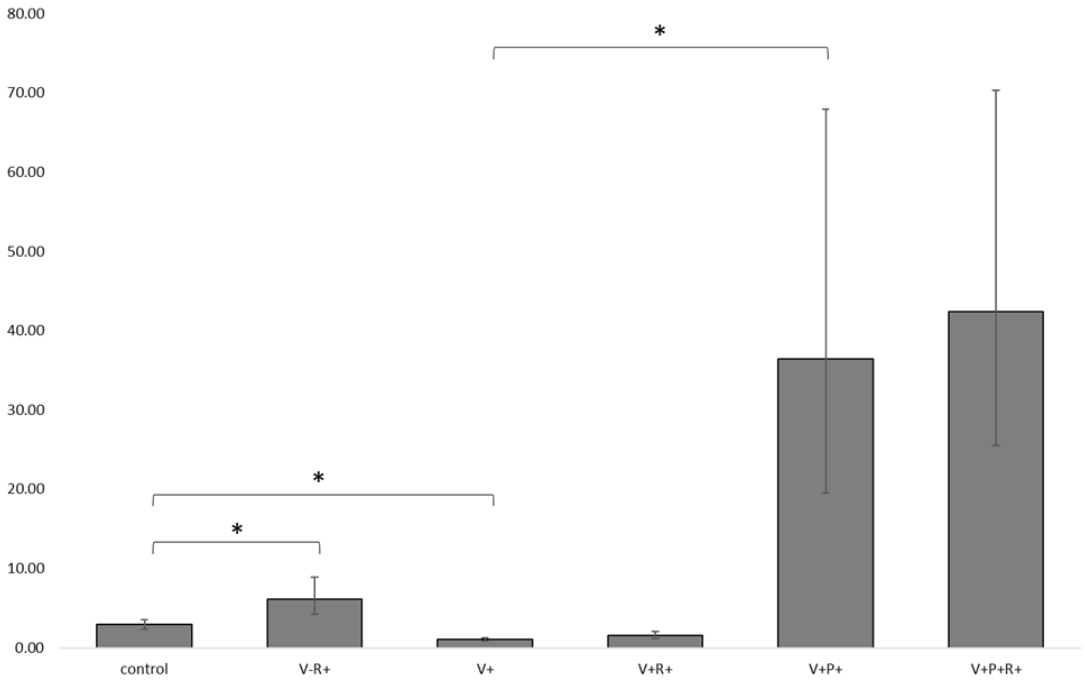

Figure 15. Relative expression of Vitellogenin in the experimental groups. *Comparison marked with asterisk are significantly different.*

As for the genes in the mTOR pathway, *Varroa* influenced IRS-1 and ATG2 (Fig. 16). In particular, mite infestation up-regulated IRS-1 (Fig. 17B; control vs. V+; Mann-Whitney U test: n1 = 12 ; n2 = 12; U = 37; *P* = 0.022; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.032; P<(i/m)Q = 0.022; significance = confirmed) and down-regulated ATG2 (Fig. 17E; control vs. V+ ; Mann-Whitney U test: n1 = 12 ; n2 = 10; U = 11; *P* = 0.001; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.008; P<(i/m)Q = 0.001; significance = confirmed).

A significant effect of pollen on the relative expression of ILP-1 (up-regulation: Fig. 17A; V+ vs. V+P+; Mann-Whitney U test: n1 = 12 ; n2 = 12; U = 22; *P* = 0.002; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.02; P<(i/m)Q = 0.002; significance = confirmed) and PI3K (down-regulation: Fig. 17C; V+ vs. V+P+; Mann-Whitney U test: n1 = 12 ; n2 = 12; U = 27; *P* = 0.001; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.028; P<(i/m)Q = 0.001; significance = confirmed) was observed.

Rapamycin at 500 μM concentration, increased both DWV relative load (Fig. 14; control vs. V-R+; Mann-Whitney U test: n1 = 4; n2 = 4; U = 3; *P* = 0.074; Benjamini – Hocberg procedure: Q = 0.1; (i/m)Q = 0.08; P<(i/m)Q = 0.074; significance = confirmed) and Vg expression (Fig. 15; control vs. V-R+; Mann-Whitney U test: n1 = 12 ; n2 = 9; U = 31; *P* = 0.050; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.06; P<(i/m)Q = 0.050; and Vg expression) but did not influence any mTOR gene when supplied alone. However, in combination with *V. destructor* and pollen, rapamycin influenced four genes in the mTOR pathway. ILP-1 (Fig. 16), was up-regulated in mite infested bees treated with rapamycin (Fig. 17A; V+ vs. V+R+, Mann-Whitney U test: n1 = 12 ; n2 = 12; U = 30; *P* = 0.008; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.024; P<(i/m)Q = 0.008; significance = confirmed); IRS-1 (Fig. 16), was down-regulated in mite infested bees fed with sugar and pollen (Fig. 17B; V+P+ vs. V+P+R+, Mann-Whitney U test: n1 = 11 ; n2 = 12; U = 18; *P* = 0.002; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.016; P<(i/m)Q = 0.002; significance = confirmed); finally, mTOR (Fig. 16) was up-regulated in mite infested honey bees treated with rapamycin (Fig. 17D; V+ vs. V+R+, Mann-Whitney U test: n1 = 12 ; n2 = 12; U = 20; *P* = 0.001; Benjamini – Hochberg

procedure: Q = 0.1; (i/m)Q = 0.012; P<(i/m)Q = 0.001; significance = confirmed). Lastly, rapamycin up-regulated the expression of ATG2 (Fig. 16) in varroa infested honey bees (Fig. 18E; V+ vs.V+R+, Mann-Whitney U test: n1 = 10 ; n2 = 11; U = 7; *P* < 0.000; Benjamini – Hochberg procedure: Q = 0.1; (i/m)Q = 0.08; P<(i/m)Q < 0.000; significance = confirmed).

Tab.2 resume all multiple comparison of genes involved in mTOR pathway.

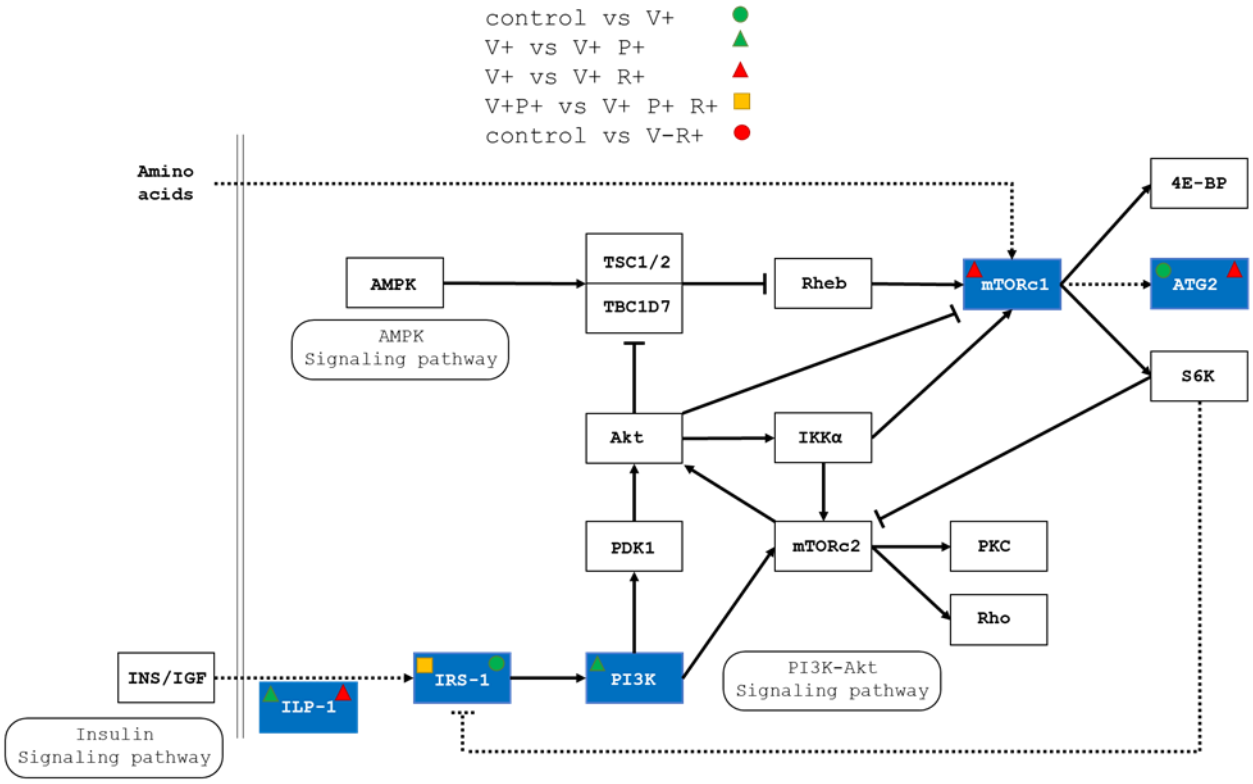

Figure 16. Studied genes of the mTOR pathway (in blue) as affected by rapamycin, Varroa *and pollen (see legend of colours and symbols).*

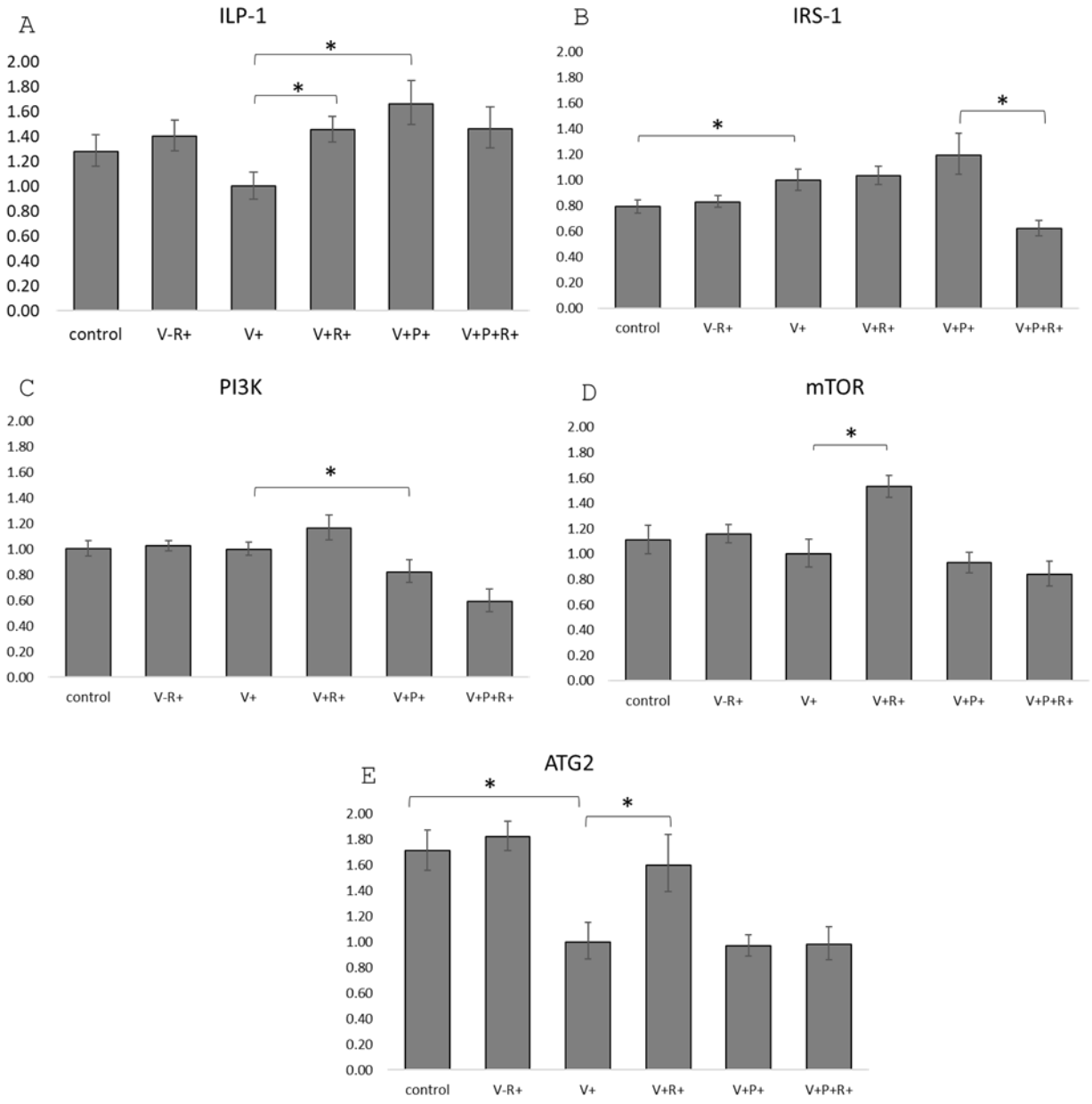

Figure 17. Relative gene expression of members of the mTOR pathway. Asterisks mark significant differences ($p<0.05$).

Table 2. Statistics associated to the comparison of relative gene expression of the analysed genes in the mTOR pathway.

gene		Comparison	n1	n2	U	p value	fdr (Q)	(i/m)Q	P<(i/m)Q	significance
ILP-1	1	control vs. V+	12	12	48	0.8293	0.1	0.1	-	negative
	2	V+ vs. V+P+	12	12	22	0.00195	0.1	0.02	0.00195	positive
	3	V+ vs. V+R+	12	12	30	0.00766	0.1	0.024	0.00766	positive
	4	V+P+ vs. V+P+R+	12	12	58	0.20946	0.1	0.052	-	negative
	5	control vs. V-P+	12	12	72	0.5	0.1	0.096	-	negative
IRS-1	1	control vs. V+	12	12	37	0.02165	0.1	0.032	0.02165	positive
	2	V+ vs. V+P+	12	11	48	0.13397	0.1	0.044	-	negative
	3	V+ vs. V+R+	12	11	63	0.42676	0.1	0.084	-	negative
	4	V+P+ vs. V+P+R+	11	12	18	0.00157	0.1	0.016	0.00157	positive
	5	control vs. V-P+	12	12	58	0.20946	0.1	0.052	-	negative
PI3K	1	control vs. V+	12	12	65	0.34305	0.1	0.076	-	negative
	2	V+ vs. V+P+	12	11	27	0.00819	0.1	0.028	0.00819	positive
	3	V+ vs. V+R+	12	12	50	0.10201	0.1	0.04	-	negative
	4	V+P+ vs. V+P+R+	11	12	44	0.08787	0.1	0.036	-	negative
	5	control vs. V-P+	12	12	69	0.43125	0.1	0.088	-	negative
mTOR	1	control vs. V+	11	12	59	0.3333	0.1	0.068	-	negative
	2	V+ vs. V+P+	12	11	52	0.19444	0.1	0.048	-	negative
	3	V+ vs. V+R+	12	12	20	0.00134	0.1	0.012	0.00134	positive
	4	V+P+ vs. V+P+R+	11	12	57	0.28982	0.1	0.064	-	negative
	5	control vs. V-P+	11	12	59	0.3333	0.1	0.068	-	negative
ATG2	1	control vs. V+	12	10	11	0.00062	0.1	0.008	0.00062	positive
	2	V+ vs. V+P+	10	11	44	0.21929	0.1	0.06	-	negative
	3	V+ vs. V+R+	10	11	7	0.00036	0.1	0.004	0.00036	positive
	4	V+P+ vs. V+P+R+	11	12	61	0.37914	0.1	0.08	-	negative
	5	control vs. V-P+	12	12	69	0.43125	0.1	0.088	-	negative

3.2.3. Effects of cold stress on mite infested honey bees

To assess the combined effect of low temperatures and *V. destructor* infestation on bees, we maintained mite infested individuals at 32 °C and compared their survival with that of bees exposed to a single stressor at a time.

We recorded a negative effect of the two stressors combined (Fig. 18A; control vs. T+V+, Log-rank test: Chi-Square = 28.387 , d.f. = 1, $P < 0.000$; V+ vs. T+V+, Log-rank test: Chi-Square = 85.40, d.f. = 1, $P = 0.003$; T+ vs. T+V+, Log-rank test: Chi-Square = 16.76 , d.f. = 1, $P > 0.000$).

We noted that sugar consumption was negatively influenced both by mite infestation and temperature but not by the interaction between the two stressors (Fig. 18B; Scheirer-Ray-Hare test: varroa: p-value < 0.000; temperature: p-value = 0.025; interaction varroa * temperature: p-value = 0.615).

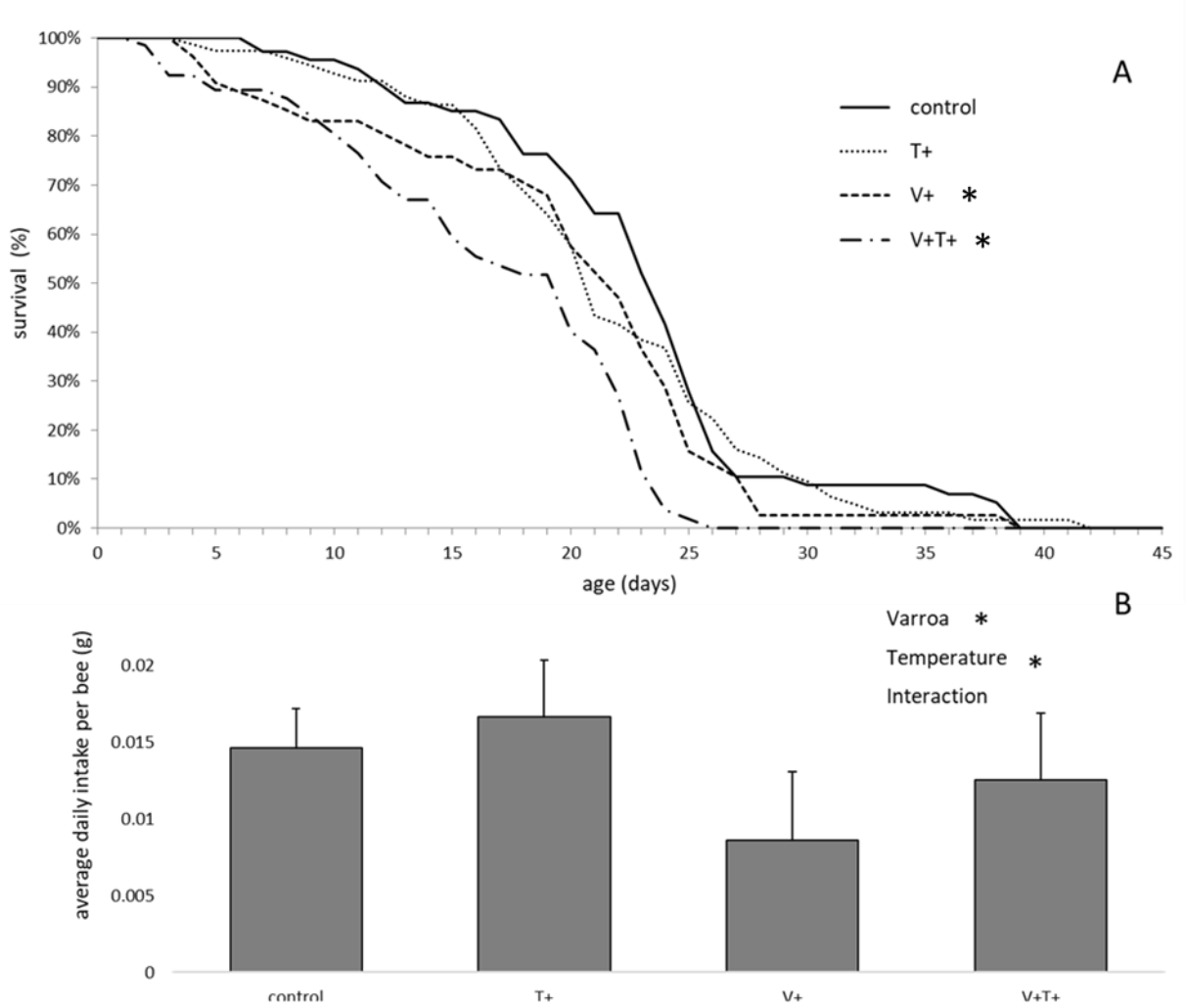

Figure 18 A- Honey bee survival. B- Average daily intake of sugar in control bees (uninfested, mantained at 34 °C), uninfested bees mantained at 32 °C (V-T+), mite infested honey bee maintained at 34 °C (V+T-) and infested bees maintained at 32°C (V+T+). Varroa*, Temperature and Interaction, refers to the Scheirer-Ray-Hare test. Only the effects of* Varroa *was statistical significant in influencing the average daily intake.*

To further explore the effect of these two stressors at the physiological level, we analysed the expression of some relevant genes.

DWV relative load was influenced by mite infestation but not by temperature (Fig. 19; Scheirer-Ray-Hare test: varroa: p-value < 0.05; temperature: p-value > 0.05; interaction varroa * temperature: p-value > 0.05).

Apidaecin relative expression was influenced by Varroa infestation but not by temperature (Fig. 20A; Scheirer-Ray-Hare test: varroa: p-value < 0.05; temperature: p-value > 0.05; interaction varroa * temperature: p-value > 0.05). Defensin expression was not influenced by any stressor (Fig. 20B; Scheirer-Ray-Hare test: varroa: p-value > 0.05; temperature: p-value >0.05; interaction varroa *

temperature: p-value > 0.05). We also investigated the expression of one gene involved in thermal stress (i.e. Heat shock protein Hsp90), a key player in the insulin/insulin-like signalling pathway (i.e. IRS1) and vitellogenin that proved to be a good marker of mite infestation and is involved in immunity. Heat shock protein Hsp90 was influenced by temperature but not by mite infestation or the interaction between factors (Fig. 20C; Scheirer-Ray-Hare test: varroa: p-value > 0.05; temperature: p-value < 0.05; interaction varroa * temperature: p-value > 0.05), while IRS-1 was up-regulated in *Varroa* infested bees (Fig. 20D; Scheirer-Ray-Hare test: varroa: p-value < 0.05; temperature: p-value > 0.05; interaction varroa * temperature: P < 0.05). Vitellogenin (Vg) was down-regulated at low temperature but not by mite infestation (Fig. 20E; Scheirer-Ray-Hare test: varroa: p-value > 0.05; temperature: p-value < 0.05; interaction varroa * temperature: P < 0.05).

