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UNIVERSITÀ DEGLI STUDI DI SASSARI
CORSO DI DOTTORATO DI RICERCA
Scienze Agrarie

Curriculum in monitoraggio e controllo degli ecosistemi agrari e forestali in ambiente mediterraneo

Ciclo XXIX

Social Immunity in honeybee: behavioral, chemical and microbiological aspects

dr. Michelina Pusceddu

Coordinatore del Corso	Prof. Antonello Cannas
Referente di Curriculum	Prof. Alberto Satta
Docente Guida	Prof. Alberto Satta
Tutor	Prof. Ignazio Floris

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1. INTRODUCTION

Defensive strategies in honeybees

Colonies of social insects can be compared by a number of similarities to multicellular organisms that, due to their level of organization, have been conceptualized, since the early 1900s, as superorganisms (Wheeler, 1911).

A century later, Strassmann & Queller (2010) proposed the definition of multicellular organism for all biological entities in which components contribute in ensuring the survival of the overall group members, as also occurs in honeybee colonies (Moritz & Southwich, 1992; Thauz, 2008). In these insects, one of the main areas of cooperation among individuals is that toward the development of a social immune system of the colony. The "social immunity" is the set of collective defense mechanisms implemented by bees to combat predators, parasites and pathogens, which constantly threaten the survival of the hive (Cremer *et al.,* 2007). In addition to the collective defense mechanisms used by *Apis mellifera*, individual defense systems can also be used simultaneously, increasing thus the resistance or tolerance against the hive intruders (Cremer & Sixt, 2009). Resistance is the ability of a system to resist infection through mechanical, chemical and physiological barriers, as well as appropriate defense responses once the infection has occurred. Conversely, tolerance against pathogens or parasites is the ability of hosts to withstand the infection and/or infestation, and is ensured by a compromise between energy costs, damages and immune response of the insect (Baracchi & Turillazzi, 2014).

1.1. Individual Immunity System

Honeybees have fewer genes involved in the immune response than several species of solitary insects, and hence this implicates less flexibility in the ability to recognize and resist pathogens (Evans *et al.,* 2006; Weinstock *et al.,* 2006). This finding suggests that individual defenses in social insects may be compensated by the collective defense mechanisms that emerge at the colony level (Cremer *et al.,* 2007). Furthermore, reproduction affects the immune defenses on the level of inter- and intra-colony variability; therefore the queen bee is assumed to mate multiple times to achieve higher genetic variability, thus increasing resistance to diseases (Seeley & Tarpy, 2007; Tarpy, 2003).

The first line of individual defense in bees consists of mechanical barriers, represented by the integument (epicuticular layer, cuticle, epidermis, basal membrane), as well as the internal layers of the gut (peritrophic matrix and epithelium) that disfavor adhesion and penetration of pathogens in the organism. Another line of individual defense consists of physiological inhibitors that induce pH variations, and glandular secretions with bactericidal and/or fungicidal action such as those produced by salivary, mandibular and hypopharyngeal glands (Crailsheim & Riessberger-Galle, 2001).

Once these system defenses are breached, cellular and humoral responses are activated. The cellular immunity is the action of haemocytes to recognize, engulf and neutralize foreign bodies. Indeed, the total count of these cells allows to estimate the cellular immunocompetence of an individual (Williams, 2007; Wilson-Rich *et al.,* 2008). On the other hand, the humoral response consists in the production of antimicrobial substances (peptides and proteins) and in retrieving an increased production of hemocyte cells, and is quantified by the number of fatty substances from which peptides and proteins with antimicrobial activity are mainly synthesized (Evans *et al.,* 2006). At present, four antimicrobial peptides have been identified in honeybee: apidaecin, abaecin, hymenoptaecin and defensins. In particular, the defensins are presented by two isoforms, defensin 1 and 2. Defensin 1 is produced by the salivary glands and is involved in the social immune system of the honeybee. In contrast, defensin 2 is produced by the adipocytes and haemocytes, and it is therefore a component of the individual immunity (Ilyasov *et al.,* 2012).

In addition, the production of antimicrobial peptides with a gradually higher bactericidal capacity has been crucial for the evolution of sociality in honeybees. Indeed, the antimicrobial agents of the most primitive semi-social species of Apoidea are stronger than those found in solitary species (Stow *et al.,* 2007). This suggests that over the course of evolution and with the increase of both group size and affinity relations among individuals, there has been a tipping point in which disease control has become an imperative necessity (Stow *et al.,* 2007). This reinforces the assumption that the presence of individual defense mechanisms in social insects are not to be considered as separate entities from social immune systems.

A further individual defensive strategy in honeybees is ensured by intestinal symbionts located in the rear intestine acquired in the first 3-5 days after emerging due to the interactions between different individuals of the colony (Anderson *et al.,* 2016). Behaviors

such as cell cleaning, grooming, trophallaxis and oral-fecal route are essential in emerging bees to enhance the composition of the adult intestinal microbial community (Martinson *et al.,* 2012; Powell *et al.,* 2014). Most of the strains isolated in honeybees belong to the genera *Lactobacillus*, *Bifidobacterium* and *Bacillus* (Alberoni *et al.,* 2016). The intestinal bacterial flora and in particular the host-symbiotic bacteria make a positive contribution to nutrition, immunity and physiology (Hamdi *et al.,* 2011). The nutritional support to the host is due to the fact that some symbiotic species have genes that encode enzymes that are involved in the decomposition of lignin and cellulose, which are essential in a plant-based diet for energy absorption (Newton *et al.,* 2013). Furthermore, the microbial flora produces other nutrients needed by the host as fatty acids, amino acids, vitamin B and secondary metabolites (Brodschneider & Crailsheim, 2010; Gündüz & Douglas, 2009). The protection against pathogens and/or parasites is yet another important trait frequently associated to a balanced intestinal flora. Indeed, a significant contribution to the host protection is provided by the antagonist activity of intestinal flora and its interaction with humoral and systemic immunity (Hedges *et al.,* 2008; Jaenike *et al.,* 2010). More specifically, microorganisms may play a role in the protection of their host by either stimulating the immune system of bees or inhibiting pathogens and parasites through the production of antimicrobial compounds (Alberoni *et al.,* 2016). For example, the results of Sabaté *et al.* (2009) pointed out that, in honeybees, there is antagonistic action of endogenous bacteria against *Paenibacillus larvae* and *Ascosphaera apis*.

