UNIVERSITA' DEGLI STUDI DI UDINE

Department of Scienze AgroAlimentari, Animali e Ambientali

PhD in Agricultural Sciences and Biotechnologies

XXIX Cycle

Stress Factors and Honey Bee Health

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Academic Year: 2015-2016

CONTENTS

Chapter 1: **general introduction**

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1.1. Biology of the western honey bee (*Apis mellifera* L.) with special emphasis on the aspects that are relevant to the present work

Due to their pollinating activity, honey bees (*Apis mellifera* L.) play a fundamental role in the maintenance of agricultural and natural ecosystems.

One third of human food is derived from plants pollinated by insects; in fact, 70 % of the main crops for human consumption require pollinators for seeds' production and to enhance quality and yields (Klein et al., 2007). Honey bees, mainly *Apis mellifera*, remain the most economically valuable pollinators of crop monocultures worldwide (McGregor, 1976; Watanabe, 1994), providing an ecosystem service in the form of crop pollination estimated to be 153 billion Euros a year worldwide and 22 billions in Europe (Gallai et al., 2008).

The honey bee is a social insect belonging to the order of the *Hymenoptera*, that exhibits a combination of individual traits and social cooperation (Winston, 1987). Bees live in colonies consisting of tens of thousands individuals organized in three castes: a fertile queen, sterile workers and reproductive drones.

Wax combs, the substrate for virtually all colony activities as brood rearing, food storage, queen care and social interactions, consist of hexagonal cells with different dimension: smaller cells that are used for brood rearing and food storage, larger cells used to rear drones, and queen cells built along the lower edge of the combs (Schneider, 2015).

The three castes present some morphological differences allowing an easy distinction (fig.1).

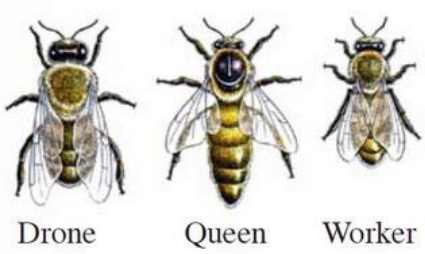

Fig 1: honey bees 'castes.

Gender in honey bees is determined by haplodiploidy: fertilized diploid eggs develop into females while unfertilized eggs develop into males. A fertilized egg has the potential to develop in a fertile queen or a sterile worker, two castes genetically identical differing only for the type and amount of food received after the first three days of larval development (Schneider, 2015). Nurse workers feed larvae with a protein-rich food secreted by the hypopharyngeal and mandibular glands (Snodgrass, 1925). If a larva receives a large amount of this brood food throughout the developmental stages it will become a queen; if the same larva receives smaller quantity of worker-made secretions plus pollen and honey during the latter part of the development it will become a worker (Schneider, 2015).

Regardless of the different castes, all individuals show the same four developmental stages: egg, larvae, pupae and adult (Schneider, 2015).

Eggs (1.5 mm longs and with a diameter of 0.3 mm) are laid by the queen at the bottom of the brood's cells. After three days from deposition, a small larva emerges and worker bees start immediately to take care of it. The larva develops very quickly and undergoes five moults; during the last larval stage, workers cap the cell with wax and the larva starts spinning a silk cocoon. Into the sealed cell, the larva develops into a pupa, starts the metamorphosis and, 12 days after sealing, it emerges from the cell. The total developmental time from egg to adult is 16 days for queens, 21 for workers and 24 for drones.

Life expectation is very different among the different castes: usually a worker lives 4-6 weeks during the summer but can lives 4-5 months during the winter, the drones survive for 3-5 weeks while the queen typically lives 1-3 years (Schneider, 2015).

Adult workers perform all the labour of a colony during their lifetime but exhibit an age-related bias in task performance that is known as temporal polyethism (Winston, 1987).

Newly emerged workers are small and harmless but in few days, they acquire a darker pigmentation and become more self-confident. Younger workers start to dedicate to in-hive duties as cleaning cells; when they are 4 day old, they become nurses and start feeding older larvae; then later, according to the hypopharyngeal glands' development, they feed the younger larvae.

Later, workers are involved in wax production, food storage and other in-hive duties; finally, from about 20 days of age, workers become foragers involved in nectar and pollen collection (Seeley, 1982; Johnson, 2008).

This polyethism schedule is not perfectly rigid, as bees can engage in other duties if necessary according to the colony's needs.

The behavioural transitions described above are associated with complex physiological changes guided by the expression pattern of different genes (Schneider, 2015). In particular, as compared with forager nestmates, nurse bees show different expression levels of genes involved in response to stimuli, glands development, lipids' storages (Ament et al., 2011) and juvenile hormone, a hormone that plays a key role in regulating the age-influenced division of labour, synthesis (Robinson, 1987, Huang and Robinson, 1992, Schulz et al., 2002).

The activities of all the individual bees inside the colony are coordinated by chemical communication ensuring, by means of different pheromones, the reproductive dominance of the queen, the workers' behavioural maturation, the coordination of colony reproduction through swarming and many other functions (Grozinger, 2015).

1.2. Honey bee colony collapse and the role of *Varroa destructor* in undermining bee health

In the last decade, managed bee colonies have experienced a period of severe crisis in both Europe and United States (Le Conte et al., 2010, VanEngelsdorp et al., 2010). Extensive colony losses concurrently reported all over the northern hemisphere (Neumann and Carreck, 2010) represent an important concern for the apiculture and the whole agriculture.

It is now accepted that there is more than one factor threatening the health of colonies; colony losses have in fact a multifactorial origin with parasites and pathogens playing an active role which is reinforced by the effects of pollution, pesticides, banalization of agricultural landscapes and depletion of natural environments (Goulson et al., 2015; Di Prisco et al., 2013), Among the above listed stress factors, the mite *Varroa destructor* Anderson & Trueman plays a major role. The ectoparasitic mite was originally confined to the Eastern honey bee, *Apis cerana*; after shifting to *Apis mellifera,* the mite spread worldwide with deleterious effects for the beekeeping industry (Rosenkanz et al., 2010).

At the individual level, the mite causes water and weight losses (Annoscia et al, 2012), decreased flight performance (Kralj and Fuchs, 2006; Kralj et al., 2007), behavioural modifications (Annoscia et al., 2015) and accelerated behavioural maturation (Downey et al., 2000). Moreover, *Varroa* indirectly causes secondary infections triggered by bacteria invading the bee through the mite's feeding hole (Vanikova et al., 2015) and vectors various honey bees' viruses (Boecking and Genersch, 2008) whose replication can be triggered by the mite (De Miranda and Genersch, 2010).

At colony level, a moderate infestation reduces the growth of the bees' population even if other symptoms are not evident (Rosenkranz et al., 2010). Beyond a certain threshold of infestation, the colony collapses irremediably; in fact, untreated infested colonies die within six month to two years of infestation (Le Conte et al., 2010).

1.3. Biology of the mite *Varroa destructor*

Varroa destructor is a mite characterized by sexual dimorphism with bigger red-brown female individuals (1.1-1.7 x 1.5-1.9 mm) and smaller grey males (0.8 mm). The shape is ellipsoidal and flattened and females have a stinging-sucking mouth apparatus that, in the adult male is transformed into an organ for the transfer of the spermatophora.

Fig.2: *Varroa* family (Huang, 2012).

The mite's life cycle is strictly synchronized with that of honey bees and *Varroa* lacks of a free living stage (Rosenkranz, 2010). The cycle can be dissected into two moments (fig. 3): a reproductive phase into brood's capped cells and a phoretic phase on adult bees.

The reproductive phase occurs exclusively into the capped brood cells; an adult fertilized female enters in a brood cell, preferring drones' ones, immediately before selaing and, after a short while, starts sucking the haemolymph from a hole made in the larva's body. About 60 h after cell invasion, the mite lays the first unfertilized egg that will develop into a male (Ifantidis, 1983, Rehm and Ritter, 1989). Subsequent fertilized eggs develop into female individuals.

Newly emerged protonymphs emerge 48 hours after the oviposition and follow the mother on the ventral side of the developing bee pupa sucking haemolymph from a communal feeding site (Donzè et al., 1998). The developing mite molts into a deutonymph and then into an adult. The total cycle from egg to adult last 6-7 days for males and 6-9 days for females (Accorti et al., 1983).

Varroa mite become sexually mature immediately after the last molt (Rosenkranz et al., 2010). Males reach maturity earlier and wait for the first adult female, to mate.

Males and nymphal stages can be found only in the sealed brood cells; instead the fertilized adult females, once emerged from the cell with the young bee, start the phoretic phase consisting in a period of time spent attached to an adult bee, often between the abdominal sternites (Pettis et al., 2015). During this time, the mites can invade other colonies or other hives via drifting bees.

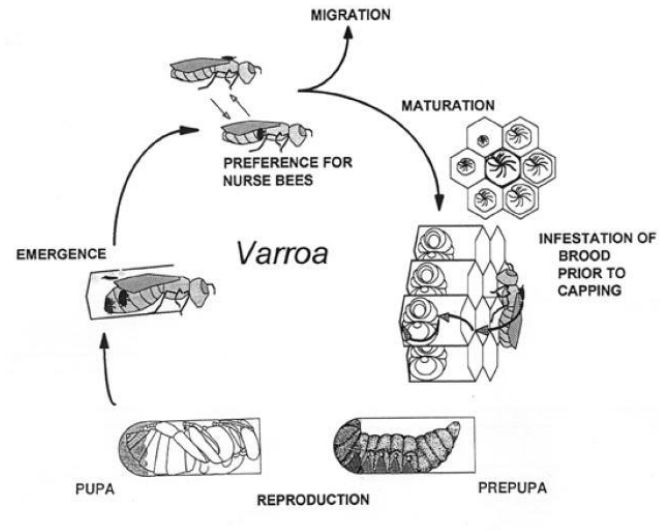

Fig.3: *Varroa* life cycle. After a phoretic phase on the adult bee, mite adult females invade the brood cells immediately before sealing, start sucking haemolymph from the larva and reproduce (Donzè et al., 1998).

1.4. The deformed wing virus (DWV)

The Deformed wing virus is one of the most common viruses found in the hive (Chen and Siede, 2007) and one of the main viruses associated with the collapse of honeybee colonies. Originally isolated in the early 80s (Bailey and Ball, 1991), the virus is now distributed worldwide, and appears to be strictly associated with *V. destructor* (Ball, 1983; Ball and Allen, 1988; Bowen-Walker et al., 1999; Nordstrom et al., 1999; Ribiere et al., 2008; Sumpter and Martin, 2004; Tentcheva et al., 2004b).

The DWV is made of a 30 nm icosahedral particle (Lanzi et al., 2006) containing of a single positive-stranded RNA genome included in a capsid made of three major structural proteins (Bailey et al, 1991; Lanzi et al., 2006). The genome organisation of the DWV is typical of the iflaviruses, a genus of the picorna-like family iflaviridae (De Miranda and Genersch, 2009).

DWV is transmitted between bees by both horizontal (faecal-cannibal-oral) and vertical (parents-offspring) transmission (Chen et al., 2006).

In presence of overt infections (De Miranda and Genersch, 2010), the virus causes well-defined disease symptoms including crumpled wings (fig.4), smaller boy's size, discoloration in adult bees and reduced lifespan (De Miranda and Genersch, 2010).

Fig.4: an infested honey bee with evident symptoms (crippled wings) of DWV infection (ph by R. Wilson, honeybeesuite.com).

1.5. *Varroa* mite and DWV

Recently, the dangerous interplay between DWV and *Varroa destructor* was studied in detail and the transition, triggered by the mite, from a latent infection to a devastating viral outbreak has been described (De Miranda and Gernersch, 2010).

A functional model describing how the delicate immune balance underpinning the covert viral infections can be destroyed by the mite causing an intense viral proliferation was recently described (Nazzi et. al, 2012) highlighting the central role of Dorsal-1A, a transcription factor NF-kB.

It appears that the interaction between mite and virus leads to a marked transcriptional down-regulation of Dorsal-1A indicating that the pathogen-parasite association can interfere with the immune responses regulated by this transcription factor, including antimicrobial peptides synthesis, clotting, melanisation and antiviral defence (Nazzi et al., 2012; Di Prisco et al., 2016). Under this scenario, the limited amount of this immune resource seems to be sufficient to sustain cover infections but any stress factor activating a response regulated by

NF-kB, such as the *Varroa* mite, could potentially break this delicate balance leading to an intense replication of the DWV (Nazzi et al., 2012).

Recently, a further element was added to the picture describing how DWV by disrupting NF-kB signaling, can indirectly favour the mite's trophic activity and reproduction (Di Prisco et al., 2016).

1.6. Honey bee health: defence strategies and immunity

The in-hive environment, with thousands of individuals in close contact, a constant temperature of about 34 °C, a high humidity and the presence of stored food, it is particularly suitable for the proliferation of bacteria, fungi and other pathogens (Chan et al., 2009).

Paradoxically, honey bees express only two-thirds as many immunity genes as solitary insects suggesting a major role of social immunity to contrast parasites and pathogens pressure (Chan et al., 2009). In fact, bees can keep under control natural enemies' diffusion by grooming and nest hygiene (Evans, 2006) and removing infected larvae (Spivak and Reuter, 2001). Other strategies to contrast parasites and pathogens proliferation consist in nest coating with antimicrobial material as propolis (Christe, 2003) or nest temperature raising by means of the "social fever" (Starks, 2000).

As regards the individual defence, insects adopt several strategies to combat infections and infestations, such as a hostile gut environment (Evans et al., 2006), very efficient cellular and humoral responses involving the secretion of AMPs, melanisation, clotting etc. (Hoffmann, 2003; Hultmark, 2003).

Innate immunity response in insects shows an architecture and specific orthologs components that are shared with vertebrates (Beutler, 2004). Canonical immunity pathways models for Toll, Imd, Janus kinase (JAK) STAT and JNK pathways in honey bees have been proposed by Evans and colleagues (2006) highlighting the promise of connecting data on honey bee pathology (Morse and Flottum, 1997) and a deeper knowledge on the bees' immune traits.

1.7. Honey bee health: nutrition and the role of pollen

The development and survival of the honey bee colonies are associated with the nutrients availability (Brodshneider and Crailsheim, 2010). Honey bees require carbohydrates, proteins, lipids, minerals, vitamins and water to normally develop and all these nutritional needs are satisfied by collecting nectar, pollen and water (Herbert and Hill, 2015).

A balanced and adequate nutrition plays a fundamental role in preserving honey bee health and intensive monocultures, loss of natural environments and biodiversity can undermine the bees' nutritional needs (Naug, 2009).

Bee-collected pollen, a blend of pollen grains derived from many plant species, plays an important role in preserving bee's physiology and parasite tolerance (Di Pasquale, 2003). Pollen is a source of proteins, amino acids, carbohydrates, lipids, sterols, vitamins and minerals (Roulston and Buchman, 2000; Stanley et al., 1974); workers eat 3.4-4.3 mg of pollen per day, with a peak at the age of nurses (Crailsheim et al., 1992) when they produce larval food.

Pollen influences the longevity of the individual (Haydak, 1970), enables the larval food production by nurses (Crailsheim et al., 1992), affects the resistance threshold to stress (Naug, 2009), positively influences physiological metabolism (Alaux et al., 2011; Ament et al., 2011), immunity (Alaux et al., 2010), tolerance to pathogens (Rinderer et al., 1974) and sensitivity to pesticides (Wahl and Ulm, 1974). Pollen also provides the proteins that are necessary for the development of muscles, tissues, glands (Herbert and Hill, 2015) and is the only source of lipids for the honey bees. Lipids represent an important source of energy and are precursors for further biosyntheses (Cantrill et al., 1981).

Pollen contains also sterols that are essential for the honey bees (Herbert and Hill, 2015) as precursors of hormones and components of cellular membranes. Pollen fatty acids are known for their antimicrobial properties (Manning et al., 2015) and are involved in the cuticle formation.

1.8. The problem

Varroa mite and the associated viruses represent the major threat for the modern apiculture worldwide (Rosenkranz et al., 2010; Le Conte et al., 2010).

In the years following the introduction in the US and Europe, available acaricides allowed a satisfactory control of the mite (Milani, 1999) but the intensive use of such products soon led to the selection and spread of acaricide resistant strains (Milani, 1999) such that *Varroa* is still decimating honey bee population worldwide.

Only a detailed knowledge of the effects of parasitization and associated viral outbreaks on honey bees together with a better understanding of the strategies adopted by honey bees to contrast pathogens and parasites, including immunity, high quality food intake and defensive behaviours may lead to a sustainable solution of the problem.

1.9. Scope of the thesis

The main aim of this work was to investigate how two stressors, namely the *Varroa* mite and deformed wings virus, can affect honey bee's health, under different conditions, at different levels.

Parallel to this, the role of a complete nutrition in preserving the health of bees challenged with *Varrroa* mite infestation was studied.

The availability of genomic tools allows a better understanding of the mechanisms underlaying host-parasite interactions and provides an important instrument to recognize and manage honey bee diseases (Grozinger and Robinson, 2015). Studies at the genomic level are the basis of a holistic approach aimed at achieving a deeper knowledge of the effects of parasites on the physiology of the whole organism, permitting to understand how a parasite or a pathogen undermines individual physiology. In particular, RNAseq analysis and the study of the transcriptome of bees under different *Varroa* mite infestation level, environments and scenarios is the first step to isolate groups of differentially expressed genes affected by the parasitization and potentially strong molecular biomarkers of colony decline.

For this purpose we analysed the honey bee's transcriptomes, as affected by mite infestation, under different conditions, in order to identify recurrent processes and genes affected by the mite; the results of this study are reported in **chapter 2**. Along this study, we also found a group of potentially good markers of imminent collapse and validated them by means of q RT-PCR.

The data obtained from the whole genome analysis highlighted the possible influence of *Varroa* mite on honey bee behaviour. Therefore, building upon previous observations about the reduced involvement of infested bees in the nursing acitiviy (Annoscia et al., 2015) and the evidence of typical forager gene expression pattern in infested nurses obtained from the study described in chapter 2, we carried out a set of experiments, whose results are reported in **chapter 3,** aiming at investigating how the nursing process is affected by the mite and what are the possible underlaying mechanisms explaining the observed effects.

Honey bees can rely on resources from the external environment to contrast the detrimental effects of parasite and pathogens. Among them, a complete, high quality nutrition plays a fundamental role in preserving honey bee health. Particularly interesting is the role of pollen, an important source of proteins, carbohydrates, lipids and other substance such as vitamins and minerals. In **chapter 4** we tested the hypothesis that pollen can be used as a self-medication tool by bees challenged with the *Varroa* mite. In particular, we investigated the effects of a pollen diet on infested bees' lifespan both in lab and field conditions identifying the components of pollen explaining the observed bioactivity.

The thesis incorporates also an appendix reporting other studies to which I collaborated and posters that were presented at conferences.

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Chapter 2: **transcriptional signatures of parasitization and markers of colony decline in *Varroa*-infested honey bees (*Apis mellifera*)**

Ph: Virginia Zanni

1. INTRODUCTION

The parasitic mite, *Varroa destructor* Anderson and Trueman, is a major factor underpinning losses of honey bee colonies worldwide (Nazzi and Le Conte, 2016).

The mite co-evolved with the Asian honey bee, Apis cerana, and does not cause significant damage to this species (Rosenkranz et al., 2010). However, in the last century, V. destructor shifted hosts to parasitize the western honey bee, *Apis mellifera*, and has gradually extended its distribution area to the rest of the world (Rosenkranz et al., 2010). In the United States and Europe, the mite causes significant damage to A. mellifera populations and is a major factor contributing to colony declines (Le Conte et al., 2010; VanEngelsdorp et al., 2010), though many African subspecies of *Apis mellifera* are able to tolerate *Varroa* infestations (Muli et al., 2014).

The mites damage directly and indirectly the colony by both feeding on the developing worker pupae and by facilitating the transmission and virulence of viral pathogens (Francis et al., 2013; Nazzi et al., 2012). Damage caused by the mites accelerates maturation of infested worker bees and reduces their lifespan, ultimately unbalancing the colony demographic structure and leading to dwindling and collapse (Barron, 2015).

In the years following the introduction of *V. destructor* to the US and Europe, commercially available acaricides allowed a satisfactory control of the mite (Milani, 1999). However, widespread use of these acaricides soon led to the selection and spread of resistant mite strains (Milani, 1999). To identify sustainable solutions, the possible mechanisms allowing A. mellifera colonies to better resist or tolerate the presence of the mite have been evaluated. Genetic mechanisms that allow honey bees to detect *Varroa* infested brood and remove diseased larvae (Spivak and Gilliam, 1998a, 1998b) or to tolerate *Varroa* parasitization without experiencing negative effects (Martin and Medina, 2004; Navajas et al., 2008) have been identified and evaluated. Other approaches aimed at mitigating the effects of mite infestation on the bees through supplementary nutrition or other external inputs may compensate the detrimental effect of the mite (Alaux et al., 2010).

Anyway, any alternative control strategy for coping with this parasitic challenge must be based on a detailed knowledge of the biological effects of the mite on the honey bee's physiology, in order to focus the possible interventions on the most vulnerable components of the system. Developing such detailed knowledge requires both highly controlled lab studies and validation in field colonies, and requires an approach that can be readily and precisely used at both levels.

The development of genomic tools in honey bees has considerably facilitated the elucidation of the mechanisms involved in the host-parasite interactions and has provided important tools to diagnose and manage bee diseases (Grozinger and Robinson, 2015). Genomic studies allow a holistic approach, in which the physiology of the whole organism can be studied and monitored by assessing the profile of the transcriptome in response to different conditions or treatments. In addition to understanding how a stressor, such as parasitization by *Varroa* mites, may affect the bees' physiology (and ultimately behaviour and lifespan), transcriptomic analysis can also identify potential early markers of the stress response. This approach can provide insights into how bees respond to *Varroa* parasitization, thereby allowing us to develop new strategies to mitigate the impacts of the mites, and suggesting novel tools for monitoring stress conditions in the field, so that interventions can be inititated early, before significant damage has been caused.

However, the transcriptional profile of an organism is influenced by many factors, including age, environment (e.g. the social environment of the colony), the time of day or season, and other stressors (Griffiths, 2000). Thus, to understand the biological impact of mite infestation and identify robust, field-relevant markers of the *Varroa* mite-induced stress, it is necessary to evaluate the transcriptomic responses to mite parasitization under a variety of conditions and scenarios, and identify those transcriptional responses that are consistent across these.

Here, in order to deepen the knowledge on the biological effects of mite infestation on the honey bee *Apis mellifera*, we studied the transcriptomes of worker honey bees exposed to different mite infestation levels under laboratory or field conditions, in three separate experiments. These results were mined to identify molecular and physiological pathways impacted by *Varroa* infestation, and to identify biomarkers of severe *Varroa* infestations in field-collected bees. We subsequently validated a set of potential biomarker genes using honey bees collected from a different set of field colonies.

2. MATERIALS AND METHODS

2.1. Transcriptomic analyses

2.1.1. Experimental plan and biological material

Data were obtained from three different experiments (Fig. 1). Experiment 1 was conducted in an isolated area of the Italian Prealps characterized by the absence of other colonies nearby, while Experiments 2 and 3 were carried out under lab conditions with bees from the experimental apiary of the University of Udine. In all cases, bees were *Apis mellifera* ligustica x *Apis mellifera* carnica hybrids.

In Experiment 1, pools of ten bees (referred to as "colony bees") were sampled in October from colonies where the overall infestation level was either low (in colonies where acaricidal

treatments had been applied) or very high (in colonies where no acaricidal treatments were carried out).

In Experiment 2, 5th instar bee larvae were not infested or artificially infested with one or three reproductive mites and subsequently sampled at eclosion (these bees are referred to as "newly emerged bees"). Newly emerged bees were sampled individually.

In Experiment 3, 5th instar bee larvae were not infested or artificially infested with one reproductive mite, reared for two days after emergence in a cage (these bees are referred to as "adult bees"). Adult bees were sampled individually

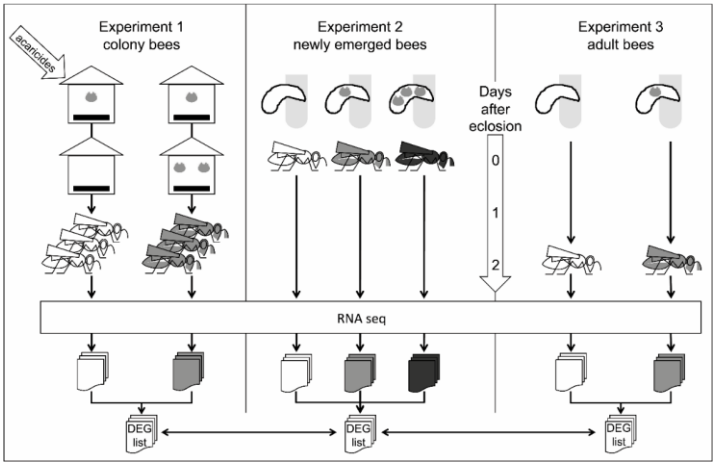

Fig. 1: experimental plan. The bees used for the transcriptomic analyses where both pools of bees from high and low mite infested colonies (experiment 1) and uninfested or mite infested individual bees, reared under lab conditions and sampled at eclosion (experiment 2) or after 2 days in cages (experiment 3).

Honey bees from high and low infested colonies (Experiment 1)

Bees used for Experiment 1 were collected during the course of a previous study carried out in 2012 (Nazzi et al., 2012). Two apiaries, made of six colonies each, were set up at the end of April in an isolated location (Porzus (UD), Italy; 46°11' N, 13°20' E), 1.6 km apart from each other. Queens were local and naturally inseminated; all hives had been treated the previous year with acaricides in order to have very low starting populations of the parasitic mite V. destructor at the beginning of the experiment. In one apiary, mite populations were kept under control during the experiment by treating the hives with prophylactic acaricides (the colonies of this apiary are referred to as "low-infested colonies"). A thymol-based product in

tablets was used from mid-August to mid-September, in presence of brood. In the other apiary, no acaricidal treatments were carried out (the colonies of this apiary are referred to as "high-infested colonies"). Note that sampling of bees from the experimental colonies was carried out a few weeks after the end of the summer treatment such that possible effect of the acaricides on the bees used for the analysis were minimized. In August, one hive in this apiary succumbed because the queen became drone layer, and was not used in further analyses.

We chose to use two separate apiaries to generate the high and low *Varroa* infestation colonies because *Varroa* mites can easily move between colonies within an apiary via adult bees drifting between colonies (Nolan and Delaplane, 2017). While there may have been limited location-specific differences between the apiaries, these were minimized by keeping the apiaries in the same geographic region (they were distant only 1.6 km) and treating the colonies in the same way.

Mite populations were monitored on a monthly basis in all colonies by assessing both the proportion of infested brood cells (n=100 cells) and the infestation of adult bees (n≈1000 bees). On the same dates both the number of brood cells and the number of adult bees were estimated; this allowed us to calculate the overall mite infestation (Nazzi et al., 2012). Samples of bees were collected in October when overall mite infestation was 35.7±13.1 and 260.1±117.9 mites per 1000 bees in the low and high-infested colonies respectively. Pools of ten bees were collected from the central area of each colony (where nurses are tipically located), transferred to the lab, and stored at -80 °C for subsequent analysis.

Infested and non infested newly emerged bees (Experiment 2)

Bee larvae were collected from brood cells that had been sealed in the preceding 15 hours and were placed into gelatine capsules (6.5 mm) at 35 °C, 75% R.H. with one, three or no mites previously collected from highly infested honeycomb frames, according to Nazzi and Milani (1994). Both bees and mites came from bee colonies maintained in 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). After 12 days, upon reaching the adult stage,

eclosing bees were sampled and stored at - 80 °C for subsequent analysis. Five bees from each experimental group were processed individually.

Infested and non infested adult bees (Experiment 3)

Non-infested and infested bees were prepared as in Experiment 2 but upon reaching the adult stage, after mite's removal, they were transferred into plastic cages maintained at 35 °C, 75% R.H. and provided with sugar candy and water *ad libitum*. After 48 hours, 4 bees from each experimental group were sampled and stored at -80 °C for subsequent RNAseq analysis.

RNAseq

Samples used for the analysis were transferred into liquid nitrogen and used for total RNA extraction by means of Tri-reagent (Ambion® Inc., TermoFisher™, Waltham, MA, USA). RNA was processed using the TruSeq mRNAseq sample prep kit (Illumina, Inc., CA, USA), starting from 2 μg of total RNA per sample. The standard mRNA sample prep from Illumina was used to produce 36 bp long tags, about 25-30 millions per sample. The sequencing reads were pre-processed by removing the adaptor sequences and low quality reads using Trimmomatic (Bolger et al., 2014). The remaining reads were aligned to the most recent honey bee genome build (Amel 4.5: Elsik et al., 2014), using Tophat2 (Trapnell et al., 2012) and annotated with the newest official gene set (OGS 3.2). Read counts for each gene were imported into R (http://www.r-project.org) for further analyses. Genes with fewer than 10 reads across all samples were removed from the analyses. The read counts were normalized using a trimmed mean of m-values (TMM) method. A generalized linear model (through the edgeR package in R (Robinson et al., 2010) was used to identify differentially expressed genes (DEGs) between the treatment groups for each of the three experiments. Genes with false discovery rate (FDR) < 0.05 were considered as differentially expressed between treatments. For each list of significantly differentially regulated genes, we conducted a gene ontology analysis to determine if any biological or functional categories of genes was significantly overrepresented using DAVID Bioinformatics Resources 6.7 (Huang et al., 2009a, 2009b). To

conduct this analysis, the *Drosophila melanogaster* orthologs of the DEGs were identified using BLAST (Altschul et al., 1990) with an e-value cutoff of 1x10-5.

2.1.2. Determination of viral infection level in the samples used in the transcriptomic analysis

To determine virus levels, each pre-processed sample was aligned to a panel of the most common honey bee viruses (Chen and Siede, 2007) using the protocol explained in the following paragraph. The read counts for each file were imported into R and normalized based on library size. A standard least squares ANOVA with a Tukey post hoc test was used to determine the significant changes in viral expression.

2.2. Validation of selected molecular markers (Experiment 4)

Experimental plan and biological material

Our results revealed that several genes related to immunity and stress response were significantly up-regulated in both colonies with high infestation levels (Experiment 1) and infested individual bees (Experiments 2-3), thus the expression of these genes may relate to a core stress response to *Varroa* mite parasitization. To determine if these expression changes were robust and could serve as potential biomarkers of mite-induced colony decline, we used quantitative real-time PCR (qRT-PCR) to assess expression levels in bees collected from a different set of eight colonies maintained in the same experimental apiary of the Dipartimento di Scienze AgroAlimentari, Ambientali e Animali of the University of Udine. These colonies were not treated with acaricides to observe the effects of an increasing mite infestation.

In this case, bees were collected from the same colonies in two different periods: June, when mite infestation is low and October, when the infestation reaches the maximum level under these environmental conditions. In particular, in June, mite infestation in the experimental

colonies, as assessed by counting the number of mites in 200-700 recently capped brood cells (0-15 hours before) in each of the eight colonies, was on average 0.24 mites/100 cells. In October, 298 and 353 brood cells were inspected in two of the eight colonies, resulting in 21.8 and 68.1 mites per 100 cells, respectively. Furthermore, 5 of the 8 colonies did not survive the winter, which is consistent with the impact of high *Varroa* infestation (Nazzi et al., 2012).