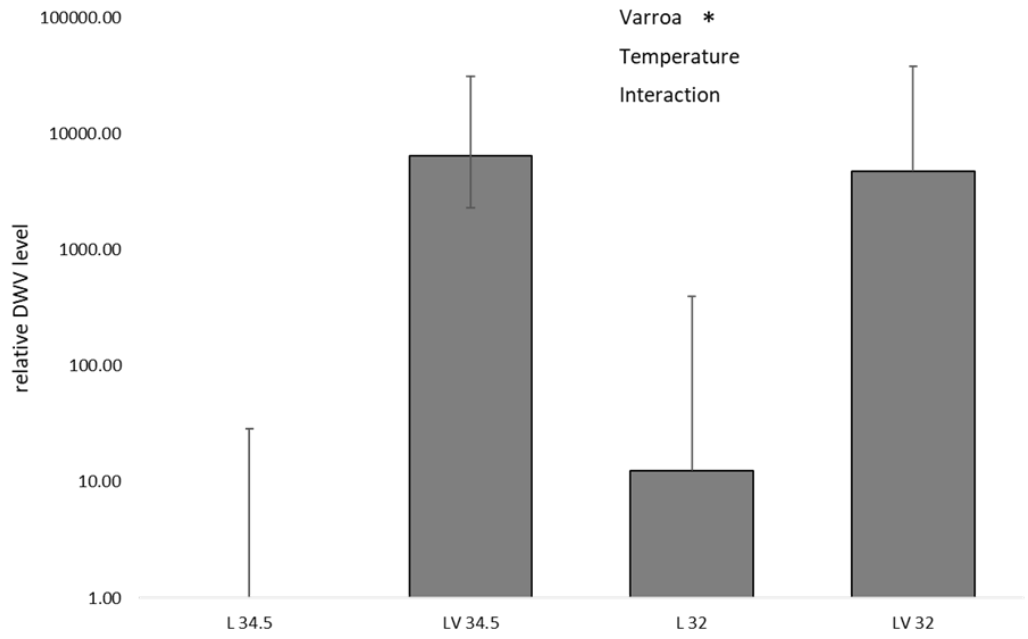

Figure 19. DWV relative expression of honey bee affected by cold stress, V. destructor *and the interaction between them. Result of the Scheirer-Ray-Hare test were reported. Asterisk refers to statistical significance (p <0.05) of the test.*

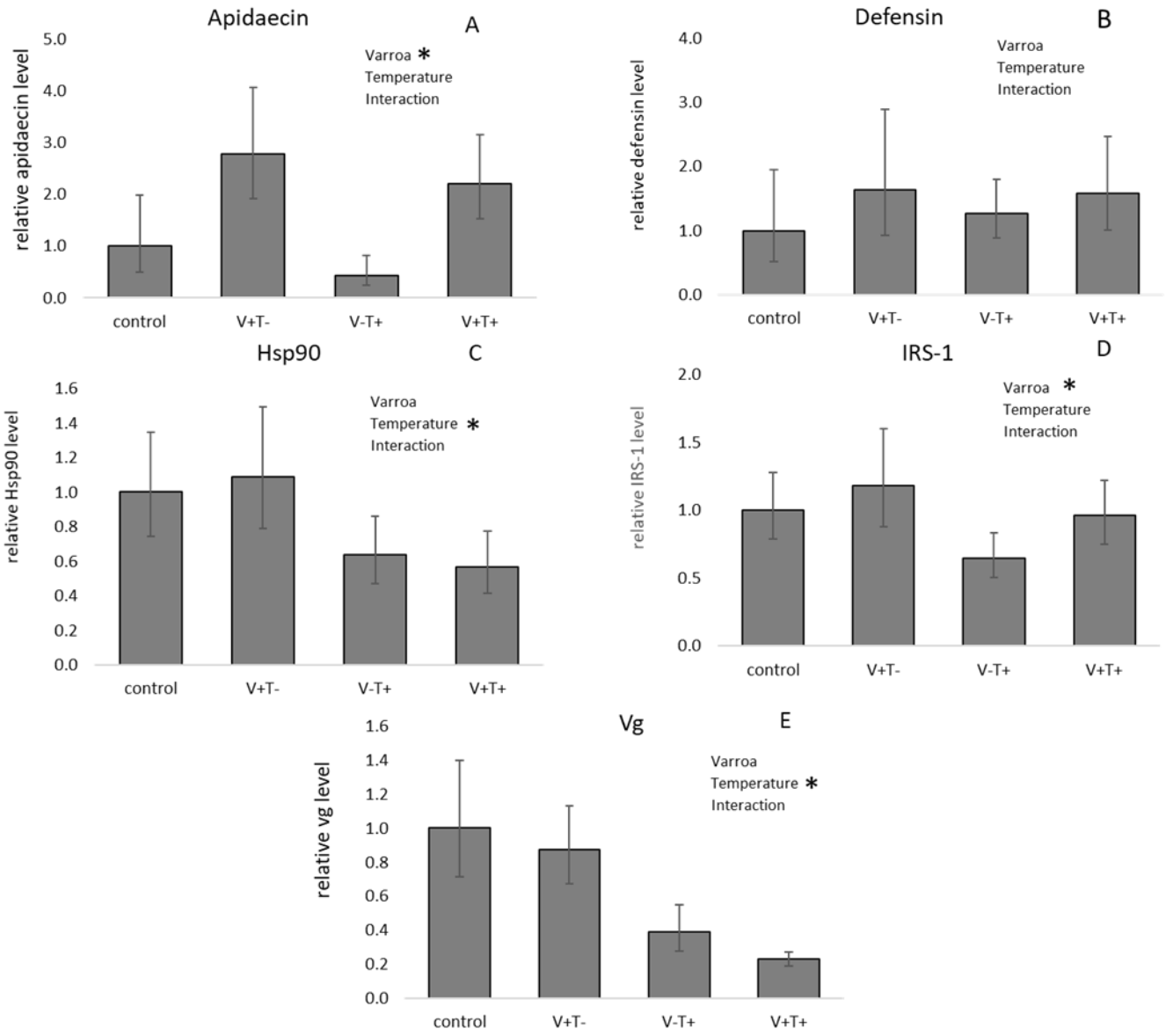

Figure 20. Relative gene expression of the several genes studied. Honey bee were affected by cold stress, V. destructor *and the interaction between them. Result of the Scheirer-Ray-Hare test were reported. Asterisk refers to statistical significance (p <0.05) of the test.*

3.2.4. Pairwise interactions between nicotine, temperature, V. destructor and pollen

In the first phase of our experimental activity we determined the effect of single factors on honey bee survival. We showed that nicotine at 50 ppm and *V. destructor* have a negative impact on honey bees lifespan while 32 °C temperature has no significant effect. Pollen instead, have a neutral or significantly positive effect depending on the seasonality of the experiments. However, under natural

conditions, a number of factors are present at a time making it necessary to understand how different factors interact with each other to influence honey bee survival. To this aim we run a fully factorial experiment including: nicotine, low temperature, *V. destructor* and pollen.

To analyse the effects of various factors on survival time, we utilized a multivariate weighted Cox proportional-hazard model. Since using more than two interacting factors tends to make the Cox regression strongly non-predictive, we started with the pairwise comparison between interaction factors and progressively deleted the non-statistically significant interactions.

```
> res.cox <- coxphw(Surv(time, status) ~ (low_temperature+varroa+pollen+nicotine)^2 - varroa:pol
len - low_temperature:pollen - low_temperature:nicotine - low_temperature:varroa, data = mydata2
, template = "AHR")
> summary(res.cox)

coxphw(formula = Surv(time, status) ~ (low_temperature + varroa +
    pollen + nicotine)^2 - varroa:pollen - low_temperature:pollen -
    low_temperature:nicotine - low_temperature:varroa, data = mydata2,
    template = "AHR")

Model fitted by weighted estimation (AHR template)

                      coef   se(coef) exp(coef) lower 0.95 upper 0.95         z            p
low_temperature  0.2919643 0.08906946 1.3390552  1.1245595  1.5944634  3.277939 1.045678e-03 *
varroa           0.6620712 0.13300655 1.9388038  1.4938896  2.5162237  4.977734 6.433303e-07 ***
pollen          -0.6037658 0.13826646 0.5467488  0.4169608  0.7169362 -4.366683 1.261478e-05 ***
nicotine         0.1475093 0.12893914 1.1589440  0.9001384  1.4921609  1.144023 2.526143e-01
varroa:nicotine -0.4195690 0.17755163 0.6573301  0.4641424  0.9309272 -2.363082 1.812367e-02 *
pollen:nicotine  0.4927955 0.18407186 1.6368857  1.1411326  2.3480135  2.677191 7.424237e-03 **

Wald Chi-square = 54.68762 on 6  df, p = 5.359947e-10


Generalized concordance probability:

                concordance prob. lower 0.95 upper 0.95
low_temperature            0.5725     0.5293     0.6146
varroa                     0.6597     0.5990     0.7156
pollen                     0.3535     0.2943     0.4176
nicotine                   0.5368     0.4737     0.5987
varroa:nicotine            0.3966     0.3170     0.4821
```

Table 2. Value and significance of the hazard ratio of different factors and interactions according to Cox.

We found that honey bees exposed to abnormal low temperatures (32 °C) and *Varroa* infestation had a significantly higher risk of death by 34% and 94%, respectively, whereas pollen significantly reduced the risk of death by 45%. We also found a significant interaction between pollen and nicotine such that the risk of death was increased by 63% when the two factors were presented together. On

the contrary, the significant interaction between varroa and nicotine caused a reduction in the risk of death by 34%.

The p-value of the overall Wald test is significant, indicating that the model is significant.

3.2.5. Combined action of HMF and acidity on honey bee survival

In the paragraph "Effects of single stressors on bee health" we showed that HMF at a low concentration (i.e. 85 ppm and 400 ppm) is not harmful to honey bees while acidity (pH = 2.91) has a negative effect on bee lifespan. In order to study the interactive effect of these two potential stressors we compared the survival of honey bees fed with an acidified sucrose solution at 2.91 pH containing HMF (85 ± 9.17 ppm) versus honey bees fed with a sucrose solution containing HMF but not acidified and an acidified solution (2.91 pH). We found that the combination of HMF and acidity has an effect not more deleterious than acidity *per se* (Fig. 21; control + HMF vs. acid + HMF, Log-rank test: Chi-Square = 93.978 , d.f. = 1, $P = 0.002$; acid vs. acid + HMF, Log-rank test: Chi-Square = 1.354 , d.f. = 1, $P = 0.245$).

Since acidification of sugar syrups appears to be critical for bee survival, and the purpose of this treatment is to obtain the inversion of disaccharide sugars into monosaccharides, we tested if feeding bees with a sucrose solution instead of glucose and fructose influences their survival.

We found that bees fed with sucrose syrup (the same recipe as that used in the previous experiments) had a longer survival than bees fed with a 1:1:1 water, glucose and fructose solution (Fig. 22; sucrose vs glu+fru, Log-rank test: Chi-Square = 7.440, d.f. = 1, P = 0.006).

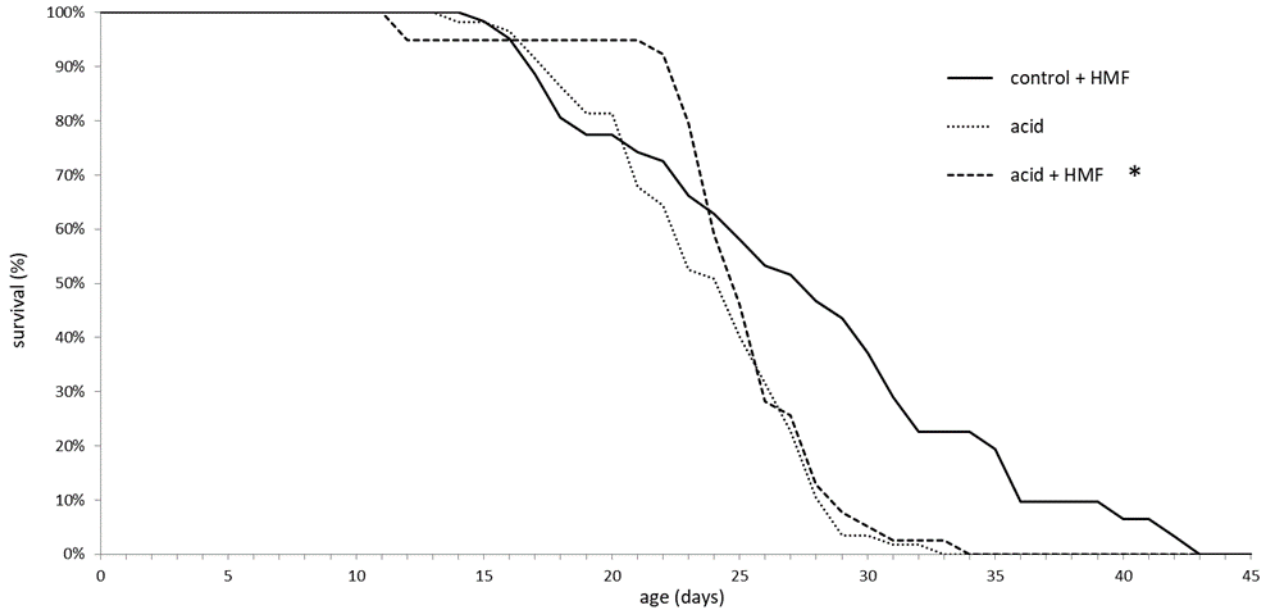

Figure 21. Survival of honey bees treated differentially with HMF, acidity and HMF + acidity.

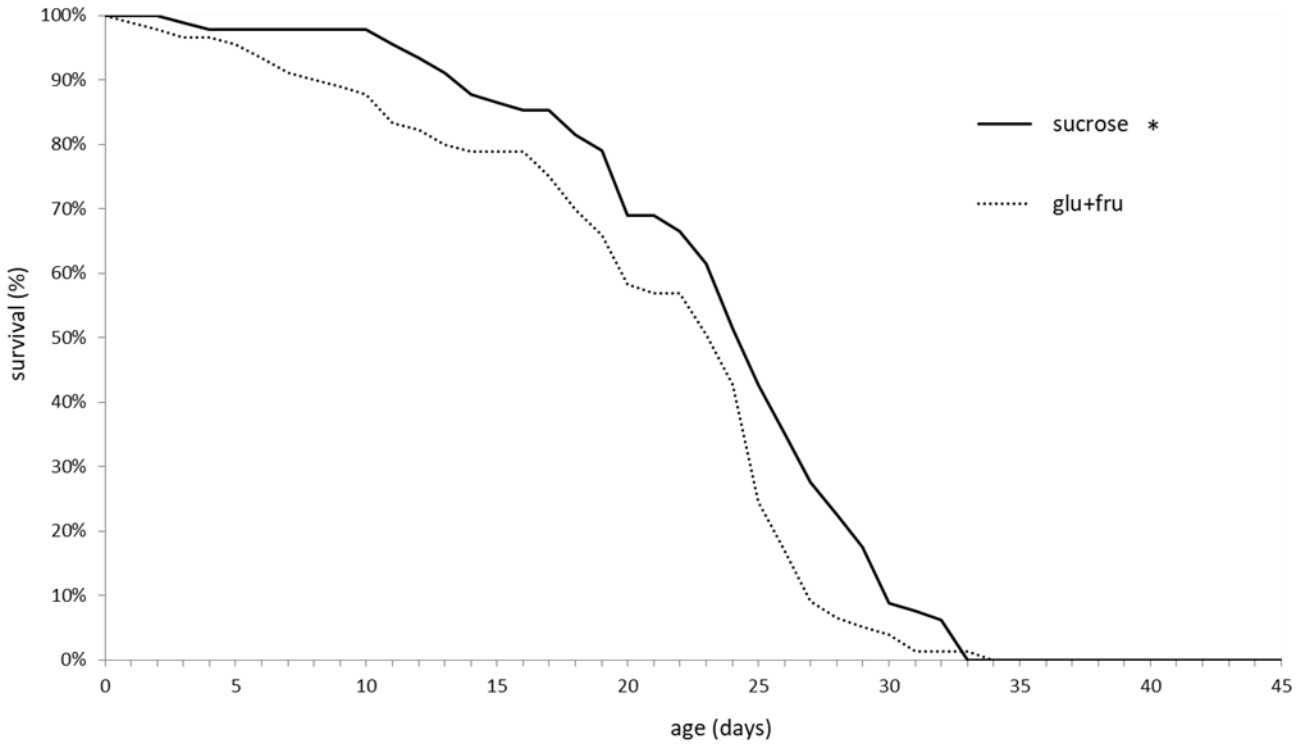

Figure 22. Survival of honey bees fed with monosaccharide and disaccharide diets.

3.2.6. Combined action of HMF and *V. destructor*

To investigate if an otherwise ineffective concentration of HMF can be harmful to mite infested bees, we treated such bees with HMF at 400 ppm and compared their survival to that of bees treated with HMF but uninfested. We noted that surprisingly 400 ppm of HMF increased the survival of mite infested bees (Fig. 23; V+ vs. V+ HMF+, Log-rank test: Chi-Square = 5.052, d.f. = 1, $P = 0.025$) while the survival of uninfested bees treated with HMF was higher than both untreated uninfested bees and mite infested (Fig. 23; V- HMF+ vs. V+ HMF+, Log-rank test: Chi-Square = 6.000, d.f. = 1, $P = 0.014$).

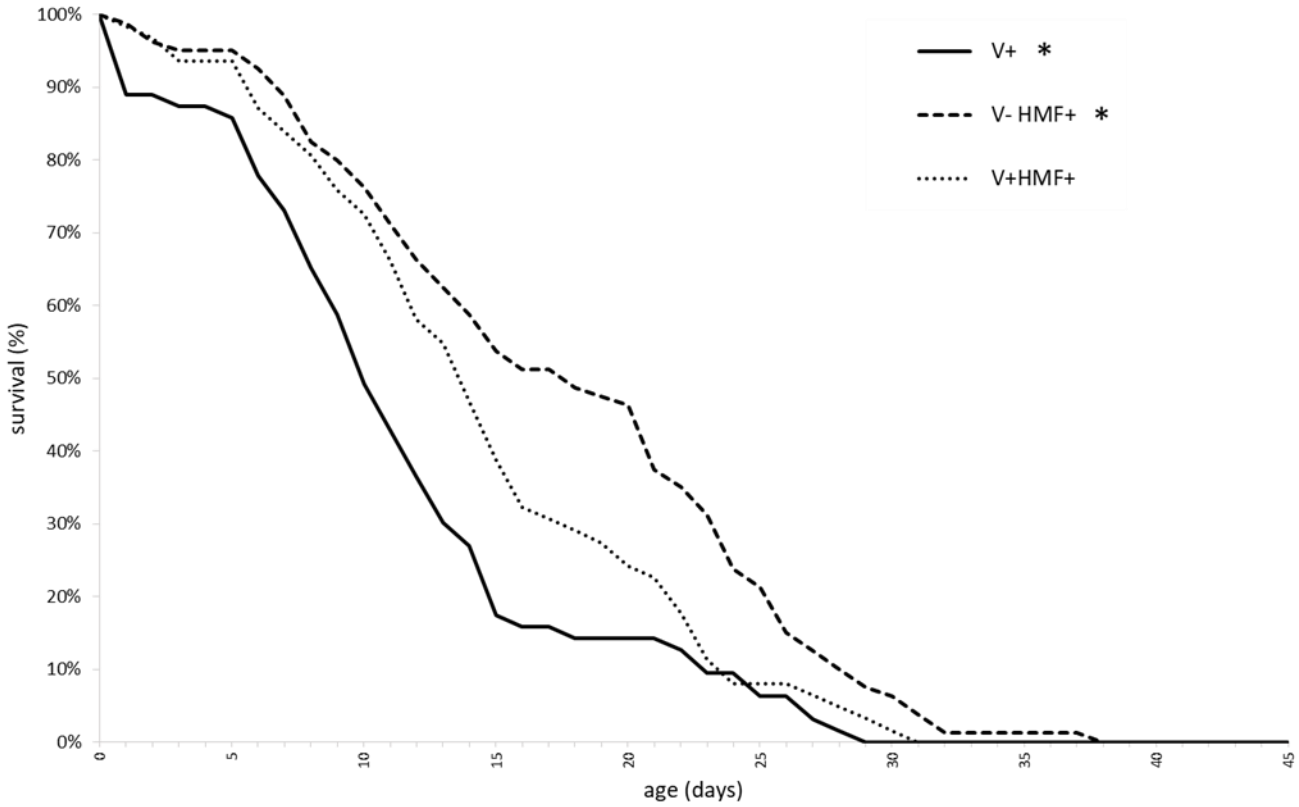

Figure 23. Survival of honey bee treated with: V. destructor*, HMF at low concentration (400 ppm) and* V. destructor *and HMF 400 ppm. Asterisk refers to statistical significance (p <0.05) compared to V+HMF+.*

4. DISCUSSION

Three years of experiments produced a considerable amount of data. For better clarity, the chapter is subdivided into paragraphs. Each paragraph refers to a single factor that is examined first for the effect that it can have on its own on bees and then for the combined effect with other factors as demonstrated along this study.

4.1. Pollen

Our study confirms the well-known beneficial effects of pollen on honey bees. In particular, pollen seems to play a fundamental role in relation to the stressors that might affect honey bee's health. Indeed, pollen did not affect the normal survival of unchallenged newly emerged adult bees (i.e. bees not exposed to any of the following potential stressors: DWV, *V. destructor*, nicotine, temperature, acidity and HMF). However, as soon as one or more stressors were added, pollen increased the lifespan compared to control. In particular, no effects of pollen on otherwise healthy bees were noted in May, when, under the conditions of the area where this experiment was carried out, most bees are DWV negative and the viral load in positive bees is low (Nazzi et al., 2012). Instead, at the end of the Summer, when viral prevalence reaches 100% and viral load is higher (Nazzi et al., 2012), a significant effect of pollen was noted.

This result implies that pollen is not fundamental *per se* for honey bee's survival under cage conditions but it is beneficial in presence of factors which can affect the homeostasis of bees. These was already noted by Annoscia et al. (2017) who demonstrated that pollen increased the survival of mite infested bees but did not do so in un-infested bees. Previous observation on the effect of pollen on mite parasitized bees were confirmed here when pollen was supplied to bees infested with one mite at the pupal stage and their survival compared to that of un-infested bees (see par. 3.2.2).

The results on the effect of pollen on bees that were infected with the virus through nutrition reported in paragraph 3.2.1. suggest that the beneficial effect of pollen on mite infested bees may be related

both to the compensation of the negative effects of mite nutrition on developing bees and a possible direct effect of pollen on the virus vectored by the mite. Clearly, it may well be that the beneficial effect of pollen on virus infected bees is related only to a compensation of the deleterious effects of virus infection rather than a possible antiviral action suggested above. This antiviral effect of pollen was already noted by DeGrandi-Hoffman and colleagues (2010) which found that worker honey bee fed with pollen had a lower DWV load than those bees fed with sugar alone, however, in a similar study, pollen increased the virus load both in *Varroa* infested and *Varroa*-free honey bees (Alaux et al., 2011). This interesting hypothesis thus, seems to be worth of further investigation. In any case, to our knowledge, for the first time we showed the net beneficial effects of a pollen diet on virus infected honey bees. Indeed, the oral injection of DWV allowed us to work with bees whose immunocompetence was challenged by the virus alone and not by other interfering factors (e.g. syringe injection triggers the immune system activating clotting and melanisation).