In general, the host-microbe interaction in social insects is the result of a long process of coevolution closely related to the stage of development, temporal polytheism and transmission through social interactions (Hughes *et al.,* 2008). Several stress factors such as nutritional deficiencies, pesticides, parasites or pathogens can cause immunosuppression, leading thus to an alteration of the composition of the microbiota (Alberoni *et al.,* 2016). The effects of the parasitic mite *V. destructor* on both the qualitative and quantitative composition of the microbiota of the individual bee and the entire colony will be treated in the fourth chapter of this thesis.

1.2. Social Immunity System

Social immunity systems refer to all collective defense mechanisms that bees and other colonies of social insects have evolved to combat the increased risk of disease transmission that arises from both social interactions and group living, and are the result of cooperation between each member of the colony (Cremer *et al.,* 2007). Some of these defense systems are preventative and are intended to restrict the transmission of diseases within the nest, whereas others are activated in case of need, when pathogens and/or pests have already penetrated into the hive. High population density, frequent physical contact between nest mates as well as a reduced genetic variability are the factors that threaten the survival of social insects. Nevertheless, social wasps have exhibited a high degree of adaptation to different environments confirming thus the effectiveness of the defense strategies evolved against pathogens (Wilson, 1971). This biological success points out that social interactions, in addition to placing individuals at risk of disease, can lead to the research of new collective defense strategies as a result of coevolutionary dynamics between host and microorganisms (Baracchi & Turillazzi, 2014). This phenomenon creates indeed a balance between both entities, characterized by constant adaptations and mutual counter-adaptations.

1.2.1. Preventive Defenses

The typical organization of social insects characterized by caste divisions of various duties, temporal polyethism and spatial division of the nest represents the first preventive defense as it regulates and limits the contact among individuals, and has been defined as "organizational immunity" (Cremer *et al.,* 2007; Naug & Smith, 2007). For instance, in ants and bumblebees, workers of the same age perform the same duties within the nest, starting their labor with nurse tasks in the center of the colony, and then drift progressively towards the periphery of the nest (Bourke & Frank, 1995; Jandt & Dornhaus, 2009). In case of a new disease transmitted by contact, this behavior, so named "centrifugal polyethism", will clearly limit the disease spread within the colony (Bourke & Frank 1995; Jandt & Dornhaus 2009). This was also demonstrated in *A. mellifera*, where the interaction pattern among the individuals of a colony, at both social and spatial levels, follows the theoretical "compartment model" in which old foragers are at the outer edges, being the most exposed to external pathogens, while young bees are present in the inner area of the nest (i.e. more protected) tending the queen and brood (Baracchi & Cini, 2014).

The ability of the beekeeper to recognize diseased or parasitized individuals at the entrance of the hive prevents the entrance of pathogens and/or parasites inside the nest (Drum & Rothenbühler, 1985; Waddington & Rothenbühler, 1976). Moreover, one of the main collective defenses of social insect colonies is the use of antimicrobial secretions directly produced by insects or collected from the environment (Sadd & Schmid-Hempel, 2006). For instance, the venom gland present in ants, wasps and bees ensures the production of compounds with antimicrobial activity and represents one of the most important sources of antimicrobial compounds produced in social Hymenoptera species (Kuhn-Nentwig, 2003). The use of venom as "external immunity" has been demonstrated in ants (Tragust *et al.,* 2013). In honeybees, the hypothesis that the venom is not only used against predators but also against pathogens and/or parasites, is supported by the antimicrobial properties of its components, in particular the melittin **(**Kuhn-Nentwig, 2003**)**. Moreover, the presence of venom increases in the comb of bees nesting in cavities, which would make them more exposed than species living out of the nesting shelters (Baracchi *et al.,* 2011). In addition, it is customary, in ants and bees, to disinfect their own nest with substances collected from the environment, which exhibit antimicrobial activity, such as fragments of solidified coniferous resin in the case of *Formica paralugubris* (Christe *et al.,* 2003) or propolis in the case of *A. mellifera* (Simone *et al.,* 2009). Currently, an important scientific debate is undergoing on the possibility that the collection of resin and use of propolis in the hive are not to be considered as exclusively a preventive defense behavior, but could be used as a self-medication behavior among individuals (Simone-Finstrom & Spivak, 2012). This topic will be the subject of extensive discussion in the third chapter of the thesis.

1.2.2. Medication or Curative Defenses

One of the main collective defense strategies in honeybees is the social fever, characterized by the increase of temperatures within the nest induced by adult bees that block the development of diseases caused by heat-sensitive pathogenic microorganisms (Starks *et al.,* 2000). This behavior has been demonstrated as a response to *Ascosphaera apis* (Starks *et al.,* 2000). Similarly, one of the most commonly used defenses to combat ectoparasites is the "grooming" behavior, defined as the removal of ectoparasites from the bee's body and occurring in two types: self-grooming and allo-grooming (Pettis & Pankiw, 1998). Studies demonstrated that grooming is a frequent behavior in *Apis cerana*, with a good percentage

of *Varroa destructor* killed after removal (35%) (Buchler *et al.,* 1992). In contrast, grooming in *A. mellifera* is rare, although this behavior increased in infested colonies (Buchler *et al.,* 1992) with a percentage of 17% and 29% of *Varroa* killed after removal in European and Africanized bees, respectively (Invernizzi *et al.,* 2015). In social insects, a key role is also assigned to the hygienic and the so called "undertaker" behaviors, which consist in detecting and removing diseased and parasitized brood and corpses from the nest, as well as in the maintenance of the latter from waste material (Arathi *et al.,* 2000); Baracchi *et al.,* 2012; Spivak & Gilliam, 1998a, b). Eventually, an extreme form of colony defense called "altruistic strategy" consists in the suicide of a diseased or parasitized group member who decides to leave the nest, preventing thus the spread of a pathogen (Rueppell *et al.,* 2010). In ants, specifically in the genus *Temnothorax*, foragers cease any kind of social contact and abandon the colony when infected by entomopathogenic fungi (Heinze & Walter, 2010). Similarly, bees presenting deformations (i.e. fringed wings) are removed or leave voluntarily the nest (Rueppell *et al.,* 2010). Moreover, in *A. cerana*, it was recently shown that the "altruistic suicide" defined as "social apoptosis" occurs also in immature workers besides adult bees (Page *et al.,* 2016). The absconding behavior is another factor contributing to the reduction of the parasite load in the hive, as observed for the coleopteran species *Aethina tumida* (Ellis *et al.,* 2003). In addition, once healthy termites identify an infected colony member, they produce vibratory alarm signals in order to isolate and surround the individual by constructing additional walls (Myles, 2002; Rosengaus *et al.,* 1999).