On planning this latter experiment, we decided to assess the effect of infestation by comparing bees from colonies at two times of the year to account for all possible confounding factors in testing the robustness of these biomarkers. In fact, in the first field experiment (Experiment 1) different colonies were sampled at the same time point so that any season effect could be excluded; however, possible colony and treatment effects could play a role. Instead, in the second field experiment (Experiment 4), we tested the response of bees from the same colony at low and high infestation levels as observed at two different time points thus excluding possible colony or treatment effect while accepting a possible season effect.

For the qRT-PCR study, pools of 10 bees were collected from the centre of each hive at each sampling period and stored at -80 °C. All bees used for the analysis were *Apis mellifera ligustica* x *Apis mellifera carnica* hybrids. For this study, we selected nine transcripts based on their biological function and the magnitude of the expression difference observed between high- and low-infested colonies in Experiment 1 (see Table 1 for a list of genes and primers). Additionally, we monitored expression of vitellogenin (*Vg*), which was differentially expressed in Experiments 2 and 3; furthermore, *Vg* has been observed to exhibit significant expression differences in previous studies comparing bees collected from surviving and non-surviving colonies in the field (Dolezal et al., 2016; Smart et al., 2016).

Primers were designed using Primer3 (Koressaar et al., 2007; Untergasser et al., 2007); *elf-S8* (Grozinger et al., 2003) and *GAPDH- 1* (Huang et al., 2012) were used as housekeeping genes. Molecular analysis was carried out at Pennsylvania State University (University Park, PA).

For each sample (eight samples from colonies with low infestation collected in June and eight samples from the same colonies with high mite infestation collected in October), the whole bodies of 10 individual bees were pooled and homogenized using a FastPrep® (Savant™TermoFisher™, Waltham, MA, USA) homogenizer. Total RNA was extracted from

each pool, according to the procedure provided with the RNeasy Plus mini kit (Qiagen®, Hilden, D, EU). The amount of RNA in each sample was quantified using a NanoDrop® spectrophotometer (ThermoFisher™ Scientific, Waltham, MA) and the quality was verified using a Bioanalyzer (Agilent Technologies, inc, Santa Clara, CA, USA). cDNA was synthesized using 150 ng of RNA and 1.5 μL of 10X buffer, 0.75 μL of 10 mM dNTP's, 0.75 of RNase inhibitor and 0.75 μL of RT ArrayScript (Ambion®, ThermoFisher™ Scientific, Waltham, MA), with water to reach 15 μL. Additional negative control samples, containing RNAase free water alone and "no RT enzyme", were included.

cDNA was synthesized by incubating the samples at 25 °C for 10 min, followed by 42 °C for 50 minutes and 70 °C for 10 minutes; samples were then maintained at 4 °C. Each sample was analyzed using qRT-PCR with the primers reported in Table 1. A master mix was prepared for each gene mixing 5 μL of SYBR® green dye (Ambion®, ThermoFisher™ Scientific, Waltham, MA), 1 μL of RNase-free water and 1 μL each of forward and reverse primers. 8 μL of master mix and 2 μL of cDNA were added into each well of an optical 384-well plate (three repetitions for each sample); negative control (H_2O) were included in each plate. Samples ran for 2 hours and 15 minutes on an ABI prism® 7900 sequence detector (Applied Biosystems™, Foster city, CA, USA).

Relative gene expression data were analysed using the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001); a Wilcoxon signed rank test was used for the statistical analysis of the normalized gene expression data.

Tab 1: Validated transcripts and associated primers

Gene	**Class**	**Sequence**
Hymenoptaecin (Aronstein et al., 2012)	Antimicrobial peptide	F: ATGGATTATATCCCGACTCG
		R: TCTAAATCCACCATTTATTCC
Apidaecin 73 like	Antimicrobial peptide	F: TTTGCCTTAGCAATTCTTGTTG
		R: GGTGGTCTTGGTTGTGGAAT
S. alvi complete genome	Gut symbiont bacteria	F: AATCATCCATGGCTTTTTGC
		R: CGCACCCCTTCAGTTATCAT
Zinc finger protein 717	Regulation of transcription	F: AAACCAGCAACAGGGTCAAC
		R: GTTTGTTGCCCGAATGAAGT
PGRP-S2	Toll pathway	F: GGAAGAGGATGGGACAAACA
		R: TGGACAAGAAGTGCGAGTTG
p450 *6AS10* (CYP6AS10)	Cuticle formation Stress response	F: TAATCGGAAGCTGCGTCTTT
		R: GCCTGACGAGGTTGTTCTTC
UNC 93	Toll pathway	F: GGAAGAAGCTGCCTTCTCAA
		R: TGGTTTTGATTCTGGAATTGG
Beta-1,2-grp2	Immune response	F: TGGATGGCAAAGAATTTGGT
		R: ATTGCAACATTGCCTTAGCC
eIF-S8 (Grozinger et al., 2003)	Housekeeping gene	F: TGAGTGTCTGCTATGGATTGCAA
		R: TCGCGGCTCGTGGTAAA
GAPHD-1 (Huang et al., 2012)	Housekeeping gene	F: GCTGGTTTCATCGATGGTTT
		R: ACGATTTCGACCACCGTAAC

3. RESULTS AND DISCUSSION

3.1.Transcriptomic analyses

3.1.1. Honey bees from high and low infested colonies (experiment 1)

RNAseq results provided information about the viral levels of the bees collected from high and low mite infested colonies, highlighting a higher proportion of DWV mapped reads in presence of high infestation levels (Fig. 2).

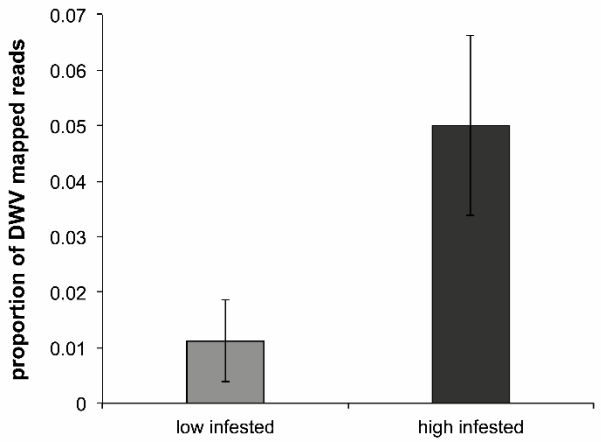

Fig.2: proportion of DWV mapped reads in bees collected from low and high infested colonies**.**

The comparison between samples collected from high- and low-infested colonies identified 174 significant ($p < 0.01$) differentially expressed genes (see Supplementary Material Table S1). Of these DEGs, 126 were up-regulated while 48 were down-regulated in high-infested colonies. Only 65 of the DEGs had *Drosophila* orthologs and were used for a gene ontology analysis; however, no significantly enriched ontology category was identified.

Many DEGs that were found to be significantly up-regulated in bees from high-infested colonies play a role in immune defence. The up-regulation of these genes could be due to the wounding of the cuticle by the *Varroa* feeding, secondary infections developing at this site, or the increased viral titers in *Varroa* infested bees that was confirmed here. These DEGs included two genes, GB51223 and GB51306, encoding antimicrobial peptides (AMPs): hymenoptaecin and apidaecin, respectively.

Up-regulation of AMPs is a common feature of the transcriptomic response of honey bees to various biotic stressors including gram positive and negative bacteria and fungi (Evans et al., 2006; Gregorc et al., 2012). Viral infection has also been found to modulate levels of AMPs in several studies, but the mechanism underlying this effect is very complex and requires further investigations (Danihlík et al., 2015).

One differentially expressed gene (GB47805) encodes for a peptidoglycan recognition protein that functions as pattern-recognition and effector molecule in innate immunity (Dziarski and Gupta, 2006), while another gene (GB45779) etraceyncodes for a UNC93-like protein, which is involved in the innate immune response by regulating toll-like receptors signalling (Kim et al., 2008). Finally, another DEG (GB40431) encodes a beta-1,3-glucan recognition protein that stimulates prophenoloxydase activation. Phenoloxidase is an enzyme promoting the melanisation of pathogens and damaged tissues in insects and other invertebrates: the conversion of the precursor in its active form is caused by various proteins including those involved in the recognition of the 1,3,-glucans of the microorganisms' cells' wall (Söderhäll and Cerenius, 1998). Phenoloxidase may also play a role in melanisation at the wound site.

Major royal jelly protein 9 and *yellow-e3* genes (GB55215 and GB55203, respectively) were both up-regulated in bees from high-infested colonies. Both are involved in the production of royal jelly, the larval food secreted from the hypopharyngeal glands of young workers. Major royal jelly 9 is also found in bee venom (Buttstedt et al., 2013) similarly to an acid phosphatase encoded by another gene (GB41302) that appeared to be up-regulated in high-infested colonies. Bees from high-infested colonies showed also a significant down-regulation of another venom component, *apamin* (GB40697), that is normally found on the cuticle because of the self-grooming activity displayed by bees and has recently been associated to a further level of social defence by Baracchi et al. (2011).

Variations in the expression level of these genes, according to mite infestation could be related to possible modifications in the self and allo-grooming behaviour in parasitized bees. Interestingly, high infestation levels were also associated with the significant up-regulation of two genes encoding for nicotinic acetylcholine receptors (GB53427, GB53428). Acetylcholine is a neuro-receptor involved in muscular contraction, and is also part of a

neural circuit regulating immunity (Olofsson et al., 2012). In the cholinergic anti-inflammatory pathway, acetylcholine binds to nicotinic acetylcholine receptor subunits, inhibiting the phosphorylation of NF-kB inhibitors and other signalling cascades; in mammals, this inhibition leads to the inhibition of pro-inflammatory cytokines release, regulating the magnitude of the host response to infection of injury (Tracey, 2009).

In honey bees, it has been shown that some neonicotinoid insecticides, agonists of nicotinic acetylcholine receptors, have the potential of disrupting the delicate balance of host-virus interactions orchestrated by NF-kB, resulting in viral amplification (Di Prisco et al., 2013).

An interesting transcript which was up-regulated in bees from high-infested colonies corresponds to the *Snodgrasella alvi* complete genome, a symbiotic gut bacterium. Previous studies investigating the composition of bee's core microbiota found that *A. mellifera* workers eclose without core resident bacteria and with a few bacteria of every kind (Gilliam, 1971; Martinson et al., 2012); only newly emerged bees exposed to the natural hive's conditions develop a stable microbial community by day 4 to 6. In our study, all the infested bees, from both the field and cage studies, showed a significant up-regulation of *S. alvi* genome in comparison with the uninfested ones (see data from Experiments 2 and 3 below). Therefore, *S. alvi* seems to be present in bees before the eclosion and the impact of *Varroa* on its levels appears to be independent of the pupal- or adult-environment. Further studies are necessary to elucidate the route of acquisition of this microorganism and its possible role in infested bees given recent evidence regarding the role of gut bacterial species in the defence against bee parasites experimentally demonstrated in *Bombus terrestris* (Koch et al., 2011) and already hypothesized in *A. mellifera* (Kaltenpoth and Engl, 2014).

Expression levels of three genes encoding cytochrome P450s were significantly increased in bees from high-infested colonies: 314A1 (GB45725), 6AS10 (GB48738) and 6AS10 (GB40285). Previous studies found up-regulated expression of cytochromes in bees from colonies challenged with biotic stressors and in collapsing colonies as well (Johnson et al., 2012). P450s play multiple metabolic roles and are important for their detoxification activity against natural and synthetic toxins (Feyereisen, 2005) but they also have unknown functions other than detoxification. The role of cytochrome p450 *6AS10* in bees exposed to high levels of

Varroa mite infestation is not clear and, in general, there is some uncertainty about the role of this gene in stress response (Morimoto et al., 2011). In this case, an up-regulation of this gene because of the use of acaricides can be excluded since bees from high-infested colonies were untreated.

The transcriptome of bees from high-infested colonies included other DEGs related to behavioural maturation. Previous studies showed that mite parasitization could cause behavioural modifications in infested bees with subsequent detrimental effects at the expense of the whole colony. For instance, infested bees are less active than uninfested nestmates and, in particular, display a limited brood caring activity (Annoscia et al., 2015). One of the DEGs identified in our study was Kruppel homolog- 1 (*Kr-h1* GB45427), a transcription factor involved in the juvenile hormone pathway in bees and other insects, and its expression is significantly higher in foragers than in nurse bees (Amsalem et al., 2015; Grozinger and Robinson, 2006). The up-regulation of *Kr-h1* in worker bees from high-infested colonies suggests that behavioural maturation of the bees in these colonies may be accelerated.

Furthermore, three genes encoding for proteins involved in circadian clock related behaviours (GB52078, period circadian protein: *per*; GB42798, circadian clock controlled protein; GB42704, takeout like protein) were significantly up-regulated in bees from high- versus low-infested colonies. Increased expression of per is associated with foraging behaviour (Bloch et al., 2001), again suggesting that the bees from the high-infested colonies may be more forager-like.

In order to test if the gene expression pattern observed in bees from high-infested colonies is consistent with the accelerated behavioural maturation hypotized above, we compared our deg list with a list of genes that are typically regulated according to bees' polyethism (Ament et al., 2011). As highlighted in Fig. 3, a notably higher number of overlapping reads was found when the transcriptome of bees from high-infested colonies was compared to that of foragers.

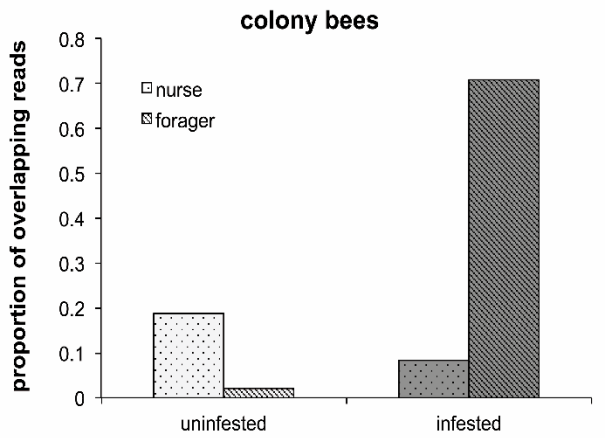

Fig.3: comparisons between high and low infested colonies' DEGs overlaps with foragers and nurses genes (Ament et al., 2011). The data above is represented as proportions of the genes from each group in the total number of overlapping genes for each experiment.

Many genes involved in metabolism were also significantly up-regulated in bees from high-infested versus low-infested colonies. These include genes involved in carbohydrate metabolism, protein modification by glycosylation, proteases and lipid metabolism.

Several genes associated with transport through the membranes and intracellular vesicle trafficking were also differentially expressed between the two groups; these genes encode for ion channels, ATP dependent transporters, cargo proteins, translocation apparatus and regulation of secretion in neural and endocrine cells.

Due to their peculiar tasks, foragers have different energetic demands and nutrition from nurse bees (Toth and Robinson, 2004); therefore, the observed differences in the expression of genes involved in metabolism could also be regarded as a side effect of the accelerated behavioural maturation commented above.

Genes involved in muscle activity and locomotion were also differentially expressed between bees from high- and low-infested colonies. These included: GB50137, which encodes for a molecular motor protein kinesin F, down-regulated in high-infested colonies; the aforementioned GB45779, which encodes for UNC93 protein involved in regulation or coordination of muscle contraction in *C. elegans*, up-regulated in presence of high infestation levels; GB45180, which encodes for myosin-1-like, an ATP dependent motor protein involved in muscle contraction, down-regulated in bees from high-infested colonies. The differential expression of these genes may be correlated with the previously observed effects of mite

infestation on the behaviour of infested bees which show a reduced activity within the colony (Annoscia et al., 2015). Altered expression of these genes may also result from the impairment of nutritional uptake associated to the parasitization or disease (Aronstein et al., 2012).

3.1.2. Infested and non infested newly emerged bees (Experiment 2)

DWV infection levels in newly emerged bees, as assessed from the number of reads mapping to viral genome, are reported in Fig. 4. Despite the high within-group variability, the infection level showed a general increase according to mite infestation.

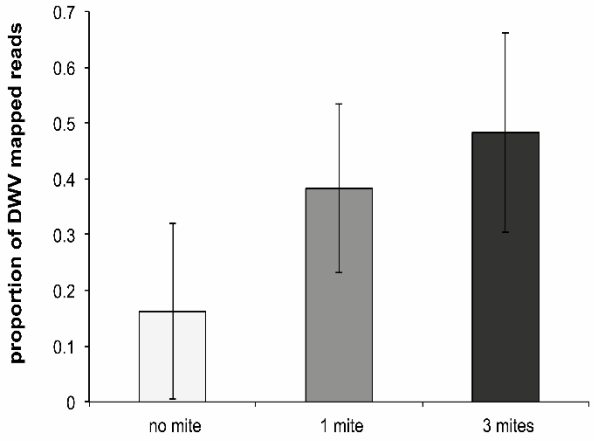

Fig 4: proportion of DWV mapped reads in newly emerged artificially infested bees.

The analysis of the transcriptome of newly emerged bees, non-infested or infested with 1 or 3 mites, identified 254 genes (Table S1) significantly differentially expressed across the three treatment groups ($p < 0.01$). Ninety-three DEGs among the three treatment groups had Drosophila orthologs and were used for a gene ontology analysis but there were no significantly overrepresented gene ontology categories.

As in the Experiment 1, *Varroa* parasitization influenced the expression pattern of several genes involved in immunity and behavioural maturation. These included a beta 1.3-glucan recognition protein 1 (GB42815) and a peptidoglycan recognition protein (GB47805) that are both possible indicators of bacterial infections and were down-regulated in infested bees.

Mite infestation also affected the expression of vitellogenin gene (GB49544) which plays a central role in honey bee worker behavioural maturation, in that levels of vitellogenin are higher in nurses versus foragers, and decreasing levels accelerate behavioural maturation of workers (Amdam et al., 2011; Nelson et al., 2007). Vitellogenin also plays a significant role in immunity as a zinc transporter (Amdam et al., 2004a). Notable, in newly emerged bees of Experiment 2, vitellogenin was down-regulated in case of mite infestation, further suggesting that these bees may be experiencing an accelerated behavioural maturation.

The comparison between differentially expressed genes in newly emerged bees according to mite infestation and genes that are regulated in forager bees showed only a limited overlap, possibly due to the very young age of the bees used in this experiment (Fig. 5). On the other hand, as expected, there was a high overlap between genes regulated in uninfested bees and those regulated in nurses.

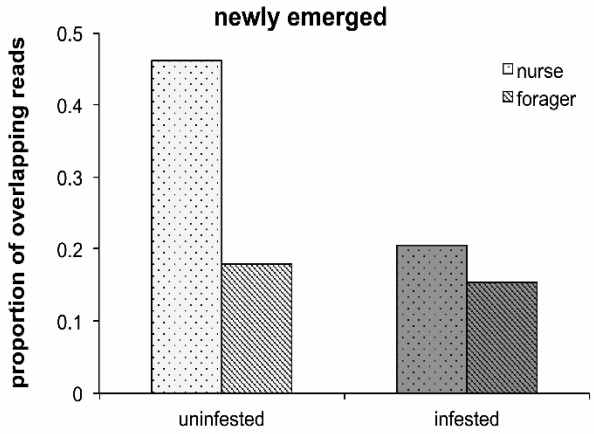

Fig.5: comparisons between infested and uninfested newly emerged bees DEGs overlaps with forager genes and nurse genes (Ament et al., 2011). The data above is represented as proportions of the genes from each group in the total number of overlapping genes for each experiment.

3.1.3. Infested and non infested adult bees (experiment 3)

In the adult uninfested bees, the proportion of mapped reads related to DWV was almost null (0.1%) while 45% of reads mapped to the DWV genome in the infested individuals (Fig.6).

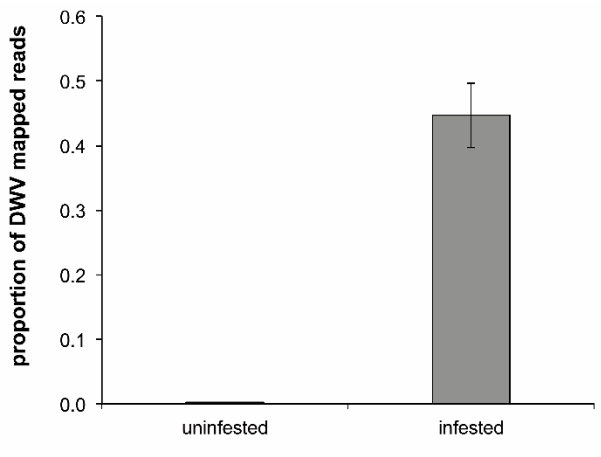

Fig.6: proportion of DWV mapped reads in adult uninfested and infested bees.

Transcriptomic analysis of adult (2 days old) artificially infested bees revealed significant differences in the expression level of 1333 genes (Table S1) according to the infestation. In total, 292 genes were down-regulated while 1041 were up-regulated in *Varroa* infested versus non-infested bees.

Only 355 DEGs had Drosophila orthologs and were used for the gene ontology analysis. Some ontology categories were found to be enriched ($p < 0.05$) only in the case of genes up-regulated as a consequence of mite infestation, including DNA binding and transcriptional regulation, neuron development and differentiation, cell and cell parts morphogenesis.

As in Experiment 1, several DEGs play a role in immune function (Evans et al., 2006; Ryabov et al., 2014). A deeper analysis of the immunity related genes showed how the infestation by mites, or the concurrent viral infection, affects the expression of Dorsal-2 (GB42472), a transcription factor of the NF-kB family playing a central role in the Toll pathway (Evans et al., 2006).

Interestingly, in infested bees, the down-regulation of dorsal was paired with the up-regulation of cactus (GB44055), a NF-kB inhibitor whose signal dependent degradation is needed for dorsal activation and the subsequent transcription of several antimicrobial peptides (Evans et al., 2006).

According to Nazzi et al. (2012), dorsal plays a critical role in antiviral defence in bees such that its down-regulation is linked to increased DWV replication. The role of this gene in antiviral defence has been supported by a study showing that a treatment with the

neonicotinoid insecticide Clothianidin, that indirectly targets the same gene, results in DWV viral burst (Di Prisco et al., 2013). Actually the role of dorsal in antiviral defence seems not to be restricted to bees, since it has been shown that the Toll-Dorsal Pathway is required for resistance to viral oral infection in the fruit fly as well (Ferreira et al., 2014). The observed effect on dorsal appears to be particularly interesting in view of the increased viral infection in mite infested bees confirmed in this study.

Moreover, *Varroa* infestation was associated to the up-regulation of a few antimicrobial peptides similarly to what observed in bees from high-infested colonies in Experiment 1: in particular, hymenoptaecin (GB51223), abaecin (GB47318) and apidaecin (GB47546).

Additionally, the peptidoglycan recognition protein S3, another member of the Toll immunity pathway, was down-regulated in infested bees.

Also argonaute 3 (GB49909), encoding for a piwi protein involved in the transposons silencing (Brenneche et al., 2007), and a RISC loading complex (GB47214; Brutscher et al., 2015) were differentially expressed in infested bees, suggesting an involvement of RNA interference, which is interesting in view of the role of this further pathway for antiviral response and the well known mite induced viral replication that has been confirmed here.

The down-regulation of a nicotinic acetylcholine receptor appears interesting for the already mentioned interplay between immunity and nervous system (Olofsson et al., 2012). As regards the components of bee venom with a potential role in social immunity previously discussed (Baracchi et al., 2011), *Varroa* infestation causes the down-regulation of two genes: a venom carboxylesterase-6- like (GB52052) and a venom acid phosphatase (GB41338).

A large group of genes, differentially expressed according to infestation, are involved in the nervous system functioning in response to olfactory stimuli (odorant and gustatory receptors) and bio ammines stimulation (octopamine receptor 2 (GB43263) and D2-like dopamine receptor (GB42577)). This could account for the behavioural modifications, observed under natural conditions, in nurse bees infested at the pupal stage (Annoscia et al., 2015); in particular, genes involved in biogenic amines modulation could be regulated in response to chronic stressors accelerating the onset of foraging in the honey bees (Even et al., 2012).

Finally, as for the regulation of genes related to energetic pathways, we observed an up-regulation, in presence of *Varroa*, of an insulin gene enhancer protein (GB45757). This gene is part of the Insulin/insulin-like growth factor signalling pathway (IIS), a nutritional pathway, immunity related, involved in the regulation of worker division of labour (Ament et al., 2008). *Varroa* infestation caused the up-regulation of another nutritional related gene, namely Malvolio (*Mvl*, GB54097). Malvolio is a manganese transmembrane transporter involved in sucrose responsiveness whose up-regulation in case of *Varroa* mite infestation was previously observed (Alaux et al., 2011). Malvolio appears also to influence behavioural maturation and seems to be involved in a precocious foraging (Ben-Shahar et al., 2004).

As with the Experiments 1 and 2, a comparison between genes differentially expressed according to mite infestation and genes associated with nursing and foraging behaviour was carried out. The obtained picture (Fig. 7) matches quite well that obtained with newly emerged bees, likely for the reasons already discussed.

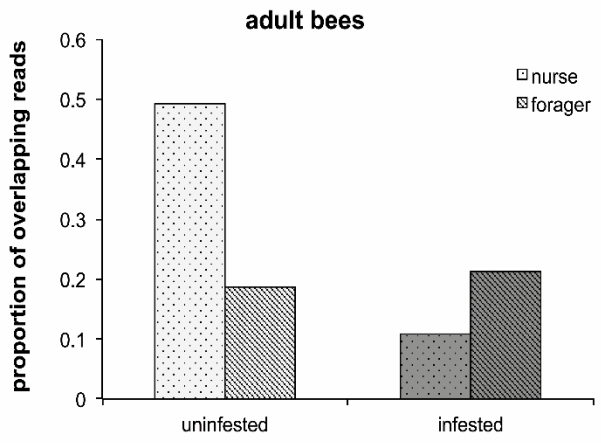

Fig.7: comparisons between infested and uninfested adult bees DEG's overlaps with forager genes and nurse genes (Ament et al., 2011). The data above is represented as proportions of the genes from each group in the total number of overlapping genes for each experiment.

One gene related to lipid metabolism was up-regulated in infested caged bees (GB50321, a lipid A export ATP binding/permease protein), while two genes were down-regulated, a phospholipase B1 (GB42475) and a pancreatic triacylglycerol lipase like (GB43510). Furthermore, the down-regulation of a fatty acid amide hydrolase (GB8850) and the up-regulation of acyl-CoA Delta desaturase like (GB40659) were observed in infested bees. Lipid

metabolism is involved in behavioural maturation (Toth and Robinson, 2005); old bees show an increase of lipid loss during the shift from nurses to foragers (Ament et al., 2011) according to a different resources demand.

A difference in the expression of genes related to lipid metabolism in bees challenged with *Varroa* seems to further support a mite effect on the nutritional pathways associated with the behavioural maturation.

3.1.4. Intersections between the three lists

Two transcripts were up-regulated in all experiments (Fig. 8): GB45601, corresponding to the *S. alvi* genome and GB47064, a zinc finger protein 717-like. Twenty-six genes were up-regulated both in bees from high-infested colonies and infested individual adult bees while 18 up-regulated genes were in common in both datasets corresponding to lab bees (Experiments 2 and 3). Only one gene was up-regulated in both colony and newly emerged bees.

No genes were down-regulated in presence of *Varroa* in all the experiments; 4 genes (Fig. 9), including three isoforms of the same gene, were down-regulated in both the colony bees and adults. Lab bees (newly emerged and adult bees) had two down-regulated genes in common (Fig. 9).

No genes were up or down-regulated both in newly emerged and adult infested bees suggesting a notable difference in the effects of parasitization on the bees' transcriptome according to the age which is not surprising in view of the fact that newly emerged bees were sampled immediately after eclosion, before they had access to food, social interactions and other environmental factors that certainly affect gene expression.

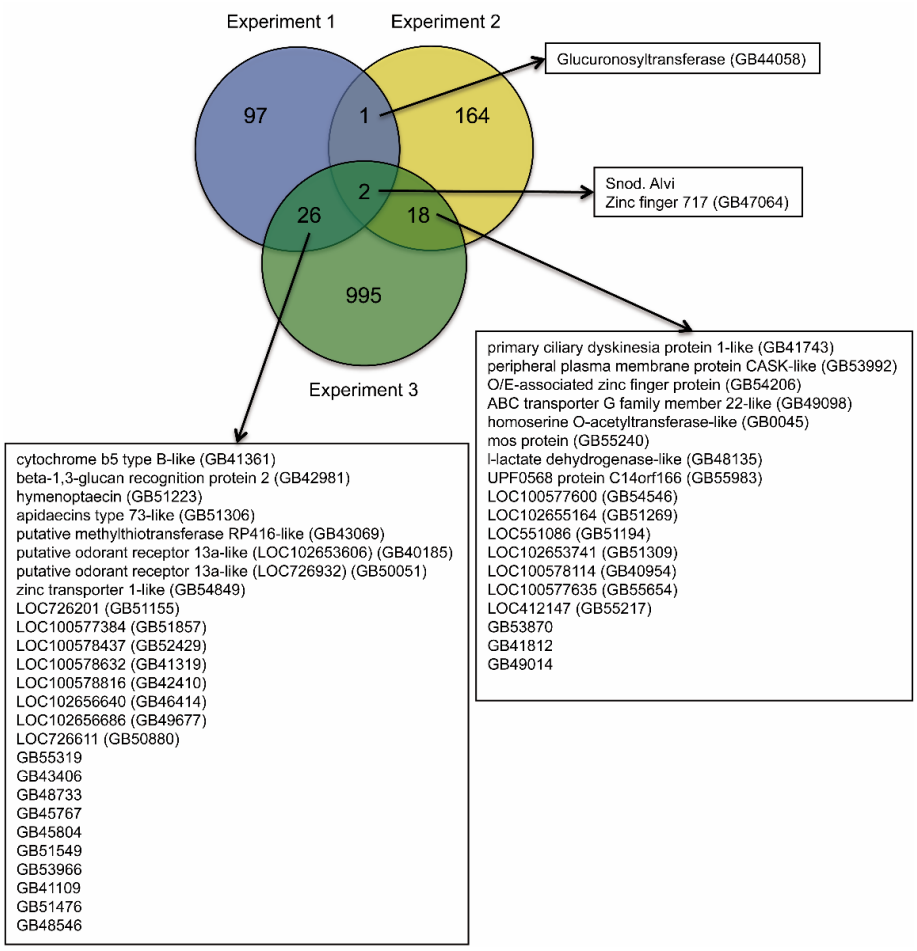

Fig.8: overlapping of the up-regulated genes among the three experiments (Experiment 1= colony bees, Experiment 2= newly emerged bees, Experiment 3= adult bees) (Venny[2.1] Oliveros, 2007-2015).