Interestingly, in early season experiment, pollen treated honey bees started to die few days before the non-treated ones (day 12 vs. day 20) but the mortality was constant and lower, compared to sugar fed honey bees. This might be explained by the fact that pollen contains compounds (i.e. flavonoids) and secondary metabolites that have to be detoxificated by the honey bees (Schmehl et al., 2014; Berenbaum and Johnson, 2015; du Rand et al., 2015). This detoxification process has a cost in terms of energy (du Rand et al., 2015), and it may be noted that, in fact, bees fed with pollen and infected with 100 and 1,000 viral copies consumed higher amounts of sugar as compared to their control without pollen.

The hypothesis that the beneficial effect of pollen comes at a cost in terms of detoxification seems to be supported by the negative interaction between pollen and nicotine observed in the multifactorial experiment that will be discussed below.

4.2. HMF and acidity

Doses of HMF similar to those reported as sublethal in the literature (Jachimowicz and El Sherbiny, 1975; Le Blanc et al., 2009) and found in our homemade sugar syrups, seem to be non-toxic for uninfested bees (see par. 3.1.2.). This result suggests that, at low concentrations, in the range of 85-400 ppm, HMF does not influence bee health. This apparent non toxicity of HMF at low doses was confirmed also with bees that were infested at the pupal stage (see par. 3.2.6.) supporting the notion that these two stressors do not interfere with each other.

Our results show that the lower acidity that can be found in homemade syrups because of lemon addition, negatively affects bees' survival, as confirmed by comparing the survival of bees fed a sugar solution acidified or not with lemon (see par. 3.1.3.). The similar results obtained after changing the acidifying agent support the notion that acidity per se, rather than any toxic compounds from lemon, is responsible for the observed effect.

However, our experiments (see appendix 6.3 "Possible side effects of sugar supplementary nutrition on honey bee health" for more information) further showed that acidified sugar solutions may reach much higher concentrations of HMF if a prolonged heating is applied and a low pH level (e.g. pH=2) is reached after lemon addition; in fact, both acidity and the amount of lemon juice are influenced by seasonality, climate and the stage of ripeness of the lemon (Bartholomew, 1923), and such low pH level can easily be reached. Since the very high concentrations of HMF that can be produced under the above mentioned conditions can be very toxic to bees (i.e. above 10000 ppm), a great care should be used while making homemade syrups. The negative effect of lemon addition obtained here suggested to test if lemon addition is really necessary; we found that sucrose can be as effective as glucose and fructose to sustain a normal survivorship under laboratory condition. This would suggest that lemon addition may be not necessary as normally thought, possibly because bees are able to invert disaccharides themselves, thanks to α-invertase (White, 1975).

The golden rule of medicine “primum non nocere” (first do no harm), attributed to Hippocrates, underlines the need of carefully considering the possible negative side effects of the treatments we may apply to sustain the health of an individual. Bees are currently exposed to a number of interacting stress factors (vanEngelsdorp et al., 2009; Hedtka et al., 2011; Dainat et al., 2012; Nazzi et al., 2012; Nazzi and Pennacchio, 2014,) that may affect bee health in a complex and often unpredictable way (Di Prisco et al., 2013; Doublet at al., 2015; Nazzi and Pennacchio, 2018). With these experiments concerning HMF and acidity we wanted to point the attention to the undesirable effects of supplementary nutrition a practice which has become very common due to the increased fragility of bees underlined above.

4.3. Nicotine

Nicotine is a natural xenobiotic that is detoxicated by honey bee’s metabolism (du Rand et al., 2015). Our data show a detrimental effect at 50 ppm while at the lower concentration of 0.1 and 1 ppm nicotine slightly increased the survival of honey bees (see par. 3.1.4.). These results confirm the findings of Kohler et al. (2012) who found that 50 ppm of nicotine are detrimental for caged honey bees.

The negative effect of nicotine may be related to an interference of the alkaloid with food utilization, as it happens for tobacco hornworms, in which dietary nicotine reduces the efficiency of food conversion (Bentz and Barbosa, 1992). On the other hand, the increased survival of bees fed with low concentrations of the compound support the dose-response relationship observed in bees between feeding and the concentration of nectar secondary metabolites: low concentration of some alkaloids are preferred to sugar-only controls, while high concentration inhibits ingestion (Hagler and Buchmann, 1993; Singaravelan et al., 2005).

When presented together with other factors, nicotine revealed a very interesting pattern of activity. In fact, this alkaloid was ineffective when taken alone but very effective when supplied to bees exposed to *V. destructor* or fed with pollen. In particular, when honey bees were exposed to both

Varroa and nicotine, the hazard ratio (HR) (probability of death) was lower suggesting that nicotine can be positive for mite infested bees. The beneficial effect of nicotine is difficult to explain; our first hypothesis refers to the antiviral effects of nicotine. It has been demonstrated that the alkaloid decreases the Hepatitis C Virus (HCV) by acting on the well conserved MAPK pathway (Yamashina et al., 2008). According to this mechanism, nicotine could have decreased the virus load in mite infested bees fed with nicotine, enhancing their survival; this interesting hypothesis will need to be tested with further analyses.

A second and more straight-forward hypothesis, implies the energetic metabolism of honey bees. Nicotine increases the resting metabolisms (McGovern and Benowitz, 2011) and its detoxification by the honey bee increases the energy demand (du Rand 2015). This could counteract the observed *Varroa* induced anorexia by stimulating the sugar daily intake; this in turn could provide the energy that is necessary to better cope with the negative effects of the ectoparasitic mite.

The negative effects of the interaction between pollen and nicotine may result since pollen constituents, in part, can regulate detoxifying genes expression affecting the metabolic pathways of phytochemical and pesticides (Gong and Diao, 2016).

Thus our results might be explained because, in some case, the compounds contained in pollen (e.g. bacteria and secondary metabolites), are detoxified by the same pathway of nicotine, entailing a flooding of these process. Indeed, it has been suggested that the small number of detoxification genes presents in *A. mellifera*, may limit the capacity to metabolize multiple toxin simultaneously that results in a reduction in lifespan (du Rand et al., 2015).

In general, our results confirm that the response of honey bees to xenobiotics is complex involving detoxification, oxidative and general stress response (Gong and Diao, 2016).

4.4. Temperature

A 2.5 degrees lower temperature did not affect honey bee lifespan, but it stimulated a higher intake of sugars. Since honey bees can regulate their body temperature by contracting the indirect flight muscles, it is reasonable to think that a lower external temperature, may activate muscle contraction, which is an energy demanding activity likely implying an increased sugar consumption. The possible interaction of his factor with lower temperature will be discussed in the next paragraph.

4.5. *V. destructor* and DWV

The detrimental effects of *V. destructor* parasitism on honey bees have been extensively studied, and our results confirm previous data. However, this is the first study which reports a reduced sugar intake in mite parasited bees and a lower average body temperature in mite infested bees (see par. 3.1.6.). In principle, this might be related to the detrimental effect of *Varroa* parasitism on fight muscles development in honey bees. Indeed, an impaired anatomy of mite infested honey bees could lead to a decreased thermoregulatory capacity with a consequent decrease in energy demand. However, this hypothesis does not seem to be supported by the similar weight of the thorax in mite infested honey bees as compared to uninfested bees. Instead, this evidence points to a possible effect of the reduced sugar intake resulting from a kind of physiological anorexia triggered by mite infestation. A disease associated anorexia associated to viral infections has been observed in caterpillars of the African armyworm *Spodoptera exempta* (Povey et al., 2013); however, in our case, the observed anorexia does not seem to be related to the viral infection associated to mite infestation since we found no differences in sugar consumption in artificially virus infected bees (see par. 3.2.1.).

Contrary to what observed in this study, Pusceddu et al. (2018) did not notice any effect of mite parasitization on sugar intake of mite infested bees; however, in that study bee were parasitized at the

adult stage and both the effect of parasitization at that stage and the resulting response could be different.

The impact of *V. destructor* on the feeding of honey bees may have notable consequences. A lower sugar intake can lead to a decrease in energy availability negatively influencing honey bee homeostasis, and, in particular, immunity (DeGrandi-Hoffman and Chen, 2015). The connection between nutrition and immunity has been demonstrated in numerous organisms where the immune function is affected by caloric restriction (Franca et al., 2009; Cotter et al., 2011).

Moreover, a reduced energy intake involves a reduced and probably slower capacity to cope with thermal stress. Therefore, it appears that the decreasing temperature observed during the cold season and the extreme weather episodes likely related to climate change, can further enhance the detrimental effects of *V. destructor* infestation. The pollen effect on Varroa parasitism, documented here, replicates the findings of Annoscia et al. (2017), where pollen was beneficial for caged infested honey bees. However, for a deeper understanding, we tested the hypothesis that this beneficial effect depends on the positive contributions of pollen to the energetic metabolism of bees and thus on the better functioning of the homeostatic mechanisms. To this aim, we altered the functioning of the energetic pathways with rapamycin. Contrary to our expectations, rapamycin had no effect on the survival of mite infested honey bees fed with pollen; this suggests that the beneficial effects of pollen are likely related to other unexpected mechanisms.

Our molecular studies, aiming at measuring the expression of vitellogenin and a number of genes in the mTOR pathway and assessing DWV infection level, provided some interpretative clues.

In particular, we noted that rapamycin consistently decreased DWV load in varroa infested bees both in presence of pollen or not. We hypothesize that this may be related to the increased autophagy stimulated by this compound. In fact, autophagy can degrade intracellular pathogens and virus (Levine, 2005; Deretic and Levine 2009; Sumpter and Levine, 2010) and mite infestation is clearly associated to higher viral load, as observed here in agreement with previous studies (de Miranda and Genersch, 2010; Nazzi and Le Conte, 2016; Wilfert et al., 2016). This hypothesis is consistent with

the up-regulation of ATG-2, a gene implicated in autophagy and life span extension (Tsukada and Ohsumi, 1993), observed in varroa infested bees treated with rapamycin; however, this effect was not noted in pollen fed mite infested bees suggesting caution with this interpretation.

As for the effect of pollen on virus infected bees that was observed in this study, we speculate that it may result from the enhanced synthesis of antimicrobial peptides that are likely involved in antiviral response as observed by Annoscia et al. (2019); in fact, Danihlik et al. (2018) observed that pollen fed bees have an higher production and expression of antimicrobial peptides.

The up-regulation of IRS-1, a key gene responding to the nutritional status of the organism, observed in mite infested bees is likely related to the necessity to cope with the increased energy demand associated to the reduced calorie intake caused by V. *destructor*.

Vitellogenin expression was greater in mite infested bees, confirming previous results and strongly supporting the validity of this protein as a marker of mite infestation (Dolezal et al., 2016; Smart et al., 2016; Zanni et al., 2017). Furthermore, pollen influenced the expression of vitellogenin in mite infested bees, most likely for the increased availability of nutrients for its production (Bitondi and Simoes, 1996; Di Pasquale et al., 2013).

Since pollen is the only source of amino acids, it is not surprising that it influenced the mTOR pathway of mite infested bees regulating the ILP-1 and PI3K genes.

With this study we show how unpredictable are the relation between stress factors and we should consider that these interactions are just the result of four factors combined. Natural environment instead, are characterized by several of these factors that might affect honey bee health. Since an analytic study of all the factors that can affect honey bee health is unimaginable, the attention should be focused on the metabolic process accounting for the observed interactions in order to develop one or several models that could help to predict the outcome of such interactions.

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6. APPENDIX: OTHER WORKS AND COLLABORATIONS

6.1. Attachment 1

Annoscia D., Brown S.P., Di Prisco G., De Paoli E., Del Fabbro S., Frizzera D., Zanni V., Galbraith D.A., Caprio E., Grozinger C.M., Pennacchio F. and Nazzi F. (2019). Haemolymph removal by *Varroa* mite destabilizes the dynamical interaction between immune effectors and virus in bees, as predicted by Volterra's model. Proc. R. Soc. B, 286, 20190331.
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Haemolymph removal by *Varroa* mite destabilizes the dynamical interaction between immune effectors and virus in bees, as predicted by Volterra's model

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The association between the deformed wing virus and the parasitic mite *Varroa destructor* has been identified as a major cause of worldwide honeybee colony losses. The mite acts as a vector of the viral pathogen and can trigger its replication in infected bees. However, the mechanistic details underlying this tripartite interaction are still poorly defined, and, particularly, the causes of viral proliferation in mite-infested bees. Here, we develop and test a novel hypothesis that mite feeding destabilizes viral immune control through the removal of both virus and immune effectors, triggering uncontrolled viral replication. Our hypothesis is grounded on the predator–prey theory developed by Volterra, which predicts prey proliferation when both predators and preys are constantly removed from the system. Consistent with this hypothesis, we show that the experimental removal of increasing volumes of haemolymph from individual bees results in increasing viral densities. By contrast, we do not find consistent support for alternative proposed mechanisms of viral expansion via mite immune suppression or within-host viral evolution. Our results suggest that haemolymph removal plays an important role in the enhanced pathogen virulence observed in the presence of feeding *Varroa* mites. Overall, these results provide a new model for the mechanisms driving pathogen–parasite interactions in bees, which ultimately underpin honeybee health decline and colony losses.

1. Introduction

Efficient pollination is vital for crop production [1] and the honeybee is the prevailing managed insect crop pollinator. Honeybees suffer from a range of adverse factors [2]; in particular, the deformed wing virus (DWV) is implicated in the substantial colony losses reported in many parts of the world [3] and the parasitic mite *Varroa destructor* plays a key role in virus transmission and replication [4,5]. Moreover, previous studies have demonstrated that the spread of *V. destructor* contributed to turning a widespread viral infection into a devastating epidemic [3].

The capacity of the *Varroa* mite to transfer DWV was proved by Ball [6] and later confirmed under field conditions [7]; these authors also provided preliminary evidence for the replication of the virus within the mite, which was later confirmed [8]. However, the mite does not act only as a vector of the virus, thus increasing the pathogen's prevalence, but can also trigger uncontrolled replication in infected bees, which undermines colony survival [9]. Several mechanisms have been proposed to explain the role of the mite as an activator of the virus, based on experiments or samplings carried out under different settings and variable conditions. Initially, increased replication was attributed to a direct immune-suppressive action exerted by the mite [10]. Based upon field experiments aiming at assessing the impact of *Varroa* infestation on bees, we showed that the immune challenge represented by the feeding mite amplifies existing viral infections through an escalating bee immunosuppression, perpetuated by the increasing DWV abundance [9]. Two additional mechanisms accounting for the higher viral load observed in mite-infested bees were proposed. First, it was suggested that higher infection levels, leading to crippled winged bees, are linked to the active replication of the virus within the infesting mite [8]. Second, based on the study of a region of the RNA-dependent RNA polymerase (RdRp) gene of DWV, during a *Varroa* invasion into a previously mite-free area, the possibility that the mite can select for a single virulent strain adapted to mite transmission, was proposed [11]. This facilitation seems to take place also at the individual level when a mite infests a honeybee, where either parasitization or artificial injection favours the replication of a single quasi-clonal DWV strain within the bee [12].

However, the available data can also support additional models on how mite feeding can influence the viral titre in bees. In particular, the significant increase in the viral titres in bees infested by three mites versus a single mite [9] and previous observations about the effects of multiple mite infestations on the proportion of symptomatic bees [13] suggests that feeding intensity may play a role. This could be the result of the injection of increasing amounts of mite derived immune suppressing factors into the bee's haemolymph [14]. However, when more *Varroa* mites parasitize the same bee, they make a single wound into the bees' cuticle to access the haemolymph and feed from the same opening [15,16], thus likely eliciting the same response in terms of melanisation and clotting, but subtracting a substantially higher volume of haemolymph. This, in turn, could be responsible for the increased viral replication observed in case of multiple infestation. The possible role of haemolymph removal on DWV dynamics seems to be confirmed by the proliferation of DWV that can be observed after simple wounding with capillary needles and the resulting bleeding from the open wounds [17]. The mite feeding on honeybee fat body rather than on haemolymph, recently claimed [18], does not challenge the established view that this parasite feeds upon the internal fluids, which could well be enriched with nutrients released by extra-oral digestion of fat body.

On a purely theoretical background, it is possible to hypothesize that the concurrent removal of virus particles and circulating antiviral immune effectors by the blood-feeding mite can generate a dynamic response similar in principle to that observed when both prey and predators are constantly removed from a predator–prey system [19]. This apparently counterintuitive circumstance was first explained by Volterra, at the beginning of the last century [19], for describing the unexpected fluctuations of certain fish species in the Adriatic Sea. The proposed model clearly showed that the subtraction of both predators and prey, through fishing, could result in the proliferation of the prey [19].

In summary, in spite of the large body of evidence about the effect of mite infestation on the dynamics of viral infection in the honeybee and the importance of the *Varroa*–DWV association for honeybee health, there are still multiple hypotheses on the major mechanisms underpinning the higher viral load observed in mite-infested bees, that are not mutually exclusive. In this study, to further contribute to the analysis of the mechanisms underlying the viral proliferation in mite-infested bees, we carried out controlled laboratory experiments to test the hypothesis that mite feeding 'per se' can destabilize viral immune control through the removal of both viral 'prey' and immune 'predators', triggering viral replication. We assess the impact of controlled bleeding on viral proliferation; we also evaluate if the resulting viral load is in part or totally owing to any of the other mechanisms described in the literature. This type of micro-ecological analysis of host–pathogen interactions has broad implications in the research area of animal parasitology.

2. Results

(a) Viral infection in mite-infested honeybees

To clarify the role of the mite in the dynamics of viral infection in honeybees, we evaluated the presence and abundance of DWV in adult bees that were artificially infested with one mite as mature larvae or were not infested with mites as controls (figure 1*a*,*b*); viral presence and titres were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR) with sequence-specific DWV primers. Furthermore, a subset of these bees (figure 1*b*) was subjected to next generation sequencing (NGS) which allowed us to confirm that the bees were infected with DWV and the sequences were greater than 98% identical with a published sequence obtained from a sample collected in the same apiary in 2006 (i.e. NC_004830.2; electronic supplementary material, figure S1) and clearly separated from other genotypes of DWV (i.e. NC_006494.1) or recombinants that were associated with higher virulence in other studies (electronic supplementary material, figure S1) [12,20]. In particular, sequencing revealed that the viral genotype present in this area can be regarded as DWW type A [21,22].

We found that 80% of individuals not exposed to mite feeding ($n = 40$) were DWV infected. However, the prevalence of DWV in bees infested by a DWV-infected mite ($n = 27$) was higher at 96% (electronic supplementary material, figure S2; $\chi^2_1 = 3.681$, $p = 0.055$).

Viral load was higher in bees parasitized by mites compared to control bees (figure 1*b*; median viral load in mite-infested bees ($n = 32$) $= 1.91 \times 10^4$ DWV genome copies; median viral load in uninfested bees ($n = 40$) $= 8.06 \times 10^3$ DWV genome copies; Mann–Whitney $U = 482$, $n_1 = 40$, $n_2 = 32$, $p = 0.037$). DWV infection levels in uninfested bees showed a great variability ranging from 10^3 to 10^6 DWV genome copies per bee (figure 1*b*). However, DWV infection levels showed even greater variability in mite-infested bees; in fact, most mite-infested bees showed infection levels falling within the same interval as that

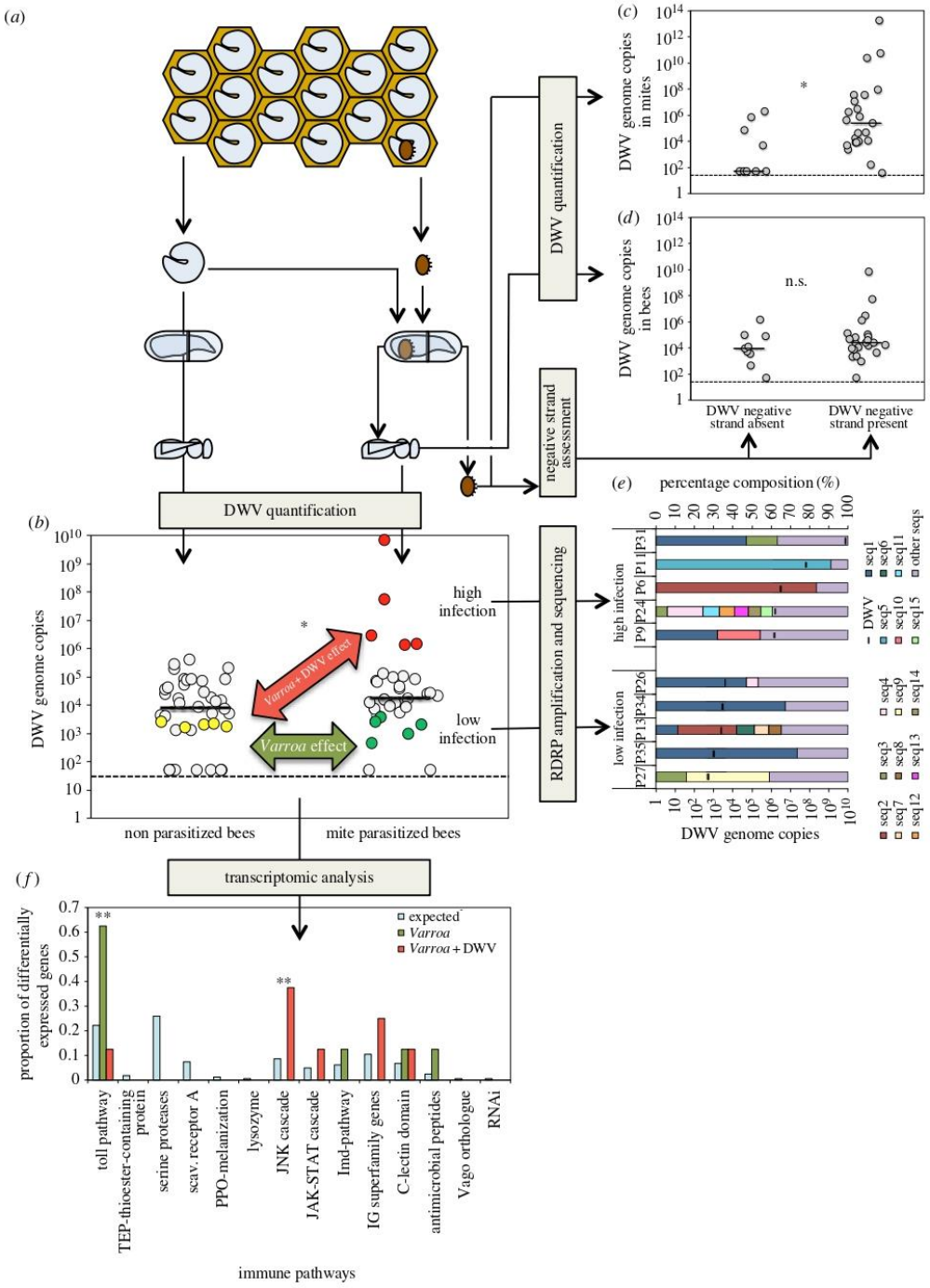

Figure 1. (*Caption overleaf.*)

recorded in uninfested bees, but a few specimens largely exceeded the upper limit of this interval, reaching 10^{10} viral genome copies per bee (figure 1*b*). Consequently, the distribution of viral loads was very skewed in mite-infested bees (skewness of the distribution of viral loads in mite-infested bees ($n = 30$) = 5.48, skewness in uninfested bees ($n = 32$) = 2.50). Individual bees sampled later in the field season, when the DWV prevalence and the basal infection rate are higher [9], and artificially infested with one mite, showed a similar skewed distribution of infection levels, with some individuals displaying very high DWV infection levels (skewness of the distribution of viral loads in mite-infested bees

Figure 1. (*Overleaf.*) Evaluation of existing hypotheses about the role of *Varroa* mite in increasing virulence of DWV: methods and results. (*a*) Individual bees naturally infected with DWV were artificially infested with one *Varroa* mite or left uninfested. (*b*) Viral load in individual bees infested with one mite or left uninfested as a control. In this and following similar figures, the dashed line represents the lower detection limit for the methodology used; the solid lines represent the median viral load. The samples used for the transcriptomic analysis are marked with different colours: yellow (uninfested-low virus infected bees), green (mite-infested-low virus infected bees) and red (mite-infested-high virus infected bees). An asterisk marks a significant difference at $p < 0.05$. (*c*) DWV genome copies in *Varroa* mites where an active replication was detected (DWV negative strand present) or not (DWV negative strand absent). An asterisk marks a significant difference at $p < 0.05$. (*d*) DWV genome copies in bees infested by mites where an active replication was detected (DWV negative strand present) or not (DWV negative strand absent). (*e*) Prevalence of different DWV variants in infected bees with variable virus infection levels. The thick vertical lines represent DWV genome copies observed in each sample. (*f*) Effect of the *Varroa* mite and the combination *Varroa*-DWV on the expression of genes of the canonical immune pathways. The proportion of differentially expressed genes in each pathway, as resulting from the comparison: uninfested-low viral infected bees versus mite-infested-low viral infected bees (i.e. *Varroa* effect) and from the comparison: uninfested-low viral infected bees versus mite-infested-high viral infected bees (i.e. *Varroa* + DWV effect), is reported as well as the proportion of immune genes belonging to that pathway (i.e. expected). Two asterisks mark significant differences at $p < 0.01$ between expected and observed proportions.