The behavioral defenses that honeybees have evolved against wasps, their natural enemy, need further consideration. Indeed, as seen so far, defenses against parasites, pathogens and predators can be divided in two similar levels: preventive (warning signals) and effective (agonistic response to attack) defenses. Of warning signals, those of chemical type, namely the emission of insect-alarming pheromone, stimulate an aggressive response in the nest mates (Collins & Kubasek, 1982). Conversely, vibrational signals cause a bioacoustic effect that temporarily interrupts feeding of the colony under attack (Tan *et al.,* 2016). Other warning signals are of visual type, such as "shimmering" in which bees would flip their abdomens upwards, producing waves once in contact with the wasp at the nest entrance (Tan *et al.,* 2012a). Such warning signals may induce the closest bees to the predator to change their behavior until gasping the wasp (Seeley *et al.,* 1982). As a matter of fact, the best-known example in collective defenses against a predator is represented by "balling", which

consists in the formation of a winter cluster of honeybees around the predator, which is bound to succumb due to thermal increase (heat-balling) (Ono *et al.,* 1995). This phenomenon may occur because wasps and hornets have a lower lethal thermal threshold than bees (Papachristoforou *et al.,* 2007). In the genus *Apis*, this behavior was described in various species such as *A. cerana*, *A. mellifera* and *Apis dorsata*, but with significant differences in the heat-generating mode, duration (Tan *et al.,* 2005), temperature reached, number of bees involved and sacrificed (Tan *et al.,* 2012b; 2016). High temperature within the winter cluster could also not reach the lethal threshold for wasps and hornets, however they will perish due to an increase of CO_2 in hemolymph (asphyxia-balling) (Papachristoforou *et al.,* 2007; Sugahara & Sakamoto, 2009). Nevertheless, honeybees do not always show collective responses to wasp attacks, and sometimes, in case of a worker bee under attack, only one or few group companions, called "helpers", will intervene. Such case is called "agonistic support", which is considered a classic example of altruistic behavior in which an individual is involved in the support of another group companion in conflict, facing therefore a potential risk while the recipient reaps the benefits of the support given (Schino *et al.,* 2007). Relative to balling, agonistic support "given by a few individuals" has been less studied in insects and will be subject of detailed study in the second chapter of the thesis.

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CHAPTER 2

Agonistic interactions between the Italian honeybee (*Apis mellifera ligustica*) and the European wasp (*Vespula germanica*) reveal context-dependent defense strategies

Michelina Pusceddu[a], Ignazio Floris[a], Franco Buffa[a], Emanuele Salaris[a], Alberto Satta[a]

[a]Dipartimento di Agraria, Sezione di Patologia Vegetale ed Entomologia, Università degli Studi di Sassari, Viale Italia 39, 07100, Sassari

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2.1. INTRODUCTION

Cooperation among individuals is observed in many phylogenetically diverse taxa and has underpinned the evolution of sociality in the animal kingdom (Szathmary & Maynard Smith, 1995; Wilson, 1975). The main advantages of sociality, promoted by natural selection, include more efficient vigilance against predators, a better ability to identify food sources, and the greater survival of developing brood. However, life within a group also presents certain disadvantages, one of the most significant being the ease with which predators can detect prey. For this reason, nest protection to reduce vulnerability is another central aspect in the evolution of sociality (Hermann, 1984; Shorter & Rueppell, 2012). Eusocial insects such as the honeybee (*Apis mellifera*) adopt numerous general and behavioral defense mechanisms against their predators. General mechanisms include nest architecture, site and visibility, as well as species-dependent morphological adaptations such as the size of an individual (Seeley *et al.*, 1982). In contrast, behavioral defenses are specific to particular enemies and require the prior identification of the predator based on olfactory, visual or tactile cues, recognition of movement, and information from previous encounters (Breed *et al*., 2004; Tan *et al*., 2012a; Wood & Ratnieks, 2004). Behavioral defense can also depend on agonistic behavior by the invader during an encounter, and in eusocial insects, on the caste to which the occupant and/or intruder belong (Breed *et al*., 1978). In the latter case, individuating and blocking specific predators in honeybee societies is the responsibility of guard bees. These bees adopt specialized behaviors that dissuade attacks by invertebrate predators and conspecifics from other colonies, thus preventing the loss of food and brood, and they also recruit "soldiers" to defend the nest against more aggressive predators (Breed *et al.*, 1990; 1992) Defense behavior in the Asiatic honeybee (*Apis cerana*) was recently shown to vary not only according to the predator, but also based on the context in which the attack takes place, e.g. minimal danger caused by an attack on a single forager contrasting with the substantial threat caused by an attack at the nest entrance (Tan *et al*, 2016).

Wasps are major invertebrate enemies of honeybees, invading hives to steal honey, pollen, larvae and adults to provide sugar and protein for themselves and their offspring (Baracchi *et al*., 2010; Matsuura & Sakagami, 1973). Current research focuses on the defense mechanisms used by *A. mellifera* against the Asian predatory wasp (*Vespa velutina*) due to its predatory success (Tan *et al*., 2007) and the damage caused by its introduction into Europe

(Monceau *et al*., 2014). However, the study of relationships between sympatric species is also necessary even though the predators are less dangerous (Markwell *et al*., 1993) because such relationships underpin the evolution of defense behaviors (Arca *et al*, 2014; De Grandi-Hoffman *et al*., 1998). We therefore investigated the predator–prey relationship between two sympatric species of social Hymenoptera, namely the Italian honeybee (*Apis mellifera ligustica*) and the European wasp (*Vespula germanica*) also known as the German wasp or German yellowjacket, in a representative area of the European Mediterranean region (Sardinia, Italy). We evaluated the effectiveness of behavioral displays of attack and defense which have co-evolved in these two species, the defense mechanisms in various danger contexts, and the real damage and disturbance caused by this predator to the honeybee colony under attack.