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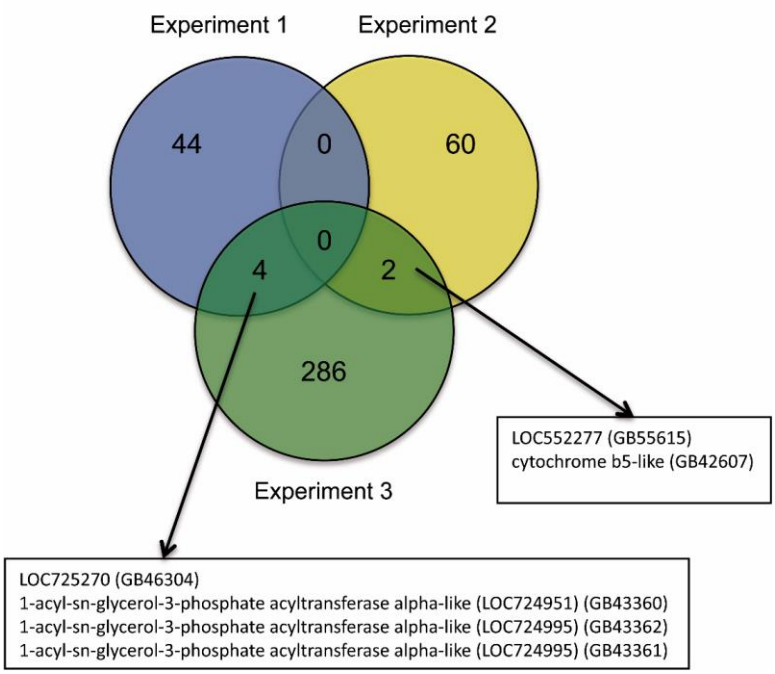

Fig.9: overlapping of the down-regulated genes among the three experiments (Experiment 1= colony bees, Experiment 2= newly emerged bees, Experiment 3= adult bees) (Venny[2.1] Oliveros, 2007-2015).

3.2. Confirmation of the expression pattern of some genes and their possible use as biomarkers of *Varroa* induced stress.

Several genes were found to be differentially expressed across the experiments, which suggested they could serve as biomarkers of *Varroa*-induced stress on worker bees. In particular, we focused on the following genes: hymenoptaecin, *PGRP-S2*, *UNC93*, *S. alvi*, *apid 73*, zinc finger 717-like, *Beta-1,2-grp2*, cytochrome p450 *6AS10*, vitellogenin.

In order to test the suitability of these genes as potential biomarkers of mite induced stress, in Experiment 4 we analysed their relative expression, using qRT-PCR, in bees collected from a new set of colonies. In particular, we compared their expression in bees sampled from eight colonies in June, when the infestation level is still low, and October, when the number of infesting mite approaches its maximum under the environmental conditions where the experiment was carried out and the mite induced decline of bee colonies becomes apparent (Nazzi et al., 2012).

Five of the candidate genes (i.e. hymenoptaecin, *PGRP- S2*, *UNC93*, p450 *6AS10* and the glucan recognition protein) displayed an expression pattern consistent with the results obtained from the transcriptomic analysis, although significance was not reached. Among the candidate genes, two (antimicrobial peptide apidaecin and zinc finger protein 717) showed a trend opposite to that observed in the samples used for the transcriptome analysis. Unfortunately, *S. alvi* showed an abnormal amplification curve and was discarded. Vitellogenin levels were significantly lower ($p < 0.05$) in bees collected from high-infested colonies compared to the low-infested ones (Fig. 10).

Vitellogenin is associated with a variety of functions in adult worker bees (Page and Amdam, 2007). Levels are higher in nurse bees if compared with forager bees, and vitellogenin serve as a storage protein that can be used by nurses to produce brood food (Amdam et al., 2003). Additionally, high vitellogenin titers keep juvenile hormone levels low, thereby slowing behavioural maturation to foragers and potentially initiating the production of long-lived winter bees (Doke et al., 2015; Fluri et al., 1982). Previous studies have found that parasitization with *Varroa* produced workers that accumulate significantly fewer

haemolymph proteins, including vitellogenin (Amdam et al., 2004b). Other studies found that vitellogenin served as good predictive marker of colony health status and winter survival (Dainat et al., 2012; Smart et al., 2016).

In our transcriptomic study, vitellogenin was not significantly differentially expressed between controls and infested samples in Experiment 1, but it was significantly up-regulated in the infested adults (Experiment 3) and down-regulated in newly emerged artificially infested bees (Experiment 2) and in bees collected from infested colonies for the qRT- PCR analysis. Notably, these colonies were sampled in October: at this stage, the colonies should have produced winter bees with high levels of vitellogenin (Doke et al., 2015). The fact that levels were low as compared to bees collected from the same colonies in June further suggests that *Varroa* parasitization had a significant impact.

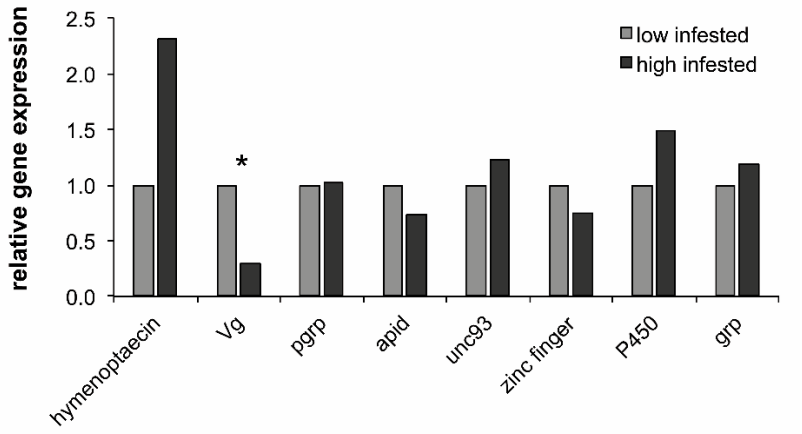

Fig. 10: relative gene expression of the possible infestation's markers (analysis performed on pools of 10 workers bees collected from the centre of eight hives in June, when the infestation level is still low and October when it's higher. Statistics: Wilcoxon signed rank test.).

4. CONCLUSIONS

The possible effects of mite infestation at the genomic level are complex and involve a large number of different biological processes and pathways. Overall, bees exposed to high infestation pressures, both under field and lab conditions, showed an altered expression of genes involved in immunity and response to stress, behaviour (particularly behavioural maturation), nervous system functioning and energetic metabolism (Fig. 11). In particular,

we observed significant effects on the canonical immune pathways, primarily Toll, and a generalized impact on AMPs level. This reaction, previously observed in other studies, is likely related to secondary infections triggered by the mite.

The differential expression of genes such as RISC suggests that viruses are also involved, although their effects on the immune system in honey bees have not been described in detail so far (Galbraith et al., 2015).

Parasitization by *Varroa* mites affects the expression of several genes involved in behavioural maturation and nervous system. We observed that infestation, probably indirectly through the virus, modifies the expression of genes involved in the response to stimuli, locomotion and circadian clock behaviour. Interestingly, supposedly nurse bees collected from the centre of high-infested colonies during times of active foraging showed gene expression patterns that are typical of older bees. This phenomenon confirms an accelerated behavioural maturation upon parasitization already observed (Downey et al., 2000) and well matches the lower nursing activity of infested bees reported by Annoscia et al. (2015).

These findings are also supported by an other study that highlighted an overlap between nurses and foragers' chemical profile according with the mite abundance increase (Cervo et al., 2014).

The observed effect of parasitization on the bee's energetic metabolism in newly emerged and young adult bees was somewhat predicted, since the mite removes substantial amounts of haemolymph - and the nutrients that it carries - as it feeds on the pupal stages of the bees. In the colony, a high infestation level (as in Experiment 1), could result in impaired metabolic processes of individual bees that were parasitized as pupae, or a reduced population size and a related reduction in the capacity to collect sufficient stores; this, in turn, causes suboptimal nutritional conditions affecting metabolism.

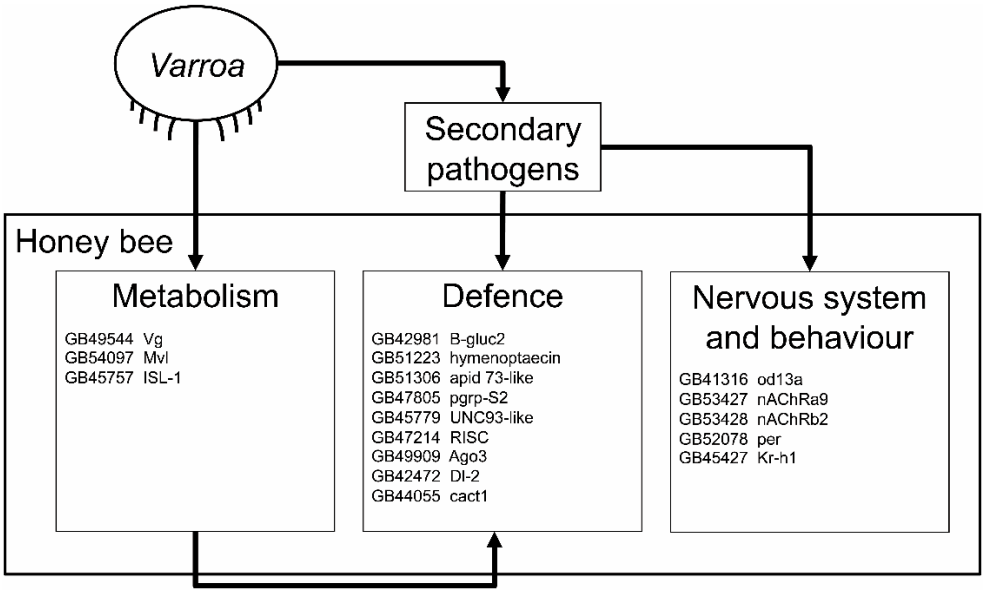

Fig.11: direct and indirect pressures from *Varroa* mite on bee's physiology.

To our knowledge, this study is the first to assess the effect of mite infestation in a number of different situations, both under field and lab conditions. This experimental plan helped to minimize the effect of possible confounding factors allowing the study of the most likely biological effects of the parasite on its host. Some notable effects of infestation on bee's transcriptome were confirmed and, in particular, the impact on immunity, nervous system, behavioural maturation and energetic pathways. We suggest that the observed effects on nervous systems are likely caused by secondary pathogens facilitated by the mite and so are most effects on immunity. On the other hand, a direct effect on the nutritional balance, caused by haemolymph subtraction was noted which could, in turn, indirectly affect immunity. Some interesting differences in gene expression between infested newly emerged and older adult bees maintained under lab conditions were noted suggesting an important effect of age on the observed patterns.

Our transcriptomic study and the subsequent field validation highlighted five genes that are good candidates as markers of infestation: *PGRP-2*, p450 *6AS10*, hymenoptaecin, the glucan recognition protein and *UNC93*. Furthermore, the reliability of vitellogenin, a yolk protein previously identified as a good marker of colonies' survival that is involved in caste division of labour, immunity response and development was confirmed here.

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Chapter 3: **how does *Varroa* mite affect honey bees' behaviour? Mechanisms underlaying reduced nursing in infested bees**

Ph: Pietro Zandigiacomo

1. INTRODUCTION

Parasites make up more than half of the living species of plants and animals on earth and play an essential role in molding natural communities as well as the evolution and behavior of the host species (Price, 1980). Parasites can modify the host's behavior to improve their reproductive potential and survival (Thomas et al., 2005; Hughes et al., 2012) but modification can also be a simple side effect of parasitization. The parasites' ability to modify host's behavior can have very complex effects in social insects as the honey bees, characterized by an organization based on an age related division of labor of the working caste (Schneider, 2015) whereby sterile workers perform a range of tasks, including cell cleaning, brood and queen caring, comb construction, colony defense and foraging, according to the age (Johnson, 2010). In general, the temporal polyethism schedule involves the younger individuals performing various tasks inside the colony and the older workers carrying out colony defense and foraging (Winston, 1987). Normally, newly emerged bees, younger than four days, are involved in cell cleaning whereas, between four and twelve days, when brood food glands in the head are functional (Gary, 2015), workers become nurses.

From the twelfth day of age to the twenty-first, workers are involved in the nest maintenance and food storage and, finally, when older than 21, become foragers (Johnson, 2008).

The behavioral transitions described above is associated with profound physiological changes (Schneider, 2015). A fundamental role in this process is played by the juvenile hormone, whose hemolymph's levels are low in newly emerged and young bees but increases with age, reaching a maximum in correspondence of the transition to foraging (Elekonich et al., 2001).

This complex system appears to be rather sensitive to perturbations and stressors that can alter the normal schedule with severe consequences on colony homeostasis (Bordier et al., 2016).

The parasitic mite *Varroa destructor* is regarded as the most harmful biotic stressor for honey bees (Rosenkranz et al., 2010) and is now accepted that it plays a fundamental role in the decline of honey bees' colonies observed in the last decade all over the northern hemisphere (Neumann and Carreck, 2010).

Mite infestation, in fact, reduces the lifespan of honey bees through several detrimental effects on bee's physiology, by reducing weight and water content (Annoscia et al., 2012), metabolism, vitellogenin titer (McDonnell et al., 2013) and response to stimuli (Iqbal and Mueller, 2007). In addition, the mite causes modifications to the polyethism schedule of worker bees' accelerating the shift to foraging (Downey et al., 2000).

Since the parasite can transmit and facilitate the replication of the deformed wing virus (Nazzi et al., 2012), it is particularly challenging to dissect the role of the mite and the virus in the observed effects. However, since, in practice, the two parasites are closely associated (Di Prisco et al., 2016) this has a limited importance.

Previous studies highlighted the effcts of parasites such as *Nosema ceranae* on temporal polyethism (Natsopoulou et al., 2015) and the potentially alterations of the in-hive activities associated to *Varroa* mite infestation (Annoscia et al., 2015). In particular, a modification in nursing behavior was highlighted by Annoscia et al. (2015) who observed a reduction in brood caring activity in the infested nurses.

Under normal conditions, nursing is a "multi-step" process (fig.1). Bee larvae release a brood pheromone, including e-beta ocimene (Maisonasse et al., 2009) and a blend of methyl esters of fatty acids (Le Conte et al., 1994), that stimulates larval food production, by means of the hypopharyngeal glands located in the worker's head, and brood caring (Le Conte et al., 2001). On reaching the twelfth day of age, changes associated with juvenile hormone titer causes the transition to other within hive tasks (Robinson, 1987). Therefore, the observed effect of the *Varroa* mite on nursing (Annoscia et al., 2015) could be the results of the perturbation of different aspects of this process and a comprehensive understanding of the phenomenon requires the investigation of the influence of the mite on any of these aspects. Unfortunately, the effects of stressors on honey bees task-related behavior have been largely studied at genomic level (e.g. expression of genes encoding for vitellogenin and JH esterase) whereas physiological as well as behavioral studies are comparatively rare.

We are interested in understanding how infestation affects the behavior of the in-hive bees and test if the possible modifications could be linked to a precocious behavioral maturation highlighted by previous studies assessing gene expression pattern. To do so, we first monitored the in-hive activity of artificially infested bees to confirm previous observations on the influence of the mite on the nursing activity of bees, then

- we tested the response of artificially infested nurse bees, collected from the hive or maintained under lab conditions, to the brood pheromone;
- we measured the hypopharyngeal gland's size in artificially infested nurse bees reared under lab and field conditions;
- we quantified the juvenile hormone concentration in artificially infested nurses reared under lab and field conditions.

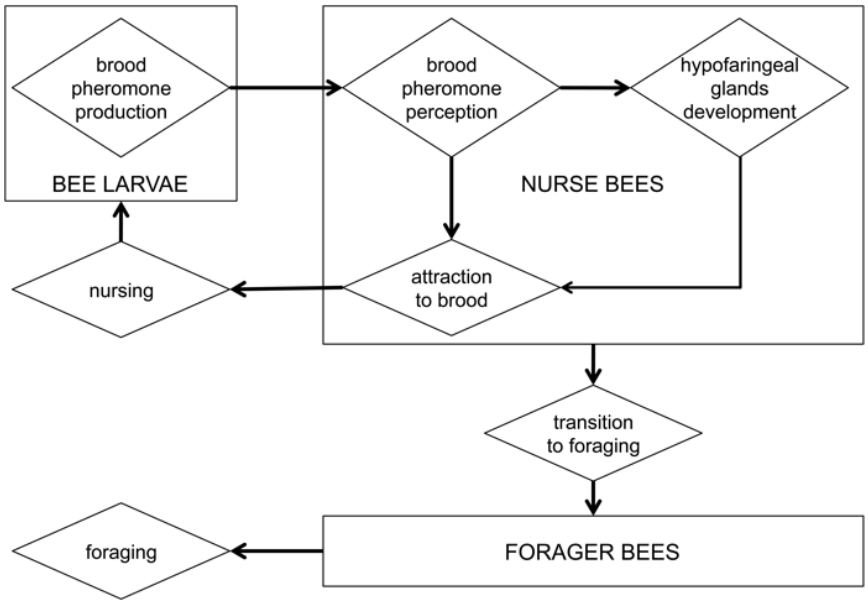

Fig.1: nursing as a multistep process and possible interferences caused by *Varroa destructor* mite

2. MATERIALS AND METHODS

2.1. Biological material used in the lab experiments

Honey bees and *Varroa* mites used for the lab experiments were collected from the experimental apiary of the Department of Scienze AgroAlimentari, Ambientali e Animali of the University of Udine (Via delle Scienze 206, Udine, Italy 46°04'54.2"N+13°12'34.2"E).

Previous studies indicated that local colonies are hybrids between *A.mellifera ligustica* Spinola and *A.mellifera carnica* Pollmann (Comparini and Biasolo, 1991; Nazzi, 1992).

Bees were artificially infested with one mite at the 5th instar larval stage (L5), the last stage before pupation. L5 larvae were collected from honeycomb frames containing brood that had been sealed by workers in the preceding 15 hours and infested with mites collected from the same combs (Nazzi and Milani, 1994). An equivalent number of larvae was maintained uninfested as control.

Infested and control larvae were inserted into gelatin capsules (Agar Scientific Ltd, UK. 6.5 mm i.d.) and maintained for 12 days into a climatic chamber at 34°C, 75% RH, in the dark.

Immediately after the eclosion, newly emerged bees were caged, put again into the climatic chamber and fed with sugar candy, pollen and water ab libitum.

Seven infested bees and an equivalent number of control bees were collected 7 days after the eclosion, upon the reaching of the nursing age, in order to carry out the brood pheromone response test followed by the hemolymph collection and the hypopharyngeal glands' measurement.

Lab experiments were repeated three times.

2.2. Biological material for the field experiments

Bees used for the field experiment were collected from the same experimental apiary; the infestation and rearing procedures were the same as described above. Two days after the eclosion, bees were marked with a numbered tag (2 mm diameter).

At the third day of age, bees were introduced in the observation hive; observations started on day four.

The procedure was repeated four times in the period from June to October using more bees possible each time (see also tab. 1).

2.3. Observation of the behavior of infested bees under field conditions

In order to observe the activity of infested and control bees, marked individuals were introduced into an observation hive housing a single nest comb and a honey comb (Dadants-Blatt size) separated by a queen excluder Glass walls on both sides of the observation hive, normally obscured with wooden panels, allowed the direct observation of the bees' activities. The observation hive hosted a small colony (about 4.500 individuals) established in the same year of the experiment. The marked bees' activities in the observation hive were monitored daily, at the same time of the day, for 20 minutes for each side of the comb.

At the age of seven days, bees were sampled to carry out the test in the olfactometer, the hypopharyngeal glands' measurement and the hemolymph collection.

We observed the following activities (Annoscia et al., 2015):

- **Brood care**: bees were observed inside a cell containing a larva or an egg;
- **Storage**: bees were observed inside a cell containing honey or pollen;
- **Ventilation**: bees were observed fanning the wings;
- **Trophallaxis**: bees were observed touching with the proboscis that of another bee;
- **Dance**: bees were observed doing waggle or round dances to indicate the direction of a source of food;
- **Pollen collection**: bees had a charge of pollen in the pollen basket;
- **Movement**: bees were observed moving around without performing any of the above mentioned activities;
- **Still**: bees were resting.

The observations obtained were divided in two groups according to the bees' age:

- newly emerged bees, normally cell cleaners, less than 5 days old (observed the same day of the introduction into the hive);
- nurses, 5-12 day old (Natrsopoulou et al., 2015).

The proportion of bees involved in different activities was compared with a Cochran-Mantel-Haenszel test.

The acceptance rate of infested and uninfested bees into the observation hive was calculated as number of bees saw at least once during the days next the introduction into the hive and tested using Cochran-Mantel-Haenszel test. The experiment was repeated four times.

2.4. Response to the brood pheromone

A four armed (Vet et al., 1983) olfactometer (24 cm of diameter) was used to test the response to brood pheromone in infested and uninfested bees reared under lab and field conditions. The treated arm was connected to a test tube containing three living 3rd instar worker larvae and one strip of wet filter paper to avoid the dehydration of the larvae; the other three arms (controls) were connected with test tubes containing only the wet strip of filter paper.

The bioassay was performed at environmental temperature (25 °C) under a cool daylight lamp (1040 lumen) with a constant air flow (flow=100 ml/min through each arm).

Seven infested nurses (7-8 day old) bees and the same number of control bees were observed for 10 minutes and the brood pheromone response was calculated as the time spent into the treaded arm by means of a dedicated software (Olfa. Nazzi, 1996)

The time spent into the treated arm by infested and control bees was compared by means of a T Student paired test (comparison between time spent by the same bee into the treated and control arms).

The test the suitability of the bioassay, we first assessed if there was a clear difference in the response of field collected nurses and foragers in the same bioassay. Field collected nurse and foragers were also used to test the analytical methods used for the study of JHIII concentration in bees.

For this purpose, nurse and forager bees were collected from a hive of the experimental apiary of the University of Udine (see above) during the first week of August 2016, a year characterized by a very low level of infestation in the summer. We classified as foragers, bees bearing a pollen load in the back legs while nurses were identified as such when observed leaning into a comb cell containing a bee larva.

Seven foragers and nurses were collected and used to test the differences in the brood pheromone response by means of the four armed olfactometer.

After the bioassay, the hemolymph of the bees was collected for juvenile hormone quantification. The experiment was repeated twice.

Then the bioassay was used to compare the response of mite infested and uninfested bees maintained under lab and field bees. The experiment was repeated three times both with lab and field bees.

2.5. Hemolymph collection and processing for JHIII quantification

For the extraction of hemolymph we centrifuged (15 minutes, 3000 RPM) bees which had the mouth and anus plugged with a cap of wax and cut legs, antennae and wings.

The hemolymph, collected from pools of five bees in order to obtain a significant amount of material (at least 40 ul per experimental group), was weighed and transferred into a clean glass tube with 1,5 ml of methanol then the juvenile hormone was extracted, isolated and derivatizated for the GC-MS analysis according to Munyiri and Ishikawa, 2004.

500 ng of Farnesol (Sigma Aldrich Co, Missouri, USA) were added to the sample before derivatization as an external standard for the quantification of JHIII.

The extraction of the hormone was performed adding 1,5 ml of 2% NaCl solution to the sample and 0.5 ml of hexane for three times. Purification of the hormone was conducted using a Pasteur pipette packed with 1 g of aluminum oxide, activity grade III previously conditioned with hexane (Munyiri and Ishikawa, 2004). The extract was loaded and washed with 2 ml of 10% ether in hexane, eluted in 2 ml of 30% ether in hexane and dried under a stream of nitrogen.

The JHIII was derivatized to its methoxyhdrine in 50 ul of deuterated methanol (Sigma Aldrich Co, Missouri, USA) and 1 ul of trifluoroacetic acid at 60°C for 15 min. Derivatized JHIII was loaded on the new aluminum oxide column, washed with 30% ether in hexane and eluted with 2 ml of 50% ethyl acetate in hexane.

The solution was concentrated to about 10 ul under a stream of nitrogen and 1 ul aliquot was subjected to GC/MS analysis (Method by Okuda et al.1996, as modified by Munyiri and Ishikawa, 2004).

2.6. JHIII detection and quantification by means of gas chromatograph-mass spectrum

The quantification of juvenile hormone into the processed hemolymph was performed by means of GC-MS system with a heated gold quadrupole (Agilent 5977E MSD, Agilent

Technologies, California, USA) equipped with a Capillary Column VF-5ms (30 m x 0.25 mm x 0.25 um), 5 % phenil 95% dimethylsiloxane (Laufer et al., 2013).

The oven temperature was programmed linearly from 90°C (held for 2 minutes) to 250 °C (held for 8 minutes) at a rate of 10°C/min, the total run time was 26 minutes. Column pressure was 12.8 psi; helium was used as carrier gas at a flow of 1.2 ml/min at a split ratio of 10:1.

Injection temperature was 250 °C. Spectra were obtained under 70 eV.

To assess the response factors and chromatographic behaviour, we injected different concentrations of derivatized commercial JHIII (Sigma Aldrich®, USA) and Farnesol (Sigma Aldrich®, USA) together with standard hydrocarbons (from C8 to C30).

All the runs were carried out in SIM and SCAN with a solvent delay equal to 3.5 minutes and gain factor 10.

The injection of the sample was performed by means of an automatic liquid sampler.

Each sample was analyzed three times.

The quantitative analysis was based on the ion intensity of the diagnostic Ions (m/z=76 and 225; Teal et al., 2000) in SIM mode (Selected Ions monitoring).

Chromatogram analysis was carried out using Agilent MassHunter Workstation software (Agilent Technologies Inc. September 2011).

2.7. Hypopharyngeal glands size

After hemolymph extraction, the hypopharyngeal glands were dissected from the bees' heads following the Coloss BeeBook protocol (Chapter 3.3.2.1. The glands). Glands were then put on a microscopy slide and, observed by means of an optical microscope (magnification 25x) and photographed with a Nikon® D7000 camera.

The microscope pictures of the gland collected from five infested and as many uninfested bees were analyzed by means of Image J software (Rasband, 1997) and the area of 10 acini per gland was calculated when possible. The areas of the acini from infested and control bees were compared performing an unpaired T test analysis.

The experiment was repeated three times both with lab and field bees.

3. RESULTS

3.1. Influence of mite infestation on the behaviour of bees under field conditions

3.1.1. Acceptance of bees in the observation hive

The number of bees introduced into the observation hive for each replication of the experiment is reported in tab.1. The acceptance rate of mite infested bees, calculated as the number of bees observed at least once out of the total number of bees introduced into the observation hive, was significantly lower than that of the uninfested ones (p=6.3326E^{-9}, Cochran-Mantel-Haenszel test, fig.2).

Tab.1: Number of accepted bees after the introduction into the observation hive for each replication

	Uninfested bees		Infested bees	
	Introduced	Observed at least once	Introduced	Observed at least once
1 replication	117	83	61	24
2 replication	82	55	66	46
3 replication	20	15	31	9
4 replication	88	71	51	19
Total	307	224	209	98

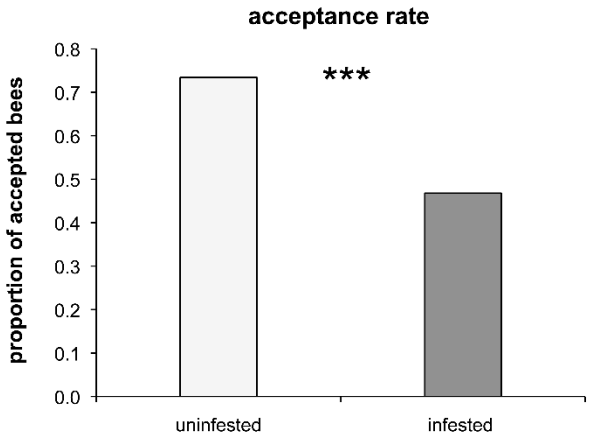

Fig.2: proportion of uninfested and infested bees accepted into the observation hive.

3.1.2. Nursing and other in-hive activities

In order to compare the tendency of infested and uninfested nurse bees to tend the brood, we considered as nurse bees the individual of 5-7 days of age. This choice is based on literature data (Natsopoulou et al., 2015) as corroborated by observing, under the conditions of this experiment, the behavior of uninfested bees from day three (introduction into the observation hive) to day twelve. In fact, under the conditions of this experiment, the proportion of healthy bees tending larvae approached 50% after 5 days and started declining after day 8 (black square, figure 3).

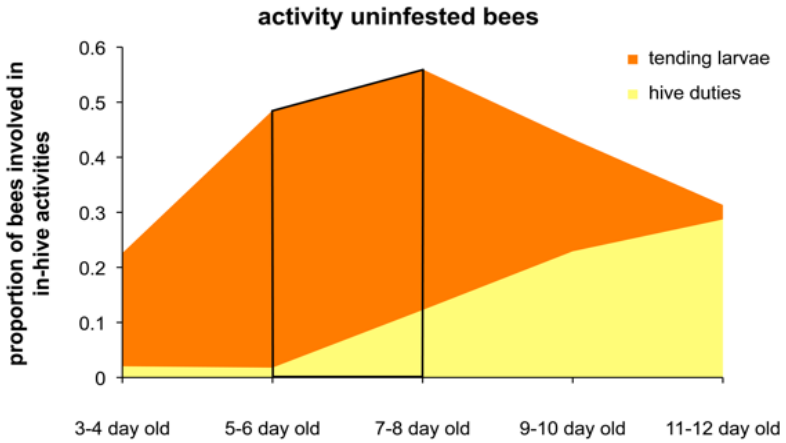

Fig. 3: proportion of uninfested bees involved in tending larvae (TL, orange) and to other in-hive duties (HD, yellow).

As regards the brood care tendency, infested nurse bees appeared to be less involved in tending larvae than the uninfested nestmates. Due to a certain variability, that is likely related to the varying environmental conditions affecting brood rearing along the season, the difference only approached significance (Cochran-Mantel Haenszel test, p= 0.092, fig.4) but the observed trend was the same in all four replications confirming previous observations by Annoscia et al. (2015).

Tab.2: proportion of bees observed while tending larvae

	Proportion of nurses tending larvae into the observation hive	
	Uninfested	Infested
1 replication	0.50	0.35
2 replication	0.67	0.41
3 replication	0.17	0.14
4 replication	0.59	0.56

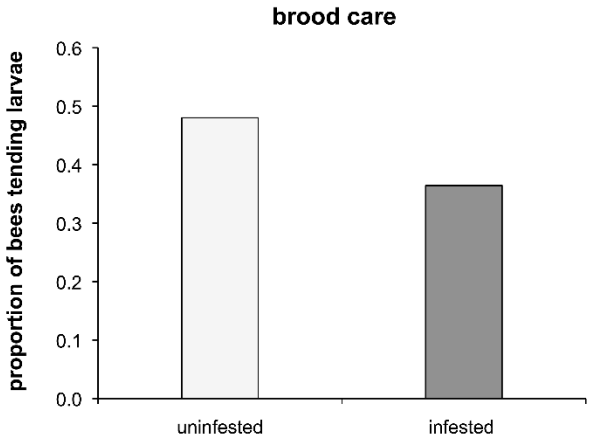

Fig.4: proportion of infested and uninfested nurses involved in tending larvae.

The days before reaching the nursing age, infested bees showed a higher tendency, albeit not statistically significant, to take care of larvae as compared to uninfested nestmates; the trend was the same in all replications (Cochran-Mantel-Haenszel test, p=0.08, fig. 5).