($n = 58$) = 5.66; electronic supplementary material, figure S3). Moreover, re-evaluation of previous data demonstrating the effect of single and multiple mite infestations on viral loads in bees [9] revealed a similar underlying distribution, with a higher median viral infection in mite-infested bees and the distribution of viral loads becoming increasingly sparse (electronic supplementary material, figure S4).

In summary, the DWV infection data show that the higher viral load observed, on average, in *Varroa*-infested bees is owing to a change in the distribution of individual viral levels, which is right skewed, owing to the presence of a sub-population of highly infected bees. Similar results were previously observed using a different experimental approach [12].

(b) Viral replication in mites

To study the vector role of *Varroa*, we evaluated the mites infesting the experimental bees above (figure 1*a*) and found that their infection levels were generally higher than those in the bees themselves (median viral load in mites ($n = 32$) = 4.60×10^{04}). A significant correlation was found between the mites' viral load and viral load of the bees they infested (electronic supplementary material, figure S5; $n = 32$, Spearman corr. coeff. = 0.531, $t_{30} = 3.433$, $p = 0.002$). However, this result cannot be unequivocally interpreted, because the observed correlation could be owing either to the fact that a highly infected mite, harbouring an intense viral replication, can inject higher amounts of viral particles, or that a mite infesting a highly infected bee can acquire more virus while feeding.

Active replication of single-stranded positive RNA viruses results in the synthesis of the complementary negative strand that is used as a template for the production of viral copies. Therefore, to assess the importance of viral replication within the mite on the level of bee infection, we assessed the presence of DWV negative strands in the mites used for the artificial infestation of bees (figure 1*a*). As expected, the mites containing DWV negative strands had a significantly higher infection level than those where no negative strands were found (figure 1*c*; Mann–Whitney $U = 42$, $n_1 = 9$, $n_2 = 23$, $p = 0.005$). However, when we examined whether the viral replication in the parasite was related to the viral load in the host, we found that the infection level of bees infested by mites where an active viral replication was detected was not significantly different from that measured in bees infested by mites which did not apparently harbour an actively replicating virus (figure 1*d*; Mann–Whitney $U = 80$, $n_1 = 9$, $n_2 = 23$, $p = 0.157$).

(c) Composition of the viral mutant cloud

Short replication time and limited correction capability in RNA viruses favour rapid genetic changes, so that, even in a single host, a virus population normally consists of an ensemble of different genetic sequences. Previous studies focusing on the viral RdRp highlighted an important effect of mite parasitization on viral diversity [11]. Therefore, to assess the importance of this factor in the higher viral load observed in mite-infested bees, we amplified and sequenced by NGS the viral region encoding the virus RdRp, in five highly infected bees and five bees with low infection levels (average DWV genome copies per bee of 1.41×10^9 and 1.95×10^3, respectively) that were obtained from the previous experiment (figure 1*b*). From 74 to 559 different variants were reconstructed in each sample, based on a number of viral reads ranging from 40 107 to 160 842 (electronic supplementary material, data S1). We found no obvious common sequence in low versus high virus infected bees: the most represented sequence was present in six samples from both the low and highly infected groups, at prevalences ranging from 11 to 74% (figure 1*e*; electronic supplementary material, data S1). Thus, a link between viral load and molecular diversity was not found, at least at the level of RdRp sequence variation (figure 1*e*; electronic supplementary material, figure S6).

(d) Effects of mite infestation and viral infection on the transcriptome of honeybees

To disentangle the effect of *Varroa* mite parasitization from that of DWV infection on the immune response of bees, we studied the expression of immune genes in bees exposed to a different combination of stress factors (figure 1*b*; electronic supplementary material, data S2). In particular, to assess the influence of the mite (i.e. *Varroa* effect), we compared the expression level of immune genes in five uninfested bees bearing a low viral infection (average DWV infection = 2.04×10^3, yellow circles in figure 1*b*) and five mite-infested bees bearing a similar low viral infection level (average DWV infection = 1.95×10^3, green circles in figure 1*b*). Next, to assess the influence of the combination *Varroa*–DWV (i.e. *Varroa* + DWV effect), we compared five uninfested bees bearing a low viral infection with five mite-infested bees bearing a high viral infection level (average DWV infection = 1.41×10^9, red circles in figure 1*b*).

We found that different immune pathways were differentially affected by *Varroa* mite alone and the replicating virus in the presence of the mite (figure 1*f*; electronic

supplementary material, data S2). Overall, infestation with mites, at low viral infection levels, caused significant changes in expression (i.e. upregulation) of genes involved in the Toll pathway, while very high DWV infection levels associated with *Varroa* infestation caused significant changes in expression of genes involved in the JNK pathway (figure 1*f*; electronic supplementary material, data S2), although this latter causal link is not as strong as the former since it may result both from an effect of high viral infection on immune expression and vice versa. Thus, the impact of *Varroa* mite feeding on bee immune response is different from the impact of the high viral titre stimulated by the mite. Furthermore, this experimental design, allowing the separation of the mite effect from that of the virus, confirmed that immune suppression by the mite [10] did not play a major role under these conditions.

(e) Immune-virus 'predator–prey' dynamics within the host

In 2012, we proposed a series of mathematical models describing how within-host viral dynamics are controlled by the immunological response, which in turn can be modified by the presence of the virus and other stress conditions, such as mite feeding or pesticide exposure [9,23]. The simplest model consistent with the observation of divergent outcomes (low-cryptic or high-overt infection) required a threshold immune-suppressive effect of DWV. Given this assumption, any factor that depletes the immune system (e.g. increasing mite load) will lead to a gradual increase in a stable DWV set-point until, for sufficiently large depletion, a critical transition to unbound viral replication will follow, leading to overt symptoms and ultimately host death. We hypothesized that, in case of mite infestation, immune depletion may result from the activation of competing immune reactions cross-modulated by shared networks of transcriptional control and, in particular, the melanisation and clotting reactions triggered at the mite's feeding site, which are under the control of a NF-kB transcription factor that is involved also in antiviral response [9,24].

In the electronic supplementary material, figure S7, we replicate the theoretical analysis from [9], illustrating that the low stable viral equilibrium and the high unstable equilibrium (solid and dashed black lines, main figure) converge as the extent of immune depletion y increases, ultimately leading to unconstrained growth of viral titre.

In the current analysis, we now examine the impact of perturbations around the low stable equilibrium. Specifically, we ask: what happens to the coupled viral and immunological dynamics when initially stable levels of V and I are transiently perturbed away from their stable equilibrium by the loss of haemolymph? The two grey dots in the electronic supplementary material, figure S7 represent a 20% drop in haemolymph volume under different initial bee health settings (differing values of y). This quantity, consistent with one mite and its offspring feeding over 12 days, was evaluated from available data on mite feeding during the reproductive phase [25] and total haemolymph volume [26]. The two inset time-series diagrams illustrate that, while the healthier bee (lower y) returned to its prior equilibrium state (left inset diagram), the less healthy bee (higher y) was driven into the unstable runaway regime by the same proportionate degree of haemolymph loss. These results illustrate that the simultaneous removal of both virus and immune effectors can lead to the destabilization and subsequent runaway increase in viral titre.

(f) Haemocytes as antiviral barriers in honeybee's haemolymph

In the model above, we assumed that loss of haemolymph results in a perturbation of the levels of both viruses and antiviral immune effectors contained in the bee's blood. To confirm this assumption, we first analysed the honeybee's haemolymph by qRT-PCR and found 10^3–10^8 DWV particles μl^{-1}. Then, to demonstrate that haemocytes play an important role as antiviral barriers in the haemolymph, we engaged the circulating haemocytes in an intense cellular immune reaction and measured the impact of haemocyte depletion on viral replication, similarly to as recently performed in *Drosophila melanogaster* [27]. The injection of chromatographic beads into white-eye bee pupae, naturally infected by DWV, resulted in an intense encapsulation response by haemocytes (electronic supplementary material, figure S8*a*), which was associated with a concurrent increase in viral load (electronic supplementary material, figure S8*b*; Mann–Whitney $U = 15$, $n_1 = n_2 = 10$, $p = 0.004$). This suggests that in bees, like in flies, the depletion of functional haemocytes negatively affects the antiviral defence barriers and demonstrates the important role of these cells as antiviral effectors.

(g) Effects of the increasing haemolymph subtraction on viral proliferation

To verify the hypothesis that haemolymph subtraction can trigger viral proliferation by perturbing the dynamics of virus and immune effectors, we carried out another laboratory experiment by artificially infesting mature bee larvae with one mite or three mites, using non-infested bees as controls, and by assessing both the viral infection level and immune response at eclosion by RNAseq. We observed that higher DWV titres are associated with heavier mite infestations (figure 2*a*; electronic supplementary material, data S3; Kruskal–Wallis: $H_2 = 6.41$, $p = 0.041$) and likely with the removal of higher amounts of haemolymph, in accordance with the results reported above (electronic supplementary material, figure S4). The lack of a differential immune response in multiple versus single mite-infested bees observed in this case suggests that haemolymph loss, rather than an increasing mite-induced immune suppression, can generate an increasing level of viral infection (electronic supplementary material, figures S9A,B and data S3; note that no differentially expressed genes were found in the comparison: one versus three mites, whereas 66 and 50 differentially expressed genes were found, respectively, from the comparisons: no mite versus one mite and no mite versus three mites).

The possibility that the removal of increasing amounts of haemolymph could have affected the cross-talk between metabolism and immunity, as a consequence of nutrient subtraction, seems to be ruled out by the transcriptional data, since the genes involved in nutrient use and metabolism are not significantly differentially regulated across the treatment groups (electronic supplementary material, data S4). Furthermore, the analysis of the whole transcriptome of the bees used in this experiment does not support the occurrence of dramatic physiological changes associated with haemolymph removal [28].

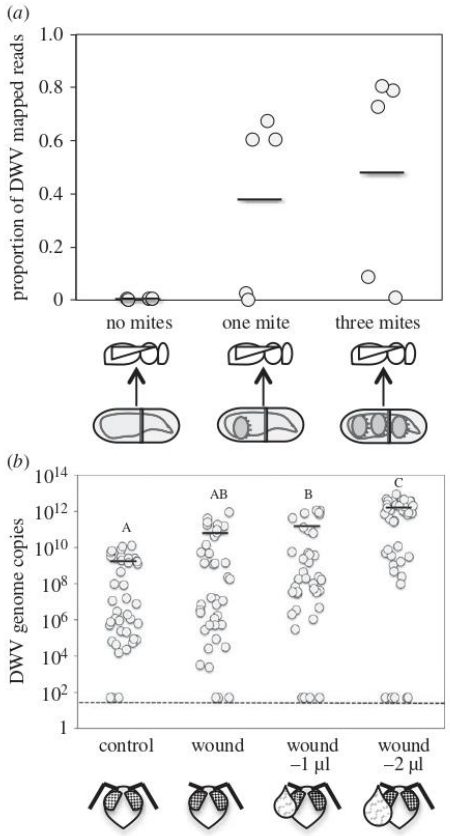

Figure 2. Increased feeding by *Varroa* mite as well as increased subtraction of haemolymph cause increased DWV infection in bees. (*a*) Viral load, as the proportion of reads mapping to DWV genome, in naturally infected bees artificially infested with no mites, one mite or three mites; the horizontal solid lines represent the average viral load. (*b*) The number of DWV genome copies in naturally infected bees after the removal of 1 or 2 µl of haemolymph through a wound is reported along the corresponding viral infection in control bees and wounded bees with no haemolymph subtraction. Different letters mark experimental groups significantly differing from each other ($p < 0.001$); consistently with the statistical analysis used here, the horizontal solid lines represent the average viral load.

To further corroborate our hypothesis, we assessed the impact of haemolymph subtraction in the absence of mite feeding by comparing viral replication in naturally infected bee pupae from which different amounts of haemolymph were removed with a microcapillary tube from a cut antenna, using wounded or untreated bees as controls. Our results demonstrated that viral load varied across treatments, with a clear dose-dependent response, positively linking the volume of removed haemolymph to the viral titre measured using quantitative real-time PCR 4 days after bleeding (figure 2*b*; $\chi^2_3 = 107.34$, $p < 0.001$). In particular, the viral infection in bees to which 2 µl of haemolymph were removed was approximately 10 times higher than that observed in bees which had only 1 µl of haemolymph removed from a single wound (figure 2*b*; Tukey's test, $p < 0.001$), suggesting that subtraction alone can play a role.

This quantity of haemolymph is consistent with the amount that a mite can subtract in about 1 day [25,26] and probably insufficient to trigger a metabolic syndrome related to nutrient subtraction. At this time point (i.e. 4 days after bleeding), a significant decrease in the expression of *Dorsal 1A*, a gene encoding a protein in the NF-kB family, indicating an active immune suppression by the DWV [9], was observed in the bees belonging to the experimental groups which had haemolymph removed (electronic supplementary material, figure S10; Mann–Whitney $U = 65$, $n_1 = 20$, $n_2 = 20$, $p < 0.001$).

Four days later, the viral infection was similar to control in all experimental groups apart from the one which had 2 µl of haemolymph removed (electronic supplementary material, figure S11). This is consistent with the long-term immune suppression and related unbounded viral replication ensuing after a critical threshold of viral titre is surpassed, as predicted by our model [9].

3. Discussion

Several mechanisms have been proposed to explain the higher viral load observed in bees infested by the *Varroa* mite. However, the predictions of those models are not supported consistently across experiments, including the ones performed here. In this study, we propose a micro-ecological model based on the destabilization of virus population and immune effectors by the removal of haemolymph; this mechanism, which is strongly supported by our results, is not mutually exclusive to the previous models, but complements them well.

DWV prevalence in uninfested bees (i.e. 80%) is consistent with available data about DWV infection in honeybee eggs and larval food [29–31] and clearly indicates that trans-ovarial and trans-stadial transmission, as well as viral acquisition by feeding upon contaminated food during the pre-imaginal life, play an important role in the spread of DWV infection within the hive (electronic supplementary material, figure S12). The higher proportion of infected bees among those infested by a mite, together with the presence of replicating viruses within the mites, confirms the role of *V. destructor* as a vector of the virus (electronic supplementary material, figure S12). More importantly, our results highlight the fundamental role of the mite for the increased virulence of DWV in infected honeybees. Collectively, our experimental data allow us to conclude that, under the conditions of our experiment, the capacity of the mite to host the viral pathogen replication [8] (electronic supplementary material, figure S13A) appears to be of limited importance for the dynamics of DWV infection in bees. The similar composition and structure of the mutant clouds, observed in low and highly infected bees, do not support an important role of viral diversity at the level of RdRp in the modulation of observed levels of DWV virulence at individual level (electronic supplementary material, figure S13B), as proposed earlier [11,12] but recently questioned [32]. Our transcriptomic study further suggests that immunosuppression by the mite [10] (electronic supplementary material, figure S13C) does not play an important role, as previously proposed [17]. Instead, on the basis of our experimental and theoretical results, we conclude that the stress resulting from mite feeding has the potential of destabilizing the equilibrium between the pathogen and the bee's immune control [9] (electronic supplementary material, figure S13D). Here we confirm our previous hypothesis,

based on the depletion of a shared immune resource [9,24] and further show that the intensity of mite feeding can affect the progression of viral infection through a dynamic process triggered by the concurrent removal of the virus and antiviral effectors, which is well described by models proposed for predator–prey interactions (electronic supplementary material, figure S13E).

In 1926, the mathematician Vito Volterra, to explain the unexpected fluctuations of certain fish species in the Adriatic Sea, developed his famous model, which clearly showed that the subtraction of both predators and prey, through fishing, could result in the proliferation of the latter [19]. Here we suggest that the pure subtraction of haemolymph—containing both virus and immune factors—from the host, by the feeding mite (electronic supplementary material, figure S13E), could trigger the proliferation of DWV which can be sustained by the depletion of a shared immune resource [9,24] and progressively reinforced by the viral-induced immunosuppression taking place as soon as the pathogen surpasses a critical threshold [9].

The model we propose here implies that haemolymph contains both virus and immune effectors whose density can be altered by the feeding activity of the mite. The presence of the virus within the circulating haemolymph is confirmed by our data and the significant correlation between viral infection in bees and the mites which fed upon them. Furthermore, several possible proteins and cells can act as antiviral effectors circulating in the haemolymph of bees and other insects. In particular, circulating antimicrobial peptides certainly play a still uncharacterized role in the immune response to viruses, being constantly upregulated upon virus infection [33]. In *Drosophila*, convincing evidence has been recently provided regarding the contribution of haemocytes to antiviral defence through phagocytosis [27] and the involvement in RNAi [34]. Our observation that higher viral loads can be observed after engaging the circulating haemocytes in an intense cellular immune reaction suggests that haemocytes play a similar role in the antiviral response of honeybees.

In general, our conceptual hypothesis represents the most parsimonious interpretation of the mite role in the enhanced virulence of the virus and provides the logical framework for future experiments aiming to unravel the intimate molecular mechanisms involved. To our knowledge, this 'micro-ecological' perspective of the immune interactions has not been proposed so far for any other blood-feeding parasite and associated pathogens. In systems, such as the honeybee–*Varroa* mites interaction where the parasite removes a substantial amount of blood from the host, this model could probably play a significant role. Thus, these results lay the groundwork for future research into the role of these predator–prey dynamics in other systems, and studies of the underlying molecular and physiological mechanisms. Furthermore, this study provides key insights into the crucial role played by *Varroa* mite in the re-emergence of DWV, an endemic pathogen of honeybees that plays a key role in the current widespread crisis of the beekeeping industry.

4. Material and methods

(a) Viral infection in mite-infested bees and mites, and effects on bees' transcriptome

In order to study DWV infection in bees and the infesting mites, we artificially infested honeybees from our experimental apiary [35] (see the electronic supplementary material, Material and methods for more details) with one or no mites as previously described [36] (see the electronic supplementary material, Material and methods for more details, including sample sizes). Sequencing of DWV, quantitative DWV analysis, analysis of DWV mutant cloud, DWV negative strand quantitative analysis and the transcriptomic study of bees were carried out using standard methods. Briefly, quantitative DWV analysis was carried out by means of qRT-PCR, using sequence-specific primers, whereas all other analyses were performed by NGS techniques, described in detail in the electronic supplementary material, Material and methods. As a rule, transcriptomic analyses were carried out on five samples per experimental group; full-length genome sequencing of the virus was done on the genetic material obtained from two highly infected bees, whereas the study of the viral mutant cloud is based on 10 virus infected bees.

DWV concentration in the honeybee haemolymph was quantified as described above on a sample obtained as described in electronic supplementary material, Material and methods.

(b) Role of haemocytes in the antiviral response of honeybees

To assess if haemocytes are involved in antiviral response in honeybees, we saturated phagocytosis by injecting a suspension of chromatography beads into white-eye pupae, naturally infected by DWV, and measured the viral load 48 h later.

White-eye honeybee pupae were manually extracted from a sealed brood comb, taken from a colony at the end of autumn when, according to previous studies, virus prevalence reaches 100% in all colonies [9]. CM Sepharose fast flow chromatography beads (Pharmacia), suspended in 2 µl of phosphate buffered saline, were injected into the haemocoel of honeybees using a Hamilton syringe equipped with a sterile 30 gauge needle. Then bees were maintained on sterile filter paper in small Petri dishes in an incubator (34°C, 75% relative humidity (R.H.), dark).

After 48 h, 10 bees per experimental group were sampled for DWV quantification by qRT-PCR; a few other bees were sampled for microscopic analysis. The experiment was carried out once using 40 bees per experimental group.

(c) Study of the effects of an increasing haemolymph subtraction on viral replication

This experiment was designed to assess the effect of the removal of an increasing haemolymph volume, in the absence of feeding mites, on the dynamics of DWV titre in naturally infected honeybees. Last instar bee larvae were collected from a brood comb as described above and maintained in an incubator (34°C, 75% R.H., dark) until the white eyes stage, which occurred about 4 days after the collection from brood cells sealed in the preceding 15 h. Then, four experimental groups, made of about 30 pupae each, were established. One group (control) was left untreated, whereas all the other bees had the right antenna cut, at the level of the scapum, using fine scissors; pupae bleeding after cutting were discarded. Bees of one group (wound) had the wound sealed with a cream containing Sulfathiazole (2%) and Neomycin sulfate (0.5%) to prevent secondary infections. Bees of the remaining two groups (wound −1 µl' and 'wound −2 µl) had the wound sealed as above, after removing either 1 or 2 µl of haemolymph, with a microcapillary tube precisely graduated with 1 or 2 µl of ethanol, dispensed through a micropipette. By subtracting increasing amounts of blood, we tried to assess the effect of pure haemolymph subtraction, while maintaining constant the impact of wounding and the resulting immune reaction.

The choice of the volume of haemolymph to be subtracted was dictated by available data showing that *V. destructor* can

consume as much as 0.7 µl of bee haemolymph every 24 h [25], which, according to our data, can contain up to 10^3–10^8 DWV particles μl^{-1} according to the infection level.

After treatment, bees were kept in a Petri dish, lined with sterile filter paper and maintained under dark, at 34°C, 75% R.H., until eclosion. After 4 or 8 days, 10 bees from each experimental group were sampled to assess the viral titre as described below. To account for the variability across colonies and genotypes, the experiment was repeated four times: on two colonies in Udine (Northern Italy) and two colonies in Napoli (Southern Italy).