2.2. MATERIALS AND METHODS

2.2.1. Experimental apiary

The experimental apiary was set up in the northwest of Sardinia inside the experimental farm (latitude 40°46'23", longitude 8°29'34") of the Department of Agriculture of the University of Sassari, where no specific permission was required to carry out our experiments, during March 2014. The apiary comprised 18 *A. mellifera ligustica* colonies maintained in new Dadan-Blatt hives containing 10 combs each. They were checked every week to confirm the presence of the queen as well as pollen and nectar provisions. We also monitored the sanitary status for evidence of microbial infections and varroosis (Pappas & Thrasyvoulou, 1988). The study did not involve endangered or protected species.

2.2.2. Behavioral observations

Agonistic events between *V. germanica* and *A. mellifera ligustica* were examined in two different contexts, one at the hive entrance where defense is thought to be initiated by the guard bees and the other on the ground close to the hive where weakened and dead bees are present. The behavioral observations were based on the “all occurrences sampling” method (Altmann, 1974) in which we recorded the frequencies of a series of behavioral events as set out in the ethogram described below.

Attacks at the nest entrance were recorded in 2014 and 2015 during September and October, when the predatory activity of wasps is more intense due to their higher nutritional requirements during reproduction and rearing of offspring (Turillazzi, 2003). Each colony was recorded for two 15-min sessions per day using a Canon LEGRIA HF R506 video camera placed ~20 cm from the opening of the hives. Recordings were taken during the hottest part of the day (between 9:30 am and 15:30 pm) when the wasps were most active. A total of 279 h of video footage was recorded (63 h in 2014 and 216 h in 2015) and all 18 colonies were observed for the same duration (15.5 h). Subsequently, two operators independently screened the video recordings using a slow motion system (VLC software, v2.2.0) and the agonistic behaviors observed were used to establish an ethogram as described below. The ethogram was supplemented with further attack and defense behaviors not observed by us but reported in the literature for similar species, or in these two species facing different antagonists. This approach allowed us to evaluate the repertoire of agonistic behavior between *V. germanica* and *A. mellifera ligustica* in a larger context following an evolutionary approach. The frequency (number of events per unit of time) was reported for all the recorded attack and defense behaviors.

Attacks at ground level (only on individuals still alive and close to the hive) were monitored in 2015 on the same colonies, concurrently with some of the observations at the nest entrance. These observations were conducted by sight, without using the video camera, for a total of 32 h. Two operators simultaneously observed the ground surface under three hives in two sessions per day, each lasting 10 min. The frequency (number of events per unit of time) was reported for all the observed attack and defense behaviors.

2.2.3. Effect of predator attacks on bee foraging activity

The 15-min video clips taken at the nest entrance in each colony were used to evaluate the disturbance caused by wasps on the foraging activity of the honeybees. We compared the frequency of pollen foragers entering the hive 5 min after wasp attack (attack context) with the frequency at random times before the attack (control context) over a fixed 2-min interval. The comparisons were carried out for 27 agonistic events observed in 2015 to account for any interference that prevented us counting the number of pollen foragers, e.g. continuation of balling, successive attacks, or other bees blocking the view of the video camera.

2.2.4. Agonistic support

To determine whether there was a correlation between the degree of agonistic support and the intensity of predator aggression, all attacks at the nest entrance were divided into two behavioral categories described as *threats* (attacks in which the defender did not make physical contact with the predator) and *fights* (where physical contact was involved) (Johnson & Hubbell, 1974; Nieh *et al*., 2005). For each agonistic event, we recorded the duration of the attack, the number of supporters intervening to help a nestmate under attack (in the case of individual support) and any observed cases of balling.

2.3. ETHOGRAM

2.3.1. Wasp attack

Behaviors detected in this study

Attack **–** The wasp swoops down to the landing board or to the ground, grasps the bee from above with its forelegs and starts biting it (usually between the head and thorax).

Fight **–** The predator and prey are involved in a physical encounter which may include instances of biting, aggressive gripping, and spinning on a surface or in flight.

Entering the hive **–** A wasp may be able to enter the nest if it is overlooked by the bees, following antennation or following a struggle.

Predation **–** The wasp kills the honeybee. The wasp usually goes on to dismember and consume the honeybee or to carry off parts to its offspring (see below). In some cases, the wasp may also eat the contents of the honey stomach (Baracchi *et al*, 2010).

Sequestration **–** After predation, and having divided the honeybee into three parts (head, thorax and abdomen), the wasp flies off with one of them, usually the thorax (Coelho & Hoagland, 1995; Free, 1970).

Retreat **–** The wasp escapes when the attack has not been successful and one or more honeybees defend themselves effectively.

Known behaviors not detected in this study

Coalition attack – A coalition comprising a wasp and other conspecifics launches an attack. This behavior has been reported for the Asian giant hornet (*Vespa mandarinia*), which has developed a strategy of group hunting: certain individuals pillage while others defend the site against conspecifics from other colonies (Matsuura, 1991; Monceau *et al*., 2013).

2.3.2. Nest defense

Behaviors detected in this study

Antennation or antennal boxing – This is often the first physical contact between the occupant and invader, and most likely facilitates the recognition of intruders and conspecifics (De Wroey & Pasteels, 1978). It is defined as asymmetric when a dominant and a submissive can clearly be distinguished from the behavioral display of the two opponents or symmetric when such distinction is not possible (Denis *et al*., 2008).

Threat – The typical behavior of a honeybee in the presence of a conspecific intruder or other predator, consisting of open mandibles and the adoption of the so-called C posture (gaster flexion with or without extension of the sting) (Breed *et al*., 1978).

Agonistic support – Altruistic behavior in which an individual helps another involved in a conflict, thus facing a potential risk. It may involve a single bee or several bees (supporters) that come to the aid of their nestmates.