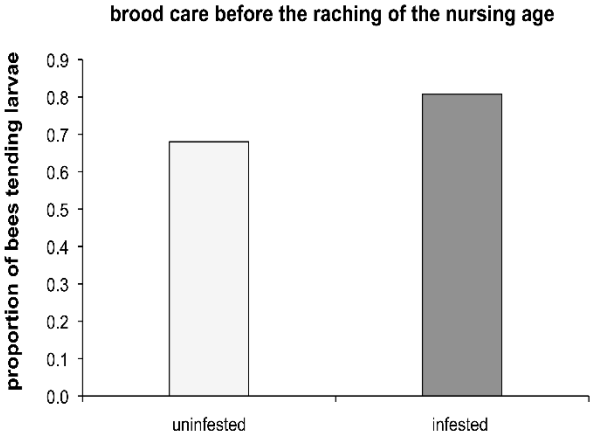

Fig.5: proportion of infested and uninfested bees before the reaching of the nursing age involved in tending larvae.

Among the bees not involved in tending larvae, a large number appeared to be inactive (i.e. still or not engaged in any activity during the observation period). In particular, infested nurses were less active than the uninfested ones; the observed difference was significant (Cochran-Mantel Haenszel test, p=0.0009, fig.6).

The involvement of bees from the two groups in hive's duties, excluding the care of larvae (i.e. food storage, ventilation, trophallaxis), was similar (Cochran-Mantel Haenszel test, p=0.11, fig.7) with infested bees being slightly more involved in hive's duties than uninfested mates.

Tab.3: proportion of active bees

	Proportion of active nurses into the observation hive	
	Uninfested	Infested
1 replication	0.66	0.31
2 replication	0.33	0.24
3 replication	0.78	0.67
4 replication	0.79	0.52

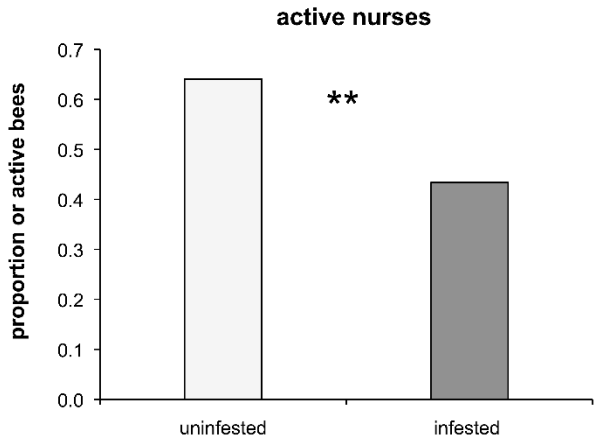

Fig.6: proportion of uninfested and infested nurses involved in activities into the hive

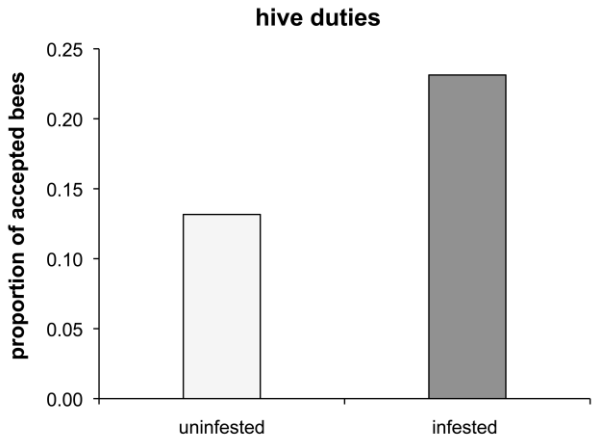

Fig.7: proportion of uninfested and infested nurses involved in other in-hive activities (food storage, ventilation, trophallaxis) into the hive

3.2. Influence of mite infestation on the behaviour of bees under lab conditions

3.2.1. Response of infested and uninfested bees to the brood pheromone

In order to test the bioassay that was adopted to study the behaviour of infested and uninfested bees under lab conditions, we compared the response of bees to the volatiles coming from bee larvae in nurses and foragers. In the star olfactometer, forager bees were slightly more mobile than nurses but the observed difference was not significant (motility calculated as number of entries in the olfactometer, unpaired t test). Overall nurse bees appeared to be more reactive to the volatiles coming from young larvae. The preference of nurse bees for the arm treated with larval odour was significant (paired t test, t=2.1, n=14, p=0.04); instead, foragers showed a significant preference for the control arms, suggesting a possible repellence (paired t test, t=3.6, n=14, p=0.003). These results confirm the suitability of this test to study how mite infestation could affect the response of nurse bees to brood pheromone.

Tab.4: Average time spent into the olfactometer's arms by uninfested and infested nurses

	Average time (min) spent into the olfactometer's arms			
	NURSES		**FORAGERS**	
	Treated	Control	Treated	Control
1 replication	2.65	1.75	1.49	2.00
2 replication	3.13	1.84	1.33	2.29
average	2.89	1.80	1.41	2.15

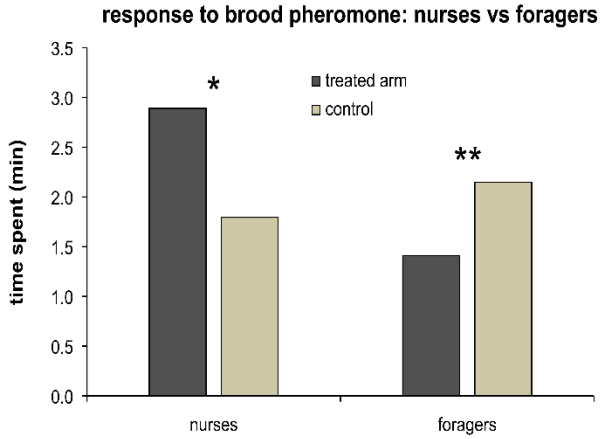

Fig.8: time spent in the treated arm (three living 3rd instar larvae) by nurses and foragers bees collected from the hive

3.2.1.1.Lab bees

In a first set of experiments, we tested the response to brood pheromone of infested and control bees nurse bees maintained under lab conditions.

Overall, bees reared under lab conditions and tested in the olfactometer after reaching the nursing age displayed a variable response (fig.9); in particular, uninfested bees did not show a clear preference for the treated arm (Paired T test, t=0.13, n=20, p=0.8) while the infested individuals tented to avoid it (Paired T test,t=3.2, n=20 p=0.004).

Tab.5: average time spent into the olfactometer's arms by infested bees and control nurses bees maintained under lab conditions.

	Average time spent (min) into the olfactometer			
	UNINFESTED bees		**INFESTED bees**	
	Treated	Control	Treated	Control
1 replication	1.45	1.48	1.10	1.33
2 replication	0.94	1.86	0.82	1.96
3 replication	2.48	1.52	1.40	1.88
Average	1.64	1.61	1.11	1.74

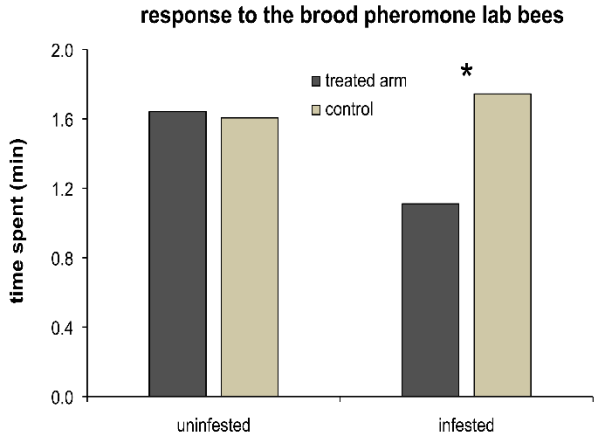

Fig.9: time spent into the treated arm (three living 3rd instar larvae) by infested and control nurses bees maintained under lab conditions.

3.2.1.2. Field bees

In a second set of experiments, we tested the response to the brood pheromone of infested and control nurse bees collected from the observation hive.

Overall, bees maintained under natural conditions showed a higher attraction to the tested stimulus. Both uninfested and infested nurse bees showed a preference for the treated arm but the tendency to approach the olfactometer's arm treated with the larval odor was higher in uninfested individuals albeit significance was not reached.

Tab.6: Average time spent into the olfactometer by uninfested and infested nurses reared under field conditions

	Average time spent (min) into the olfactometer's			
	UNINFESTED bees		**INFESTED bess**	
	Treated	Control	Treated	Control
1 replication	2.45	1.59	2.40	1.91
2 replication	2.03	1.88	2.06	1.94
3 replication	1.46	1.29	1.44	2.09
Average	2.00	1.63	2.04	1.96

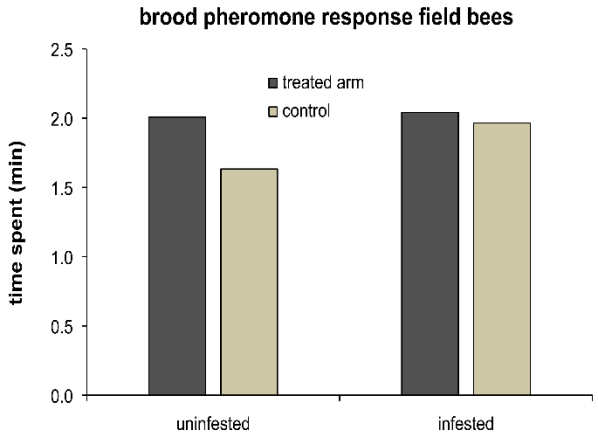

Fig.10: time spent into the treated arm (three living 3rd instar larvae) by infested and control nurses field bees

3.3. Hypopharyngeal glands development

3.3.1. Lab bees

Overall, infested bees maintained under lab conditions showed less developed hypopharyngeal glands than the uninfested ones in all the three replications (fig.11); however, differences were not statistically significant (unpaired t test: t=1.6, n=140 and 150 acini from uninfested and infested bees, respectively, p=0.1).

Tab.7: hypopharyngeal glands' size lab bees

	Average hypopharyngeal glands'size (um²)	
	Uninfested	Infested
1 replication	11.5	7.4
2 replication	6.72	6.46
3 replication	13.4	10.5
Average	10.80	8.41

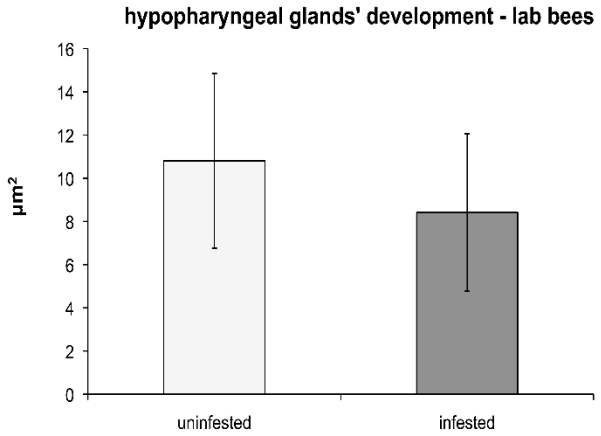

Fig.11: hypopharyngeal glands'size (area of about ten acini per bee) in infested and uninfested bees maintained under lab conditions at the adult stage.

3.3.2. Field bees

In general, nurse bees maintained under field conditions showed hypopharyngeal glands that were more developed than the individuals maintained under lab conditions (fig.13). Nurses showed significantly less developed hypopharyngeal glands if parasitized by *Varroa* mite during the pupal stage, in all the replications of the experiment (unpaired T test, t=2.7, n=145 acini uninfested bees, 129 acini infested bees, p=0.018, fig.12).

Tab.8: hypopharyngeal glands'size of bees maintained under field conditions

	Average hypopharyngeal glands'size (um²)	
	Uninfested	Infested
1 replication	21.4	18.8
2 replication	18.8	14.53
3 replication	25.3	18.3
Average	21.65	19.94

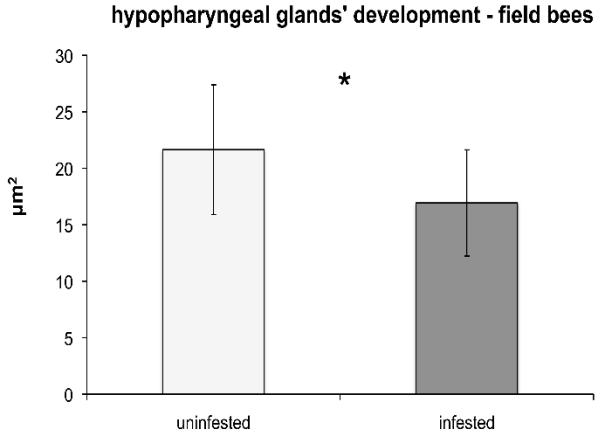

Fig.12: hypopharyngeal glands' size (area of about ten acini per bee) in field bees infested with *Varroa* mite at pupal stage and comparison with uninfested bees.

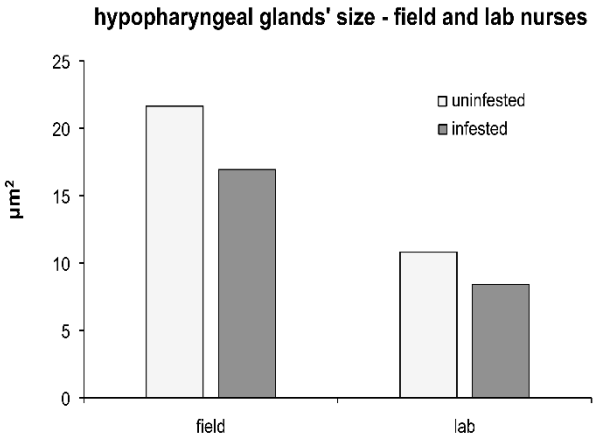

Fig.13: comparison of the hypopharyngeal glands size between field and lab bees

3.4. JHIII titer in the hemolymph

In order to test the sensitivity of our analytical method, we first compared the JH concentration in filed colleceted nurse and forager bees that should have clearly different

levels of the homone. As expected, healthy field collected forager bees had a higher titer of juvenile hormone in the hemolymph as compared to nurses; in particular we detected 1.30 and 0.75 pg /µl of hormone in foragers and nurses, respectively (Unpaired Test T. t=2.9, p=0.04).

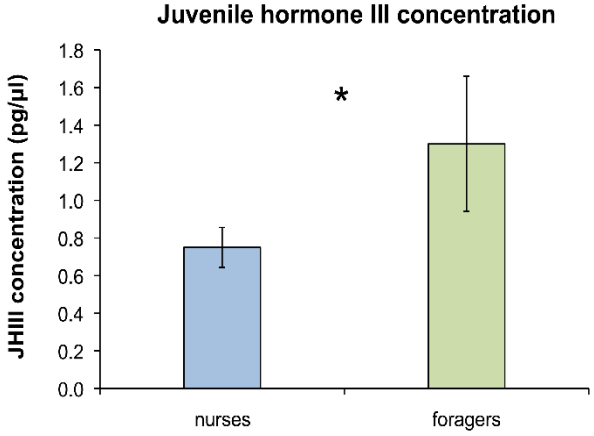

Fig.14: comparison of the JHIII titers in the hemolymph of nurse and forager bees collected from the hive.

Nurse bees reared under lab condition showed higher JHIII titers in the hemolymph as previously observed in caged bees by Lin et al. (2003); however, a significant difference between uninfested bees (1.4 pg of Juvenile Hormone III per µl of hemolymph) and infested bees (2.2 pg/µl) was noted here (Unpaired Test t, t=2.8, p=0.02) suggesting an accelerated transition to foraging in infested bees.

Bees reared under field conditions showed a trend similar to that observed in lab bees, with infested bees showing a higher concentration of JHIII in the hemolymph (unpaired Test t t=0.2, p=0.8) although significance was not reached possibly because of the lower amount of JHIII that is normally found under this conditions.

Tab 9: JHIII concentration in the hemolymph of uninfested and infested 7 day old bees reared under lab conditions (pg/µl)

	JHIII concentration (pg/ul)	
	Uninfested	Infested
1 replication	1.10	1.93
2 replication	1.40	2.24

Average	1.39	2.24

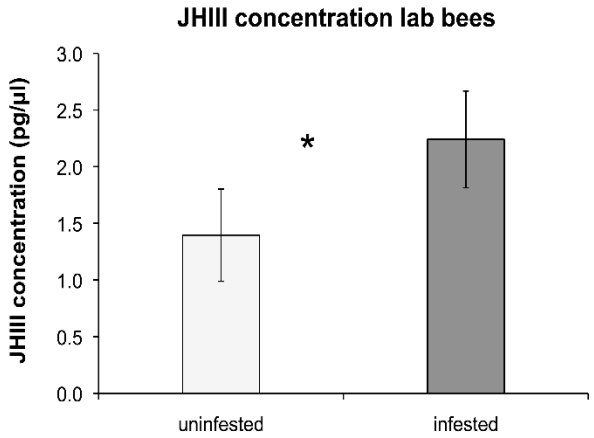

Fig.15: comparison of the JHIII titer in the hemolymph between uninfested and infested nurses reared under lab conditions

4. DISCUSSION

The monitoring of the in-hive behavior of nurse bees mite infested or not during the pupal stage, confirmed previous results regarding the reduced nursing activity of parasitized bees (Annoscia et al. 2015).

As pointed out in the introduction, nursing can be regarded as a multi-step process initiated by the perception of the brood pheromone, emitted by the larvae to communicate their nutritional needs (Free et al., 1983; Huang et al., 1991; Slessor et al., 2005), which stimulates royal jelly production by the hypopharyngeal glands and the feeding of the growing larvae. Royal jelly production is likely reinforced through a positive feedback loop, after the contact with brood. Clearly, gland development could also precede the first response to larvae, as observed in caged bees grown in absence of larval contacts; however, this fact has not important consequences on the results of the reasoning below.

In any case, due to the age regulated division of labor in bee colonies (Robinson, 1992), all the described events happen only if bees are in a given stage of their life that is the nursing stage. This is largely under endocrine control since nurse bees normally have lower levels of juvenile hormone and the transition to foraging is linked to a marked increase in the concentration of this hormone in the hemolymph (Huang et al., 1991, 1994). It has been shown

that parasitism and, in particular, mite parasitization may results in an accelerated transition of bees to foraging (Janmaat et al., 2000) with detrimental consequences for the entire colony; this has been also hypothesized based on transcriptomic results showing increased expression of genes related to this transition (Ament et al., 2011).

The higher concentration of JHIII in the hemolymph of mite parasitized bees as compared to healthy bees of a similar young age that was proved here confirms, on different bases, that mite infestation may cause an accelerated maturation leading to a precocious transition to foraging and nicely complements previous transcriptomic studies. In this study, we could not observe in the hive parasitized bees after entering the foraging stage likely because of the reduced lifespan of such bees and their higher mortality outside the colony. However, we recorded that parasitized bees in all the experimental conditions (lab and field) had less developed hypopharingeal glands as expected in forager-like bees. The accelerated maturation is further suggested by the fact that, at a younger age, infested bees seemed to be more involved in tending larvae than uninfested ones, as expected in case nursing would not be lost at all but only anticipated.

As from the response of bees to stimuli coming from the brood, we found a difference between mite infested and uninfested bees but the lack of significance does not allow any further speculation about the involvement of this further factor in the reduced nursing activity observed in this study.

At a lower infestation level, the observed reduced nursing in case of infestation could be regarded as beneficial for the colony. In fact, nurse bees infested during the pupal stage, normally showing a higher DWV infection level, are more likely to transmit the virus to young larvae by supplying contaminated larval food. Under this point of view, the limited involvement in nursing of infested bees, which normally have higher viral infection levels (Francis et al., 2013), minimizing the contact with the larvae, would reduce within hive viral transmission.

Instead, at a high infestation level the limited involvement of parasitized bees in brood caring could have important consequences at the colony level since the normal preimaginal development of bees requires a constant supply of larval jelly (Haydak, 1970). However, at

this infestation level, further mechanisms could take place to preserve colony balance. In fact, we observed a significant lower acceptance rate of infested bees meaning that those bees are actively removed from the hive and should readily be replaced following the control feedback loops causing increased brood rearing.

It has long been know that *Varroa* mite, in association with DWV, causes modifications in the cuticular hydrocarbons profile of the bees (Annoscia et al., 2012; McDonnel et al., 2013; Salvy et al., 2001). Thus, the significantly lower acceptance rate of infested bees noted here could be related to changes in the cuticular hydrocarbons profile caused by parasitization.

Cuticular hydrcarbons are involved in social recognition (Gibbs, 2002) and bees used for the experiment were from another colony and introduced into the hive at the age of three day, before reaching the nursing age. The lab manipulation and the choice of bees from another family likely caused rejection of introduced bees by the hosting family. However, the great difference between the acceptance rate of infested and uninfested bees as well as some recent evidence about infestations effects on social interactions (Cappa et al., 2016, McDonnell et al., 2013) suggest that unhealthy bees are recognized and eliminated in a further level of social defence.

CONCLUSIONS

Previous studies and ours highlighted how the behavior of infested nurse bees inside the hive is different from that of non parasitized bees; in fact, infested individuals are in general less active and, in particular, are less involved in tending larvae.

Lab and field experiments aiming to show how different steps of nursing could be affected by mite parasitization showed that the accelerated behavioral maturation, confirmed here by studying the concentration of JHIII in haemolymph of bees and the reduced hypopharyngeal glands, is certainly involved.

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Chapter 4: **prophylactic use of pollen by honey bees infested by the *Varroa* mite**

Ph: J.M.Ugalde

1. INTRODUCTION

Animal self-medication is receiving increasing attention (Richardson et al, 2015; Baracchi et al, 2015) due to its profound implications for host-parasite interactions, including the effects on parasite transmission and the evolution of parasite virulence and host defenses. It has also been suggested that the interference of humans with the ability of animals to self-medicate can increase disease risk in managed species, such as in agricultural systems (De Roode et al., 2013).

Among the growing number of animal pharmacists, honey bees occupy an important position in that their use of plant resin with antibiotic properties (Simone-Finstrom & Spivak, 2012) is well known from ancient times and may even represent the first documented example of such an important aspect of ethology.

Indeed, the peculiar conditions of the bee hive - with up to fifty thousand individuals living in close proximity, under high temperature and humidity conditions – creates significant issues in terms of parasite management. It has been suggested that bees deploy their complex social behaviors and structures to combat diseases, in processes termed “social immunity”

(Cremer et al., 2007); foraging for specific materials that can help combat diseases in the colony can represent an important component of social immunity.

The diets of honey bees (and most bee species) consist of nectar and pollen. Due to its sugar rich composition, nectar is the major source of energy for bees (Vaudo et al., 2015), while pollen serves as the main source of proteins and lipids (10-40% and 1-13% based on dry weight respectively), and provides also minor components such as vitamins, phenolic compounds and flavonoids (Campos et al., 2008). The effect of pollen diets on the survival and physiology of bees has been studied (Brodschneider & Crailsheim, 2010) but the potential of pollen as a self-medication tool for bees has not been investigated.

Alaux et al. (2010) tested whether dietary protein quantity and diversity can influence bees' immunocompetence and found that protein feeding modifies both individual and social immunocompetence as measured by assessing haemocyte concentration, fat body content, phenoloxidase activity and glucose oxidase. These results were expanded by Alaux et al. (2012), where a transcriptomic study of bees fed a rich diet of pollen and sugar versus a poor diet of sugar alone demonstrated that beside activating nutrient-sensing and metabolic pathways, pollen positively influenced longevity (Haydak, 1970) and the production of some antimicrobial peptides (Alaux et al., 2010).

The major threat to honey bees' colonies is currently represented by the parasitic mite *Varroa destructor* (Anderson & Trueman) (Nazzi & LeConte, 2016) and the viruses vectored and facilitated by this parasite (Di Prisco et al., 2016). The mite can cause direct damage to bees by removing significant amounts of hemolymph during its feeding (Bowen Walker & Gunn, 2001) and thus perturbing the energetic balance of the honey bee. Mite feeding also breaks the cuticle which plays an important role for water balance (Annoscia et al., 2012 and citations therein) and may result in secondary microbial infections at the wound site (Kanbar & Engels, 2003). Furthermore, *V. destructor* contributes to the transmission of bee viruses and can trigger viral replication (Chen & Siede, 2007; Nazzi et al., 2012; Ryabov et al., 2014). Viruses, in turn, can affect the immune defenses of honey bees with important consequences for the control of covert viral infections (Nazzi et al., 2012). Although the side effects of this immune-suppressive syndrome have not yet been studied in detail, it is likely that it may

have important consequences for the proliferation of other secondary parasites including fungi and bacteria that are widespread within bee hives (Cox-Foster et al., 2007). Indeed, the hive hosts a complex cohort of symbionts that can be propagated by the mites invading the brood cells or attaching to adult bees (Gliński & Jarosz, 1992).

In this study, we tested the hypothesis that pollen can be used for self-medication by honey bees challenged with the parasitic mite *V. destructor*. Furthermore, we characterized the compounds responsible for such an effect and, based on available data, drew hypotheses on the possible functions of those compounds to be tested in future studies. Finally, we use whole transcriptome sequencing to compare the transcriptome of bees fed rich diets of pollen and sugar versus bees fed poor diets of sugar alone to examine the molecular mechanisms that may underpin these observations.

2. MATERIAL AND METHODS

2.1. Sources of honey bees and *Varroa* mites

Honey bee larvae and *Varroa destructor* adult females used in this study were collected from 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; Nazzi 1992).

2.2. Pollen collection

The pollen used in this study was collected in May 2013, near Udine, Italy (46°00'39" N, 13°20'00" E). At the time of sampling, an extensive flowering of *Amorpha fruticosa* (Fabaceae) was reported. A palynological analysis revealed that besides *A. fruticosa*, other pollens belonging to the plants of the genus *Fagopyrum*, *Lirodendron*, *Lonicera*, *Papaver*, *Taraxacum*, *Vitis* and of the family Urticaceae were present. Harvesting was carried out using pollen traps; pollen balls were frozen at -20 °C and stored under that condition until use.
Another aliquot of pollen was freeze-dried and stored at -20 °C until analysis.

2.3. Experiments 1 and 3: lab studies with honey bees

Bee larvae from brood cells capped in the preceding 15 hours and mites from the same cells were obtained as previously described (Nazzi et al., 2012).
Individuals were collected randomly from several colonies of the experimental apiary for these studies. Larvae were transferred into gelatine capsules (Agar Scientific Ltd., 6.5 mm Ø) with no mites or one mite and maintained in an environmental chamber (34 °C, 75% R.H., dark) for 12 days (Nazzi and Milani 1994). Upon eclosion, newly emerged adult bees were

separated from the infesting mite and transferred into plastic cages (185 x 105 x 85 mm), maintained in a climatic chamber (34 °C, 75% R.H., dark).

Bees were fed with water *ad libitum* and different diets.

In Experiment 1 the diets consisted of either a sugar diet (s) or a sugar diet complemented with crude pollen (sp). In Experiment 3, diets consisted of a sugar diet complemented with freeze-dried pollen (sp), or sugar diet complemented with pollen deprived of the apolar fraction (-af) or deprived of the polar fraction (-pf). Each experiment was replicated three times.

During the experiments, cages were assessed to remove any dead bees and bees exhibiting possible symptoms of severe DWV infection, on which it makes no sense to hypothesize a possible compensatory effect of pollen, such as deformed wings accompanied by movement difficulties. Comparison of survival rates of the uninfested and infested bees fed with different diets (Experiment 1) were conducted using the log rank test without continuity correction; in this case, 22-27 bees per group were used in the three replicates.

Comparisons of bee mortality of the infested bees fed with different diets (Experiment 3) were conducted using the Mantel–Haenszel test; in this case, 52-58 bees per group were used in the three replicates.

2.4. Chemical analysis of pollen

Reagents

Triheptadecanoin, n-octadecane, 5α-cholestan-3β-ol, Sylon BFT (bis(trimethylsilyl) trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) 99:1), Supelco 37 Component FAME Mix, were obtained from Sigma-Aldrich (Missouri, US).

All other reagents and solvents were analytical and chromatographic grade. The water was obtained by a Water Milli-Q purification system (Millipore, Vimodrone, Italy).

The silylating mixture was prepared by mixing Sylon BFT and pyridine in the ratio 1:1 (v/v).

HCl 5% (w/v) was prepared by adding 1 ml of acetyl chloride to 10 ml of chilled methanol (Ichihara e Fukubayashi, 2010). HCl 8% (w/v) was prepared by mixing 0.97 ml of commercial conc. HCl (37%, w/w) with 4.15 ml of methanol (Ichihara e Fukubayashi, 2010). Thin-layer chromatography (TLC) silica gel plates, 20 x 20 cm, 0.25 mm thickness, were provided by Merck (Darmstadt, Germany). Bond Elut silica, 1g, Solid Phase Extraction (SPE) columns were purchased from Agilent Technologies Italia (Cernusco s/N, Italy).

Lipid extraction

Lyophilized pollen was ground with mortar and pestle and an aliquot of 10 g was extracted with 100 ml of dichloromethane by sonication for 15 min at room temperature. After decantation of the solvent, the residue was re-extracted with 60 ml of dichloromethane under the same conditions. The pooled extracts were filtered on a Bückner filter and the solvent was removed under reduced pressure in a rotavapor at 40 °C. The lipidic fraction was weighed and then dissolved in chloroform and brought up to a volume of 20 ml in a volumetric flask. The lipid concentration of this solution was 21.73 mg/ml.

Saponification

An aliquot (4 ml) of the lipidic solution (86.9 mg, lipid), was transferred in a Teflon-lined screw cap Pyrex tube and the chloroform evaporated under a nitrogen stream. Then 800 μl of triheptadecanoin in n-hexane (4.25 mg/ml), 1000 μl of n-octadecane in n-hexane (1.00 mg/ml) and 350 μl of a 5α-cholestan-3β-ol methanolic solution (1.01 mg/ml) were added as internal standards for the quantitation of fatty acids, hydrocarbons and sterols respectively. After the evaporation of the solvent under nitrogen, 3 ml of a 2 M KOH ethanolic solution were added and the mixture heated at 95-98 °C for 30 min in a water bath. After cooling to room temperature, 3 ml of H_2O were added and the insaponifiable fraction extracted with three 3 ml aliquots of n-hexane. In case of formation of an emulsion the mixture was centrifuged. The pooled organic phases were washed with water, dried over anhydrous sodium sulfate and the solvent removed under nitrogen stream.

Purification of hydrocarbons and sterols

The unsaponifiable solids were dissolved in 1 ml of n-hexane and loaded onto a silica SPE column previously conditioned with 5 ml of n-hexane. The hydrocarbons fraction was eluted with 10 ml of n-hexane followed by 10 ml of a mixture of n-hexane/diethyl ether (1:1, v/v) for the elution of the sterols fraction.

The hydrocarbon fraction was reduced to dryness under nitrogen and dissolved in 1 ml of n-hexane prior to GC and GC-MS analysis. See below for further details.

The sterol fraction was reduced to dryness under nitrogen and re-dissolved in 200 μl of chloroform. 120 μl of this solution were deposited onto a TLC plate together with a solution of 5α-cholestan-3β-ol as reference standard. The plate was eluted with a mixture of n-hexane/diethyl ether (65:35, v/v). The band containing the sterols was visualized under UV light (λ = 254 nm) after spraying with an ethanolic solution of 2,7-dichlorofluorescein and identified by comparison with the spot of 5α-cholestan-3β-ol. The band was scraped off and sterols were extracted with two 0.5 ml aliquots of chloroform. The solvent was then removed under nitrogen.