Ten bees from each experimental group, from the second replicate of the experiment, carried out in one location, were also used to assess the expression level of *Dorsal 1A*, a gene in the NF-kB family, indicating an active immune suppression by the DWV [9]. Sample processing was as explained below whereas dorsal analysis was carried out as described in ref. [9].

(d) Statistical analysis

The statistical analysis of data was carried out using standard methods [37,38] described in detail in the supplementary material, Material and methods.

(e) Simulations

In [9], we presented a series of models capturing the coupled within-host dynamics of viral copy number (V) and a shared immune currency (I). The most parsimonious model analysed further in the current paper included an immunosuppressive effect of high viral load, as described by the following ordinary differential equations

$$\left.\begin{aligned} \frac{dV}{dt} &= (1-I)V \\ \text{and}\quad \frac{dI}{dt} &= x - yI + z(1-V)V. \end{aligned}\right\} \tag{4.1}$$

These equations describe the within-host growth of a pathogen population V and its controlling immunological counterpart I. In this dimensionless form [9], the units of time are rescaled to the maximal growth rate of the virus, the units of viral density to the density that halts immune proliferation and the units of immune density to the density that halts viral proliferation. The dynamics of V is shaped by the maximal rate of pathogen replication (scaled to 1), which is counteracted by immunological control. The dynamics of I are shaped by an intrinsic production rate x, a rate of decay y and an activation/suppression parameter z. This model implementation ensures that the sign of the impact of the virus on immune dynamic (immunostimulatory or immunosuppressive) will depend on viral titre, V. Specifically, we assume that at low densities, the pathogen is a net activator of immunological activity, whereas at high densities (whenever $V > 1$), the pathogen becomes immunosuppressive.

To study the impact of an episodic removal of haemolymph on viral level, we conducted phase portrait and time-series analyses of equations (4.1) under differing scenarios of haemolymph removal and concurrent initial reductions in both viral and immunological titres V and I. In this case, we applied a 20% drop in haemolymph volume which is consistent with available data on the reduction observed in mite-parasitized bees [25,26].

Data accessibility. This article has no additional data.

Authors' contributions. D.A. designed and performed the research, analysed data and wrote the paper; S.P.B. designed the research, analysed the data and wrote the paper; G.D.P. designed and performed the research and analysed the data; E.D.P. analysed the data; S.D.F. analysed the data; D.F. performed the research; V.Z. performed the research; D.A.G. analysed the data; E.C. performed the research; C.M.G. designed the research and wrote the paper; F.P. designed the research and wrote the paper; F.N. designed and performed the research, analysed the data and wrote the paper.

Competing Interests. We declare we have no competing interests.

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6.2. Attachment 2

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ORIGINAL ARTICLE

Honeybees use various criteria to select the site for performing the waggle dances on the comb

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Abstract
After returning to the hive, successful honeybee foragers dance on the surface of the comb, where they interact with dance followers. It has been shown that bees establish a specific site for their waggle dances that is likely marked with chemical signals. By recording the site where dances take place on the comb in a single-frame observation hive, we investigated the relative importance of three different criteria for the selection of the dance floor by bees, including the distance from the hive entrance, the cell filling, and the chemical marking by bees and found that all these criteria play a role, albeit their importance does not seem to be equal.

Significance statement
The existence of a dance floor, where forager bees perform most waggle dances after returning to the hive, was first reported by von Frisch and later confirmed by various authors; however, the factors affecting the choice of a certain site, for this purpose, by bees have not received so far sufficient attention. Besides confirming the existence of a specific site on the comb where bees prefer to dance, we clarified the criteria used by bees for establishing this site, showing that both the distance from the entrance, the quality of the comb, and the chemical marking by bees play a role.

Keywords *Apis mellifera* · Dance floor · Semiochemicals · Waggle dance

Introduction

Forager bees communicate to nestmates the distance and direction of a food source by performing, on the comb surface, in the darkness of the hive, the so-called waggle dance (von Frisch 1967). As with any form of animal communication, two parties are involved: the dancer and the dance followers that must be connected for the communication to take place (Wilson 1975). Consequently, the efficiency of the engagement process and, in turn, of the whole foraging activity, is increased if a particular site on the comb, which is recognized by both dancers and dance followers, is established. In fact, bees preferably perform their waggle dance on a specific site that was called the "dance floor" (Körner 1940; von Frisch 1967). Seeley and Towne (1992) showed that foragers prefer to dance in the vicinity of the hive entrance, with most dances occurring at a distance between 4 and 18 cm from the entrance. On the other hand, Tautz and Lindauer (1997) suggested that the dance floor is marked by chemical compounds that do not necessarily come from the food source where the bees collected their load. The possible involvement of chemical signals is further suggested by other studies; in particular, it has been shown that foragers release hydrocarbons that are used for recruiting other foragers (Thom et al. 2007), and Gilley (2014) found that the waggle-dance hydrocarbons play an important role in honeybee foraging recruitment, by stimulating foragers to perform waggle dances following periods of inactivity.

It was also demonstrated that the nature of the floor on which the bees dance (i.e., sealed or empty comb cells) influences the recruitment of nestmates to a food source (Tautz

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1996); similarly, illumination seems to influence the recruitment success (Tautz and Rohrseitz 1998). However, although it is likely that more dances are carried out on the most suitable position in terms of recruitment success, the data reported above do not necessarily imply that forager bees would preferentially select open or illuminated portions of the comb for dancing.

In sum, it appears that bees establish a dance floor that is used by foragers to perform their dances and where other foragers congregate to pick up the information transmitted by the first. It is also clear that several factors may influence the position of this dance floor within the hive, although their respective role has not been described in detail.

This work's aim was to investigate the criteria used by bees for the selection of the site used as a dance floor. For this purpose, after confirming the existence of a dance floor, we tested the importance of the following factors: distance from the hive entrance, cell filling of the comb, and possible chemical marking.

Materials and methods

Bees and hive

Experiments were carried out using a single-frame observation hive (45 × 52 × 4.5 cm) hosting a queenright honeybee colony (*Apis mellifera* L.) of about 3000 individuals, as estimated from pictures of the comb according to Marchetti (1985). The colonies, which were established in spring, performed well until the end of the trial, the following autumn, and appeared to be completely self-sufficient in terms of food gathering and bee number. The observations carried out in the framework of the first experiment (see below) revealed that the behavior of both the queen and the workers was perfectly normal, following the standard pattern.

Colonies used in this study were allowed 1 month to settle before the beginning of each experiment. The entrance of the hive was a circular hole (3.5 cm) drilled in the small side of the box, at 4 cm from the bottom.

In order to facilitate the relocation experiments described below, a "modified comb" was used. The standard wooden frame (42 × 28 cm) was subdivided into six squares (13 × 12.5 cm) by two pairs of vertical and one horizontal wooden bar (vertical bars 13 × 0.9 cm each; horizontal bar 41.5 × 0.9 cm); each square contained one moveable panel made of a 13 × 12 cm piece of comb containing about 500 worker cells per each side included in a frame made of four wooden bars. The size of the panels matched the area of the dance floor estimated by Tautz and Bujok (2006). Panels were labeled as in Fig. S1. Two positions for the entrance were used in the experiments as explained below. Because of the framing, the distance separating two adjacent panels was about 2.3 cm; dances performed on the wooden bars were not considered.

In all the experiments, the response variable that was recorded was the number of dances carried out on each panel on both sides of the comb, which was later translated into the proportion of dances carried out on each panel. For this purpose, dances were directly counted on both sides of the observation hive by one or two operators working in series or at the same time, respectively. We considered waggle dances only and regarded a dance as such when the waggle phase was repeated at least twice.

Several bee colonies were used in the experiments as detailed in the description of each experiment.

Experimental plan

Using the setup described above, we studied the following:

- Position of the dance floor
- Distribution of "potential dancers" on the comb
- Role of the distance from the hive entrance for the selection of the dance floor
- Role of cell filling for the selection of the dance floor
- Role of the possible marking of the dance floor by means of chemical signals

Position of the dance floor

To test if bees preferentially use a certain portion of the comb for their waggle dances, we compared the number of dances performed on six different portions of the comb (denoted as "panels" in the following text, see Fig. S1).

To assess the possible influence of the time of the day on the distribution of dances, observations were carried out every 1 h, from 9.00 to 15.30, on three different days of good meteorological conditions. The observation lasted 15 min for each side of the comb; during the observations, one side of the comb was illuminated to daylight but not to direct sunlight, whereas the other remained obscured.

For this experiment, the same colony was used for all the replicates (colony 1).

Distribution of "potential dancers" on the comb

Our first experiment showed that dances are concentrated on the panels closer to the hive entrance. Since it has been shown that forager bees tend to congregate in this area, regardless of their dancing activity (Baracchi and Cini 2014), we wanted to test if the distribution of dances in our experiment simply matched the distribution of forager bees within the experimental hive and could therefore be considered as a side effect of the latter. For this purpose, at eclosion, we marked 99 bees

with a numbered tag and released them into an observation hive, similar to that used for the other experiments; in this case, a normal comb was used, but the position of bees, with respect of the six panels mentioned above, could be assessed thanks to three lines marked on the glass walls of the hive. Then, every day, at the same time, for 1 month, we noted the position of marked bees; we also noted if any marked bee was performing the waggle dance and considered the age at which the first waggle dance was observed as a rough indication that, from that age onwards, marked bees could potentially be involved in foraging activities and thus could be observed while performing the waggle dance on the comb.

It is known that different bees can initiate foraging at different ages, and this age is influenced by several factors (Robinson 1992); furthermore, foragers can revert to in-house activities, if needed (Johnson 2010). However, the aim of this experiment was not to establish the distribution of forager bees on the comb but rather to test whether the position of bees potentially involved in the behavior we studied (i.e., the waggle dance) matched or not the distribution of dances observed in this study.

This experiment was carried out twice using two different colonies in two following years (colonies 2 and 3).

Role of the distance from the hive entrance for the selection of the dance floor

To establish the importance of the distance from the hive entrance as a criterion for the selection of the dance floor, we sealed the entrance used in the first experiment ("position of the dance floor"; entrance 1, Fig. S1) and created a new one on the same side of the observation hive, such that the entrance was closer to another panel (entrance 2, Fig. S1); then, the colony was allowed to settle for 1 day. Subsequently, the number of dances performed on each panel was recorded from 11.00 to 13.00 and the proportion of dances on each panel was compared to the distribution observed during the first experiment, during the same period of time, when the entrance position was different. The experiment was replicated three times.

Clearly, for purely geometric reasons, both with the entrance in positions 1 and 2, there are portions of a panel, that is regarded as "far from the entrance" (e.g., the bottom left corner of panel A1, with entrance 1), that are at a smaller distance to the entrance than some portion of the panel that is regarded as "closer to the entrance" (e.g., the upper right corner of panel B1, with entrance 1); however, our classification is perfectly adequate for the most part of each panel. Therefore, for practical reasons, we summed up all the dances observed on a given panel, disregarding the exact position where the dance was carried out.

For this experiment, the same colony was used for all the replicates (colony 1).

Role of the cell filling for the selection of the dance floor

To assess the importance of comb type as a criterion for the selection of the dance floor, we carried out another experiment which was also used to collect some preliminary information on the possible role of chemical marking by dancers.

For this purpose, at 8.30 of five different days, the panel occupying the most preferred position for dancing (i.e., close to entrance 1) was exchanged with any of the other five panels which, at the time of the experiment, contained a different proportion of sealed and open cells containing brood, honey, pollen, or nothing. Subsequently, the dances were counted during a period of 1 h, from 11.00 to 12.00, and pictures were taken of each side of all panels to record the filling of comb cells in the shortest time possible so as not to introduce any possible bias related to manipulation. Then, within a 15-min period of time, panels were replaced to their original positions and the dances counted again for 1 h (Fig. S2a; note that this second part of the experiment was carried out for the purpose of testing the possible marking of panels). Later, based on the pictures, we counted the proportion of cells that contained sealed brood, open brood, honey (either open and sealed), or pollen; we also recorded the number of cells that could not be classified due to the unfavorable angle of view.

For this experiment the same colony was used for all the replicates (colony 1).

To confirm the importance of cell filling as a criterion of choice, we carried out another experiment using panels that were markedly different for the content of the cells and were obtained from the same colony (later called "mother colony") that was used to create the nucleus hosted in the observation hive. For this purpose, we provided the mother colony with three modified combs, as described above, where each panel consisted of empty wax cells. After 2 weeks, we inspected the hive; at this time, cells were either empty or they contained eggs, uncapped larvae, sealed brood, uncapped honey, or sealed honey, according to the needs of the colony. The panels that appeared to be homogeneous by type of filling (i.e., the proportion of cells of one type surpassed 50%) were removed and carefully inserted into a new modified frame to obtain a comb made of the following six panels: 2 fresh brood, 1 capped brood, 1 open honey, 1 sealed honey, 1 empty cells. Due to brood aging and honey consumption/collection, cell filling could vary over time. However, in our experiment, we always used panels that had at least 50% of the cells of the same kind, apart from one of three replicates with sealed honey, when this proportion was slightly lower.

Subsequently, we carried out the experiment as described below. First, the comb assembled as above was inserted into the observation hive together with the worker bees and the queen collected from the same hive; then, we let the colony to settle for a couple of days. The day preceding the

observations, at evening, the panel to be tested was photographed and moved to the most favored position for dancing on the comb (i.e. positions B1, with entrance 1) and the entrance was closed after the sunset. The following day, early in the morning (at about 8.00), the entrance was opened and the dances were counted for 1 h, starting from 9.30 after opening the entrance.

The same routine was repeated as long as each type of comb could be tested at least three times. For this experiment, one colony was used (colony 4).

Role of the possible marking of the dance floor by means of chemical signals

In order to verify the possible marking of the dance floor, by means of chemical signals released by bees, we carried out an experiment whereby we first allowed bees to "mark" a panel, while in the favorite position, and then, we moved it to a less favored position to test the preference of dancers for the panel in the new position (please note that this experiment was simply the prosecution of the first described in the section "Role of the cell filling for the selection of the dance floor")

Since the results of this experiment suggested that only panels from the first column were preferred when marked, after replacing to their original position—which confirmed the importance of the distance from the entrance as a criterion of choice—to confirm the importance of marking by dancing bees, we carried out another experiment whereby we first allowed bees to dance on a panel while in the most preferred position and then replaced it to the adjacent position, within the same first column, and counted the dances again.

For this purpose, the panel to be marked by bees was put in front of the entrance every morning at 8.30. To prevent that any panel was marked before the beginning of the experiment, the entrance of the hive was closed the night before and reopened at 8.30. The first observation of dances was carried out between 11.00 to 12.00, then panels occupying positions B1 (closest to the entrance in this set of experiments) and A1 were exchanged and another round of observations was carried out between 12.15 and 13.15 (Fig. S2b). The experiment was replicated three times.

For this experiment, the same colony was used for all the replicates (colony 1).

In order to prove that marking was done by means of unknown chemicals left by forager bees after returning to the hive, we carried out another experiment whereby we compared the proportion of dances on panels in the most and less favored position of the first column (i.e., B1 and A1, respectively), after treating the panel occupying the less favored position with an extract of forager bees and the other with the solvent used for the extraction. We hypothesized that treating with the solvent should not alter the preference for B1, while treating A1 with the foragers' extract should reduce the preference for B1, inducing bees to dance preferably on A1. Therefore, our experiment involved testing first the solvent; then, the extract for three times and then the solvent again in five different days.

The extract of foragers was prepared as follows. One hundred fifty foragers (recognized because carrying a pollen load) were collected on the entrance of the hive after returning to the mother colony and stored at − 20 °C. For each replicate of the experiment, 50 foragers were cleaned from the pollen load and immersed in 40 ml of diethyl ether for 1 h; then, the extract was withdrawn and another 10 ml of diethyl ether was added to bees and recovered after gently stirring the vial. Subsequently, the solvent was evaporated under a gentle stream of nitrogen, recovered with 6.5 ml of acetone and stored at − 20 °C until use.

To prevent the marking of the dance floor before the experiment, the entrance was closed at night. Each morning at 8.30, panels B1 and A1 were taken and bees shaken off with a brush. Then, panels were treated by spraying 2.5 ml of either the extract (corresponding to about 15 foragers equivalent) or the solvent on each side of panels A1 and B1 with a TLC plate glass sprayer and relocated in their positions. The entrance remained closed until 9.30, to allow bees to settle down, then dances were counted on each panel of the comb for 1 h, starting at 10.30 in the first replicate of the experiment and at later times (i.e., 11.30 and 14.20) in successive experiments due to reduced flying activity related to poor weather conditions.

For this experiment, one colony was used (colony 4).

Statistical analysis of data

The role of the following factors: time interval, side of the comb and panel of the comb in the first experiment was tested by means of GLM on the square transformed data; the analysis was carried out using Minitab 16 Statistical Software (2010); fixed factors were time interval, side of the comb and panel; replication was regarded as random factor. Normality, residuals, and homoscedasticity were assessed before considering the results. Post hoc comparisons were carried out by Tukey test. In this and the following experiments, the proportion of dances carried out on different panels was compared using Kruskal–Wallis; for paired comparisons, the Mantel–Haenszel test was used.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Results

Position of the dance floor

In the first replication of the experiment, 177 dances were counted during the whole observation period, whereas in the second and third replications, 117 and 146 dances were counted, respectively; the difference in the number of dances in different days appeared to be significant (GLM: factor: replication, $df = 2$, $F = 4.01$, $P = 0.02$). In general, a similar number of dances was recorded throughout the observation period, from 9.00 to 15.30 (GLM: factor: time interval, $df = 6$, $F = 0.76$, $P = 0.606$), but a significant difference was noted between the number of dances carried out on one side of the comb and the other (GLM: factor: side of the comb, $df = 1$, $F = 31.55$, $P < 0.001$).

Dances appeared to be unequally distributed on panels (Fig. S3a); in fact, on both sides of the comb, more than 90% of dances were performed on the panels located in the first column of the comb (panels A1 and B1), with the panel closer to the hive entrance (i.e., B1, in this set of experiments) hosting twice as many dances as the other panel from the same column (A1). Only a few dances were observed on the panels occupying the second column of the comb (panels A2 and B2), and none was observed on the third (panels A3 and B3). The difference among the six panels with respect to the number of dances performed was statistically significant (Fig. S3a; GLM: factor: panel, $df = 5$, $F = 239.15$, $P < 0.001$) and, in particular, the difference between both A1 and B1 and all the other panels, on both sides of the comb (see lettering on bars in Fig. S3a).

Despite the difference between the number of dances carried out on the two sides of the comb, the proportion of dances on the different panels was remarkably similar on the two sides (Fig. S3b); therefore, for subsequent analyses, we considered the sum of the dances carried out on both sides of each panel and calculated the proportion of these dances relative to the sum of dances observed on the entire comb. The proportion of dances varied considerably among panels (Kruskal–Wallis test: adj $H = 15.46$, $df = 5$, $P = 0.009$) and, in particular, the proportion of dances carried out on panels A1 and B1 appeared to be different from that recorded on all other panels (Fig. 1).

The distribution of dances among panels was not affected by time (GLM: interaction interval of time × panel: $df = 30$, $F = 0.70$, $P = 0.875$). Instead, the interaction between side of the comb and panel was significant (GLM: factor: side of the comb × panel: $df = 5$, $F = 11.62$, $P < 0.001$) likely due to the fact that, despite the overall difference between the two sides, we observed no dances on certain panels on either side of the comb.

Distribution of "potential dancers" on the comb

Seventy-six marked bees out of 99 were observed at least once during the first replicate of the experiment so that, over the whole period, 523 observations of individual bees could be carried out; instead, in the second replicate of the experiment, 53 marked bees were observed at least once and 716 observations of individual bees could be carried out over the whole period. Due to the limited number of marked bees, only a few dances were observed in this experiment; in the first replicate of the experiment, the first dance was recorded when bees were 11 days old, followed by three other dances carried out before the bees reached 31 days of age, reflecting also the poor weather conditions which reduced the foraging activity of bees in the following period of observation. In the second replicate of the experiment, the first dance was recorded when bees were 15 days old, followed by 18 other dances that were observed until bees had 31 days of age.

In the first replicate, the proportion of bees older than 10 days, that, according to our observation, could be regarded as "potential dancers" on the different portions of the comb, showed an uneven distribution (Kruskal–Wallis test: adj $H = 41.06$, $df = 5$, $P < 0.001$) with the lower part of the comb, closer to the entrance, occupied preferentially by those bees, and, in particular, panels B1 and B2 (Fig. S4).

In the second replicate of the experiment, the proportion of "potential dancers" (i.e., bees older than 15 days) on the different portions of the comb, showed again an uneven distribution (Kruskal–Wallis test: adj $H = 51.79$, $df = 5$, $P < 0.001$) again with the lower part of the comb, closer to the entrance, occupied preferentially by those bees, and, in particular, panels B1 and B2 (Fig. S4).

Overall, this experiment revealed a similar picture with the higher proportion of potential dancers concentrated in panels B1 (0.31 and 0.37) and B2 (0.31 and 0.32) in the first and second replications, respectively.

Role of the distance from the hive entrance

The distribution of dances on the different portions of the comb, which was observed in the first experiment, matched quite well their distance from the hive entrance, with the panels located closer to the entrance being more used for dancing. Therefore, to test the hypothesis that the distance from the entrance plays a role in the selection of the dance floor by dancing bees, we modified the position of the hive entrance, so that another panel (A1) was closer to it, and then observed the position of dancing bees.

In total, we observed 75 dances, nearly equally distributed among the three replicates; again, most dances were observed in the first column of the comb but the most used panel, in this case, was the upper one (i.e., A1) which was located near the new entrance (Fig. 2). The comparison between the proportion

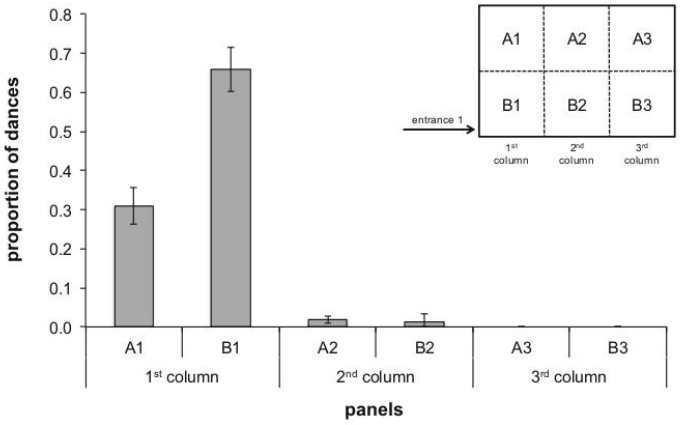

Fig. 1 Proportion of dances carried out on each panel of the comb (both sides). The error bar represents the standard deviation among different replicates of the same experiment

of dances carried out on the same panel before and after moving the entrance revealed a significant difference for panels located in the first column (Mantel–Haenszel test: panel A1, entrance 1 vs entrance 2: $U = 0$; $n1 = 3$, $n2 = 3$, $P = 0.025$; panel B1, entrance 1 vs entrance 2: $U = 0$; $n1 = 3$, $n2 = 3$, $P = 0.025$).