Balling – The formation of a ball of bees around a wasp until the latter is killed or becomes harmless. In *heat-balling* the wasp succumbs to the heat inside the ball because hornets and wasps have a lower thermal tolerance than bees (Ono *et al*., 1995; Tan *et al*., 2012b). In *asphyxia-balling*, the heat inside the ball can be lethal to the predator but it dies due to the increased concentration of CO_2 in the hemolymph which causes asphyxiation (Papachristoforou *et al*., 2007; Sugahara & Sakamoto, 2009).

Killing and removal of the predator – Wasps can be killed by a single bee sting or the stings or several bees, or by balling (see above). The dead or dying wasp is then removed from the nest or landing board.

Known behaviors not detected in this study

Bee carpet – A large proportion of the colony regroups on the landing board and along the sides of the hive, forming a "bee carpet" (De Grandi-Hoffman *et al*., 1998). This behavior

in *A. mellifera ligustica* has been observed against the European hornet (*Vespa crabro*) (Baracchi *et al*., 2010).

Shimmering or shaking signal **–** When a wasp is seen, the guard bees simultaneously vibrate their abdomens for a few seconds from side to side, emitting a loud hissing noise (De Grandi-Hoffman *et al*., 1998). This behavior has been observed in the giant honeybee (*Apis dorsata*) (Kasterberger *et al*., 2008; 2012), the dwarf honeybee (*Apis florea*) (Seeley *et al*., 1982), *A. cerana* (Abrol, 2006), *A. cerana nuluensis* (Tan *et al*., 2007) and *A. mellifera cypria* (Papachristoforou *et al*., 2011) against *V. velutina* and the oriental hornet (*Vespa orientalis*), and also in *A. mellifera ligustica* toward *V. crabro* (Baracchi *et al*., 2010). Shimmering is considered to be a visual signal for the predator and seems to have evolved in order to dissuade the latter from attacking, i.e. it is an honest alert signal that reduces the likelihood of predator success (Kastberger *et al*., 2008; Tan *et al*., 2012a).

Interruption of foraging **–** The interruption of foraging activity in a colony under attack by *V. velutina* has been reported in *A. cerana* (Tan *et al*., 2005) and *A. mellifera cypria* (Papachristoforou *et al*., 2011).

Retreat into the nest **–** Complete retreat into the nest during an attack has been described in *A. cerana* (Tan *et al*., 2007) and *A. mellifera cypria* (Papachristoforou *et al*., 2011), as well as the Cape honeybee (*A. mellifera capensis*), the African honeybee (*A. mellifera scutellata*) and the Carniolan honey bee (*A. mellifera carnica*) (Kastberger *et al*., 2009).

Attack **–** The switch from nest defense to attack within a larger perimeter is one of the greatest differences between European and Africanized bees (Collins *et al*., 1982). Moreover, the response of the African line to the same stimulus is faster, more aggressive, and involves the recruitment of more nestmates for the attack (Arca *et al*., 2014). Attack behavior has also been observed in *A. mellifera cypria* towards *V. orientalis* (Papachristoforou *et al*., 2011).

2.4. STATISTICAL ANALYSIS

The disturbance of foraging activity was measured by comparing the number of pollen foragers in the attack context to the number of pollen foragers in the control context using the Wilcoxon signed rank test (paired comparisons). A chi-squared test was used to measure

the proportional difference in support events (individual agonistic support and balling) between the *threat* and *fight* categories. To reduce the chance of a type I error, continuity correction was used for the chi-squared tests because the sample size was less than 200 (Sokal et al., 1981).

The Wilcoxon rank sum test (unpaired comparisons) was used to compare the number of supporters in the *threat* and *fight* categories (excluding balling). We also tested for correlation (non-parametric Spearman correlation) between the number of supporters and the duration of attacks. To reduce the chance of a type I error in this analysis, we used Bonferroni correction in the case of multiple testing with significance set at $\alpha = 0.05/2 = 0.025$. All tests were carried out using R v3.0.2 implemented with library (exactRankTests) and library (coin).

2.5. RESULTS

2.5.1. Wasp attack

We observed 68 attacks at the hive entrance in 279 h of video footage, specifically 11 attacks in 2014 (63 h) and 57 in 2015 (216 h) representing ~0.24 attacks per hour. The most frequent outcome was wasp escape (55 events, 80.9%) and the least frequent was bee predation (1 event, 1.5%). On three occasions (4.4%) the wasp was observed entering the hive and coming out alive. On another three occasions it was not possible to confirm the fate of the wasp because observation session terminated while the wasp was still inside the hive. The average attack time was 3.5 ± 0.4 s.

We observed 465 attacks at ground level in 32 h only targeting isolated bees (~14.5 attacks per hour). In this case, the outcome was more balanced. Bee predation was observed 226 times (48.6%) and in 91 of these cases sequestration also occurred. The wasp was chased away 239 times (51.4%), which is a much lower proportion compared to hive entrance attacks. The attack behavioral display data are summarized in Table 1.

2.5.2. Nest defense

We did not observe a collective attack against any of the colonies so our data only represents defense behaviors against individual wasps. Among the 68 agonistic events observed at the hive entrance, 28 cases (41.2%) involved a single bee defending itself successfully causing

the wasp to flee. In the remaining 40 attacks (58.8%), other bees from the same nest came to the rescue. Agonistic support was exclusively individual in 90% of cases, with an average of 1.9 ± 0.2 supporters per attack, and was collective in 10% of cases, resulting in balling. In six cases (8.8%), the wasp was killed and removed from the landing board. Agonistic support of the bees under attack was never observed among the 465 ground level attacks close to the hive. The defense behavioral display data are summarized in Table 2.

2.5.3. Disturbance of foraging

We did not observe any disturbance of foraging activity when the colony was under attack. Indeed, there were no statistically significant differences between the frequency of foraging in the attack context (24.2 ± 4.1) and in the control context (23.0 ± 3.4) in 2015 ($U = 154$, $N1 = N2 = 27$, $P = 0.6378$).