Sterols were derivatized to their corresponding trimethylsilylethers prior to GC and GC-MS analysis. Derivatization was carried out with 300 μl of pyridine/Sylon BFT (1:1, v/v) for 1 hour at room temperature. The silylating reagent was then removed under nitrogen and the sample dissolved in 0.5 ml of n-hexane.

Fatty acids

Five drops of a methyl orange ethanolic solution was added to the aqueous phase, left after the extraction of the insaponifiable fraction, and the solution was acidified with sulfuric acid (20% v/v) until the development of red color. Fatty acids were then extracted with three 3 ml aliquots of n-hexane. Phase separation was induced by centrifugation. The pooled organic phases were washed with water, dried over anhydrous sodium sulfate and reduced to dryness under a stream of nitrogen. The fatty acids (about 30 mg) were finally dissolved with 4.5 ml of a mixture of n-hexane/dichloromethane in the ratio 2:1 (v/v).

Fatty acid methyl esters (FAME)

The fatty acids methyl esters were prepared according the methods reported by Ichihara and Fukubayashi (2010) with minor modifications.

Method A. About 10 mg of fatty acids (1.5 ml of the fatty acids solution) were transferred to a teflon-lined screw cap Pyrex tube. After the evaporation of the solvent under nitrogen, 2 ml of a HCl 5% (w/v) methanolic solution were added and the mixture heated at 98-100 °C for 60 min in a water bath. After cooling to room temperature, 1 ml of water was added and FAME were extracted with 2 ml of n-hexane. The organic phase was washed with water and dried over anhydrous sodium sulfate.

Method B. About 5 mg of fatty acids (0.75 ml of the fatty acids solution) were transferred to a Teflon-lined screw cap Pyrex tube. After evaporation of the solvent under nitrogen, 0.2 ml of toluene, 1.75 ml of methanol and 0.05 ml of a HCl 8% (w/v) methanolic solution were added. The mixture was then heated at 98-100 °C for 5 min in a water bath. After cooling to room temperature, 1 ml of water was added and FAME were extracted with 1 ml of n-hexane. The organic phase was washed with water and dried over anhydrous sodium sulfate.

Method C. The same procedure of Method B was used with the only difference that the esterification was carried out at 45 °C for 60 min in an oven.

All the analyses were performed in triplicate.

Gas chromatography (GC)

A Carlo Erba HRGC gas chromatograph mod. 5160, equipped with a flame ionization detector and a split-less injector (Carlo Erba Instruments, Rodano, Italia), was used.

For the analysis of FAME, a fused silica capillary column SP2380 (poly(90% biscyanopropyl/10% cyanopropylphenyl siloxane)), 30 m x 0.32 mm i.d., 0.20 μm film thickness, (Supelco, Sigma–Aldrich, Milan, Italy) was used. The column temperature was programmed from 140 °C to 220 °C at 3 °C/min and held at 220 °C for 5 min. The injector and detector temperature was 260 °C. The carrier gas was helium at a flow rate of 1.0 ml/min. Split ratio was 1:30 and the injection volume 1 μl.

For the analysis of hydrocarbons a fused silica capillary column SPB-5 (poly(5% diphenyl/95% dimethyl siloxane)) 30 m x 0.32 mm i.d., 0.25 μm film thickness, was used (Supelco, Sigma–Aldrich, Milan, Italy). The column temperature was programmed from 80 °C to 300 °C at 5 °C/min and held at 300 °C for 15 min. The injector and detector temperature was 300 °C. The carrier gas was helium at a flow rate of 1.5 ml/min. Split ratio was 1:50 and injection volume 1 μl.

For the analysis of sterols as trimethylsilylether derivatives, a fused silica capillary column SPB-5 30 m x 0.32 mm i.d., 0.25 μm film thickness, was used (Supelco, Sigma–Aldrich, Milan, Italy). The column temperature was programmed from 260 °C to 280 °C at 2 °C/min and held at 300 °C for 30 min. The injector and detector temperature was 300 °C. The carrier gas was helium at a flow rate of 1.2 ml/min. Split ratio was 1:30 and the injection volume 1 μl.

Gas chromatography–mass spectrometry (GC–MS)

A Shimadzu gas chromatograph coupled to a quadrupole mass spectrometer QP-2010 (Shimadzu Corporation, Kyoto, Japan) was used. The column was a 30 m x 0.25 mm i.d., 0.25 μm film thickness, fused silica SPB 5 (Supelco, Sigma–Aldrich, Milan, Italy). The transfer line and ion source temperatures were 300 and 200 °C respectively. The mass spectrometer operated in electron impact ionization mode at 70 eV.

For the analysis of FAME, the column temperature was programmed from 80 °C to 250 °C at 5 °C/min, from 250 °C to 300 °C at 10 °C/min and held at 300 °C for 20 min. The injector temperature was 280 °C. The carrier gas helium at a flow rate of 1.0 ml/min (constant flow). Split ratio was 1:30 and injection volume 1 μl.

For the analysis of hydrocarbons, the column temperature was programmed from 60 °C to 300 °C at 5 °C/min and held at 300 °C for 15 min. The injector temperature was 300 °C. The carrier gas was helium at a flow rate of 1.0 ml/min (constant flow). Split ratio was 1:30 and injection volume 1 μl.

For the analysis of sterols, the column temperature was programmed from 260 °C to 280 °C at 2 °C/min and held at 280 °C for 30 min. The injector temperature was 300 °C. The carrier

gas was helium at a flow rate of 1.0 ml/min (constant flow). Split ratio was 1:30 and injection volume 1 μl.

2.5. Experiment 2: evaluation of the effects of pollen supplementation in the field

To verify the effect of dietary pollen on the survival of bees maintained in standard Dadant-Blatt hives, we performed a field trial in which the survival of bees was compared between colonies fed with an additional dose of pollen (n = 4) and control colonies which did not receive such treatment (n = 4). The treated group received 50 g of dehydrated pollen in pellets, every week, for four weeks; pollen was placed in a bowl between the lid and the frames of the hives. The two groups of colonies were homogeneous in terms of colony strength (i.e. number of bees per colony) and infestation (i.e. number of mites per bees). At the beginning of the experiment, all colonies were made pollen-free by removing all frames where more than a quarter of the surface was occupied by pollen reserves; on average, two frames of pollen per colony were removed.

Throughout the experiment, we measured adult bee populations' size, *Varroa* levels, and adult bee mortality. The adult bee populations in the experimental hives were estimated three times, before, during and at the end of the trial, by counting the number of ''sixth of frames'' covered by bees in each hive at sunset and calculating the overall bee population, considering that one fully covered sixth of comb corresponds to 253 adult bees (Marchetti, 1985). Infestation of *V. destructor* was estimated on weekly basis during all the experiment, by counting the number of mites that have naturally fallen on a sticky bottom board placed in each hive (Coloss BeeBook, 4.2.4. Natural mite fall). To assess honey bee mortality, dead bees found in cages placed in front of the colonies were counted on weekly basis; bee mortality was calculated by averaging the number of dead bees in the time interval elapsed since the last sampling date. This value was then referred to the mean bee population in that period, obtained by considering the initial and final bee population (Nazzi et al., 2012).

2.6. Experiment 4: RNAseq and transcriptomic analysis

To evaluate the impact of *Varroa* infestation and diet on transcriptome profiles using RNAseq we collected four infested and as many uninfested individual two-day-old bees for each diet in Experiment 1 (sugar only and sugar complemented with pollen) and stored the bees at -80 °C, for a total of four individuals per treatment and four treatment groups.

Frozen samples used for the analysis were transferred into liquid nitrogen and used for total RNA extraction by means of Tri-reagent® (MRC Inc, USA). RNA was processed using the TruSeq mRNAseq sample prep kit (Illumina, Inc., CA, USA), starting from 2 μg of total RNA per sample. The standard mRNA sample prep from Illumina was used to produce 36 bp long tags, about 25-30 millions per sample, for 16 samples (IGA Technologies, Udine, Italy).

The sequencing reads were pre-processed by removing the adaptor sequences and low quality reads using Trimmomatic (Bolger et al. 2014).

The remaining reads were aligned to the most recent honey bee genome build (Amel 4.5: Elsik et al. 2014), using Tophat2 (Trapnell et al. 2012) and annotated with the newest official gene set (OGS 3.2).

Read counts for each gene were imported into R (http://www.r-project.org) for further analyses. Genes with fewer than 10 reads across all samples were removed from the analyses. The read counts were normalized using a trimmed mean of m-values (TMM) method. A generalized linear model (through the edgeR package in R, Robinson et al., 2010) was used to identify differentially expressed genes (DEGs) between the treatment groups.

Genes with FDR < 0.05 were considered differentially expressed between the four treatments. One DEGs list was particularly important for the purpose of the study, we conducted a *Gene Ontology* analysis to determine if any biological or functional categories of genes were significantly overrepresented using DAVID Bioinformatics Resources 6.7 (Huang et al., 2009). To conduct this analysis, the *Drosophila melanogaster* orthologs of the DEGs were identified using BLAST (Altschul et al., 1990) with an e-value cutoff of 1x10-5.

To determine virus levels, each pre-processed sample was aligned to a panel of the most common honey bee viruses (Chen and Siede, 2007) using a similar protocol as above. The

read counts for each file were imported into R and normalized based on library size. A standard least squares ANOVA with a Turkey post hoc test was used to determine the significant changes in viral expression.

3. RESULTS

3.1. Experiment 1: effect of pollen and *Varroa* parasitization on honey bee worker survival under lab conditions

To determine if access to pollen can mitigate the adverse effects of a parasitic infestation, we reared honey bee larvae inside artificial cells in presence of a *Varroa* mite (V+) or not (V-), and then maintained the emerging adults in cages under standardized environmental conditions to evaluate survival under two different diet regimes: a sugar diet (s) and a sugar diet complemented with pollen (sp), supplied *ad libitum*.

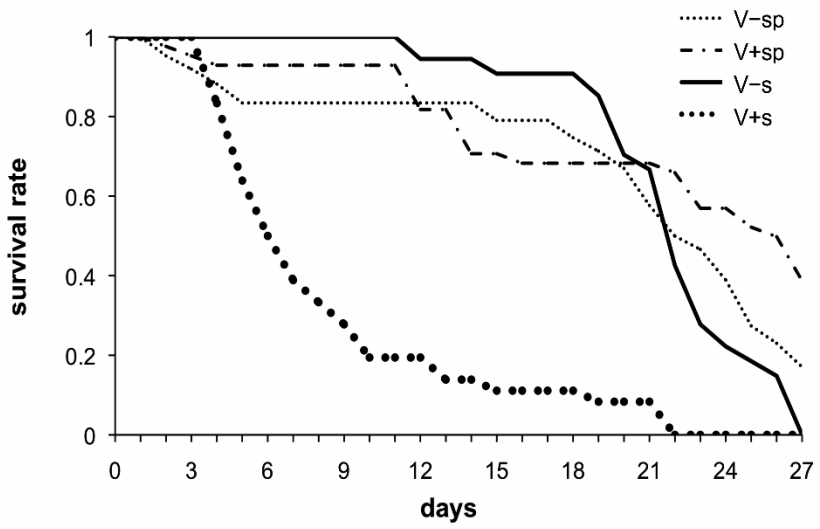

Fig. 1: Survival of bees that had been infested or not during the pupal stage (V+ and V- respectively), maintained as adults on a pollen rich diet (sp) or a standard sucrose diet (s).

Under lab conditions, pollen did not significantly increase the lifespan of healthy-uninfested bees relative to bees fed on sugar-only diets (fig. 1; (V-sp vs V-s: Log Rank (Chi Square=0.46, d.f.=1, *P*=0.49). However, in bees that were infested with *Varroa*, access to pollen significantly

increased their lifespan relative to parastitized bees reared with sugar alone (fig. 1; V+s vs V+sp: Log Rank (Chi Square=26.8, d.f.=1, P<0.001). Thus, at least under artificial conditions, pollen is not essential for bees to survive but can mitigate the adverse effects of a parasitic infestation, in fact, under the conditions of this experiment, it appears that dietary pollen can compensate for the negative impact of mite infestation.

Per capita pollen consumption, as measured by weighing the pollen consumed by bees daily, was about a few milligrams per day. A slow decline in consumption was noted both in uninfested and infested bees with infested bees consuming slightly more pollen compared than uninfested bees (fig. 2). However, no significant difference was found between the two groups suggesting that parasitized bees do not actively consume more pollen compared to uninfested bees.

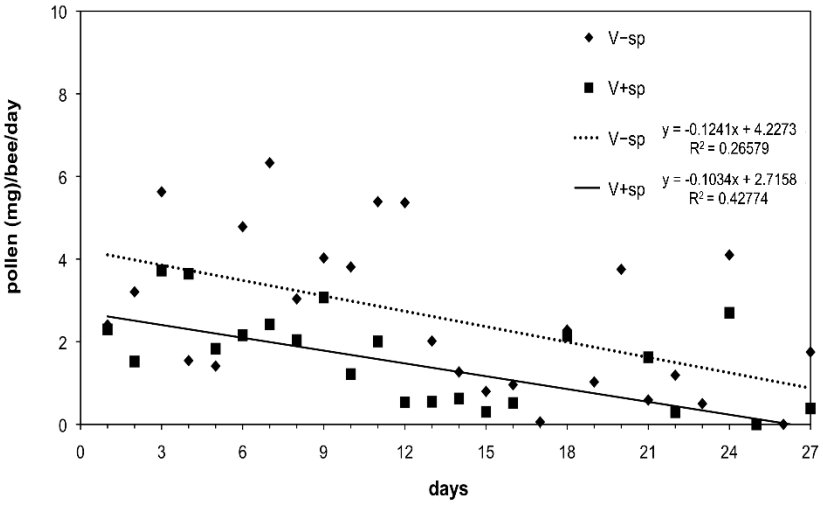

Fig. 2: Pollen consumption of infested (V+sp) and uninfested (V-sp) bees over the period of the lab trial.

3.2. Experiment 2: pollen and survival under field conditions

In this experiment, we sought to determine if the positive effect of dietary pollen on bee survival that was observed under lab conditions, could also be obtained under more complex field conditions. Thus, we removed the stored pollen and balanced the bee populations and mite infestation levels in 8 colonies in late summer. Subsequently, we provided 4 of the

colonies with 50 g of pollen per week, for one month, while we left the other 4 colonies as untreated controls.

As expected from previous studies, a gradual increase in bee mortality was observed in all colonies over the course of the experiment. After the treatment, bee mortality in colonies supplemented with pollen was slightly smaller but the observed difference was not significant (fig. 3).

At the end of September, despite a chemical treatment to control *Varroa* levels following the trial, two out of four colonies that received no pollen died but all the pollen-treated colonies survived. By the end of November, all of the colonies that received no pollen were dead, while two of the pollen-treated colonies survived. Overall, though there is a great amount of biological variability in these field studies, these results suggest a positive effect of dietary pollen on bee survival under field as well as under lab conditions.

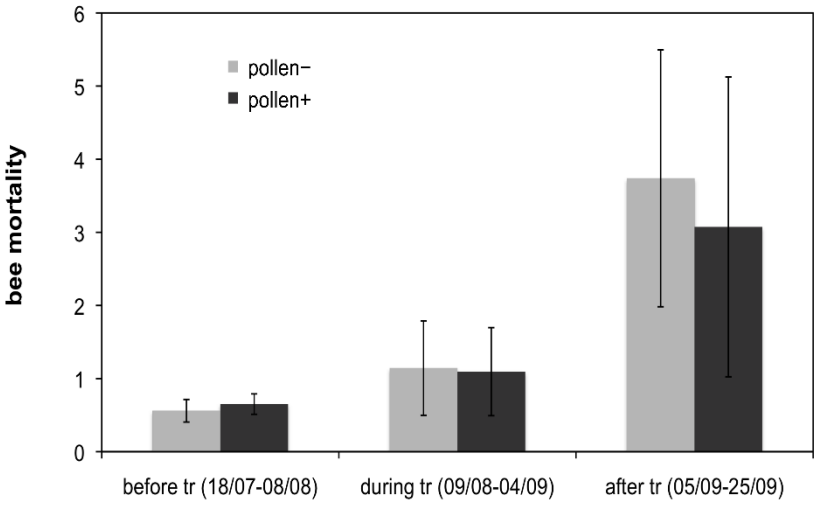

Fig. 3: Bee mortality in experimental colonies supplied or not with extra pollen in late summer.

3.3. Experiment 3: effect of pollen components on survival of infested bees

To gain insight into the active ingredients underpinning the positive effects of dietary pollen on the survival of infested bees under lab conditions, we washed the pollen with two

different solvents to remove either the apolar (p-af) or the polar components (p-pf), and fed this pollen to infested (V+) bees.

The mortality of infested bees fed with sugar and pollen deprived of the apolar components (V+sp-af) was significantly higher than that observed in bees fed complete pollen (V+sp).

The effect became evident after three weeks (fig. 4; Mantel Haenszel (V+sp vs V+sp-af: weeks 1-2, Heterogeneity Chi-2=0.212, d.f.=2, *P*=0.710; weeks 3-4, Heterogeneity Chi-2=0.566, d.f.=2, *P*=0.067; weeks 4-5, Heterogeneity Chi-2=0.165, d.f.=2, *P*=0.036).

Since lipids represent the most important component of the apolar fraction of pollen, this results suggests a possible role of lipids in the observed beneficial effect of pollen.

A similar, but not significant, effect was noted when comparing the survival of infested bees fed with sugar and pollen with (V+sp) or without the polar components (V+sp-pf) (Mantel Haenszel (V+sp vs V+sp-pf: weeks 1-2, Heterogeneity Chi-2=0.483, d.f.=2, *P*=0.919; weeks 3-4, Heterogeneity Chi-2=0.320, d.f.=2, *P*=0.998; weeks 4-5, Heterogeneity Chi-2=0.499, d.f.=2, *P*=0.087).

In conclusion, a significant effect of apolar components on the survival of infested bees was observed, whereas the possible effect of polar compounds remains unclear.

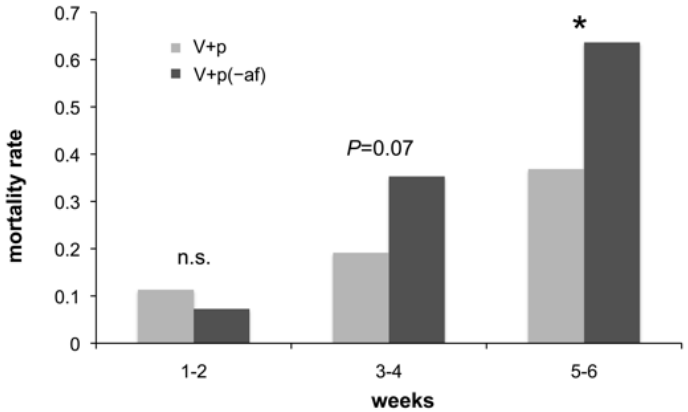

Fig. 4: Mortality of the infested bees fed with a complete pollen diet (V+p) and with pollen without apolar fraction (V+p(-af)).

3.3.1. Composition of the apolar fraction of pollen

To assess the composition of the apolar fraction of pollen used in the experiments, the extract was treated with sodium hydroxide; the unsaponifiable fraction was separated on a silica column and analyzed by means of GC-MS whereas the saponifiable fraction was first methylated and then analyzed.

Lipids represented 3.9 ± 0.2% (n = 2) of the dry weight of the pollen used in this study, which contained 24.2 ± 0.3% (n = 3) water. The apolar fraction of pollen contained fatty acids, hydrocarbons and sterols.

Fatty acids were determined by three different methylation procedures that showed a good repeatability both within each method and among the different methods, with correlation coefficients of 0.999. Identified fatty acids are reported in the table 1.

The main fatty acids found in the pollen used in this study were palmitic acid (28%), linolenic acid (23%), myristic acid (15%), linoleic acid (14%) and oleic acid (12%). Unsaturated fatty acids (C18:1 + C18:2 + C18:3) represented about 50% of total fatty acids.

Both saturated and unsaturated hydrocarbons from C21 to C33 were present. In particular, the predominant hydrocarbons were the n-alkanes with an odd number of carbon atoms from C23 to C29. The percentage of unsaturated hydrocarbons was lower than that of the corresponding saturated hydrocarbons in the case of C23, C25 and C27, while an opposite trend (i.e. a higher percentage of unsaturated hydrocarbon compared to the corresponding saturated hydrocarbon) was noted for C29, C31 and C33. The main unsaturated hydrocarbon was C31:1 (13%), followed by C33:1 (11.5%) and C29:1 (9.2%). The unsaturated hydrocarbons accounted for 43% of the total hydrocarbons. Generally, at least two chromatographic peaks were present for each alkene, probably due to the presence of isomers with the double bond at different positions of the hydrocarbon chain.

The main sterols of the pollen used here were Δ5-avenasterol (31.1%), β-sitosterol (27.7%), 24-methylenecholesterol (15.2%), stigmasterol (7.95%), campesterol (5.4%), cholesta-5,24-diene-3-ol (4.83%), cholesterol (3.93%).

Tab.1: Fatty acids isolated from the apolar fraction of pollen by means of three different methylation procedures.

Fatty acids	
C10:0	Capric acid
C12:0	Lauric acid
C14:0	Myristic acid
C16:0	Palmitic acid
C16:1	Palmitoleic acid
C18:0	Stearic acid
C18:1n-9	Oleic acid
C18:2n-6	Linoleic acid
C18:3n-3	Linolenic acid
C20:0	Arachid acid
C20:1n-9	Eicosenoic acid
C22:0	Behenic acid
C24:0	Lignoceric acid

3.4. Experiment 4: RNAseq and transcriptomic analysis

3.4.1. Effects of pollen on gene expression in bees parasitized or not by the mite

The RNAseq analysis of our experimental groups generated four DEGs lists.

A number of genes showed significantly different expression levels according to the pollen feeding or *Varroa* mite infestation (Table 2) but there were interesting commonalities and discrepancies between treatments (figure 5).

Tab.2: genes significantly differentially expressed ($p < 0.01$) according to the treatment (V+= *Varroa* infested bees, V-= uninfested bees, P+ = pollen + sucrose fed bees, P- = sucrose fed bees).

DEGs lists	**Regulated genes**	**Up-regulated**	**Down-regulated**
V+P+/V+P-	393	133	260
V-P+V-P-	330	153	177
V+P-/V-P-	1333	1041	292
V-P+/V+P+	391	264	127

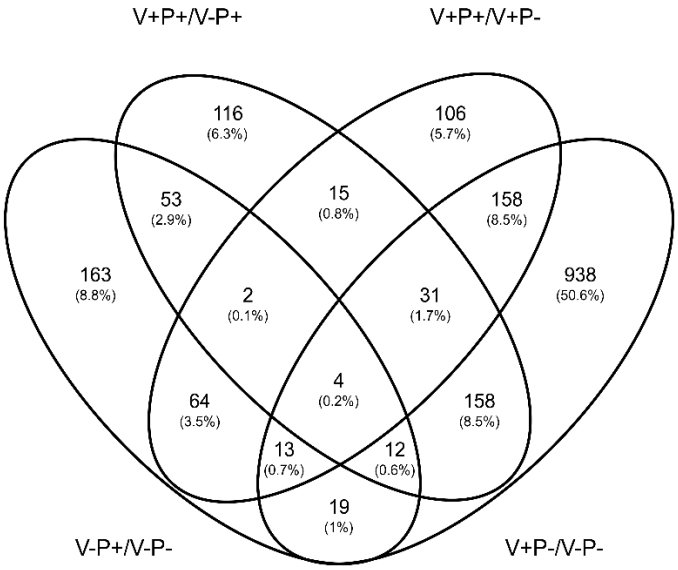

Fig.5: genes in common between the four DEGs list (Venny[2.1])

The gene expression profiles according to the treatment were used for a principal component analysis (fig. 6); on the PC plot, parasitized bees fed with pollen (P+/V+) tended to cluster closer to unparasitized bees fed or not with pollen (P-/V- and P+/V-) as compared to parasitized bees fed with sugar only (P-/V+); further suggesting, at the level of global gene expression, that a pollen rich diet can somehow compensate the deleterious effects of mite parasitization.

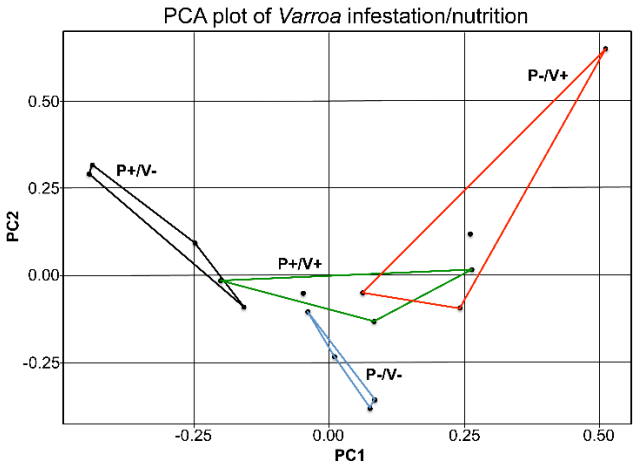

Fig.6: principal component analysis (R; groups are circled arbitrarily. V+= infested bees, V-= uninfested bees, P+= bees fed with sucrose + pollen, P-= bees fed with sucrose only).

According to the objectives of our study, we then focused our attention on the genes that showed differential expression in infested bees fed with pollen as compared to infested bees

fed with sugar only, after subtracting genes that were regulated according to parasitization regardless of diet and diet regardless of parasitization (fig. 5).

This group contained 106 significantly (p<0.01) regulated genes. A Gene Ontology analysis of the 53 DEGs with *Drosophila* orthologs, that was performed to identify possible functional components regulated by pollen feeding in parasitized bees, revealed 12 functional annotation clusters. The ontology categories revealed by the analysis primarily regarded polysaccharides, amine and carbohydrate metabolic processes, chitin metabolic process, polysaccharide and carbohydrates binding. However, only one term was significantly enriched (dme00520), corresponding to a KEGG pathway involved in amino sugar and nucleotide sugar metabolism. The pathway is involved in the formation of polysaccharides components of chitin, one of the main component of insect exoskeleton.

A more detailed analysis of the differentially expressed genes between the two diets in presence of *Varroa* mite infestation revealed some recurring themes. In particular, genes related to lipid metabolism and potentially involved in cuticle formation were significantly regulated in pollen-fed infested bees (Tab.3), including Acyl CoA desaturase-1 (GB48194), lipid storage droplets surface-binding protein 1 (GB47140), chitinase-3 (GB43173), lipase-3 (GB41760), *Apis mellifera* "mummy" (GB44897), cuticular proteins 6 and 17 (respectively GB40566 and GB46310), apidermin 2 (GB53119), GB52854, cuticular protein analogous to peritrophins 3-E.

In order to further characterized the observed response, we carried out a comparison of our DEGs and a group of genes potentially involved in a stress response triggered by a wound on the cuticle found in a previous study by Richard et al. (2012). Three "cuticular integrity" genes were in common between ours and Richard and colleagues' study and resulted to be regulated by pollen assumption (see Tab. 3). Lipid droplets storage protein 1, involved in energy homeostasis and lipid metabolism (Beller et al., 2006), *TRAM* a traslocating chain-associated membrane protein and groucho a NF-kB corepressor for several transcription factors including dorsal. Other genes involved in lipid metabolism and cuticle formation affected by a pollen diet in presence of mite infestation are reported in the table 4

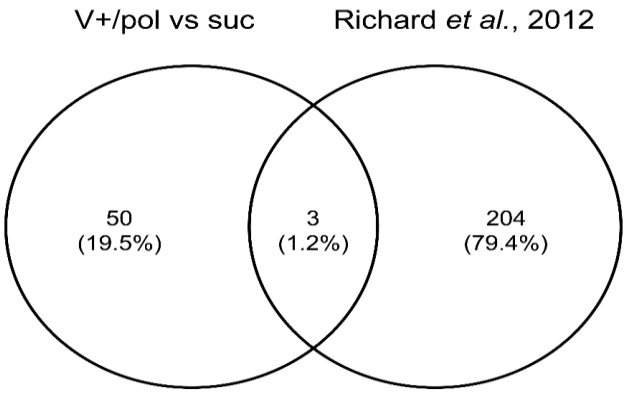

Fig.7: genes in common between the DEGs list corresponding to infested bees fed with the two different diets and the list of "cuticle integrity" genes obtained by Richard and colleagues' study.

Tab.3: genes related to "cuticle integrity" (Richard et al., 2012), significantly regulated by a pollen diet in presence of infestation.

Gene ID (Beebase)	Gene name	Pathway/function	Pollen effect
GB50944	*TRAM*	Translocating chain-associated membrane protein	Up-regulation
GB47140	*Lsd-1*	Lipid droplets storage protein	Up-regulation
GB48608	*Gro*	Transcriptional repressor	Down-regulation

Tab.4: other genes involved in lipid metabolism and cuticle formation regulated by pollen feeding in presence of infestation.

Gene ID (Beebase)	Gene name	Pathway/function	Pollen effect
GB53119	*Apd-2*	Apidermin, cuticular protein	Up-regulation
GB41760	*Lipase-3*	Lipid metabolism	Up-regulation
GB48194	*Acyl-CoA*	Desaturase	Up-regulation
GB44897	*Mummy*	Chitin and glycan synthesis	Up-regulation
GB47140	*Lsd-1*	Lipid store droplet protein	Up-regulation
GB52854	*Cpap-3e*	Cuticular protein	Down-regulation
GB40566	*CPR-6*	Cuticular protein	Down-regulation
GB46310	*CPR-17*	Cuticular protein	Down-regulation
GB43173	*Cht-3*	Chitinase	Down-regulation

Additionally, several genes that have previously been linked to immune and stress responses were regulated in infested bees fed with pollen, supporting the hypothesis concerning the role of a complete diet in providing energy and materials to be used for mouting an immune response in bees challenged with the mite and the vectored pathogens.

A comparison between our pollen regulated genes and a group of genes know to be involved in the immunity canonical pathways (Ryabov et al., 2014) showed five immunity genes regulated by pollen feeding in presence of infestation (Tab.5). Other genes not included in the Ryabov list (Tab.6) but potentially related to stress response are a venom acid phosphatase Acph-1-like (GB41302), a capa receptor-like (GB55629), a DRR1-related protein (GB51281), an endoplasmin (GB41867), and a dnaJ homolog subfamily B member 11-like (GB51659).

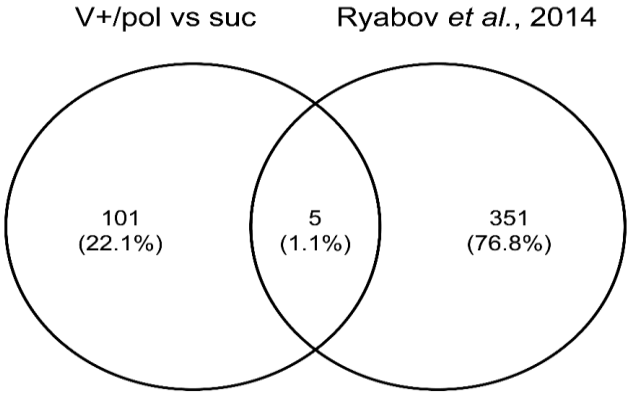

Fig.8: common genes between the DEGs list corresponding to infested bees fed with two different diets and the list of genes related to canonical immunity pathways (Ryabov et al., 2014).