Role of cell filling

In order to test the preference of dancing bees for comb portions differing for the content of the cells, we carried out two experiments which basically involved comparing the proportion of dances carried out on a panel occupying the normally preferred position for dancing, according to the content of the cells included in the panel.

In the first experiment, we observed that most panels were used by bees to dance after relocation in position B1, which is normally the most preferred for dancing, regardless of their original position and quality. However, a difference could be noted between the panels occupying the first or second column and those from the last column (see solid line in Fig. S5). In particular, the panel originally occupying position B3, which, according to the pictures taken during relocation, contained only empty cells, was never used for dancing (Fig. S5).

Since this experiment suggested that subtle differences did not affect the preference of dancers for a certain comb but big differences may do, we carried out a replicated experiment whereby we tested the proportion of dances carried out on panels that were markedly different, which we obtained from the colony that was used to prepare the hive under study.

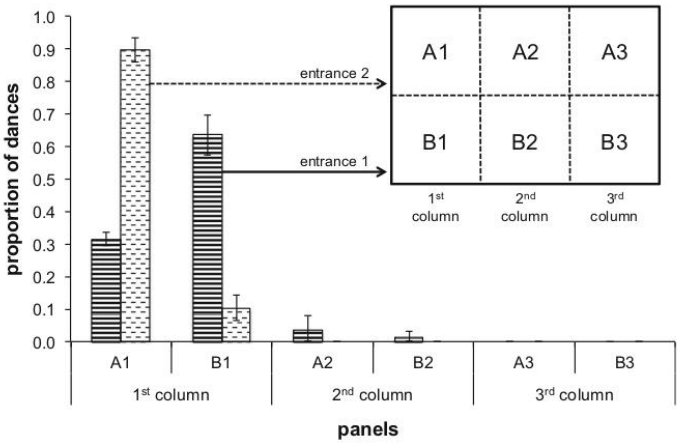

Fig. 2 Proportion of dances carried out on each panel of the comb (both sides) before and after moving the position of the hive entrance. The error bar represents the standard deviation among different replicates of the same experiment

In this case, a significant difference was found among combs differing for their cell filling (Kruskal–Wallis test: adj $H = 11.24$, $df = 5$, $P = 0.047$); in particular, panels composed of cells hosting brood at different stages were preferred over empty combs or combs containing honey (Fig. 3).

Role of the possible marking by bees

To test if the comb is marked by dancers with some long-lasting signals that may affect the subsequent use for dancing, we observed the dances carried out on panels that had been replaced to their original position, at different distances from the hive entrance, after staying 3.5 h near the entrance hole (Fig. S2a).

We noted that only the panel originally located in the first column, far from the entry (i.e., panel A1), once replaced in its position, after staying close to the entry, hosted more dances than before, albeit the difference was not significant, whereas no dances were recorded on panels located in the second and third columns (i.e., panels A2, B2, A3, B3) after replacement in that position despite a significant number of dances had been performed on those panels while in the most favored position (Fig. S6).

To further investigate the possible presence of marking signals on the panels used for dancing by foragers, keeping into account the results of the previous experiment, we carried out another test, where we first let the bees dance on a panel in the most favored positions, then we moved it to a less favored position, but still within the first column, and compared the proportion of dances observed in this new situation.

The results obtained after the relocation demonstrate that the comb on which the bees performed more dances while in favored position (B1) is also preferred when it is transferred to a normally less favored position (A1), further away from the entry of the hive (Fig. 4). The difference in the proportion of dances performed on the comb far from the entrance before and after marking by the dancers was statistically significant (Mantel–Haenszel chi-square = 17.002, $df = 2$, $P < 0.001$).

To confirm that the comb is marked by means of chemical signals, we tested if an extract of incoming foragers can affect the preference of dancers for a certain portion of the comb. We observed that treating a panel with the solvent used for the extraction did not alter the preference of foragers for dancing (Fig. S7a); instead, the treatment with an extract of incoming forager influenced the preferences of dancers for that panel (Fig. S7b) leading to a significant preference for A1 despite the less favored position (Mantel–Haenszel chi-square = 0.718, $df = 2$, $P < 0.001$).

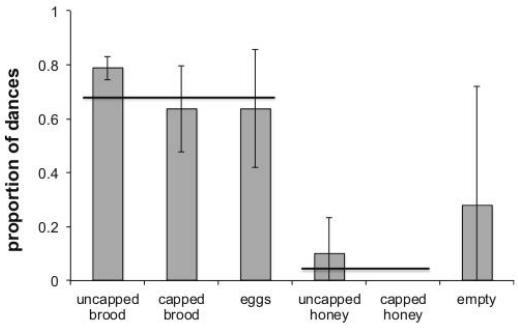

Fig. 3 Proportion of dances carried out on panels that were markedly different for the content of the cells when located in the most preferred position for dancing (i.e., B1, both sides). The solid lines represent the average proportion of dances in the following homogeneous groups: brood cells, cells containing honey, and empty cells. The error bars represent the standard deviation among different replicates of the same experiment

Discussion

All the results of this study were obtained using observation hives containing a single comb; similarly, other authors dealing with the same subject used observation hives that were more or less different both from standard hives and the nest of feral colonies (Seeley and Towne 1992; Tautz 1996; Tautz and Lindauer 1997; Tautz and Rohrseitz 1998). However, according to our observations, the colonies appeared to be fully comparable, under any respect, to standard colonies, and thus, conclusion could be generalized.

Normally, the nest of cavity nesting *Apis* species is composed of multiple, parallel frames; therefore, our single-frame observation hive should be regarded as a cross section of a standard nest. Although possible differences with a standard hive cannot be excluded, we believe that our observations, and in particular the conclusions regarding the factors affecting the location of the dance floor, are not probably much affected by this circumstance. Also, not all experiments, albeit fully replicated, were repeated on different colonies; although possible colony-specific differences cannot be excluded, our conclusions most likely have a general value.

The difference in the number of dances we recorded in our observation hive in different days is not surprising and likely depends on minor changes in weather conditions and forage availability, according to several studies showing that the foraging activity depends on external temperature, light, wind, and nectar flow (Gary 1967; Nùnez 1977; Riessberger and Crailsheim 1997).

On the other hand, the similar number of dances observed from late morning to early afternoon in the same observation date, in the absence of significant changes in weather conditions along the day, indicates that, in the season when the experiments were carried out, the foraging activity likely begins before and finishes after the end of the observation period. Moreover, this observation indicates that the distribution

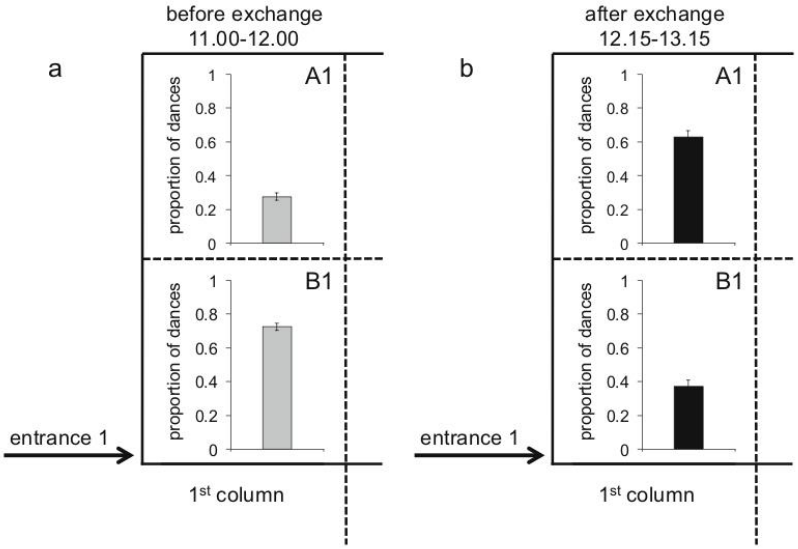

Fig. 4 Proportion of dances carried out on the panels of the first column before and after exchanging position. During the first part of the experiment, most dances occurred on the panel located near the entrance (**a**), but when this panel was moved far from the entrance, foragers continued dancing preferentially on it (**b**)

of dances recorded after the relocation of comb portions in the subsequent experiments was not affected by the elapsed time but only by the factors under study.

Since the two sides of the comb were similar under any respect (i.e., the study of the cell content carried out in the context of the fourth experiment revealed a similar composition of the two sides), the unequal distribution of dances between the two sides of the comb may depend on the orientation of the observation hive and the higher temperature that may be reached during summer by the side of the hive facing sun for most of the day. However, the fact that the proportion of dances on different panels did not vary between the two sides of the comb suggested that the location of the dance floor on the comb was not affected, and, for the purpose of this study, the number of dances on both sides of each panel could be considered together.

The distribution on the comb of bees that, according to their age, could be involved in foraging and thus observed dancing during our experiments, which did not perfectly match that of dances, suggests that the uneven distribution of dances observed in this and previous studies is not a simple side effect of the first. Thus, the observed uneven distribution of dances on the comb clearly show that foragers prefer to dance on a specific site, as already noticed during early studies (Körner 1940; von Frisch 1967) and later confirmed (Seeley and Towne 1992; Tautz and Lindauer 1997). The portion of the comb that is preferentially selected for dances appears to be that closest to the hive entrance, suggesting that the distance from the hive entrance plays a role in the choice of the dance floor. This was clearly confirmed by the experiment in which the position of the entrance hole was changed obtaining a clear

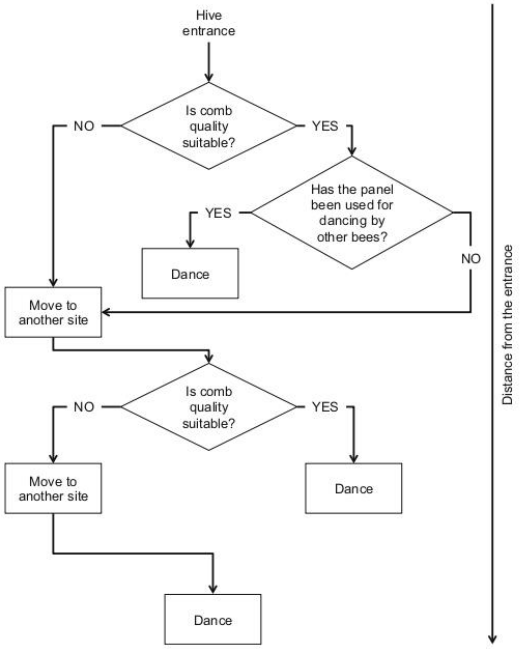

Fig. 5 Flow diagram illustrating the proposed criteria used by bees in the selection of a site for dancing. After entering the hive, in case the comb is suitable, the bee likely tests the possible chemical marking by other dancers; if the panel is marked, the dance is performed, if not, the bee moves to another site and performs the same kind of tests; however, after reaching a certain distance, a position is selected even in the absence of marking

change in the preference of bees. This conclusion matches previous observations carried out under natural conditions (von Frisch 1967); the ease of access and the illumination of the area located near the entrance could explain, at least in part, this preference (Tautz and Rohrseitz 1998).

In general, a similar proportion of dances was observed after moving to the most preferred dancing site, different portions of comb that had similar proportions of cells of different types but a clear difference was noted when combs that were markedly different were compared; this suggests that cell filling, or other unknown factors related to comb quality, play a role as a selection criterion, which can be more important than the distance from the entrance.

Apparently, our results, indicating that brood cells are preferred for dancing irrespective of their capping, seems to be not in line with those previously reported (Tautz 1996) regarding the higher recruitment success on combs made of open cells as compared to sealed cells. However, it is worth noting that Tautz (1996) compared the number of unmarked bees reaching a feeding station, after some bees from the same colony were trained to that station and allowed to dance on a comb made of open or sealed cells (i.e., the recruitment success), and not the number of dances performed by foragers after coming back to the hive, that is the parameter assessed in this study.

When panels that had been used for dancing by bees, once in proximity of the entrance, were moved to another position, they appeared to be not more attractive to dancing bees unless the new distance from the entrance was limited; in fact, in the latter case, bees preferred to dance on the panel used before, even if it was no longer close to the entrance hole. This suggests that markers are released on the area of the comb used for dancing, but their effect is limited as compared to that of the distance from the entrance hole.

This latter experiment showing that returning foragers prefer to dance on a specific site, previously used for that purpose, regardless of its relocation into a new position, provided that it is not too far from the entrance, suggests that bees use more than just spatial cues to find the dance floor. In particular, the results presented here clearly points to the existence of marking chemicals already hypothesized by Tautz and Lindauer (1997). Our last experiment with panels treated with the extract of incoming foragers seems to confirm this hypothesis; the hydrocarbons that are used for recruiting other foragers to the dance floor, identified by Thom et al. (2007), may be involved but other compounds could also be used for this purpose.

Overall, our data confirm the existence of a dance floor within the hive and help to clarify the criteria used by bees for establishing this site. Furthermore, the results suggest a possible hierarchy of various factors. In particular, it seems that both cell filling and the distance from the hive entrance play a critical role, with the first criterion apparently occupying a predominant position (Fig. 5). Particularly interesting appears to be the demonstration of a chemical signature of the dance floor which, however, seems to be less important than the abovementioned criteria (Fig. 5). Further studies will deal with the chemical nature of those markers in order to clarify the identity of the substances used by bees for marking the dance floor.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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6.3. Attachment 3

Frizzera D., Del Fabbro S., Ortis G., Zanni V., Bortolomeazzi R., Nazzi F., Annoscia D. Possible side effects of sugar supplementary nutrition on honey bee health.

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Possible side effects of sugar supplementary nutrition on honey bee health

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ABSTRACT

Food shortage, along with biotic stressors is a leading factor of winter honey bee colony losses. To support honey bee colonies, beekeepers normally supply homemade syrups which could contain compounds (e.g. hydroxymethylfurfural, HMF) with possible negative side effects. In this study we investigated the toxicity of HMF at doses consistent with literature data both to healthy bees and bees challenged with their most important ectoparasite (i.e. *Varroa destructor*). To strengthen available data on HMF concentration in sugar syrups, we also investigated HMF formation in homemade 2:1 inverted sugar syrup, considering, in particular, the influence of temperature or boiling time on different homemade sugar syrups according to their acidity.

We show that doses of HMF similar to those reported as sublethal in the literature appear to be non-toxic even to mite infested bees. However, the amount of HMF that can be found in homemade syrups, which increases with temperature and acidity, can be much higher and can cause significant bee mortality. Moreover, we highlighted the detrimental effect of syrups acidity on honeybee survival. Our results suggest a responsible approach to homemade colony nutrition.

Keywords: **honey bee,/hydroxymethylfurfural,/sugar syrup/acidity**

1. INTRODUCTION

Nutrition plays a fundamental role in maintaining strong and healthy honey bee colonies. 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 (Standifer et al., 1977). Honey

bees gather these substances by collecting nectar, pollen and water from the natural environment. However, in some periods of the year and in some areas, natural resources can be limited and not match the colony's needs. For this reason, beekeepers normally sustain colonies with additional sources of carbohydrates (Haydak, 1970; Brodschneider and Crailsheim, 2010; Krainer et al., 2016), using homemade inverted sugar syrups, high fructose corn syrup (HFCS) or starch syrup (Jachimowicz and El Sherbiny, 1975; LeBlanc et al., 2009; Brodschneider et al., 2010; Brodschneider and Crailsheim 2010; Krainer et al., 2016). Additional sources of proteins, consisting of pollen supplements or pollen substitutes (Standifer et al., 1977), can also be provided. Carbohydrate rich supplementary food provides an alternative source of energy, increases colony strength, prevents starvation and may reduce wintering losses (Emsen and Dodologlu, 2014). Indeed, a mixture of sucrose and water is commonly used to feed honey bees (Free and Spencer-Booth, 1961; Barker, 1971; Semkiw and Skubida, 2016) especially in the Autumn–Winter period in temperate areas, when honey bees may suffer from low nectar flow and bad weather. The most common diet consists in inverted sugar syrup obtained by mixing sugar and water in a 2:1 ratio to which a variable amount of an acidifying agent is added (Bailey, 1966; Standifer et al., 1977; Genc and Aksoy, 1993); very often, beekeepers produce this food themselves, boiling a water sugar solution acidified with vinegar or lemon juice.

Supplementary nutrition, especially in the Autumn period, has eventually become standard practice in temperate climates since important winter colony losses caused by a number of interacting stress factors and, in particular, the ectoparasite *Varroa destructor* and the associated pathogenic deformed wing virus (DWV), are common (Genersch et al., 2010; Nazzi et al., 2012). In fact, several lines of evidence suggest that the capacity of the colony to face both biotic and abiotic stressors can be enhanced by maintaining a high colony strength through a convenient supply of nutrients (Haydak, 1970; Michener, 2007; Annoscia et al., 2017). In general, these recent advancements fit well within an integrated concept of colony health, including both the potential stressors the bee colony must cope with and the available resources (Nazzi and Pennacchio, 2014).

However, a responsible approach to bee health, requires that also the possible detrimental side effects of any intervention, including supplementary nutrition, are investigated. This issue was investigated for some types of sugar syrup as HFCS, starch syrups and inverted sugar syrup and sugar candy (Barker and Lehner, 1978; Rinderer and Baxter, 1980; Severson and Erickson, 1984; Von der Ohe and Schönberger, 2002; Ceksteryte and Racys, 2006; Le Blanc et al., 2009; Sammataro and Weiss, 2013; Smodiš Škerl and Gregorc, 2014; Semkiw and Skubida, 2016). However, despite the use of 2:1 sucrose – water sugar syrup is very common (Bailey, 1966; Standifer et al., 1977; Genc and Aksoy, 1993) only limited scientific information is available on its possible side effects. In fact, to our knowledge, only Bailey (1966) and Jachimowicz and El Sherbiny (1975) thoroughly studied the possible side effects of a supplementary sugar nutrition based on inverted 2:1 sucrose – water solutions. In particular, Bailey found that 2:1 acid-hydrolysed carbohydrates are toxic to bees but their mode of action remained rather obscure. Moreover, Bailey excluded the possibility that hydroxymethylfurfural (HMF) and/or its degradation compounds (i.e. laevulinic acid and formic acid), at the concentration found in syrups (0.04 - 0.2 %) could be the cause of the recorded toxicity. HMF, is an organic compound consisting of a furan ring containing both an aldehyde and an alcohol function, which has been proved to be harmful to adult bees at 150 ppm (Jachimowicz and El Sherbiny, 1975) and 8000 ppm in sugar solution (Krainer et al., 2016), 250 ppm in HFCS syrup (LeBlanc et al., 2009) and 915 ppm in sugar candies (Smodiš Škerl and Gregorc, 2014); while negative effects on larvae were observed at concentrations higher than 750 ppm (Krainer et al., 2016). This compound can be formed both through the Maillard reaction and the thermal and acid-catalyzed degradation of sugars and carbohydrates (Zirbes et al., 2013; Krainer et al., 2016). Thus, HMF can be found in many foods and, in particular, HMF in honey represent a quality-determination compound (Spano et al., 2008); indeed, HMF is normally absent in fresh honey but concentration increases with time, storage methods and excessive heat (Tomasini et al., 2012). Nevertheless, HMF toxicity risk in sugar syrup is still debated and unclear (Zibres et al., 2013). Indeed, Zirbes et al. (2013) states that it

is currently impossible to establish a maximal concentration limit for HMF in relation to honey bee health and standardized experiments are currently lacking.

To further contribute to understanding the real harmfulness of HMF, we investigated the toxicity of HMF at doses consistent with literature data (Jachimowicz and El Sherbiny, 1975; LeBlanc et al., 2009, Krainer et al., 2016) both in healthy bees and bees challenged with their most important ectoparasite (i.e. *V. destructor*). To strengthen available data on HMF concentration in sugar syrups, we also investigated HMF formation in homemade 2:1 inverted sugar syrup, considering, in particular, the influence of temperature or boiling time on different homemade sugar syrups according to their acidity. Finally, we fed honey bees with these syrups to disentangle the role of various factor (e.g. HMF, acidity, other possible compounds) on honey bees survival.

2. MATERIALS AND METHODS

2.1.Honey bees and *Varroa* mites used in this study

Experiments were carried out between May 2016 and September 2018. Newly emerged adult bees and mites 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 local honey bee colonies are hybrids between *Apis mellifera ligustica* Spinola and *Apis mellifera carnica* Pollmann (Comparini and Biasiolo, 1991).

2.2.Homemade syrups preparation

Homemade syrups were prepared according to a standard recipe which suggests to add the juice obtained from a lemon to a 2:1 sucrose/water solution obtained by dissolving 1800 g of sucrose (brand: “Maxi”, 100% sucrose) in 900 mL of water (brand “Sant’Anna”, dry residue: 22 mg/L, water hardness: 0.9 °F) and then to stir the mixture while heating. Since heating time differ from one recipe to another, with some suggesting to boil the solution for up to 30 minutes, we prepared our homemade

syrups at three different temperatures (25 °C, 50 °C and 110 °C, in this latter case the solution was left to boil for 10 minutes).

To assess the role of lemon juice, two groups of solutions were created: in one group we added a dose of lemon corresponding to 1/10 of ten squeezed lemons (variety: Femminello, organic) while the other group was prepared without lemon.

The concentration of HMF was quantified using a reflectometer (Reflectometer RQflex®plus Reflectoquant®) one day after the preparation of homemade syrups since the compound needs about 24 hours to reach a stable concentration. Three technical replicates were made for each sample. pH was assessed at room temperature (20°C) using a pHmeter (XS 8 series, resolution ± 0.1/0.01 pH). The experiment was replicated three times.

2.3.Effects of homemade syrups on the survival of honey bees

To investigate the possible side effects of wintering supplementary food on the survival of bees, we fed honey bees with homemade syrups produced in the previous experiment ("Homemade syrups preparation").

To this aim, the day before the experiment several combs containing emerging bees were randomly collected from the apiary and stored overnight in a climatic chamber (34.5 °C, 75% R.H., dark). The day after, newly emerged honey bees were transferred into plastic cages (185 x 105 x 85 mm) and maintained under the same controlled conditions. Bees were fed *ad libitum* with water and the homemade syrups. Homemade syrups consisted in: 2:1 sucrose solution produced at 25 °C (labeled as "L-25 °C" in figures), 2:1 sucrose solution with lemon produced at 25 °C ("L+25 °C"), sucrose solution, boiled for 10 minutes at 110 °C ("L- BOILED"), sucrose solution with lemon, boiled for 10 minutes at 110 °C ("L+ BOILED"), 2:1 sucrose solution with lemon and HMF ("L+25 °C HMF"), 2:1 sucrose solution with HMF ("L-25 °C HMF"). The concentration of HMF in L+25 °C HMF and L-25 °C HMF corresponded to the concentration of HMF found in the sucrose solution added with

lemon and boiled for 10 minutes at 110 °C (L+ BOILED). The concentration of HMF was 95 mg/L, 83 mg/L and 77 mg/L in the three replicates of the experiment, respectively.