2.5.4. Agonistic support

The agonistic events most commonly supported by nestmates either individually or by balling were those involving physical contact (*fights*) rather than warning behavior (*threats*). Accordingly, we observed a statistically significant difference between the number of supported threats and the number of supported fights as shown in **Figure 1** (*chi-squared* = 13.07, $df = 1$, $P = 0.0003$). Moreover, when balling events were excluded, the average number of supporters was significantly higher in fights than threats, as shown in **Figure 2** ($U = 221$, $N1 = N2 = 32$, $P = 0.00001$). There was also a positive correlation between the number of supporters and the duration of attack ($S = 20653$, $P = 0.000007$, $rho = 0.53$). Agonistic support was observed only at the hive entrance, not at the ground level.

2.6. DISCUSSION AND CONCLUSIONS

Our data revealed that *V. germanica* attacks *A. mellifera ligustica* infrequently on the landing board of the nest so there is a low risk of predation, and cases in which the predator managed to overcome the barrier of guard bees, enter the hive, pillage it and escape, were extremely rare. Instead, the predatory activity of *V. germanica* is clearly directed towards the bees at ground level, which are weak or isolated. This specialized form of attack, as opposed to a direct attack on the hive, achieves high predation efficiency and confirms that *V. germanica*

is a scavenger in the apiary ecosystem (Coelho & Hoagland, 1995). This phenomenon can be explained by the optimal foraging theory, which postulates a trade-off between energy returns and mortality due to predation (Free, 1970; Kasper *et al*., 2008; D'Adamo & Lozada, 2003). Our observations revealed a compromise between the reward obtained and the risk taken by the wasp, with a direct attack on the hive entrance attracting a greater risk than attacks on isolated bees. Accordingly, the isolation of foragers is essential to improve the hunting effectiveness of hornets in the vicinity of *A. dorsata* nests because this species is extremely effective in repelling hornets by shimmering (Kastberger *et al*., 2008).

Our observations also indicated that *V. germanica* is predominantly a solitary predator, because we found no evidence of coordinated attacks involving other conspecifics. In contrast, competition for food and pillaging among wasps were observed during predation. This probably reflects the individual and independent foraging typology of this species (D'Adamo & Lozada, 2003) hence individuals from different colonies can find themselves at the same foraging site thus explaining why each individual defends its own prey (Free, 1970). In contrast to other predators such as *V. velutina* and *V. crabro* (Monceau *et al*., 2013), *V. germanica* has never been observed attacking forager bees in flight and returning to the hive, only bees on the ground or on the landing board.

The relatively weak predation practiced by *V. germanica* is confirmed by the ability of between one and three bees to repel an attack without recourse to truly collective defense strategies such as balling. Indeed, balling by *A. mellifera ligustica* against *V. germanica* has never been reported before, and we observed this behavior only four times throughout our observation period. In contrast, balling is deployed much more frequently against Asian wasps (Arca *et al*., 2014; Tan *et al*., 2005). This is important because balling often kills some of the participating bees in addition to the predator, suggesting that *A. mellifera ligustica* regulates its defense behavior depending on the intensity of the threat in order to prevent unnecessary sacrifices (Helfman, 1989; Tan *et al*, 2012a). We also saw no evidence of alternative collective defense strategies such as a bee carpet or shimmering, which are often deployed against *V. crabro* (Baracchi *et al*., 2010). Again this suggests that *A. mellifera ligustica* adjusts its defense strategy in response to different predators, and can likewise be interpreted as a trade-off between the involvement of the colony in collective defense (with the associated risks discussed above) and the danger the predator represents.

Our study also revealed that more intense agonistic events (i.e. fights rather than threats) attract stronger support from nestmates, and showed a correlation between the degree of support and the duration of attack. This suggests that that *A. mellifera ligustica* can adapt its defense behavior according to the context of the danger (Tan *et al*., 2016). This hypothesis is further supported by the different defense responses observed at the hive entrance and on the ground. Whereas hive entrance attacks usually attracted supporters in the events we observed because such attacks are recognized as a potential danger for the entire colony, attacks against fallen individuals were never supported because these individuals were no longer recognized as nestmates.

The different nestmate-recruiting capacity observed in different behavioral danger categories probably reflects the emission of warning signals such as alarm pheromones by the bee under attack (Breed *et al*., 2004; Tan *et al*., 2016). In contrast to recent observations in colonies of *A. mellifera ligustica* attacked by *V. velutina* in Liguria, Italy (Cervo, personal communication), we never observed the interruption of foraging or complete retreat into the nest. We can therefore exclude the possibility that vibration stop signals are used to recruit supporters (Tan *et al*., 2016). Indeed, we found no evidence that *V. germanica* disrupts *A. mellifera ligustica* foraging activity, providing more support for the hypothesis that the prey–predator relationship between these two sympatric species has reached a state of balance and that *V. germanica* need not be considered a threat to apiculture. However, to exclude the threat to bee foraging activity completely, further observations are required in areas with a greater density of wasp colonies.

Docile characteristics are preferred when selecting genetic lines of *A. mellifera* and several methods have therefore been developed to evaluate the aggression of reared honeybees. It follows that an understanding of the agonistic behavioral displays in *A. mellifera* against natural enemies could be used to develop more effective tests to replace the current evaluation methods, which have been called into question (Zakour & Bienefeld, 2013). Indeed, the method recommended by Apimondia (International Federation of Beekeepers' Associations) is based on subjective evaluation by the operator on a four-point scale, where 1 is most aggressive and 4 is most docile, thus establishing the protective equipment the beekeeper must use (Ruttner, 1972). This method does not account for climatic, chemical, visual, social and environmental variables that can play a role in the aggressive behavior of

a colony, and is therefore too subjective and difficult to reproduce. A much more selective method has been developed to distinguish between aggressive and docile states in *A. mellifera* colonies representing the subspecies *carnica*, *scutellata* and *capensis* (Kastberger *et al.,* 2009). Our data could also facilitate the selection of genetic lines of honeybees that are less hostile to humans while maintaining aggressive behavior towards their natural enemies.

In conclusion, our study provides insight into the mechanisms of attack and defense deployed by *V. germanica* and *A. mellifera ligustica* both in terms of predator–prey coevolution (Futuyama, 1986) and in terms of potential defense strategies that can be used by native bees against alien species such as *V. velutina.*

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2.8. TABLES AND FIGURES

Table 1. Attack behavioral display observed in colonies of *A. mellifera* under attack by *V. germanica*.