Tab.5: genes involved in canonical immune pathways (Ryabov et al., 2014) significantly regulated by pollen feeding.

Gene ID (Beebase)	**Gene name**	**Pathway/function**	**Pollen effect**
GB48662	*Toll*	Toll signaling pathway	Up-regulation
GB41428	Defensin-1	AntiMicrobialPeptide	Up-regulation
GB50865	*AmSCR-B3*	Scav.receptor A	Down-regulation
GB49363	*AmSCR-B5*	Scav.receptor A	Down-regulation
GB54246	Corin-like	Scav.receptor A	Down-regulation

Tab.6: genes involved in various types of stress responses significantly regulated by pollen feeding.

Gene ID (Beebase)	**Gene name**	**Pathway/function**	**Pollen effect**
GB41302	*Acph-1*-like	Venom acid phosphatase	Up-regulation
GB51281	Discoidin domain receptor family	Stress inducible actin interacting protein	Up-regulation

GB55629	Capa receptor like	Desiccation stress	Up-regulation
GB51659	DnaJ homolog	Co-chaperone (heat shock proteins)	Up-regulation
GB52854	*Cpap-3e*	Cuticular protein	Up-regulation
GB41867	Endoplasmin like	Endoplasmatic reticulum degradation	Up-regulation

3.4.2. Effect of pollen feeding on viral loads

Using the RNA sequencing results, we examined the number of viral reads, related to the 11 viruses most commonly found in the hive (Chen and Siede, 2007) in the infested bees fed with sucrose or with a complete diet (sucrose + pollen). The RNAseq analysis revealed, on average, 0.1% of viral reads in uninfested bees, regardless of diet (Fig.9). Instead, the proportion was higher in parasitized bees, reaching 45% in infested bees fed with sugar only as compared to 21% in bees fed with a pollen rich diet.

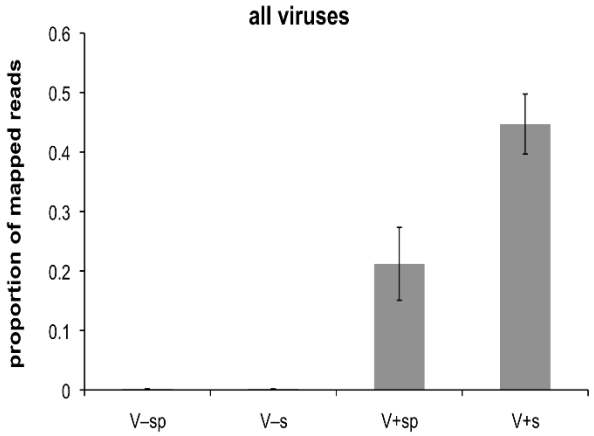

Fig 9: proportion of mapped reads, in infested and uninfested bees (V+, V-) according to the diet (s, sp), related to the 11 most common viruses typically found in the hive (p<0.0001).

Overall, Deformend Wings Virus (DWV) was the most prevalent virus, representing 75 % of the total mapped viral reads in the case of infested bees fed with a sucrose diet, and 70% in infested bees fed with a sucrose plus pollen. If attention is restricted to this virus only the

overall pattern remains the same as that discussed above with a 35% of DWV mapped reads in bees fed with sugar only and a 15 % in bees fed with a complete diet (Fig. 10).

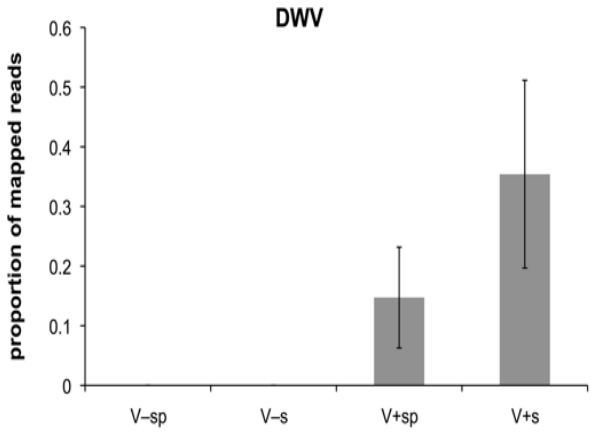

Fig.10: proportion of mapped reads corresponding to Deformed Wing Virus in infested and uninfested bees (V+, V-) according to the diet (s, sp). (p=0.0003).

4. DISCUSSION

Our results indicate that access to dietary pollen can mitigate the negative impact of *Varroa* mite infestation and the related viral infection in caged honey bees under laboratory conditions. Interestingly, access to dietary pollen did not improve the survival of uninfested bees, suggesting that pollen is not critical for bee survival and health under these controlled conditions. Dietary pollen is important for nurse bees to produce lipid rich larval jelly to feed to developing larvae (Keller et al., 2005), but since there were no larvae present in the cage, this did not play a role in this study.

Previous studies have found a slight negative effect of dietary pollen on caged bees as they age consistent with the fact that older forager bees do not consume pollen and have altered digestive processes that may not be able to manage a high pollen diet (Crailsheim et al., 1992). This is consistent with the steady reduction in pollen consumption that we observed during the course of our study.

Parasitized bees did not consume higher quantities of pollen compared to unparasitized ones indicating that active compounds from pollen are used for a prophylactic medication, whereby potentially active substances are taken up by both infected and uninfected

individuals alike, to prevent parasite infection or the detrimental effect caused by them, in contrast with the therapeutic use of substances, which implies an active use of useful substances by individuals challenged by a parasite.

Bee colonies normally show strong differences in terms of pollen collection with some strains being more active in pollen foraging compared to others (Abou-Shaara, 2014). Such a variability, as well as other uncontrolled factors, may have confounded the results of the field study which, however, seem to confirm our conclusion about the potential of pollen for decreasing the impact of mite infestation on bee colonies. In fact, despite the effect on bee mortality was at most limited, colonies treated with pollen showed enhanced survival suggesting a promising practical use of pollen for the prevention of colony losses.

Pollen is very chemically complex, serving as the primary source of proteins and lipids, while providing vitamins, minerals, phenolic compounds and flavonoids (Campos et al., 2008). Our studies indicated that the apolar, lipidic compounds found in pollen play a key role in prolonging the lifespan of parasitized bees. However, our experiments do not rule out the possibility that other compounds from the polar fraction may play a role; this matter is certainly worth of further investigation.

The lipid content of the pollen used in this study, as extracted with dichloromethane, was around 4% of the freeze dried pollen similarly to what observed by other authors using slightly different analytical methods (i.e. 4.8-7.2% (Serra Bonvehí and Jorda, 1997); 8.7 ± 1.5%, 5.5 ± 2.3% and 6.2 ± 2.5% (Szczesna, 2006); 4.3-6.3% (Feás et al., 2012); 6.4-7.4% (Di Pasquale et al., 2013); 5.30-9.12% (Mărgăoan et al., 2014)).

The apolar fraction of the pollen is still quite chemically complex. Several fatty acids were identified in the pollen used in this study including capric, lauric, palmitoleic, stearic, arachidic, eicosenoic, behenic and lignoceric acid, the most represented being palmitic (28%), linolenic (23%), myristic (15%), linoleic (14%) and oleic acid (12%). Unsaturated fatty acids represented about 50% of total fatty acids.

Percentages of linolenic acid similar to those found in this study were reported by other authors (Bonvehí and Jordá, 1997; Yang et al., 2013); instead, higher values (i.e. 46%, 36%,

49% and 33%) were reported by Szczesna (2006) and Mărgăoan et al. (2014). The percentage of palmitic acid (28%), which represents the main saturated fatty acid in our pollen, is, on the contrary, similar to that reported by these authors.

The pollen used in this study is characterized by a higher content of myristic acid (15%) compared to percentages reported in the literature (Serra Bonvehí and Escolá Jordá, 1997; Szczesna, 2006; Yang et al., 2013; Mărgăoan et al., 2014), which are generally lower than 5%. Higher percentages of this fatty acid were reported by Hassan (2011) in date palm pollen (13%) and by Yang et al. (2013) in rape bee pollen (21%). All authors agree that there can be large variations in fatty acid composition of pollens depending on botanical origin, geographical area, collection time and processing and storage conditions.

Based on the identity of the compounds identified in the active fraction of pollen and the biology of bees parasitized by the *Varroa* mite, it is possible to draw some hypotheses on the possible prophylactic role of pollen, setting the ground for future researches aiming at testing such concepts with appropriate experiments (fig. 11).

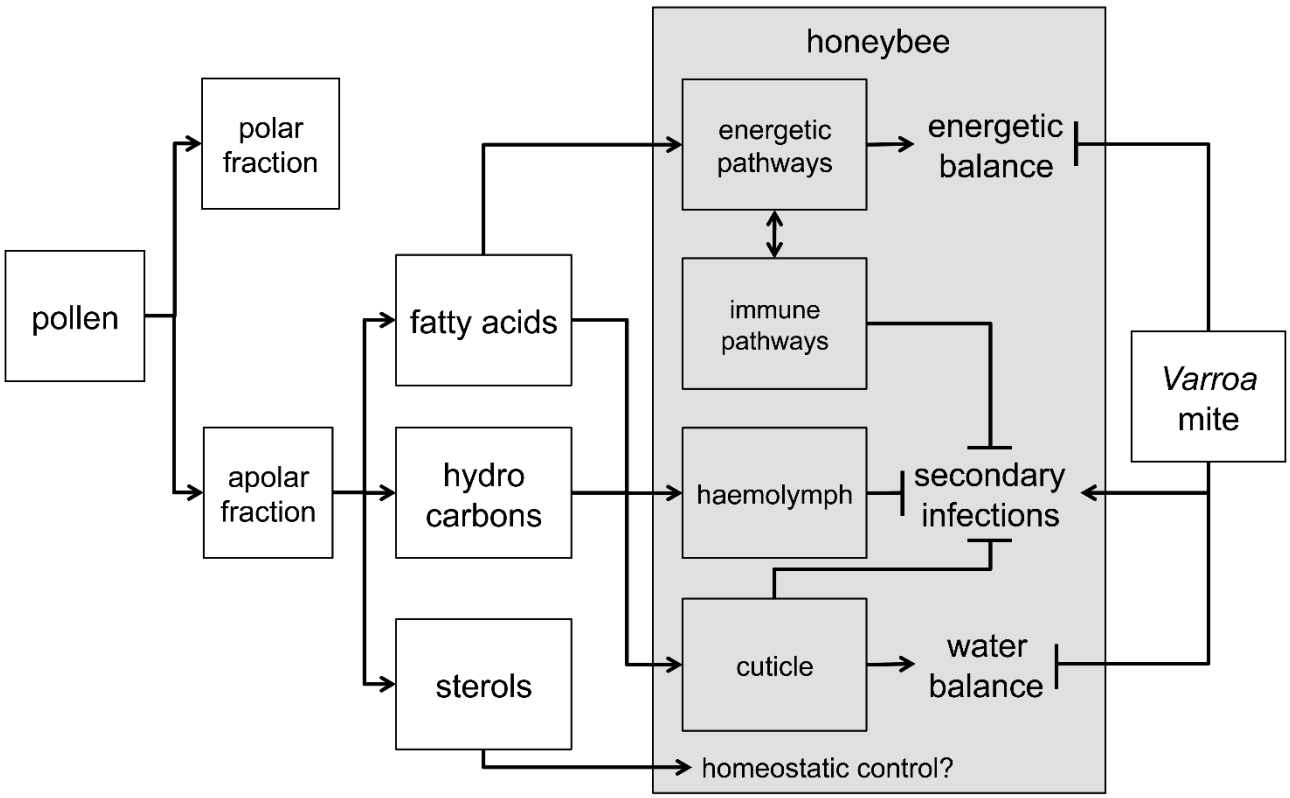

Fig. 11: diagram reporting some hypothesis about the possible prophylactic role of pollen.

Since fatty acids could be used as an energy source complementary to sugars, through the beta oxidation pathway (Metzler; 2003), it could be speculated that increased survival of parasitized bees fed with pollen may be related to the reconstitution of lipid energetic stores depaupered because of the feeding activity of the mite (Bowen-Walken &Gunn, 2001). However, Bowen-Walker & Gunn (2001) comparing the composition of infested and uninfested bees noticed that only proteins and carbohydrates are affected by parasitization whereas lipids apparently are not.

Similarly, worth of consideration would be the possibility that the exploitation of pollen components in the energetic pathways can release energy and materials to be used to sustain the immune defenses necessary to control the secondary pathogens facilitated by the *Varroa* mite. Activation of the insulin pathway following a pollen rich diet has already been shown in the honey bee (Alaux et al., 2011) and the strict link between energetic pathways and immunity has been proved in *Drosophila* (Unckless et al., 2015). Certainly, this is a research path that deserves more attention and future research effort.

Fatty acids are also involved in membrane formation and cellular integrity (Cooper, 2000), for this reason, in principle, it is possible that pollen lipids play a role in the mitigation of cellular damage inflicted by the mite at the pupal stage. Fatty acids and other lipids could also be important for their possible direct beneficial effects. In particular, in arthropods, the cuticle represents the first barrier against possible invading parasites such as bacteria and fungi entering through any solution of continuity of the integument. It has been shown that in some insects cuticular fatty acids are involved in the resistance towards fungi (Gutierrez et al., 2015) whereas data about bacteria are scarcer. Furthermore, unsaturated fatty acids are well known for their antibiotical activity (Feldlaufer et al., 1993) and the pollen used in this study appears to be particularly rich in such compounds. Both fungi and bacteria are widespread in the hive and can be associated to the *Varroa* mite. In particular, Vanikova et al. (2014) studied the *Varroa* mite-associated bacterial population and found that is

dominated by Gram-positive bacteria of *Bacillus* and *Microbacterium* genera while Gram-negative bacteria were represented by members of *Brevundimonas* and *Rhizobium* genera. Bacteria, such as *Melissococcus pluton* and other undetermined elongate types, are normally found around the feeding hole of the *Varroa* mite (Kanbar & Engels, 2003).

Moreover, the contribution of the mite to virus-driven immunosuppression is largely accepted and this lowering of immune defenses can certainly facilitate opportunistic pathogens inhabiting the hive, including fungi and bacteria. As a matter of fact several transcriptomic analyses of parasitized bees revealed that a significant number of members of the Toll pathway, including PGRP and antimicrobial peptides, are affected upon infestation (Yang and Cox-Foster, 2005) confirming the exposition of parasitized bees to a higher risk of microbial infections.

Thus, in principle, lipids and in particular, fatty acids, acquired with the diet, could either be transferred to the cuticle where they may reinforce the first line of defense against pathogens facilitated by the *Varroa* mite or they may circulate in the hemolymph and cooperate in the control of infecting parasites reaching the hemocael. This could be tested by studying the translocation route of fatty acids ingested with the diet and their role for the control of microorganisms facilitated by the mite.

Hydrocarbons represent an essential component of the honey bee cuticle and, apart from a fundamental role in chemical communication within the colony, they exert and essential function in the prevention of water loss through the integument (Gibbs, 1998). It has been shown that mite infestation can alter the composition of the lipidic layer of the honey bee cuticle with possible consequences for water balance (Annoscia et al., 2012) and pollen contains hydrocarbons and waxes that are similar to those observed on the bees' cuticle (Annoscia et al., 2012). For this reason, a further effect of pollen could be the reconstitution of the integrity of the cuticular waterproof layer of parasitized bees.

Whether this can actually help in the maintenance of the water balance of bees could then be tested by comparing the performance under this respect of the cuticle of pollen fed bees and

control bees after confirming that pollen hydrocarbons acquired with the diet can actually reach the bee's cuticle.

Finally, sterols are component of cellular membranes, precursors of many hormones (e.g. 20-OH ecdysone) and regulate genes involved in developmental processes; they could therefore play a role in the conservation of the homeostatic balance of parasitized honey bees.

The transcriptomic analysis of bees from each experimental group, and, in particular, the comparison between infested bees fed with a pollen rich diet and uninfested bees that received a similar diet gave futher support to some of the hypotheses listed above.
In particular, the expression of a number of genes involved in the formation of cuticle components suggests that pollen components could indeed be involved in the restoration of integrity of the cuticle offended by the parasite or secondary pathogens.
Furthermore, the significant effect of pollen on lipid metabolism highlighted by our transcriptomic analysis confirms that pollen could restore the resources needed for the energetic metabolism or the build up of natural defences as highlighted by the many genes involved in innate defences whose expression was affected.

Infested bees showed much higher values of viral reads in their transcriptome than uninfested ones, with a clear prevalence of DWV, further confirming the well known relationship between *Varroa* mite parasitization and DWV replication (Nazzi et al., 2012; Di Prisco et al., 2016). On the other hand, the reduced level of DWV infection in parasitized bees fed with a pollen rich diet, already observed in previous studies (DeGrandi-Hoffman et al., 2010), is of great interest and worth of deeper investigation. We can speculate that bees receiving a complete diet can rely on a constant supply of lipids and proteins that could compensate for losses caused by the mite feeding activity, thus saving energy from metabolism and nutrient stores mobilization, in favour of immune system activation against the pathogen. Alternatively, pollen could simply provide the row material to build an antiviral defence in bees challenged with the *Varroa* mite, a resource that is not strictly

necessary in uninfested bees. In any case, more detailed studies are needed that could benefit from the data obtained through the transcriptomic analysis.

In conclusion, on the ground of the results described here, we can state that pollen represents an essential component of bees' nutrition whose properties go well beyond the supply of essential amino acids or metabolic energy. In particular, it appears that the apolar components of this food can provide important tools for the maintenance of the honey bee's homeostasis including energetic and water balance and allow the coexistence with the rich cohort of symbionts inhabiting the hive.

Further, in depth investigation into these matters appears essential in view of preserving the essential role of these insects for cultivated and natural ecosystems.

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Chapter 5: **general conclusions**

Ph: Virginia Zanni

During my research activity, I focused my attention on two important stress factors affecting the western honey bee: the mite *Varroa destructor*, the major threat for apiculture in most parts of the Northern emisphere (Rosenkranz et al., 2009), and the deformed wing virus (DWV), a deadly pathogen to which the mite is linked in a mutualistic symbiosis (Di Prisco et al., 2016). I also investigated the role of a complete nutrition to preserve honeybee health, highlighting the therapeutic effects of pollen in mite-parasitized bees.

In my research I adopted, where possible, a holistic approach; therefore, I started with the analysis of the mite's impact on the bee's transcriptome (Chapter 2). RNAseq analysis allows monitoring the changes in the organisms' gene expression pattern under different environmental and experimental conditions, providing a sensitive indication of effects that will impact physiological systems (Grozinger and Robinson, 2015).

It has been shown that *Varroa* causes important physiological alterations (Amdam et al., 2004) as well as behavioural modifications in bees (Annoscia et al 2015) that have important consequences at colony level but the underlaying mechanisms are still unclear. In order to investigate the effects of mite parasitization and the concurrent viral infection in infested bees, I analysed the transcriptome of bees collected from apiaries with different infestation

levels and artificially infested individual bees reared under lab conditions. Field collected bees highlight the effect of the mite on the transcriptome in presence of changing environmental conditions while rearing under lab conditions allows to isolate the sample from external effects; overall this approach, provides a reasonably complete picture of the problem.

The aim of the first part of my research was to elucidate which groups of gene are affected by the parasite, taking into account the presence of the virus. The results of this analysis suggest that a large group of biological processes and pathways are affected in case of parasitization; in particular, in bees exposed to the mite, both under field and lab condition, an altered expression level of genes involved in immunity, response to stress, behavioural maturation, metabolism and nervous system was observed.

Effects of mite parasitization and the concurrent viral infection, confirmed here in the experimental bees, on honey bee immune competence were notable, in particular at the expenses of genes from the Toll pathway.

The differential expression of several genes related to behavioural maturation in presence of infestation confirmed that *Varroa* could alter the expected temporal polyethism schedule (Seeley, 1982). Regardless of age, the infested bees we tested showed a gene expression profile more similar to that of foragers while controls appeared more "nurse-like". This finding implies a potentially serious damage at colony level related to neglecting of the brood and other in-hive duties (Annoscia et al., 2015), precocious foraging (Woyciechowski and Kozłowski 1998) and premature death of the adults (Rueppel et al., 2007).

Genes identified as above, showing a consistent trend across data sets, were also screened by q RT-PCR to test their suitability as potential molecular markers of colony decline. Of particular interest under this respect was the gene encoding for the vitellogenin, an egg yolk protein associated with different functions in honey bees (Amdam et al., 2011) and previously identify as a predictive marker of bee colony collapse (Dainat et al., 2012, Smart et al., 2016).

The modified expression levels of genes involved in behavioural maturation and the foragers like expression pattern showed by infested bees are reflected in the behavioural changes previously observed (Annoscia et al., 2015) and confirmed by my studies. In chapter 3 of this

thesis, I described some experiments performed to highlight the modifications in the multistep nursing process, which can be observed in case of infestation. In particular, I found that infested nurses are less active within the hive and tend to neglect the brood with potentially serious effects at whole colony level. Starting from this evidence, I investigated the behavioural modification in nurses artificially infested during the pupal stage, focusing the attention on three steps of the nursing process: the brood pheromone response, the development of the structures used for larval food production and the behavioural maturation. All the experiment were performed using both bees reared under lab and field conditions.

The higher concentration of JHIII in the hemolymph of mite parasitized bees as compared to healthy bees of a similar young age confirms, on different bases, that mite infestation causes an accelerated maturation leading to a precocious transition to foraging and this could explain the observed reduced nursing activity of infested bees observed also in this study. Despite development of hypopharingeal glands is partly controlled by contact with brood, the reduction of these glands in infested bees both under field and lab conditions, together with the similar response to brood of infested and uninfested bees, indicates that disruption of endocrine regulation of polyethism may play a major role.

The importance of the hive environment in moulding the development of the bees was confirmed in my study (Robinson, 1992); in fact, lab bees, reared without contact with brood and exposure to queen's pheromone, showed less developed hypopharyngeal glands and a lack of a defined response in the olfactometer.

Infested bees introduced into the observation hive were less accepted than healthy individuals under the same controlled experimental conditions; the already reported differences in the cuticular HC may well account for the observed difference.

Interestingly, at low infestation levels, the reduction of brood caring in infested bees could be regarded as beneficial for the colony. In fact, nurse bees infested during the pupal stage are more likely to transmit the virus to young larvae by supplying contaminated larval food. Under this point of view, their reduced involvement in nursing, minimizing the contact with the larvae, would reduce viral transmission.

Finally, in chapter 4, I focused my attention on pollen, a food resource that seems to be able to counter the detrimental effects of *Varroa* parasitization. First, we tested the effect of a complete diet composed by sugar candy and pollen on bees parasitized by *Varroa*, noting a significant increase in the lifespan of infested bees fed with pollen. Then we identified in the lipidic fraction, the component of pollen that is at least partly responsible for the therapeutic effects above described. These results allowed to formulate some hypotheses about the potential role of pollen.

A possible role of pollen in supporting the immune system, providing a reserve of nutrients and promoting the cuticle restoration is confirmed by the analysis of the transcriptome of infested bees fed with the two different diets (i.e sugar candy and sugar candy plus pollen). As a matter of fact a pollen diet seems to help to control viral replication in infested individuals.

In conclusions, through my research project, I tried to fill some gaps in the knowledge about the effects of the mite and the virus on honey bee health by moving from an holistic approach aiming to identifiy the possible effects to the analytical dissection of the mechanistic details of the involved processes.

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Appendix: **other works and collaborations**

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ATTACHMENT 1

Annoscia D., Brown S., Di Prisco G., De Paoli E., Del Fabbro S., Zanni V., Caprio E., Pennacchio F., Nazzi F. Honey bee immune-competence is the major factor controlling Deformed Wing Virus proliferation triggered by *Varroa* mite. Submitted.

Honey bee immune-competence is the major factor controlling Deformed Wing Virus proliferation triggered by *Varroa* mite

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Summary paragraph

Efficient pollination is vital for crop production[1] and the honeybee is the prevailing managed insect crop pollinator. However, domesticated bees suffer from a range of adverse factors[2]. In particular, the Deformed Wing Virus (DWV) is implicated in large colony losses reported in some parts of the world[3]; furthermore, recent studies indicated that even wild pollinator populations may be at risk of infection due to the possible spillover of the virus from managed populations of *Apis*[4]. The parasitic mite *Varroa destructor* plays a key-role in virus transmission

and replication[3,5,6]; however, there are still alternative and not fully resolved hypotheses on the major mechanisms underpinning the *Varroa*-DWV association[7,8,9,10,11]. Here we show that the increasing virulence of DWV, in presence of *Varroa* mite, depends on the disruption of the bee's immune competence, with effects that are influenced by the starting infection level. We found that neither the viral replication within the vector nor the immune-suppression by the mite play a relevant role; furthermore, the composition and structure of the viral mutant cloud in infected bees, do not support an important role of viral molecular diversity in the modulation of DWV virulence. Instead, on the ground of our results and simulations, we conclude that the stress resulting from mite feeding has the potential of destabilizing the equilibrium between the pathogen and the bee's immune defence leading to uncontrolled viral replication. These findings shed light on the dynamics of multiple parasitic interactions in bees and will pave the way for a more sustainable management of this impending pollinator crisis.

To clarify the role of the mite in the dynamics of viral infection in honeybees, we studied the presence and abundance of DWV in bees obtained from larvae artificially infested with a mite as compared to uninfested control larvae. Furthermore, where possible, we used NGS techniques to assess the genotypic identity of the Deformed Wing Virus infecting the bees used in this study (Extended Data Fig. 1).

In two cases, the high abundance of virus-derived reads allowed for the *de novo* assembly of the complete sequence of the DWV genome. The obtained DWV sequences showed 99% identity with each other and 98 and 99% identity respectively with a published sequence obtained from a sample collected in the same apiary in 2006 (i.e. NC_004830.2; Extended Data Fig. 2).

The analysis of the viral infection status of each experimental bee showed that 80% of individuals not exposed to mite feeding (n=40) were DWV infected. Nevertheless, the prevalence of DWV in bees infested by a DWV infected mite (n=27) was significantly higher and reached 96% (Extended Data Fig. 3; Chi Sq=3.681, d.f.=1, P=0.055).

Viral load was higher in bees parasitized by mites compared to control bees (Fig. 1; median viral load in mite infested bees (n=32)=1.91E+04; median viral load in uninfested bees (n=40)=8.06E+03; Mann-Whitney U=482, n_1=40, n_2=32, P=0.037).

DWV infection levels in non-parasitized bees showed a great variability ranging from 10^3 to 10^6 (Fig. 1). However, a much higher variability in DWV levels was associated with mite infestation; in fact, despite most mite infested bees showed infection levels falling within the same interval as that recorded in uninfested bees, a few specimens largely exceeded the upper limit of this interval, reaching 10^10 viral genome copies per bee (Fig. 1). Consequently, the distribution of viral loads was very skewed (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 season, when the prevalence and the basal infection rate are higher[11], and artificially infested with one mite, showed a similar right skewed distribution of infection levels (skewness of the distribution of viral loads in mite infested bees (n=58)=5.66; Extended Data Fig. 4).

Interestingly, the reconsideration of previous data demonstrating the effect of single and multiple mite infestation on viral loads in bees[11], revealed the existence of a similar underlying distribution of individual data with a higher median viral infection in mite infested bees and a distribution of viral loads becoming increasingly sparse (Extended Data Fig. 5).

To study the vector role of *Varroa*, we focused on the mites infesting the experimental bees above (Extended Data Fig. 1) and found that their infection level was generally higher than in these latter (median viral load in mites (n=32)=4.60E+04).

A significant correlation was found between the mites' viral load and that found in the bees they infested (Extended Data Fig. 6; n=32, Spearman corr. coeff.= 0.531, t=3.433, d.f.=30, P=0.002). However, this result cannot be univocally interpreted, since the observed correlation could be due 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 goes through 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[8], we assessed the presence of DWV negative strands in the mites used for the artificial infestation of bees (Extended Data Fig. 1).

As expected, the mites containing DWV negative strands had a significantly higher infection level than those where no negative strands were found (Extended Data Fig. 7; 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 (Extended Data Fig. 8; Mann-Whitney U=80, n_1=9, n_2=23, P=0.157).

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 (i.e. quasi-species). In order to study the composition of the viral mutant clouds in bees with different levels of viral infection, we amplified and sequenced the viral region encoding the virus RNA dependent RNA polymerase, by individually processing 5 highly infected bees and 5 bees with low infection levels (average DWV infection level (DWV genome copies per bee), 1.41E+09 and 1.95E+03, respectively) that were obtained from the previous experiment (Extended Data Fig. 1). 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. No single sequence was found at a relevant density (>5%) in all samples, the most represented being present in six samples from both the low and highly infected groups, at prevalences ranging from 11 to 74% (Fig. 2). A link between viral load and molecular diversity was not found (Fig. 2, Extended Data Fig. 9).

To test the role of the bee's immune response in the observed distribution of viral infection level, we studied the transcriptome of uninfested bees, bearing a low viral load, and mite infested bees bearing either a low or a high viral load after parasitization (Extended Data Fig. 1). The clustering of samples according to mite parasitization and viral infection state (Extended Data Fig. 10) underlined the similarity in the overall pattern of expression of immune genes in bees affected by the same combination of stress factors, which was confirmed by a multivariate discriminant analysis allowing a correct group classification of the majority of samples according to mite parasitization and viral infection level (percentage of correct group classification with cross validation: 80%, 80% and 40% for uninfested bees, mite infested bees with low DWV infection and mite infested bees with high DWV infection, respectively).

To disentangle the effect of the *Varroa* mite from that of DWV on the immune competence of bees, we studied the expression of immune genes in bees exposed to a different combination of stress factors (Extended Data Fig. 1; Supplementary Data 1). In particular, to assess the influence of the mite, we

compared the expression level of immune genes in five uninfested bees bearing a low viral infection (average DWV infection=2.04E+03) and five mite infested bees bearing a similar low viral infection level (average DWV infection=1.95E+03); instead, to assess the influence of the combination *Varroa*-DWV, we compared five uninfested bees bearing a low viral infection with five mite infested bees bearing a high viral infection level (average DWV infection=1.41E+09).

We found that different immune pathways were differentially affected by the *Varroa* mite and the *Varroa*-DWV combination (Fig. 3). Mite infestation appeared to influence the Toll pathway and downstream antimicrobial peptides likely due to wounding by mite feeding and the associated secondary infections, which activate the humoral and cellular responses under Toll control (note that Toll genes differentially expressed were all up-regulated, Supplementary Data 1). This *Varroa* effect negatively influences the bee immune-competence and results in viral proliferation which may increasingly affect immunity by impacting other pathways such as JNK[12] (Fig. 3).