To assess the composition of the feeding solutions as altered by the thermal treatment, an aliquot of the L+ BOILED syrup and L-25 °C syrup were analysed by High Performance Liquid Chromatography (HPLC). For this purpose, 1 g of syrup was diluted with 4 mL of water in order to reduce the viscosity of the sample before the loading on a 1 g Strata C18-E Solid Phase Extraction (SPE) column (Phenomenex, Italy) previously conditioned with 5 mL of methanol and 5 mL of water. After loading, the column was washed with 3 mL of water and this fraction containing the carbohydrates was discarded, the less polar compounds were then eluted with 4 mL of methanol. The volume of the methanolic fraction was reduced to about 0.5 mL under a nitrogen stream and the sample was then transferred to an autosampler vial for the HPLC-UV analysis.

An UHPLC Shimadzu Nexera R (Shimadzu, Milan, Italy), coupled to a SPD-M20A Photo Diode Array detector and equipped with a degasser, a thermostated autosampler and a column oven was used. The chromatographic separation was performed with an Agilent Poroshell 120 EC-C18, 4.6 × 150 mm, 2.7 μm particle size, column (Agilent Technologies, Italy), thermostated at 30 °C. Elution was carried out at a flow rate of 0.45 mL min^{-1}, using as mobile phase a mixture of water (solvent A) and acetonitrile (solvent B) with the following gradient: 0–2 min, isocratic condition at 5% B; 2–30 min, linear gradient from 2 to 95% B. The injection volume was 5 μL. HMF was identified on the basis of the retention time and UV spectrum of a standard HMF solution.

2.4.Effects of syrup acidity on the survival of honey bees

To confirm the effects of acidity on honey bee survival, newly emerged honey bees collected and reared with the same protocol used in previous experiments were fed *ad libitum* with three different solutions: 2:1 sucrose solution produced at 25 °C (labeled as “L-25 °C” in figures), 2:1 sucrose

solution with lemon produced at 25 °C ("L+25 °C") and 2:1 sucrose solution produced at 25 °C and acidified with HCl ("HCl 25 °C").

The homemade syrup solutions used here were the same as those used before, except HCl 25 °C solution that was originally an aliquote of L-25 °C acidified with HCl to reach the same pH of L+25 °C (pH=2.80).

Three replicates using three different cages were made (each replicate corresponded to one cage).

To further investigate the effects of acidity on honey bees, we performed a quantitative real time PCR (qRT-PCR) assessing the relative expression of Vitellogenin (Vg) (forward: 5'-TTGACCAAGACAAGCGGAACT-3'; reverse: 5'-AAGGTTCGAATTAACGATGAA-3'), Apidaecin (forward: 5'-TTTTGCCTTAGCAATTCTTGTTG-3'; reverse: 5'-GAAGGTCGAGTAGGCGGATCT-3') and deformed wing virus (DWV) (forward: 5'-GGTAAGCGATGGTTGTTTG-3'; reverse: 5'-CCGTGAATATAGTGTGAGG-3') relative load.

Vitellogenin was selected as a generic marker of stress (Dolezal et al., 2016; Smart et al., 2016; Zanni et al., 2017); the antimicrobial peptide Apidaecin was used to investigate the possible effects on honey bees' immune-competence, while DWV was studied since it represents a constant pathogenic threat for honey bees (Nazzi and Pennacchio, 2018).

RNA extractions were performed with Rneasy® Plus Mini Kit (Quiagen), cDNA synthesis with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega) and real-time PCR using the CFX96™ optical reaction module (Bio-Rad) and the C1000 Touch™ thermal cycler (Bio-Rad). B-Actin (forward: 5'-TGCCAACACTGTCCTTTCT -3'; reverse: 5'-AGAATTGACCCACCAATCCA-3') was used as housekeeping gene.

2.5.Toxicity of low HMF doses on healthy and mite infested bees

To assess the possible negative effects of HMF in homemade wintering food, we investigated the survival of uninfested and mite infested honey bees at doses similar to those developed in our homemade syrups and also compatible with those reported in literature.

For this purpose, sealed brood combs from several colonies of the apiary were transferred into the lab and stored in a net cage to collect emerging bees as they eclosed from brood cells; then, newly emerged bees were transferred into plastic cages (185 x 105 x 85 mm) and maintained in a climatic chamber (34.5 °C, 75% R.H., dark). Bees were fed *ad libitum* with water and different diets and mortality was recorded daily. Diet consisted in a sugar solution (glucose 61%, fructose 39%; Thom et al., 2003) added with 0, 50, 100, 200, 400 mg/L of HMF. The experiment was replicated twice. A total of fifty bees per group were used.

Since the experiments revealed no negative effect of this dose on uninfested bees, the toxicity of HMF to mite infested honey bees was studied using the highest dose tested on healthy bees. To do so, we collected mature bee larvae from brood cells capped in the preceding 15 hours and transferred them into gelatin capsules (Agar Scientific Ltd., 6.5 mm Ø) with no mites (V−) or one mite (V+) that had been collected from recently sealed brood cells (Nazzi and Milani, 1994; Nazzi et al., 2012); bees were maintained in an environmental chamber at 34.5 °C, 75% R.H., dark, for 12 days. Upon eclosion, newly emerged adult bees were separated from the infesting mite and transferred into plastic cages (185 × 105 × 85 mm), maintained in a climatic chamber at 34.5 °C, 75% R.H., dark. Bees were fed *ad libitum* with water and sugar solution (0 mg/L HMF) and sugar solution (glucose 61%, fructose 39%) with 400 mg/L of HMF (400 mg/L HMF). The experiment was replicated twice. From 62 up to 80 honey bees per experimental group were used in total.

2.6.HMF concentration in relation to pH and boiling time

Since our experiments showed that HMF concentration is enhanced in sugar syrups acidified with lemon and boiled for 10 minutes, we carried out another experiment to better study the HMF formation in relation to pH and boiling time.

To this aim, three sucrose syrups acidified with HCl at 2, 3 and 4 pH were boiled for 40 minutes at 110 °C. To follow HMF formation, every 10 minutes, from the beginning of boiling till 40 minutes after, 20 mL of each syrup were sampled to assess HMF concentration as described above.

2.7.Effects of high HMF doses on honey bees

To investigate the effect on honey bees of high HMF doses that could develop at low pH and prolonged boiling time, we studied the survival of caged honey bees fed with 10000 mg/L of HMF in sugar syrup. This concentration was selected based on the experiment described before which showed that up to 6000-12000 ppm of HMF are formed in sugar syrup after 30 and 40 minutes of boiling, respectively. Indeed, many beekeeper recipes suggest to boil syrups for 30 minutes or more. Moreover this dose is comparable to that used by Krainer et al. (2016) in their experiments.

For the purpose, bees were fed *ad libitum* with two different solutions: 2:1 sucrose solution produced at 25 °C (labeled as "SUCROSE" in figures) and 2:1 sucrose solution produced at 25 °C added with 10000 mg/L of HMF ("HMF 10000").

2.8.Effects of different monosaccharides on honey bees

To verify the need to invert sucrose in homemade syrups and thus the importance of lemon juice addition, we studied the survival of bees fed either with monosaccharide or disaccharide sugars. We therefore reared newly emerged honey bees obtained as above and provided either a water and a sucrose solution *ad libitum* (labeled as "SUCROSE" in figures) or a 1:1:1 water, glucose and fructose solution ("GLUCOSE & FRUCTOSE"). Three replicates with three different cages were made for this experiment (each replicate corresponded to one cage).

2.9.Statistical analysis

All statistics analysis were performend with Minitab 16®. Each survival curve was compared with its control using the Log-Rank test. Since in the experiment "Effects of homemade syrups on honey

bee" several groups were compared, we applied a correction according to Benjamini and Hochberg (1995), setting the false discovery rate at 0.1.

3. RESULTS

3.1. Acidity and HMF concentration of sugar syrups

In the first replicate of the experiment 10 squeezed lemons produced 490 mL of lemon juice, whereas in the second and third replication 337 and 311 mL were produced, respectively. Since we used 1/10 of the squeezable juice, 49, 33.7 and 31.1 mL of lemon juice were added respectively to the sugar solution in each replicate, reaching a pH ranging from 2.87 and 2.96 (Tab. I). However, it is worth noting that, in previous preliminary experiments, a pH as low as 1.65 in one case and 2.61 in another case was obtained, using the same lemon variety and procedure; therefore, it is safe to conclude that the addition of one lemon to one liter of a sugar solution can reduce the pH to 2 or even less. Both acidity and heating affected HMF formation but only at high temperatures (Tab. II); in fact, acidified sugars syrups, boiled for 10 minutes, reached an average HMF concentration of 85 mg/L ± 9.17 ppm (the high standard deviation could be partly related to the accuracy of the reflectometer (± 2.5)). The HLPC analysis (Fig. 1) confirmed the presence of high concentrations of HMF in the acidified boiled sucrose solution (L+BOILED); other minor peaks were observed as well in this solution that were absent from the sucrose solution produced at 25 °C (L-25 °C).

3.2.Effects of homemade syrups on the survival of honey bees

The highest survival was observed in bees fed with sugar syrup to which no lemon was added, regardless of heating (L-25 °C, L-BOILED, L-25 °C HMF; Fig. 2). In this group no significant differences were found among the three treatments, and, in particular, between L-25 °C and L-25 °C HMF (Tab. III, comparisons n. 1, 2, 3).

An intermediate survival was observed in bees fed with acidified but not boiled sugar syrup (L+25 °C, L+25 °C HMF; Fig. 2); again the addition of HMF did not affect the survival at this stage (Tab. III, comparison 4).

Finally, the lowest survival was observed in bees fed with an acidified solution boiled for 10 minutes (L+BOILED, Fig. 2).

The survival of bees belonging to each of three groups was significantly different from that of bees belonging to the other groups (Tab. III, comparisons from 5 to 15).

3.3.Effects of syrup acidity on the survival of honey bees

Since the previous experiment suggested a negative effect of acidity of the sugar syrup on the survival of bees, we tested this effect using both lemon and hydrogen chloride.

Bees fed with a sugar solution acidified to the same pH (2.80) either with lemon or hydrogen chloride showed a significantly reduced survival as compared to bees fed the same sugar solution without an acidic addition (Fig. 3; L-25 °C vs. HCl 25 °C, Log Rank (Chi-Square = 25.059, d.f. = 1, $P = 0.000$); L-25 °C vs. L+25 °C Log Rank (Chi-Square = 47.852, d.f. = 1, $P = 0.000$)). No significant difference was found between the survival of bees fed with solutions acidified with lemon or hydrogen chloride (Log Rank (Chi-Square = 1.103, d.f. = 1, $P = 0.294$)).

qRT-PCR analysis highlighted a significant lower expression of Apidaecin in honey bees fed with HCl added syrup as compared to control bees fed standard syrup (Fig. 4; L-25 °C vs. HCl 25 °C, Mann Whitney (n1 = 6 ; n2 = 6; U = 7; $P = 0.039$)). No significant differences were found in Vitellogenin expression between honey bees fed with acidified syrups and control bees. A lower DWV infection level was found in bees fed with HCl 25 °C syrup as compared to control bees, although significance was not reached in this case (HCl 25 °C vs. L-25 °C, Mann Whitney (n1 = 6 ; n2 = 6; U = 8; $P = 0.055$)); however, no difference was found in the relative viral load between control syrup and the one acidified with lemon (L-25 °C vs. L+25 °C, Mann Whitney (n1 = 6 ; n2 = 6; U = 16; $P = 0.37$).

3.4.Toxicity of low HMF doses on healthy and mite infested bees

To confirm the results of the first experiment, showing no apparent effect of the addition of HMF to the toxicity of sugar solutions, we tested if doses similar to those observed in that trial or found in the literature can affect the bees' survival.

No significant differences and no apparent negative effects on the survival of uninfested bees were observed with HMF doses similar to those found in our sugar syrups and to those reported in literature (0, 50, 100, 200, 400 mg/L of HMF) (Fig. 5A).

As expected, bees artificially infested with *V. destructor* showed a reduced lifespan as compared to un-infested bees (V+ 0 mg/L HMF vs V- 0 mg/L HMF , Log Rank (Chi-Square = 10.539, d.f. = 1, $P = 0.001$) and V-400HMF vs. V+400HMF, Log Rank (Chi-Square = 6.001, d.f. = 1, $P = 0.014$)) (Fig. 5B).

Moreover, we observed a notable difference in the shape of the curves between uninfested (Fig. 5A) and mite infested bees (Fig. 5B), with the first group of bees following a type 1 curve and the second following a type 2 survival curve, possibly caused by the different handling of bees during artificial infestation.

In any case, 400 mg/L of HMF did not negatively affect the survival of bees; actually, an increased survival was observed in infested bees (V+ 400 mg/L HMF vs. V+0 mg/L HMF, Log Rank (Chi-Square = 5.052, d.f. = 1, $P = 0.025$)). This trend was not confirmed in uninfested honey bees were the survival of honey bees treated with 400 mg/L of HMF was not different from the control (V- 400 mg/L HMF vs. V- 0 mg/L HMF, Log Rank (Chi-Square = 1.264, d.f. = 1, p-value = 0.261)). These results nicely match the results reported above and obtained in a separate experiment (Fig. 2).

3.5.HMF concentration in relation to pH and boiling time

Considering the results presented above regarding the effect of boiling acidified sugar solutions on HMF formation, and the non-significant effect of low doses of HMF, we wondered if a prolonged

heating of acid solutions may result in higher concentration of HMF that could be toxic to bees. To answer to this question, we prepared sugar syrups with different acidity (pH: 2, 3, 4) and assessed HMF formation in relation to increasing boiling time.

We found that boiling time did not strongly affect HMF formation at pH 3 and 4, causing concentrations that, according to our previous results, are non-toxic to bees. However, at pH 2, the heating process triggers the formation of a much higher HMF concentrations, ranging from 1786.7 mg/L, after 10 minutes of boiling to 12,005.3 mg/L thirty minutes later (Fig. 6A).

3.6.Effects of high HMF doses on the survival of honey bees

Feeding bees with a sugar syrup containing an HMF concentration similar to that obtained after boiling an acidic solution for a few minutes (i.e. 10000 mg/L of HMF) caused a strong significant reduction in the lifespan of bees; indeed, 100% of mortality was recorded after only 15 days, while control bees lived until the 34th day (SUCROSE vs HMF 10000, Log Rank (Chi-Square = 16.452, d.f. = 1, P = 0.000)) (Fig. 6B).

3.7.Effects of a monosaccharide based diet on honey bees

Since acidification of sugar syrups appears to be critical for bee survival, and the purpose of this treatment is to obtain the inversion of disaccharide sugars into monosaccharides, we tested if feeding bees with a sucrose solution instead of glucose and fructose influences their survival.

We found that bees fed with sucrose syrup (the same recipe as that used in previous experiments) had a longer survival than bees fed with a 1:1:1 water, glucose and fructose solution (GLUCOSE & FRUCTOSE vs SUCROSE, Log Rank (Chi-Square = 7.440, d.f. = 1, P = 0.006)) (Fig. 7).

4. DISCUSSION

Doses of HMF similar to those reported as sublethal in the literature (Jachimowicz and El Sherbiny, 1975; Le Blanc et al., 2009) and found in our home made sugar syrups when heating treatment is

restricted, seem to be non-toxic both for uninfested and mite infested bees. This result suggests that, at low concentrations, in the range of 10-400 ppm, HMF does not influence bee health, even in presence of the most common additional stressor of bees: the ectoparasite *V. destructor* and the viruses that are normally associated to it.

However, our data show that the lower acidity that can be found in homemade syrups because of lemon addition, negatively affects bees' survival, as confirmed by comparing the survival of bees fed a sugar solution acidified or not with lemon. The similar results obtained after changing the acidifying agent support the notion that acidity *per se*, rather than any toxic compounds from lemon, is responsible for the observed effect. Molecular analysis shows an interesting down-regulation of Apidaecin in bees fed with HCl acidified syrups, suggesting an interaction with the bee's immune system. However, we did not find a similar significant pattern in bees fed with lemon, supporting the view that it is not acidity but rather the quality of the acidifying agent that matters in this case.

Vitellogenin expression, which did not differ between groups, indicates that the abiotic stress of acidity has no effects on the gene expression of this lipoprotein, which, in this case, does not appear a good marker of stress. The same consideration can be drawn for DWV load, which revealed no differences between the different experimental groups.

Our experiments further showed that acidified sugar solutions may reach much higher concentrations of HMF if a prolonged heating is applied and a low pH level (e.g. pH=2) is reached after lemon addition; in fact, both acidity and the amount of lemon juice are influenced by seasonality, climate and the stage of ripeness of the lemon (Bartholomew, 1923), and such low pH level can easily be reached. Since the very high concentrations of HMF that can be produced under the above mentioned conditions can be very toxic to bees (i.e. above 10000 ppm), a great care should be used while making homemade syrups. This last data is consistent with the results obtained by Krainer and co-workers (2016) who observed that concentration of 8000 ppm of HMF is toxic for adult honey bees.

Lemon addition is normally done to facilitate the inversion of disaccharide sugars to obtain the purportedly more digestible monosaccharides, glucose and fructose. Indeed, hydrolysed sucrose is

commonly believed to be nutritionally better for honey bees (Bailey, 1966). The negative effect of lemon addition obtained here suggested to test if lemon addition is really necessary; we found that sucrose can be as effective as glucose and fructose to sustain a normal survivorship under laboratory condition. This would suggest that lemon addition may be not necessary as normally thought, possibly because bees are able to invert disaccharides themselves, thanks to α-invertase (White, 1975). However, we can not exclude that other results could be obtained under field condition, where nutritional requirements of bees can be different. Nevertheless, our results support a careful evaluation of this aspect.

A further interesting result obtained in this study is the much-reduced survival observed in bees fed an acidified sugar solution after boiling for only 10 minutes. This result can neither be explained by the negative effect of lemon addition (that it is lower), nor by the HMF concentration that could be reached in this case (that is lower than the harmful one). Indeed, our HPLC analysis showed that acidified-boiled syrups contain other substances, further than HMF, that can be related to the toxicity of these solutions, as already suggested by Bailey (1966), who found that acid-hydrolysed carbohydrates are toxic for bees due to the formation of unknown compounds in these solutions.

In conclusion, we provided convincing evidence that homemade sugar syrups can hide several possible negative side effects for bees that can impair normal survival. These negative effects can be related to the possible formation of high doses of HMF, to the acidity and to the formation of further compounds, whose identity has not been studied so far.

The golden rule of medicine “primum non nocere” (first do no harm), attributed to Hyppocrates, underlines the need of carefully considering the possible negative side effects of the treatments we may apply to sustain the health of an individual. Bees are currently exposed to a number of interacting stress factors (vanEngelsdorp et al., 2009; Hedtka et al., 2011; Dainat et al., 2012; Nazzi et al., 2012; Nazzi and Pennacchio, 2014,) that may affect bee health in a complex and often unpredictable way (Di Prisco et al., 2013; Doublet at al., 2015; Nazzi and Pennacchio, 2018). Here we wanted to point the attention to the undesirable effects of supplementary nutrition since this has become a common

practice due to the increased fragility of bees underlined above. We sincerely hope that a balanced equilibrium can be found between the need of sustain bee colonies and the risk of perturbing their normal functioning.

Figures

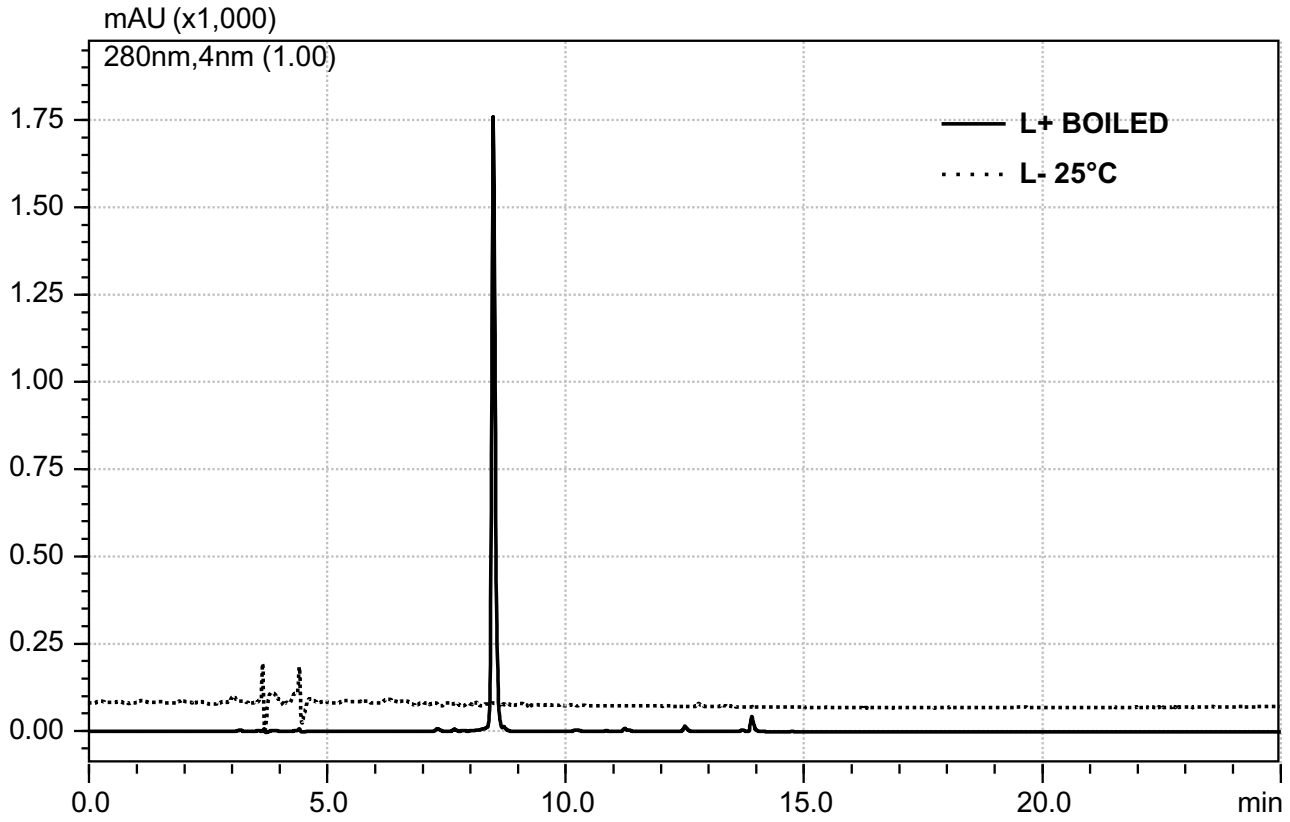

Figure 1 – HPLC analysis of an acidified boiled sugar solution and a sugar solution produced at 25 °C, no acidity.