ATTACK BEHAVIORS	HIVE ENTRANCE (68 attacks in 279 h)		ON THE GROUND (465 attacks in 32 h)	
	n	%	n	%
Antennation*	11	16.2	-	-
Predation*	1	1.5	226	48.6
Sequestration*	-	-	91	19.6
Entering the hive	6	8.8	-	-
Retreat	55	80.9	239	51.4

* Behaviors which are not mutually exclusive.

Table 2. Defense behavioral display observed in colonies of *A. mellifera* under attack by *V. germanica* when predation did not occur.

DEFENSE BEHAVIORS *	HIVE ENTRANCE (68 attacks in 279 h)		ON THE GROUND (465 attacks in 32 h)	
	n	%	n	%
Single bees	28	41.2	465	100
General agonistic support	40	58.8	-	-
individual	36	90.0	-	-
collective (balling)	4	10.0	-	-
Killing wasp*	6	8.8	-	-

* Behavior which is not mutually exclusive.

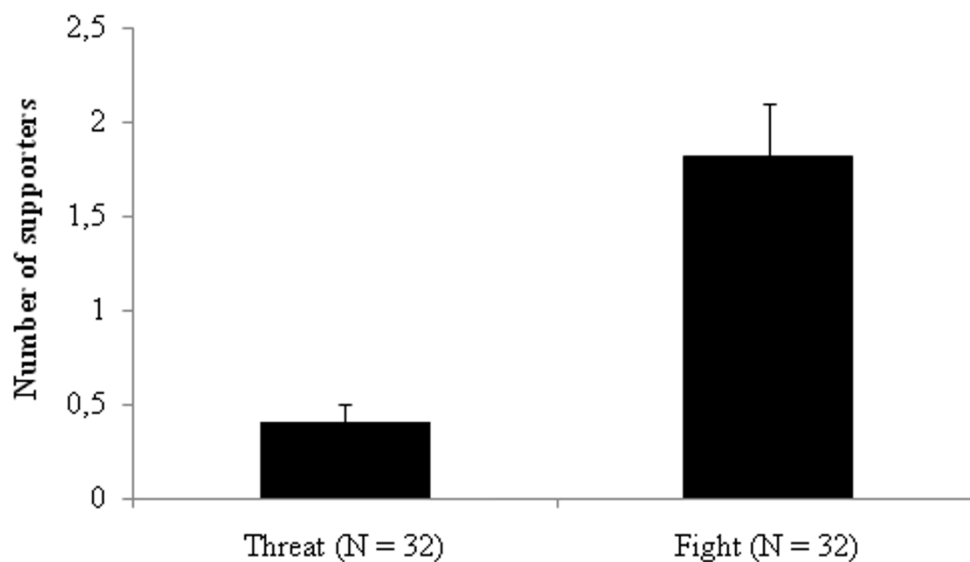

Figure 1. Number of supported and unsupported events classed as *threats* (agonistic interaction without physical contact) and *fights* (agonistic interaction with physical contact). The difference between the two groups was highly significant (chi-squared test, $P < 0.001$). N = number of agonistic events observed in 18 colonies.

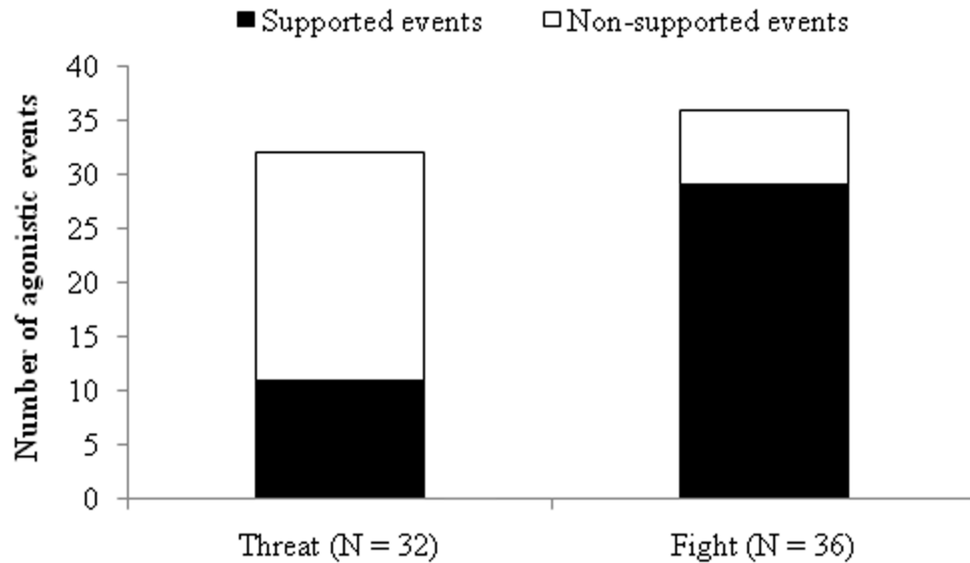

Figure 2. Number of supporters (mean ± standard error) for the agonistic events classed as *threats* (agonistic interaction without physical contact) and *fights* (agonistic interaction with physical contact). The 68 agonistic events we observed included four cases of balling which are excluded from the analysis. The difference between the two groups was highly significant (Wilcoxon rank sum test, unpaired comparisons $P < 0.0001$). N = number of agonistic events observed in 18 colonies.

CHAPTER 3

Resin foraging dynamics in *Varroa destructor* infested hives. A case of medication of kin?

Michelina Pusceddu[a], Giannella Piluzza[b], Panagiotis Theodorou[c,d], Franco Buffa[a], Luca Ruiu[a], Simonetta Bullitta[b], Ignazio Floris[a], Alberto Satta[a]

[a]Dipartimento di Agraria, Sezione di Patologia Vegetale ed Entomologia, Università degli Studi di Sassari, Viale Italia 39, 07100, Sassari
[b]Istituto per il Sistema Produzione Animale in Ambiente Mediterraneo (ISPAAM uos Sassari) Consiglio Nazionale delle Ricerche (CNR), Baldinca-Li Punti, 07100 Sassari., Italy;
[c]General Zoology, Institute for Biology, Martin Luther University Halle-Wittenberg, Hoher Weg 8, 06120 Halle (Saale), Germany.
[d]German Centre for integrative Biodiversity Reserch (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, 04103 Leipzig, Germany.