In 2012 we proposed a series of models describing how within-host viral dynamics are controlled by immunological response, which in turn can be modified by the presence of virus and other stress conditions, such as mite feeding or pesticide exposure[11,13]. 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 host death. In ref. 11 we focused on how the stable and unstable (threshold) DWV equilibria changed with increasing immune depletion, here we numerically explore the system dynamics under three scenarios of increasing immune depletion (Fig. 4) and for varying initial viral titre. When immune depletion is minimal (Fig. 4a), we find that initial viral titre only leads to explosive viral growth when the titre begins above the threshold unstable equilibrium (dashed grey line, Fig. 4a), and otherwise we see strong immune-mediated return to the stable equilibrium (solid grey line, Fig. 4a). As immune depletion increases (Fig. 4b), we see that the unstable threshold equilibrium drops, but still defines the outcome of infection (explosive growth only when initial titre is above the threshold). Finally, for the most severe depletion scenario (Fig. 4c), in addition to a reduced threshold to explosive viral growth, we see that even very low inocula can lead to explosive growth if they approach the stable equilibrium (solid grey line, Fig. 4c) too rapidly and overshoot past the nearby unstable threshold. Our numerical simulations highlight that, besides destabilizing viral regulation, the mite-induced depletion of the host immune resources can enhance the sensitivity to initial viral titre conditions resulting, after parasitization, in a few bees with very high infection levels along with a majority of bees with lower infections, according to the initial infection level of parasitized bees (Fig. 4).

The DWV sequences obtained here from an apiary where colony collapses are frequently observed, were clearly separated from other genotypes of DWV (i.e. NC_006494.1) or recombinants that were associated with higher virulence in other studies[10,14] (Extended Data Fig. 2).

The infection level in uninfested bees is consistent with available data about DWV prevalence in bee eggs and larval food[15,16] 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 (Extended Data Fig. 11). The variability in the transmission efficiency and the individual variation in immune competence account for the variable infection rate in bees before mite parasitization occurs.

On the other hand, the higher proportion of infected bees among those infested by a DWV infected mite, together with the presence of replicating viruses within the mites, confirms the crucial role of *V. destructor* as a vector of the virus[8] (Extended Data Fig. 11).
The DWV infection data show that the higher viral load observed, on average, in infested bees is due to a change in the distribution of individual viral levels, which is right skewed, due to the presence of a sub-population of highly infected bees as already observed using a different experimental setup[10].

Collectively, our experimental data allow to conclude that the capacity of the mite to favour the viral pathogen replication[8] (Extended Data Fig. 12a) appears to be of limited importance in the induction of individual viral bursts. The similar composition and structure of the mutant clouds, observed in low and highly infected bees, do not support an important role of viral molecular diversity in the modulation of observed levels of DWV virulence at individual level[10] (Extended Data Fig. 12b). Our transcriptomic study further confirms that immune-suppression by the mite[7] (Extended Data Fig. 12c) does not play an important role. Instead, on the ground of our results and simulations, we conclude that the stress resulting from mite parasitization[11] has the potential of destabilizing the equilibrium between the pathogen and the bee's immune control (Extended Data Fig. 12d) with consequences that depend upon the starting level of infection in bees; this is largely conditioned by the preceding history of the bee and the chances of vertical and horizontal transmission of the pathogen (Extended Data Fig. 11).
We believe that this new information on the interactions within the triangle bee-mite-virus contributes to clarify the crucial role of the *Varroa* mite in the re-emergence of DWV, an endemic pathogen of honey bees that is responsible for the current widespread crisis of the beekeeping industry.

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Acknowledgments
We gratefully acknowledge Michelle Flenniken for critically reading the manuscript. The research leading to these results was funded by the European Union Seventh Framework Programme (FP7/2007-2013), under Grant 613960 (SMARTBEES).

Figures

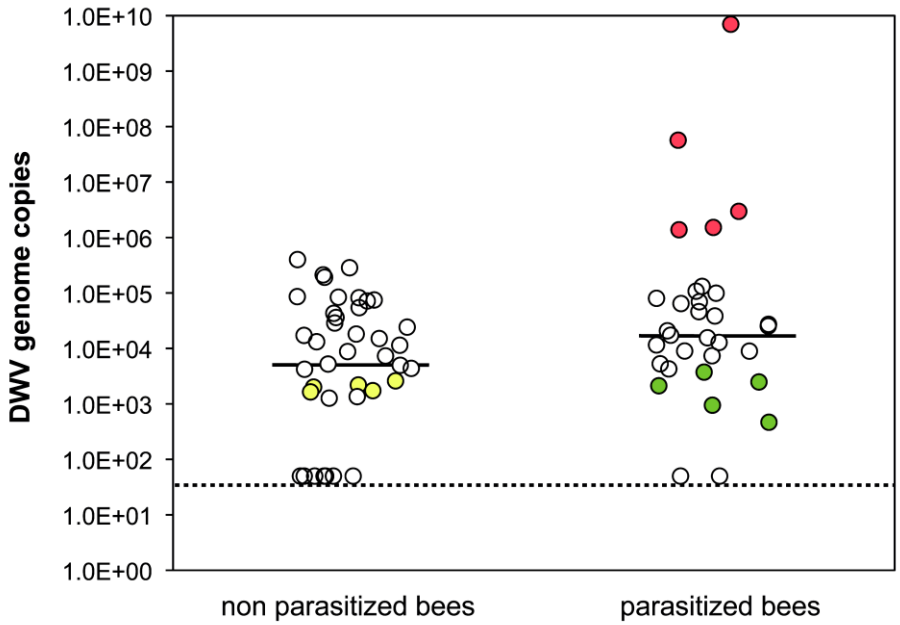

Figure 1: Viral load in individual bees artificially infested with one mite or left uninfested. The dashed line represents the lower detection limit with the used equipment. The solid lines represent the median viral load. The samples used for the transcriptomic analysis are marked with different colours: yellow (uninfested-low virus infected bees), green (mite infested-low virus infected) and red (mite infested-high virus infected).

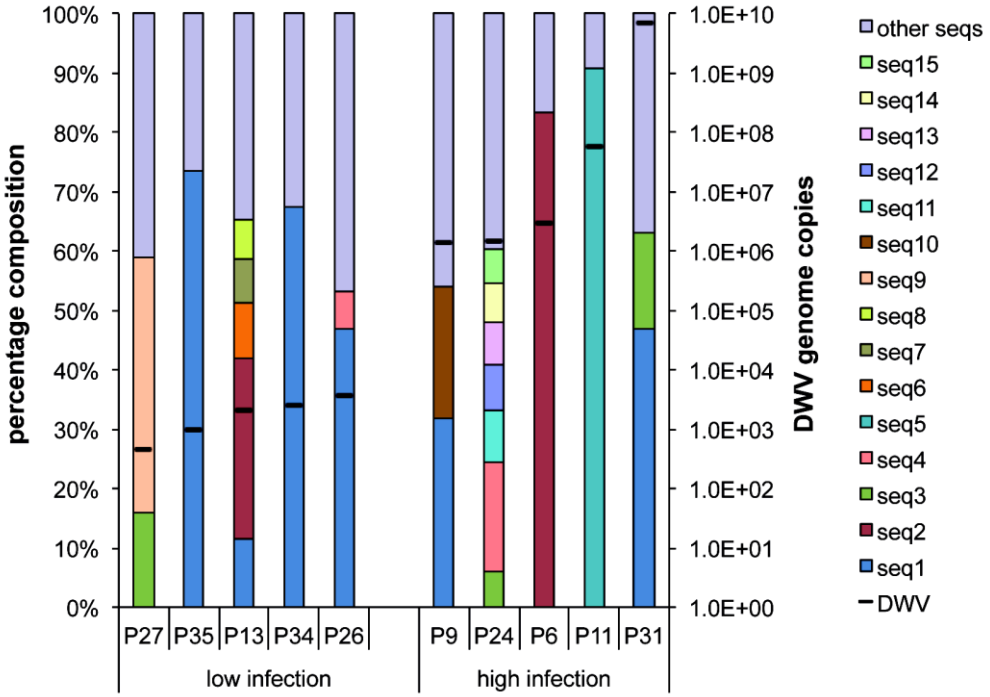

Figure 2: Prevalence of different DWV variants in infected bees. Samples are grouped according to the infection level that is reported in the graph with a hyphen.

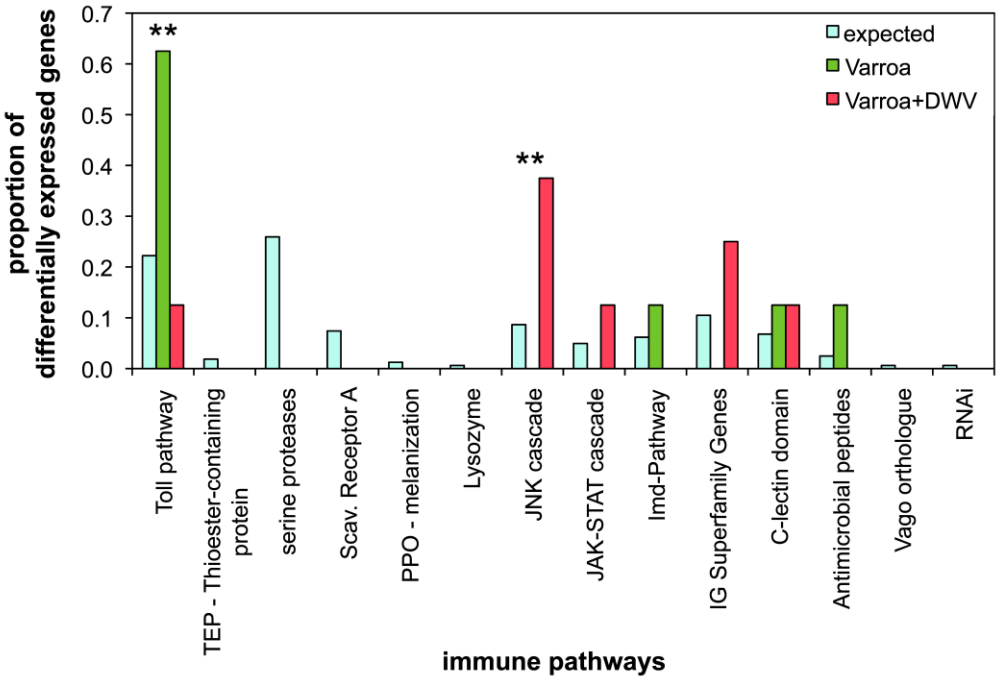

Figure 3: Effect of the *Varroa* mite and the combination *Varroa*-DWV on the expression of the genes of the canonical immune pathways. The proportion of differentially expressed genes in each pathway, as resulting from the comparison: uninfested, low viral infected bees vs mite infested, low viral infected bees (i.e. *Varroa* effect) and from the comparison: uninfested, low viral infected bees vs 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.

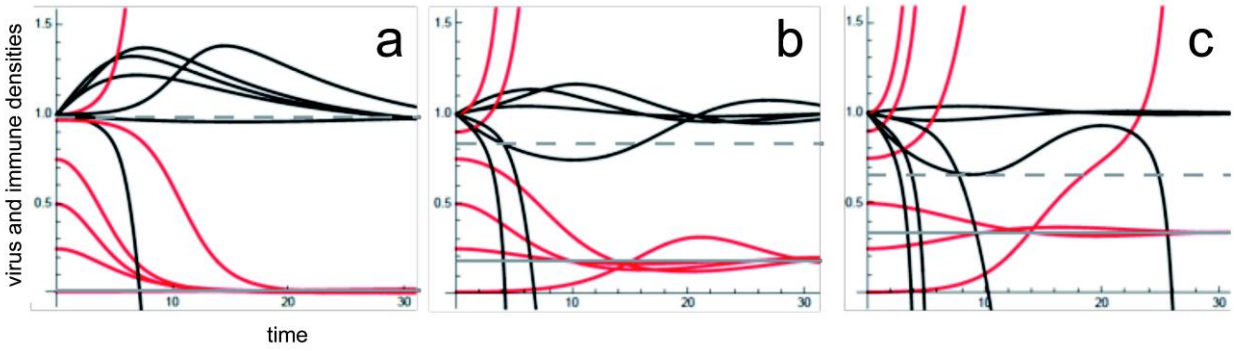

Figure 4: System dynamics under three scenarios of increasing immune depletion and for varying initial viral titre. Mite-induced depletion of host immune response can in theory destabilize viral regulation and enhance sensitivity to initial viral titre conditions. Relative viral titre (red) and immune status (black) as a function of time; stable (solid line) and unstable (dotted line) viral equilibria in grey. Model details in SM. a. Low immune depletion (y = 0.1): virus is tightly regulated to low viral steady state (V* = 0.02, solid grey line) and high threshold to dysregulation (V' = 0.98, dashed grey line). b. Intermediate immune depletion (y = 0.15) leads to increased viral stable state (V* = 0.18) with unstable threshold dropping to V* = 0.82. c. With high immune depletion (y = 0.18), the steady state (V* = 0.33) and unstable threshold (V* = 0.66) approach convergence (convergence to complete dysregulation occurs when $y = x + z/4 = 0.19$, where x and z capture baseline and virus-dependent immune activation/inhibition; here $x = 0.09$ and $z = 0.4$).

Online Content files include:
Methods
Extended Data Figs 1–12
References (17–31)
Supplementary Data 1

ATTACHMENT 2

Di Prisco, G., Annoscia, D., Margiotta, M., Ferrara, R., Varricchio, P., Zanni, V., Caprio, E., Nazzi, F., Pennacchio, F. 2016. A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. PNAS, 113, 3203-3208.

A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health

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Edited by David L. Denlinger, Ohio State University, Columbus, OH, and approved January 26, 2016 (received for review December 1, 2015)

Honey bee colony losses are triggered by interacting stress factors consistently associated with high loads of parasites and/or pathogens. A wealth of biotic and abiotic stressors are involved in the induction of this complex multifactorial syndrome, with the parasitic mite *Varroa destructor* and the associated deformed wing virus (DWV) apparently playing key roles. The mechanistic basis underpinning this association and the evolutionary implications remain largely obscure. Here we narrow this research gap by demonstrating that DWV, vectored by the *Varroa* mite, adversely affects humoral and cellular immune responses by interfering with NF-κB signaling. This immunosuppressive effect of the viral pathogen enhances reproduction of the parasitic mite. Our experimental data uncover an unrecognized mutualistic symbiosis between *Varroa* and DWV, which perpetuates a loop of reciprocal stimulation with escalating negative effects on honey bee immunity and health. These results largely account for the remarkable importance of this mite–virus interaction in the induction of honey bee colony losses. The discovery of this mutualistic association and the elucidation of the underlying regulatory mechanisms sets the stage for a more insightful analysis of how synergistic stress factors contribute to colony collapse, and for the development of new strategies to alleviate this problem.

Apis mellifera | *Varroa destructor* | deformed wing virus | mutualistic symbiosis | honeybee colony losses

The mismatch between the increasing demand for pollination in agriculture and the capacity of honey bees to provide such ecological service severely undermines the sustainability of our food production system (1). Indeed, apiculture is facing a major crisis owing to concurrent factors, such as landscape deterioration, agrochemicals, and parasites (2). Honey bee colony losses have been a major problem since the beginning of modern apiculture (3); however, in 2006, the dramatic dimension of this phenomenon attracted public attention and increasing interest in the scientific community. Several years of intense investigation did not reveal a specific causal agent, but a multifactorial origin was proposed for this syndrome, which is often associated with high levels of parasites in combination with pathogens (2, 4). It was evident that the immunocompetence of honey bees in collapsing colonies was impaired, undermining their capacity to face the biotic stress factors occurring in the hive ecosystem. Indeed, a number of studies have identified important roles of the parasitic mite *Varroa destructor* (5) and vectored viral pathogens, particularly the deformed wing virus (DWV) (6), in contributing to significant changes in the global viral landscape and a continuing decline in honey bee health (7–9).

We recently contributed to this research issue, proposing a functional model describing how the delicate immune balance underpinning the covert infections of DWV can be destabilized by *Varroa* feeding, resulting in intense viral proliferation (10). That earlier study provided evidence supporting a major role of DWV in the immune suppression process, characterized by a negative impact on a member of the NF-κB protein family (10). Separate independent work further corroborated this evidence, showing that viral infection in honey bees interfered with the expression of genes that participate in the Toll pathway (11, 12). This finding supports the hypothesis that in honey bees, and more generally in insects, inducible antiviral barriers besides RNAi-mediated mechanisms (11–18) may have an important role. Indeed, in collapsing colonies, these latter barriers under NF-κB or JAK-STAT control appear to be targeted, whereas the RNAi machinery seems to be unaffected (19).

The occurrence, often asymptomatic, of DWV in nearly all honey bee colonies (6), favored by the active vectoring activity of *Varroa* mite (5), represents a constant threat that can become a severe problem in the presence of additional stress factors, such as, among others, pesticides and poor nutrition, which can promote viral replication (8, 20). Therefore, multiple stress agents exert a synergistic action that compromises the delicate immune balance underpinning the covert DWV infections, and may well account for the multifactorial origin of colony losses.

Collectively, the available experimental evidence indicates that DWV adopts a virulence strategy, still obscure from a molecular standpoint, that targets antiviral barriers under control of the Toll pathway. This virulence strategy can have multifaceted functional implications as a consequence of the central role of NF-κB in immunity and cross-modulated physiological pathways (8), as well as in control of the behavior-specific neurogenomic

Significance

The parasitic mite *Varroa destructor* and the deformed wing virus (DWV) are linked in a mutualistic symbiosis. The mite acts as vector of the viral pathogen, whereas the DWV-induced immunosuppression in honey bees favors mite feeding and reproduction. This functional interaction underpins a rapidly escalating immunosuppression, which can be primed and/or aggravated by a wealth of stress factors that co-trigger colony losses. Our experimental results explain the pivotal role proposed for the *Varroa*–DWV association in the induction of honey bee colony losses. Here we provide a functional framework for studying the dynamics of this multifactorial syndrome and defining effective strategies to reduce its negative impact on the beekeeping industry.

Author contributions: F.N. and F.P. designed research; G.D.P., D.A., M.M., R.F., P.V., V.Z., E.C., and F.N. performed research; G.D.P., D.A., and F.N. analyzed data; and F.N. and F.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1523515113/-/DCSupplemental.

states in the brain that rely on specific transcriptional modules controlled by this transcription factor (21).

The active role of *V. destructor* in the dispersal and enhanced replication of the virus, triggered by parasite feeding (7, 10, 12, 22), indicates that the mite–virus association has clear benefits for the latter, whereas an adaptive value for the mite, if any, remains unknown. The feeding behavior of *V. destructor* is complex, characterized by prolonged use of a feeding hole created by the mother mite on abdominal sternites of the bee pupa, through which both the mother and its offspring repeatedly feed on the bee's hemolymph (23). Any humoral and cellular immune reaction in the host, such as hemolymph clotting, melanization, or encapsulation, that directly interferes with food uptake and use may in principle result in reduced mite fitness. Therefore, based on current knowledge about DWV-induced immunosuppression, a positive influence of viral infection on mite feeding and reproduction can be hypothesized. However, notwithstanding the key importance of the *Varroa*–DWV association, the intimate aspects of their interaction have been largely overlooked.

Here we focus on the functional basis of this tight association, examining the impact of DWV infection on multiple immune barriers under the Toll pathway and assessing whether this has any effect on *Varroa* mite fitness. These are very relevant issues that, if properly addressed, can provide mechanistic insights of key importance to understand the dynamics of mite–virus association and developing new strategies to alleviate its dramatic impact on honey bee colonies.

Results

DWV Infection and Honey Bee Immunosuppression. To study the DWV-induced immunosuppression, we first assessed at the phenotypic level how the cellular and humoral components of the immune response vary across different DWV infection levels, irrespective of the presence of *Varroa*, in the absence of other viral pathogens. We did this by scoring the degree of encapsulation and melanization of a nylon thread at 24 h after its implantation into the body of a fifth instar honey bee larva, whose infection level, scored as the number of DWV genome copies, was determined by quantitative RT-PCR (qRT-PCR). The melanization and encapsulation response in experimental larvae were negatively correlated with DWV titer (melanization: $\rho = -0.656$, $n = 28$, $P < 0.001$; encapsulation: $\rho = -0.390$, $n = 28$, $P = 0.040$) (Fig. 1). It is worth noting that any possible effect of mite parasitism at this stage can be virtually excluded, because mite feeding begins at the prepupal stage, within the sealed brood cell.

Melanization and encapsulation in insects are mediated by a number of genes that control the formation of a cellular capsule around foreign intruders and the deposition of melanin and other toxic molecules on their surface (24). Moreover, although the processes of clotting and of nodule and capsule formation differ in their final appearance, they share some molecular steps, and both involve melanin biosynthesis (24–26). Among the immune genes involved in these reactions is a sequence of key importance codes for a protein generating amyloid fibers that mediate encapsulation and strictly localized melanization of nonself material in Lepidoptera (27, 28). Here we cloned and characterized the cDNA of the homolog of this gene in *Apis mellifera* (GenBank accession no. KU513387), hereinafter denoted as *Amel\102*. This full-length cDNA encodes a predicted protein of 270 aa that shows 36% sequence identity with P102Hv (27) (Fig. S1). The expression profile of *Amel\102* in different tissues of honey bee larvae, assessed by qRT-PCR analysis, matched that observed in lepidopteran larvae, showing high levels of expression in the haemocytes and lower levels in the gut and fat body (Fig. 2*A*; adjusted $H = 11.52$, $df = 2$, $P = 0.003$).

To assess whether *Amel\102* is an inducible immune gene under NF-κB control, we silenced this transcription factor by RNAi and assessed the expression level of *Amel\102* by qRT-PCR. The significant down-regulation of *dorsal-1A* in honey bees exposed to dsRNA targeting this gene (Fig. 2*B*; $H = 11.57$, $df = 1$, $P < 0.001$) was associated with a drastic drop in the transcription level of *Amel\102* (Fig. 2*C*; $H = 14.29$, $df = 1$, $P < 0.001$). This clearly indicates that *Amel\102* is under NF-κB control.

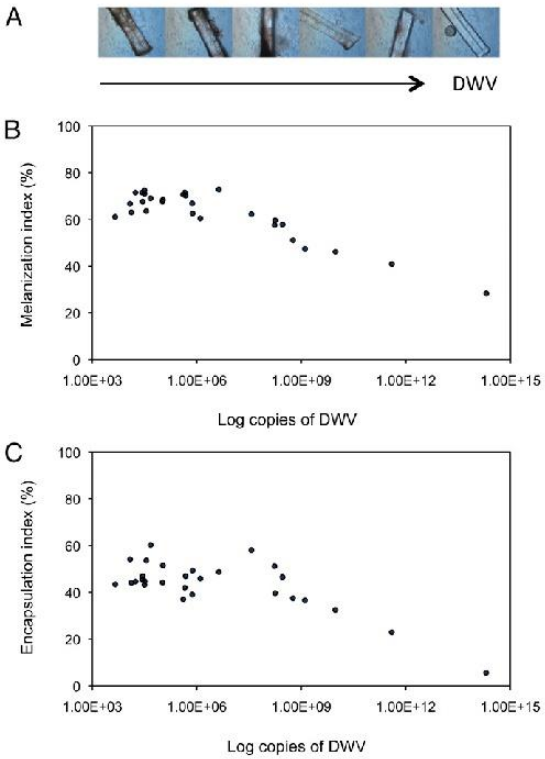

Fig. 1. Immunocompetence of honey bee larvae as affected by DWV infection. (*A*) Nylon threads at 24 h after implantation into the body of fifth instar honey bee larvae with increasing DWV infection levels. (*B*) Level of melanization of a nylon thread implant in honey bee larvae with different levels of viral infection, measured as number of DWV genome copies ($\rho = -0.656$, $n = 28$, $P < 0.001$). (*C*) Level of encapsulation of a nylon thread implant in honey bee larvae with different levels of viral infection, measured as number of DWV genome copies ($\rho = -0.390$, $n = 28$, $P = 0.040$).

To demonstrate the possible occurrence of an upstream viral action on the Toll pathway with effects on various immune responses under NF-κB control, we scored the impact of different levels of DWV infection on the transcription of *Amel\102* and *apidaecin*, a gene encoding an antimicrobial peptide under NF-κB control (29), as also supported by its strong down-regulation in response to *dorsal-1A* silencing performed in our experiment (Fig. S2; $H = 14.29$, $df = 1$, $P < 0.001$). We found that the transcription level of *Amel\102* was negatively correlated with the level of DWV infection (Fig. 3*A*; $\rho = -0.575$, $n = 28$, $P = 0.001$). This result is consistent with the expected central role of the *Amel\102* gene in melanization and encapsulation, as well as the negative effect of DWV on such processes. Moreover, a negative correlation was also found between the transcription level of *apidaecin* and the level of DWV infection (Fig. 3*B*; $\rho = -0.636$, $n = 28$, $P < 0.001$). Collectively, these results corroborate the importance of viral infection on honey bee immunosuppression, and clearly indicate that the adverse effect of DWV on the bees' immune response is caused by an upstream alteration of the Toll pathway.

Because NF-κB activation in honey bees is negatively modulated by a leucine reach repeat protein (*Amel\LRR*) (30), we

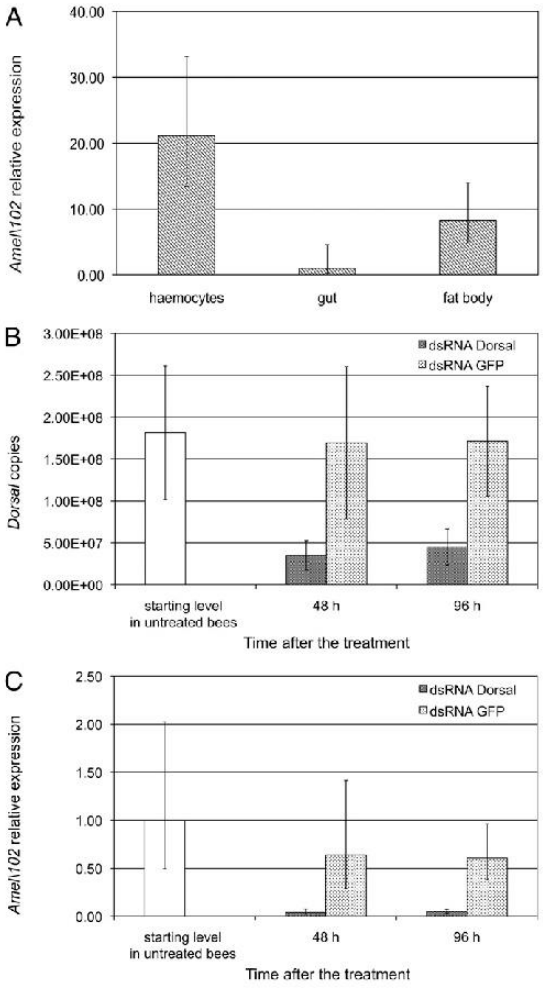

Fig. 2. *Amel\102* transcriptional profile and regulation. (*A*) *Amel\102* transcription profile in honey bee larval tissues (adjusted H = 11.52, df = 2, P = 0.003). (*B*) Transcription level of *dorsal-1A* in honey bees maintained for different time intervals on a sucrose/protein solution containing a dsRNA targeting this gene (dsRNA *dorsal*) or dsRNA *GFP* as a control. (*C*) Transcription level of *Amel\102* as affected by silencing of *dorsal-1A*. The error bars indicate the SD of the mean. The significant drop in *dorsal-1A* transcription (H = 11.57, df = 1, $P < 0.001$) was associated with a significant transcriptional down-regulation of *Amel\102* (H = 14.29, df = 1, $P < 0.001$).

investigated whether the transcription rate of its encoding gene can be influenced by the level of viral infection. Indeed this was the case, as demonstrated by a positive correlation between the number of DWV genome copies and the transcription rate of *Amel\LRR* (Fig. 3*C*; $\rho = 0.511$, $n = 28$, $P = 0.005$). This result indicates that DWV infection could have a negative effect on NF-κB activation by enhancing the transcription of its negative modulator *Amel\LRR*. The possibility that DWV adopts a virulence strategy somewhat similar to that recently described for *Salmonella*, which targets an NLR protein that negatively regulates NF-κB signaling (31), is an intriguing hypothesis meriting further research efforts.

To assess whether the observed immunosuppression was due exclusively to the action of DWV infection, we performed an additional experiment in vitro. Honey bee larvae were exposed to a controlled *Varroa* infestation, and the expression level of *Amel\102*, as affected by mite feeding, was assessed in the presence or absence of DWV infection. The lowest transcription rates of *Amel\102*, which appears to be under NF-κB control, were observed in DWV-infected larvae, irrespective of the presence or absence of *Varroa* (comparison of DWV infection levels: $F = 66.37$, df = 1, $P < 0.001$; comparison of mite infestation levels: $F = 2.74$, df = 1, P = not significant; interaction: $F = 1.69$, df = 1, P = not significant) (Fig. 3*D*). Therefore, this gene appears to be a good molecular marker of honey bee immunocompetence as affected by DWV infection. The matching pattern of variation shown by *Amel\102* transcription and the immune parameters scored above (Fig. 1) indicates that this gene, as in lepidopterans, plays an important role in the modulation of both humoral and cellular immune responses and is targeted by DWV, which adversely affects NF-κB signaling through a molecular mechanism that remains to be studied.

Effect of Honey Bee Immunosuppression on *Varroa* Fitness. To assess whether the immunosuppressant action of DWV can facilitate mite feeding and, consequently, increase mite fitness, we assessed mite reproduction in honey bees showing variable levels of DWV infection. To do so, we artificially infested fifth instar honey bee larvae with one mite each and kept them under laboratory conditions for 12 d (Fig. S3). After pupation, we noted the possible presence of offspring generated by each mother mite during the honey bee's metamorphosis, and assessed the viral infection level of the honey bee, as the number of DWV genome copies, by qRT-PCR. We replicated this experimental setup twice, resulting in 90 bee samples. The proportion of reproducing mites (i.e., fertility) increased with DWV infection level up to a threshold of 10^8 genome copies per honey bee, after which very high viral loads seemed to exert a negative impact on mite reproduction (Fig. 4*A*; equation of the curve describing the observed trend: $y = -0.0122x^2 + 0.1979x - 0.2437$, $R^2 = 0.868$, df = 3, $P = 0.048$). Overall, mite fertility was similar to that reported previously under the same experimental conditions (32), and clearly varied according to the level of DWV infection.

The occurrence of crippled wings at eclosion is a characteristic symptom of an overt virus infection in honey bees, induced by a high DWV load (6). In fact, we found a significant correlation between viral load and wing deformity in 46 samples from the previous experiment, in which the condition of wings was evident at the time of sampling ($\chi^2 = 5.894$, df = 1, $P = 0.015$). Therefore, to check the observed positive relationship between viral infection and mite reproduction, we performed an additional in vitro experiment in which we assessed the reproduction of mites feeding on immature honey bees with either deformed or normal wings at the adult stage (Fig. S4). We studied a total of 149 honey bees, 49 of which had deformed wings at eclosion. The fertility rate approached 40% in mites infesting larvae giving rise to deformed wing bees, but was limited to only 22% when the host larvae developed into normal wing bees (Fig. 4*B*; $\chi^2 = 4.64$, df = 1, $P = 0.031$). Conversely, the number of offspring generated by each reproducing mite (i.e., fecundity) did not seem to be affected (Fig. S5).

Collectively, our data demonstrate that mite fertility is enhanced by high DWV titers, and support the hypothesis that viral infection promotes *Varroa* fitness.