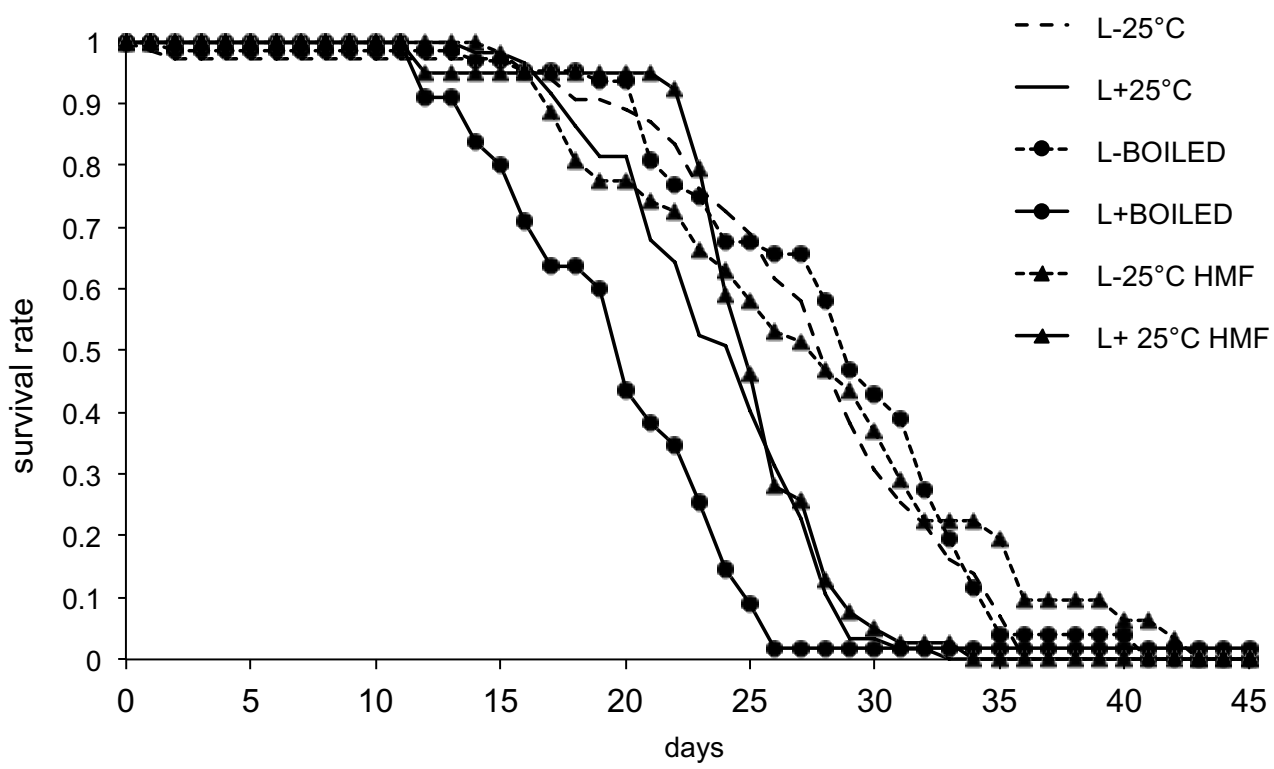

Figure 2 - Effects of different sugar syrups on honey bees survival.

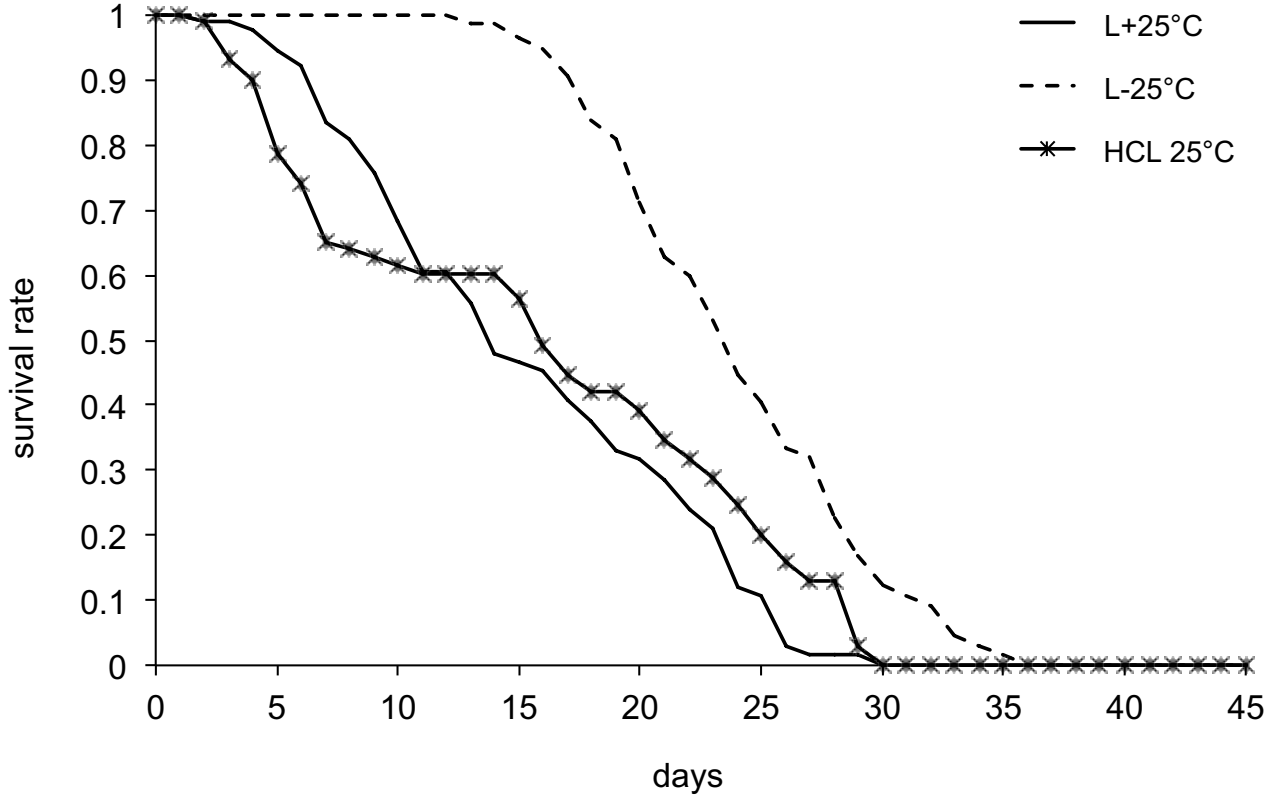

Figure 3 - Effects of acidity on honey bees survival.

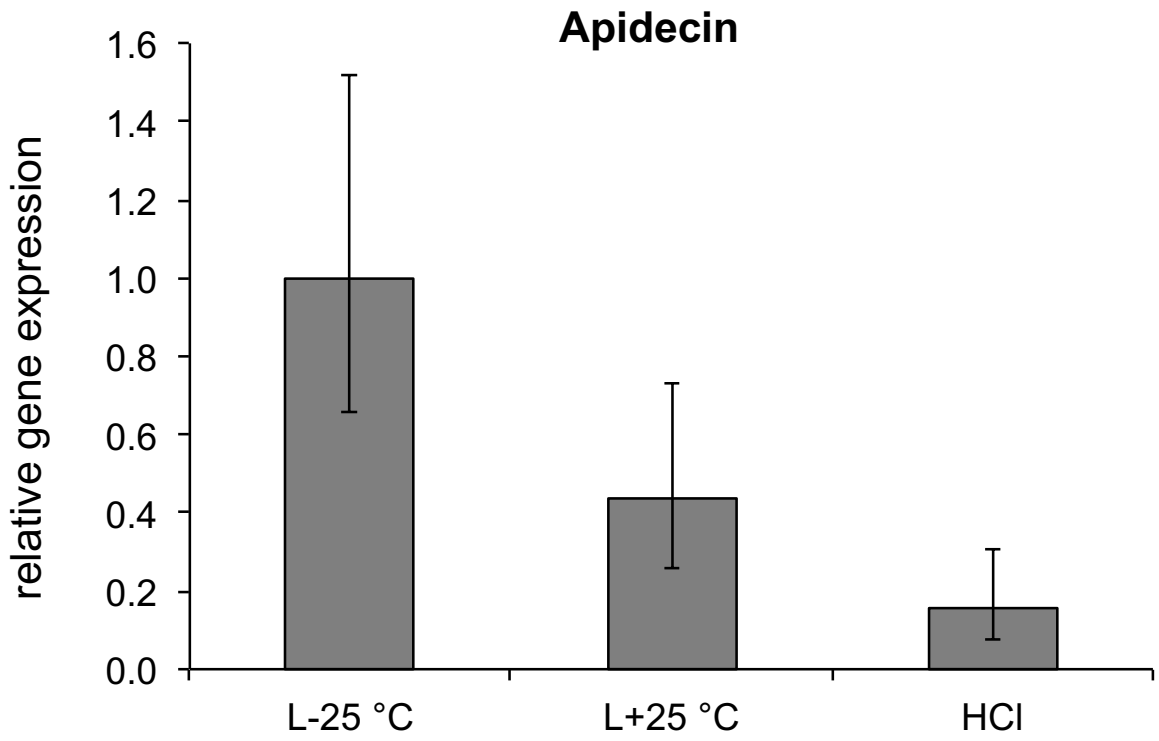

Figure 4 - Relative expression of Apidecin in honey bees treated with different acidified sugar solutions.

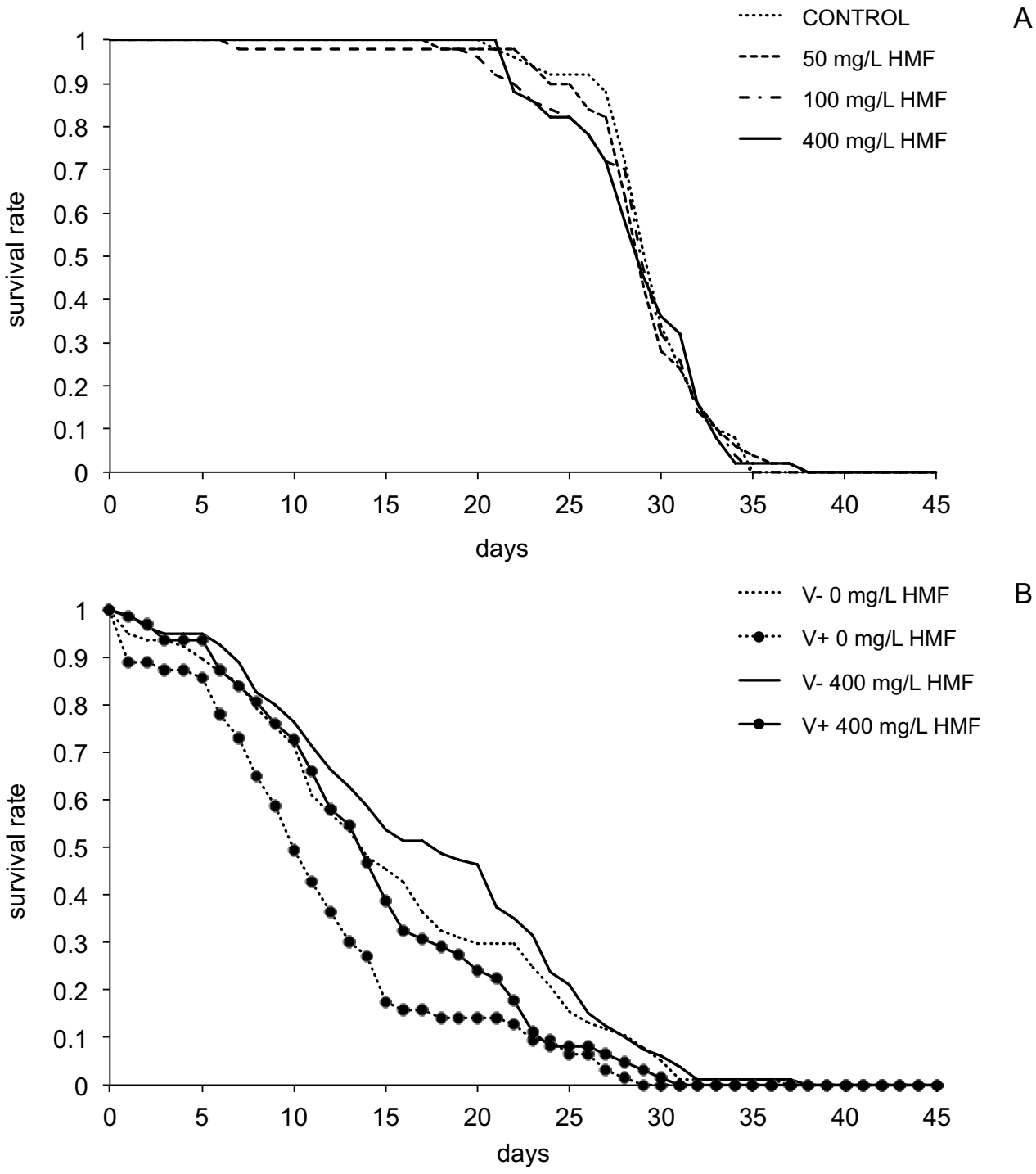

Figure 5 - Survival rate of uninfested adult honey bees fed with sugar syrup containing different concentrations of HMF (A) and survival rate of adult bees infested (or not) with 1 mite during the pupal stage and fed with a sugar syrup containing (or not) 400 mg/L of HMF (B).

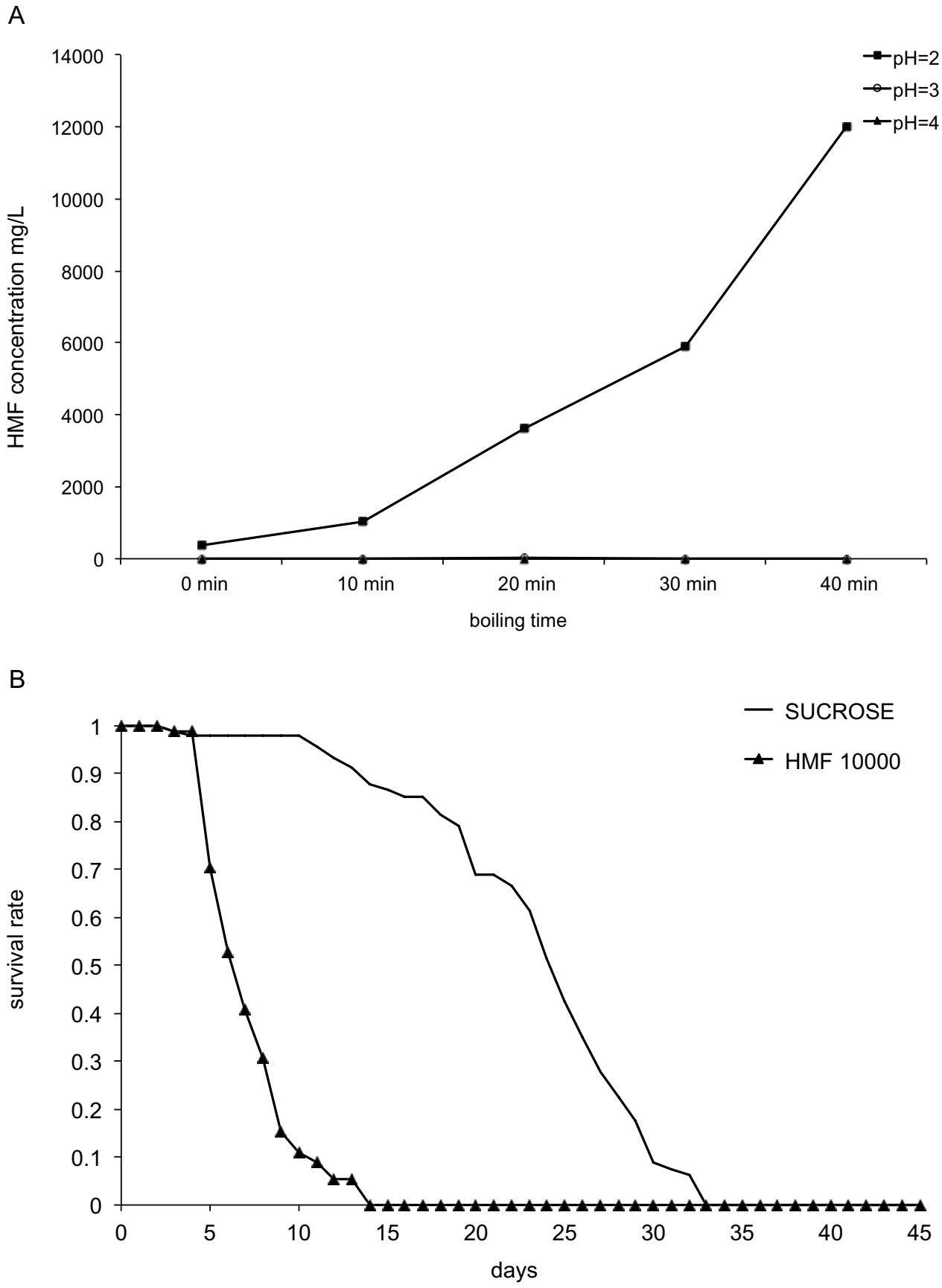

Figure 6. HMF formation in relation to heat treatment and acidity (A); survival rate of honeybees fed or not with a very high dose of HMF (B).

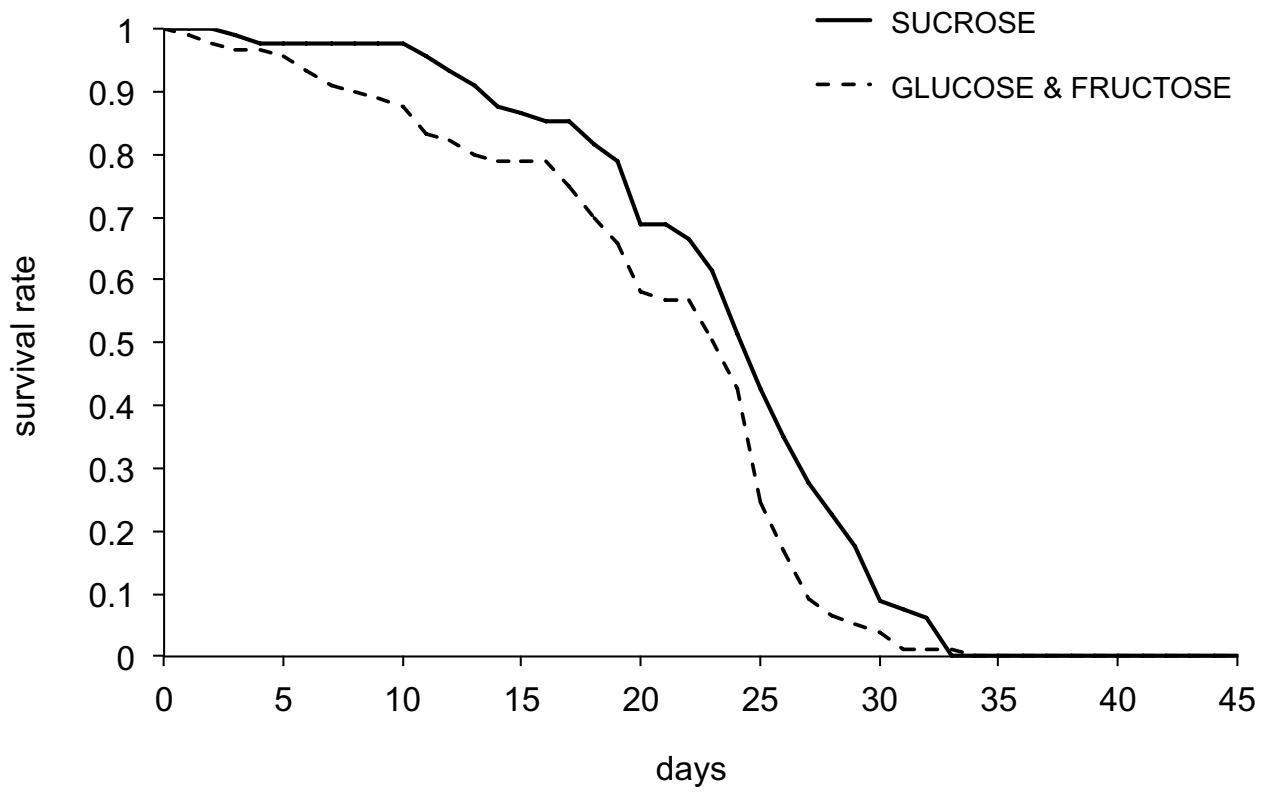

Figure 7. Survival rate of honeybees fed with a diet based on monosaccharides or disaccharides.

		25 °C	50 °C	110 °C BOILED 10'
1st REPLICATE	**L-**	7.37	7.13	8.23
	L+	2.96	2.97	3.00
2nd REPLICATE	**L-**	7.32	7.08	7.06
	L+	2.87	2.9	2.84
3rd REPLICATE	**L-**	6.05	7.01	6.30
	L+	2.87	2.75	2.88
MEAN	**L-**	6.91	7.07	7.20
STD-DEV.		0.75	0.06	0.97
MEAN	**L+**	2.90	2.87	2.91
STD-DEV.		0.05	0.11	0.08

Table 3. pH of homemade sugar solutions according to the preparation method.

		25 °C	50 °C	110 °C BOILED 10'
1st REPLICATE	**L-**	2.3	2.8	3.8
	L+	3.1	2.6	95.0
2nd REPLICATE	**L-**	2.8	3.3	1.6
	L+	1.6	3.0	83.0
3rd REPLICATE	**L-**	1.8	2.2	1.5
	L+	2.4	2.8	77.0
MEAN	**L-**	2.3	2.8	2.3

STD-DEV.		0.5	0.6	1.3
MEAN	**L+**	2.4	2.8	85.0
STD-DEV.		0.7	0.2	9.2

Table 2. HMF (mg/kg) produced in homemade sugar solutions.

	Comparison	Chi-Square	DF	*P* value
1	L-25 °C VS L-25 °C HMF	0.56708	1	0.451
2	L-25 °C VS L-BOILED	0.57005	1	0.450
3	L-BOILED VS L-25 °C HMF	0.01982	1	0.888
4	L+25 °C VS L+25 °C HMF	1.35442	1	0.245
5	L+25 °C VS L-25 °C	22.4025	1	0.000
6	L+25 °C VS L-25°C HMF	17.8190	1	0.000
7	L+25 °C VS L-BOILED	29.4064	1	0.000
8	L+25 °C VS L+BOILED	28.2390	1	0.000
9	L+25 °C HMF VS L-25°C	11.9552	1	0.001
10	L+25 °C HMF VS L-25°C HMF	93.9784	1	0.002
11	L+25 °C HMF VS L-BOILED	15.5093	1	0.000
12	L+25 °C HMF VS L+BOILED	36.2876	1	0.000
13	L+BOILED VS L-25 °C	61.1796	1	0.000
14	L+BOILED VS L-25 °C HMF	45.1172	1	0.000
15	L+BOILED VS L- BOILED	62.7928	1	0.000

Table 3. Statistical analysis (Log-Rank test) related to the survival of honeybees fed with different sugar syrups (Fig. 2).

ACKNOWLEDGEMENTS

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