Discussion

The results of this study show that honey bees with increasing DWV loads have reduced immunocompetence at both the humoral and cellular levels. The negative impact of the combined action of *Varroa* and DWV on honey bee immunity has been proposed based on the results of several studies showing variable levels of transcriptional down-regulation of immune genes in most cases. The very close relationship between the mite and the virus, along with the complexity of the results from transcriptomic studies, have generated a somewhat contrasting picture, however. The initially proposed primary role for the parasitic mite in

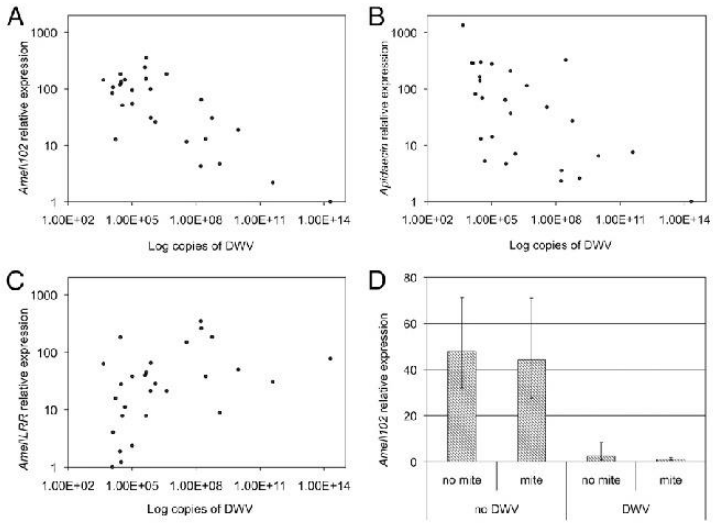

Fig. 3. Gene expression in honey bee larvae as affected by DWV infection. (*A–C*) Effect of DWV infection level on transcription of (*A*) *AmeN102* ($\rho = -0.575$, $n = 28$, $P = 0.001$), (*B*) *apidaecin* ($\rho = -0.636$, $n = 28$, $P < 0.001$), and (*C*) *AmeNLRR* ($\rho = 0.511$, $n = 28$, $P = 0.005$). (*D*) *AmeN102* transcription as affected by DWV infection ($F = 66.37$, df = 1, $P < 0.001$) and feeding activity by the *Varroa* mite ($F = 2.74$, df = 1, P = not significant; interaction: $F = 1.69$, df = 1, P = not significant). Error bars indicate SD.

the induction of this immune syndrome (33) has been challenged by several studies that apparently do not support this hypothesis (12, 22, 34–36), even though the possibility that *Varroa* feeding and its saliva may modulate immune effector molecules cannot be ruled out, as suggested by the effect of this secretion on insect hemocytes in vitro (37). Moreover, a direct immunosuppressive activity of DWV targeting the antiviral barriers under NF-κB control has been proposed in the framework of a bistable dynamic model that assumes a transition from immunostimulation to immunosuppression as the DWV titer increases and then exceeds a critical threshold (Ct) (10). This working hypothesis is in tune with more recent data indicating that in honey bees from colonies affected by colony collapse disorder, which regularly show high levels of viral infection, the immune response mediated by the RNAi machinery is apparently unaffected (19). Furthermore, immune genes in the Toll pathway are down-regulated in honey bees exposed to *Varroa* and bearing high DWV loads (12) or challenged with nonspecific dsRNAs (11).

Our present data, obtained with a more focused functional approach, demonstrate the immunosuppressive role of DWV and show that its virulence strategy for overcoming the specific antiviral barriers under the Toll pathway by disrupting NF-κB signaling inevitably has a multifaceted influence on different arms of the immune response. This has a direct impact on the fitness of the *Varroa* mite, possibly facilitating the mite's ectoparasitic trophic activity. Thus, the viral pathogen has a positive influence on mite feeding and consequently on its reproduction, as measured in this study. Given the strong impact of mite feeding on viral replication (10, 12), a loop of reciprocal stimulation of the two symbionts is evident, which largely accounts for their central role in honey bee colony losses (7, 10).

The basal level of infertility in *Varroa* mite populations can be quite high and may vary for reasons not completely understood, often related to undefined host factors (5). We suggest that the presence and level of infection by DWV may partly account for this variation, which could be due to different levels of host immunosuppression. Even though this positive effect of DWV infection on mite reproduction can be partly induced by enhanced feeding efficiency, we cannot rule out the possibility of a greater nutritional suitability of infected hosts, which is an issue meriting further research efforts aimed at fully characterizing the host regulation strategy adopted by the *Varroa* mite.

The concept of host regulation is adopted to describe a wide range of host physiological and behavioral changes induced by parasites of arthropods, which have been especially well investigated in insect parasitoids (38). These carnivorous insects, particularly parasitic wasps of the order Hymenoptera, are able to colonize and exploit living insect hosts using a wealth of virulence factors (38). The ovarian secretions injected by adult females during oviposition may contain symbiont viruses (39), which in some parasitic wasps of caterpillars are members of the unique family Polydnaviridae (39–41). The ancestor of these viral symbionts in the genus *Bracovirus* was a viral pathogen of the host, in the Nudivirus group, now stably integrated into the wasp genome and used as a tool to deliver virulence factors (41, 42). The *Varroa*–DWV association could be considered a similar symbiotic relationship, but at an early stage and with a less intimate level of integration, where the vector role of *Varroa* is paid back by a DWV-induced fitness enhancement mediated by host immunosuppression. This seems to be part of a unique evolutionary pattern promoting the "alliance" of parasitic arthropods with viral pathogens of the host to overcome its immune barriers (39).

The results of our present study shed new light on the *Varroa*–DWV association, supporting a previously unrecognized mutualistic symbiosis. This information accounts for the central importance of the mite–virus complex in the induction of honey bee colony losses, and sets the stage for studies aiming to develop new management strategies for one of the most dangerous parasite–pathogen associations for the beekeeping industry. Indeed, any environmental stress that interferes with honey bee immunocompetence and promotes DWV replication in individuals bearing covert infections (8, 10, 30) also has an indirect effect on *Varroa*, which is favored by the escalating viral-induced immunosuppression. This may account in part for the recently observed positive correlation between *Varroa* populations and honey bee exposure to neonicotinoids (43, 44), which are known to promote viral replication in DWV-infected honey bees (30); however, additional field evidence is needed to support this hypothesis. A more thorough understanding of the molecular mechanisms underlying this self-boosted process that enhances the

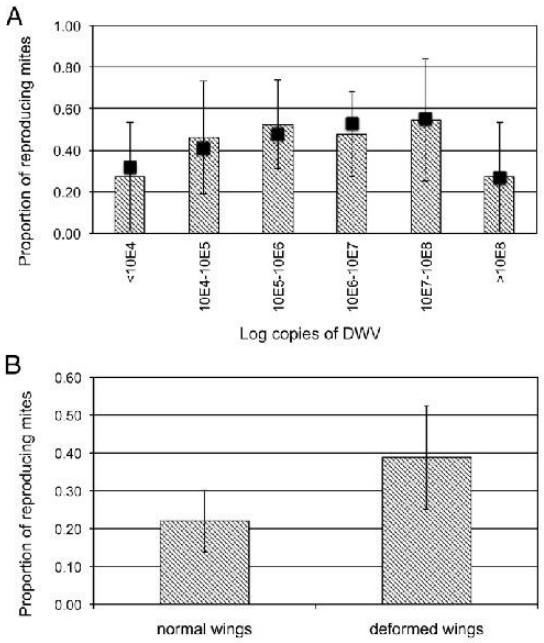

Fig. 4. Viral infection and mite reproduction. (*A*) Proportion of reproducing *Varroa* mites (i.e., fertility) on honey bees with different levels of DWV infection. The equation of the curve describing the observed trend and the correlation coefficient are $y = -0.0122x^2 + 0.1979x - 0.2437$ and $R^2 = 0.868$ (df = 3, $P = 0.048$). The black squares represent the values expected under this model. (*B*) Mite fertility on honey bees with deformed or normal wings at edosion ($\chi^2 = 4.64$, df = 1, $P = 0.031$). Error bars indicate the 95% confidence limits of the proportions.

fitness of both symbionts, as well as its modulation by interacting stress factors, will be crucial to understand the complex dynamics of honey bee colony losses and developing novel strategies to alleviate its significant negative impact on agriculture and environment.

Materials and Methods

Biological Material. The honey bees and mites used in this study were from *A. mellifera ligustica* colonies maintained in the experimental apiary of the Università degli Studi di Napoli "Federico II" and from *A. mellifera ligustica* × *A. mellifera carnica* colonies maintained in the experimental apiary of the Università degli Studi di Udine.

In brief, to obtain both the fifth instar honey bee larvae and the mites needed for artificial infestation and in vitro rearing experiments, all of the sealed cells of several brood combs were marked on the evening before the experiment. The next morning, the brood combs were transferred to the laboratory, and all of the unmarked cells that had been sealed during the preceding 12–15 h were unsealed with forceps. The frames were incubated at 35 °C and 75% relative humidity, to allow the honey bee larvae to emerge from cells together with possible infesting mites. When needed, honey bee larvae were isolated in gelatin capsules (6.5 mm diameter) together with one mite each, and then kept in an incubator at 34–35 °C and 75–80% relative humidity.

Encapsulation and Melanization Assay. Fifth instar honey bee larvae ($n = 40$) were obtained from freshly capped cells. A piece of nylon thread (0.08 mm) was inserted into the hemocelic cavity under a stereomicroscope at 20× magnification. After 24 h, the implants were removed and photographed under a light microscope (400×). Image analysis was performed using GIMP version 2.8 (GNU Image Manipulation Program; www.gimp.org). The degree of encapsulation was scored as the percentage of nonwhite pixels (i.e., covered by hemocytes). The melanization index (a percentage) was calculated as $(1 - x/255)100$, where x represents the mean degree of gray intensity (a numerical reading ranging from 0 for black to 255 for white) of the pixels in the area covered by hemocytes. The rest of the body was immediately stored at −80 °C for the subsequent molecular analysis. All samples were analyzed by qRT-PCR to assess for the possible presence of black queen cell virus and sacbrood virus, which were previously found in the same area, although at a comparatively much lower prevalence than DWV (10). A total of 28 larvae were considered for the analysis, after discarding nine larvae showing prolonged bleeding after the implantation and three larvae showing the presence of viral pathogens other than DWV.

Cloning of the *Amel\102* Gene. To clone the *A. mellifera* homolog of the *102Hv* gene, we first blasted the protein sequence of 102Hv (GenBank accession no. CBY85302.1) against the "nonredundant protein sequences" (nr) database of the honey bee genome. A sequence (XP_003251941.1) showing 36% identity with 102Hv was identified, and the corresponding cDNA was obtained by qRT-PCR and elongated by 5′ and 3′ RACE, using specific primer pairs (Table S1). This cDNA is contained in a contig (11266) of the whole genome shotgun sequence (GenBank accession no. AADG06011266.1).

Expression Analysis of the *Amel\102* Gene. We measured the expression profile of *Amel\102* in various tissues of honey bee larvae by qRT-PCR. For this, we used a micropipette to collect the hemolymph exuding from a puncture made with a sterile needle in the dorsal vessel in the fourth abdominal segment of fifth instar honey bee larvae ($n = 15$), which was transferred in 40 μL of PBS (137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer, pH 7.4). After bleeding, experimental larvae were dissected under an optical stereomicroscope (20×) to separate the gut and the fat body. Hemolymph samples were centrifuged at 600 × *g* for 6 min, to isolate the hemocytes. All tissues to be analyzed were immediately transferred in a cold extraction buffer for subsequent total RNA extraction.

To assess whether *Amel\102*, *apidaecin*, and *AmelLRR* expression is influenced by DWV infection, we performed qRT-PCR to determine transcription levels in honey bee larvae with different levels of viral genome copies in the samples used for the encapsulation and melanization experiments. To examine whether immunosuppression was due exclusively to the action of DWV infection, we performed an additional experiment in which honey bee larvae were exposed to a controlled *Varroa* infestation. At the end of the experiment, we measured the expression level of *Amel\102*, as affected by mite feeding, in the presence and absence of DWV infection. To do so, we collected fifth instar honey bee larvae from freshly capped cells and introduced them singly into gelatin capsules (6.5 mm diameter), along with a single *Varroa* mite obtained from a highly infested colony. An equal number of uninfested fifth instar honey bee larvae served as controls. Rearing capsules were then transferred into an incubator and kept at 35 °C and 80% relative humidity. After 24 h, honey bee larvae were flash-frozen at −80 °C for the molecular analysis. Ten individuals for each experimental combination of *Varroa* infestation and DWV infection were selected at random, and the transcription level of *Amel\102* was measured.

Virus Infection and Mite Reproduction. We artificially infested fifth instar honey bee larvae with one mite each and kept them in gelatin capsules at 35 °C and 75% relative humidity (32). After 12 d, at the end of the pupal stage, cells were opened and inspected under a stereomicroscope to assess the possible occurrence of honey bee wing deformity and mite reproduction. The honey bees were then stored at −80 °C for subsequent qRT-PCR to assess the viral genome copy number.

This experiment was replicated twice. A total of 90 honey bees in which the viral infection level as well as mite reproduction could be assessed were obtained and used in the analysis. Five honey bees that appeared to be uninfected were not used in this analysis.

Because the relationship between wing deformity at the adult stage and viral load is well known from the literature (6) and confirmed by the present study, we conducted another experiment in which artificially infested honey bee larvae were checked after reaching the adult stage (when wing deformity can be unequivocally assessed). The status of their wings correlated with the reproduction of the mites feeding on them. We replicated the experiment six times, obtaining 149 adult honey bees, of which 100 had normal wings and 49 had crippled wings.

qRT-PCR. qRT-PCR was performed following standard methods, as described in detail in *SI Materials and Methods*.

RNAi. Double-stranded honey bee *dorsal-1A* (*A. mellifera* Dorsal variant A, mRNA, GI:58585243, 2389 bp) was prepared using the MEGAscript RNAi Kit (Ambion), following the manufacturer's standard protocol. The target sequence

was PCR-amplified with specific primers, carrying a 5′ tail of the T7 promoter at both ends and used as template for T7-dependent in vitro transcription. The following primers were used: forward, 5′-TAATACGACTCACTATAGGGAGACA-ATCCAGCACTTATTC-3′; reverse, 5′-TAATACGACTCACTATAGGGAGCCTG-AATAGTGTTATTAGC-3′. The reaction product was subjected to DNase digestion and purified, and the final preparation was dissolved in nuclease-free water.

Individual frames were removed from the colony and stored in an incubator overnight at 34 °C and 90% relative humidity. Emerging bees were maintained as groups of 30 individuals in sterile boxes and fed daily with 2 mL of a 50% sucrose/protein solution, containing 50 μg of dsRNA of *dorsal-1A*. Controls were fed with a similar solution containing a dsRNA of mGFP6, obtained as described above. Samples were collected from five bees at the beginning of the experiment, to assess the starting level of scored parameters, and again after 48 h and 96 h of exposure to the dsRNA feeding solution. Samples were stored at −80 °C until use for RNA extraction.

The transcription levels of *dorsal-1A* and *apidaecin* and the number of DWV genome copies were determined by SYBR Green qRT-PCR, as described above.

Bioinformatic and Statistical Analyses. Alignment of *Heliothis virescens*, *Spodoptera littoralis*, *Drosophila melanogaster*, and *A. mellifera* protein sequences was performed using the ClustalW algorithm. Secondary structure prediction of *A. mellifera* P102 was carried out with InterProScan tool and the EMBOSS: garnier algorithm. Analyses were performed using Geneious version 6.1.6 (Biomatters; available from www.geneious.com).

All of the correlation analyses were carried out using the Spearman rank-sum method. The expression profile of *Amel\102* in different honey bee tissues was analyzed using Kruskal–Wallis one-way ANOVA.

In the RNAi experiments, gene expression and viral replication in bees fed with dsRNA of *dorsal-1A*, or dsRNA of GFP as controls, were compared using the Scheirer–Ray–Hare extension of the Kruskal–Wallis test. Five bees per each treatment were used in the analysis. *Amel\102* transcription as affected by DWV infection and feeding activity by *Varroa* was analyzed using the Sheirer–Ray–Hare extension of the Kruskal–Wallis test.

The polynomial curve fitting the distribution of *Varroa* mite fertility rates associated with different levels of honey bee viral infection, expressed as the logarithm of the average infection level of the bees in each class, was calculated using the least squares method. The relationship between viral load and wing deformity was assessed by logistic regression. The fertility of *Varroa* mites on honey bees with normal and crippled wings was analyzed using the χ^2 test. The 95% confidence limits of the fertility reported in Fig. 4 were calculated using the following equation: $1.96 * \sqrt{P * (1 - P)/n}$.

ACKNOWLEDGMENTS. We thank Spencer Behmer (Texas A&M University), Michael Strand (University of Georgia), and Silvia Gigliotti (Institute of Biosciences and Bioresources - Consiglio Nazionale delle Ricerche, IBBR-CNR) for their critical read of the manuscript, and Ilaria Di Lelio and Adriana Marinelli (University of Napoli "Federico II") for their help and suggestions during the development of this study. The research leading to these results was funded by the European Union Seventh Framework Programme (FP7/2007-2013), under Grant 613960 (SMARTBEES).

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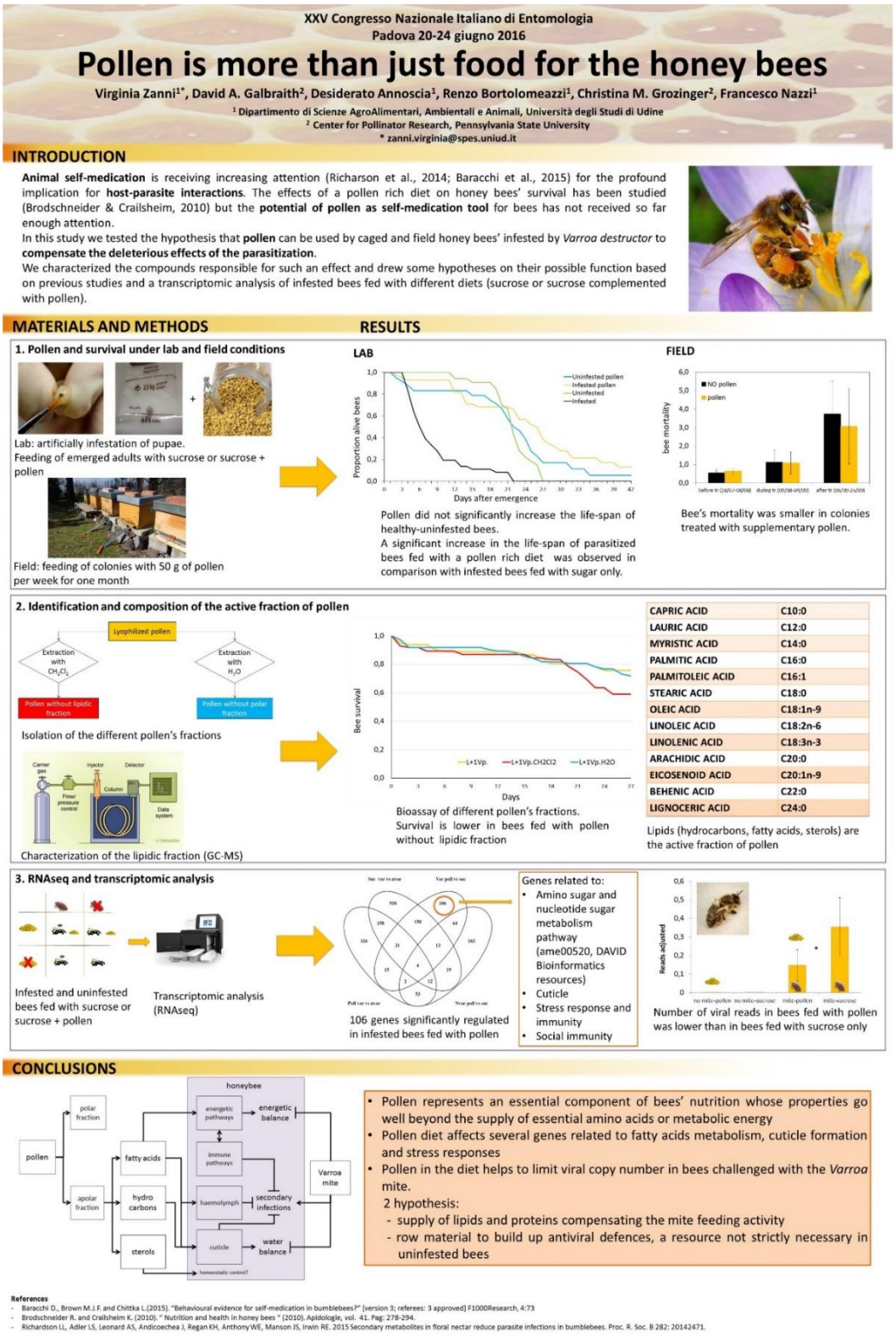
ATTACHMENT 3

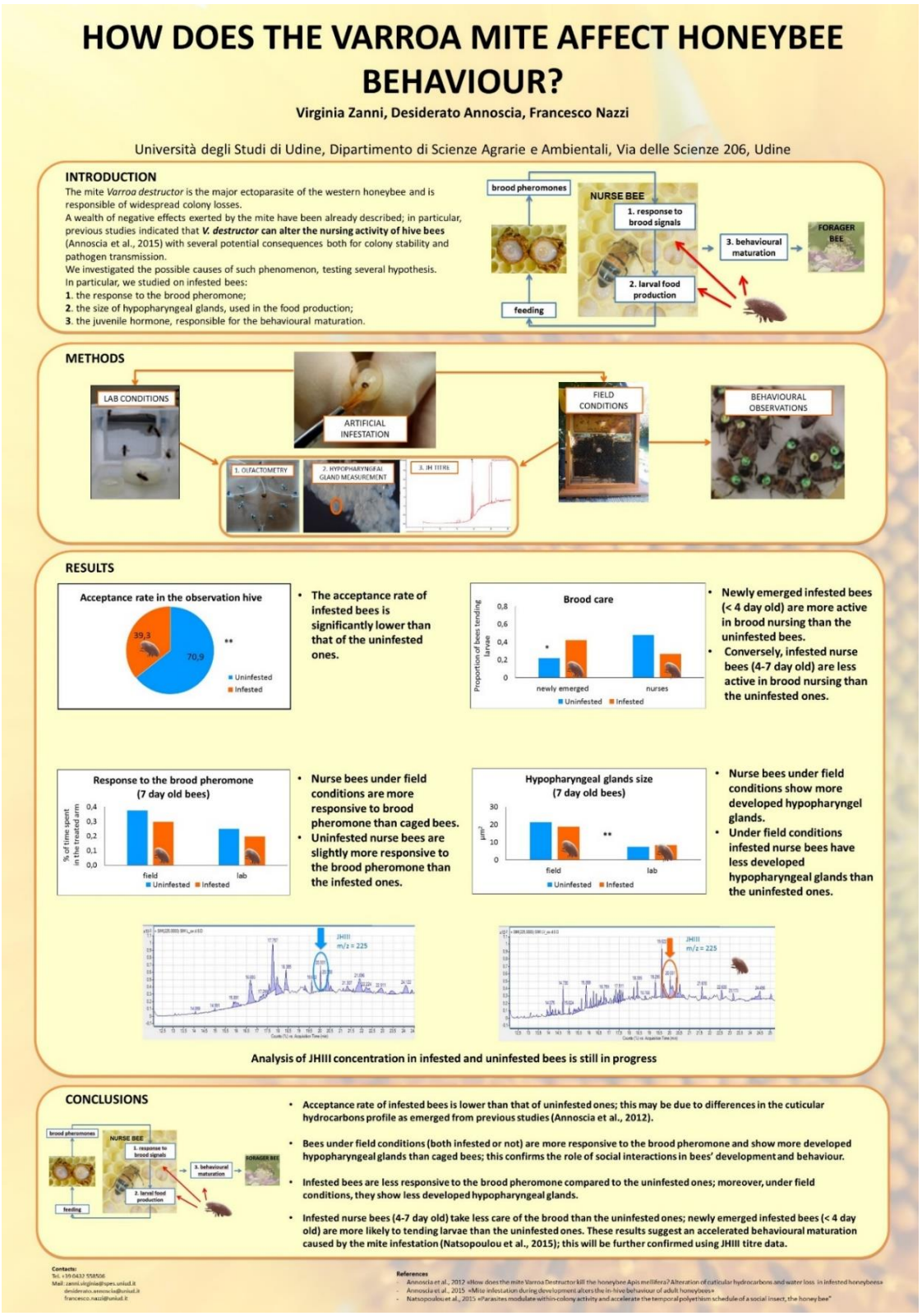
Zanni, V., Galbraith, D.A., Bortolomeazzi, R., Nazzi, F., Grozinger, C.M. Pollen is more than just food for the honey bees

XXV Convegno Nazionale Italiano di Entomologia. Padova 20-24 Giugno 2016



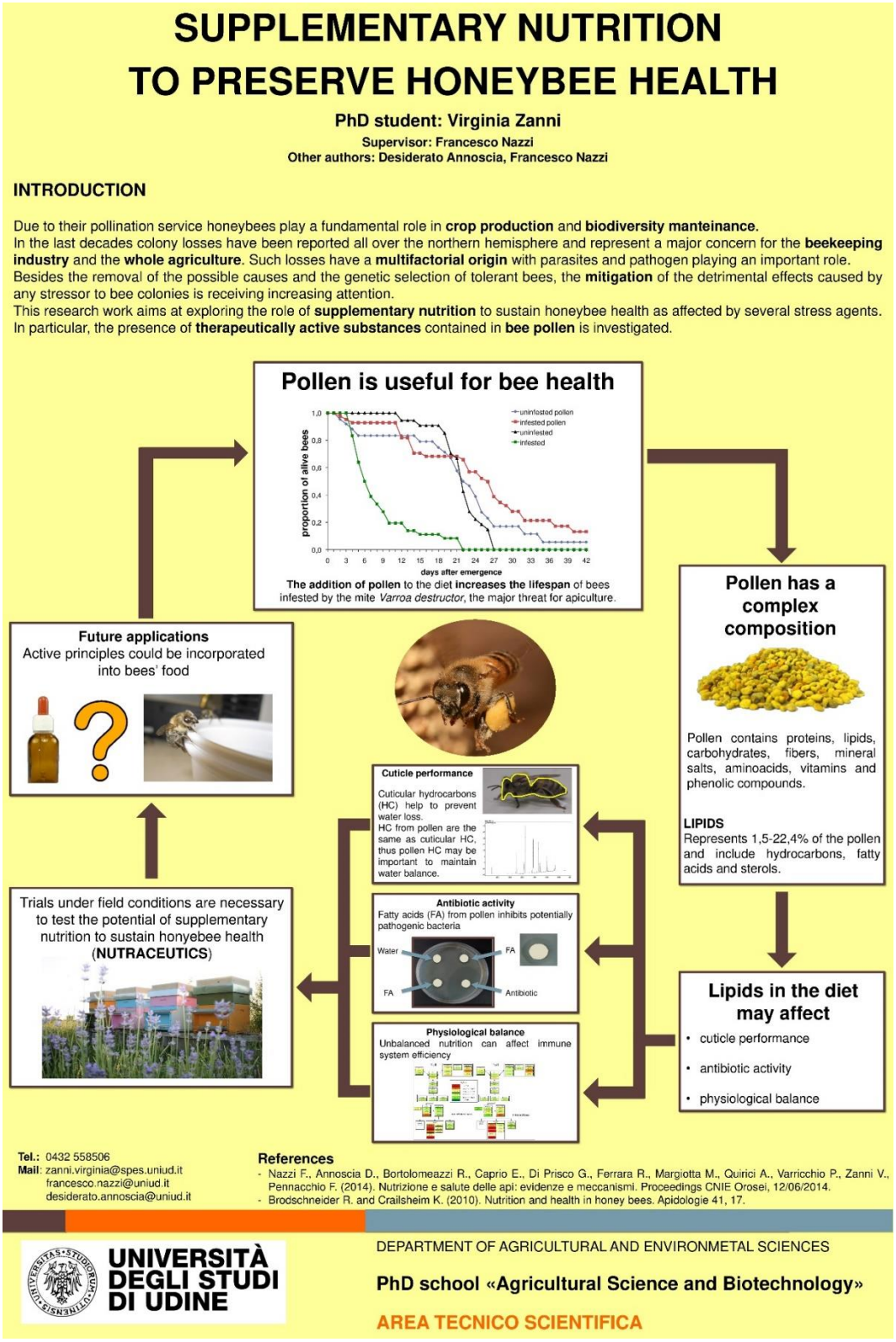
ATTACHMENT 4

Zanni, V., Annoscia, D., Nazzi, F. How does the *Varroa* mite affects honey bee behaviour? European PhD Network in Insect Science-6th Annual Meeting-Florence, 11-12-13 November 2015



ATTACHMENT 5

Zanni, V., Annoscia, D., Nazzi, F. Supplementary nutrition to preserve honey bee health. PhD EXPO 2015, Udine 18 Giugno 2015.



ATTACHMENT 6

Conference proceedings

- Annoscia, D., Di Prisco, G., Margiotta, M., Ferrara, R., Varricchio, P., Zanni, V., Caprio, E., Nazzi, F., Pennacchio, F. 2016. *Varroa destructor* and Deformed Wing Virus are linked in a mutualistic symbiosis accounting for their major role in honeybee colony collapses. Proceedings of "EurBee7, 7th European Conference of Apidology", Cluj-Napoca (Romania), 7-9 September 2016, p. 133.

- Annoscia, D., Di Prisco, G., Margiotta, M., Ferrara, R., Varricchio, P., Zanni, V., Caprio, E., Nazzi, F., Pennacchio, F. "Il parassita *Varroa destructor* e il virus delle ali deformi sono legati da una simbiosi mutualistica che rende conto dell'effetto devastante sulla salute dell'ape" XXV Congresso Nazionale di Entomologia. Padova, 20-24 giugno 2016.

- Nazzi, F., Annoscia, D., Bortolomeazzi, R., Caprio, E., Di, Prisco G., Ferrara, R., Margiotta, M., Quirici, A., Varricchio, P., Zanni, V., Pennacchio, F. 2014. Nutrizione e salute delle api: evidenze e meccanismi. Riassunti delle comunicazioni orali "XXIV Congresso Nazionale Italiano di Entomologia", Orosei, 9 – 14 giugno 2014, 136-137.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisor Prof. Francesco Nazzi for the continuos support of my studies, for his patience, motivation, friendship and immense knowledge. I could not meet better person.

Thanks to my "Uniud bees and ticks" colleagues: Desiderato Annoscia, Simone Del Fabbro, Tatiana Trifonova, Fabio Del Piccolo and our experienced beekeeper Mauro D'Agaro.

Thanks to Angela Sepulcri for the essential support with the GC-MS analysis and for the friendship that she has given me.

Thanks to all the group of Entomology of the University of Udine led by Prof. Pietro Zandigiacomo and to my colleagues from the Istitute of Applied Genomics (IGA).

Thanks to my American supervisor Prof. Christina M.Grozinger and my colleagues from the department of Entomology of the Penn State University: Dave Galbraith, Mali Doke, Gabriel Villar, Aine O'Sullivan, Antony Vaudo and Etya Amsalem.

Thanks to my beloved parents Giorgio and Donatella and all my friends for supporting me throughout my PhD experience and my life in general.

Finally, thanks to Stefano, the most extraordinary thing that this experience has given me.