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3.1. INTRODUCTION

Self-medication, defined as a specific prophylactic and therapeutic behavioral change in response to disease or parasitism, plays a main role among the variety of behavioral defense mechanisms that animals have evolved against pathogens and parasites (Lozano, 1998).

Whilst the conditions defining this adaptive behavior have over time been refined, three classic criteria were provided by Clayton and Wolfe (1993): 1) The substance in question must be deliberately contacted; 2) The substance must be detrimental to one or more parasites; 3) The detrimental effect on parasites must lead to increased host fitness. The second and the third criteria are rather self-evident: a substance that does not reduce parasite fitness or does not increase host fitness can hardly be considered medicinal. According to de Roode *et al.* (2013) it is not essential to meet the second criterion, because medication behavior may enhance host fitness by increasing tolerance to infection (allowing the host to maintain fitness despite being infected) without reducing parasite fitness (Lars *et al.* 2007). The first criterion however, is of fundamental importance as it assumes that the use or the incremented use of the medicinal substance would be a direct consequence of a parasitic and/or pathogenic action (de Roode *et al.* 2013). Singer *et al.* (2009) see self-medication as a type of adaptive plasticity resulting from behavioral changes induced by the outside environment and improving the animal survival and reproduction prospect. In agreement with these authors, because of its fitness cost, self-medication is observed only in the presence of a disease or a parasite. On this basis, an additional criterion to define self-medication was described: 4) self-medication behavior decreases fitness in uninfected animals, having a detrimental effect or a major cost for the host in the absence of parasites or diseases (Singer *et al*. 2009). Finally, de Roode *et al.* (2013) suggested that to be considered an adaptive form of medication, self-medication has to be relevant in the natural environment of the host. It follows that experiments using artificial diets to investigate medication mechanisms, are not sufficient to demonstrate their relevance in nature.

Mostly studied in higher vertebrates (Gompper & Hoylman, 1993; Gwinner *et al.* 2000; Wimberger, 1984; Wrangham & Nishida 1983), self-medication was also observed on a variety of solitary insects, such as *Grammia incorrupta* (Singer *et al.* 2009; Smilanich *et al.* 2011) and *Drosophila melanogaster* (Milan *et al.* 2012). In eusocial insects, it is necessary to distinguish between self-medication and medication of kin, which extends the self-medication concept to the colony level (Abbott, 2014). In fact, eusocial insects add to their

immunological individual defenses against pathogens and parasites (Schmid-Hempel, 2005), several evolutionary behavioral and organizational adaptations within the colony (Cotter & Kilner 2010). Some of these defense mechanisms generally prevent or limit disease transmission, while others are induced by the presence of either parasites or pathogens. This "social immunity" system results from single member cooperation toward reducing the disease transmission risks typically associated with social life (Cremer *et al.* 2007). A higher exposition to pathogens and parasites is indeed expected as a consequence of high population density, frequent physical interactions among colony members, and the continuous use of the same nesting sites with microclimatic conditions (i.e., temperature and relative humidity) favoring the development of microorganisms (Schmid-Hempel, 1998). The reduced number of immune-related genes in *Apis mellifera* in comparison with other insect species, is in line with observations on other Hymenopteran species (Barribeau *et al.,* 2015). Different social immunity behaviors have been observed on the honeybee. These include social fever (Starks *et al.* 2000), hygienic behavior (Ibrahim & Spivak, 2005), allogrooming (Pettis & Pankiw, 1998), and self-medication through ingestion (Gherman *et al.* 2014). An interesting and scarcely studied self-medication behavior (by contact or proximity) involves the collection and use of resins in the hive (Simone-Finstrom & Spivak 2012). These viscous and complex substances are normally secreted by plants that exploit their bioactive properties to protect against parasites and pathogens (Langenheim, 2013; Simone *et al.* 2009; Simone-Finstrom & Spivak 2010). After being collected from diverse plant species, resins are carried to the colony where they are mixed with wax and incorporated into the hive structure as propolis (Simone-Finstrom & Spivak 2010). The colony mechanisms regulating resin collection have not been clarified. Besides, how workers communicate the need of collecting resins to other colony members is still under investigation (Nakamura & Seeley, 2006). It was demonstrated that an increased propolis content in the hive may correspond to a decrease in its microbial load (Simone *et al.* 2009), even if such effect was not observed by Borba *et al.* (2015). On the other side, a significant down regulation of individual immune-related genes was reported (Borba *et al.* 2015; Simone *et al.* 2009). Moreover, an increase in resin collection after infections of the fungus *Ascosphaera apis* was observed, suggesting a therapeutic use of propolis in the hive (Simone-Finstrom & Spivak 2012). Nevertheless, such response does not appear to be associated with the action of the American foulbrood agent, *Paenibacillus larvae* (Simone-Finstrom & Spivak 2012).

Assuming that bacterial and fungal infection mechanisms can be different from the action of a parasite, the objective of this study was to verify if the amount of resin collected and propolis quality within the hives infested by *Varroa destructor* were different from non-infested ones. We propose two hypotheses to explain the behavior of resin foragers in response to *Varroa* parasitism: 1) an increase in the usually collected amount of resins (quantitative hypothesis); and 2) an increase in the bioactive substance content (i.e., polyphenols and flavonoids) in propolis (qualitative hypothesis). The quantitative hypothesis is based on the antiparasitic, antimicrobial and antioxidant properties of propolis (Dresher *et al.*, 2017; Huang *et al.* 2014; Marcucci, 1995), mostly associated with its polyphenolic and flavonoid content (da Silva *et al.* 2006; Siripatrawan *et al.* 2013). Acaricidal effects of propolis extracts against *V. destructor* have been reported (Damiani *et al.* 2010; Garedew *et al.* 2002). The qualitative hypothesis is based on the ability of *A. mellifera* to select different kinds of resins (Erler & Moritz 2015; Isidorov *et al.* 2016; Loenhardt *et al.,* 2009). For instance, a preference for *Baccharis dracunculifolia* (alecrim plant, Asteraceae) females versus males (Teixeira *et al.* 2005), for buds and younger leaves (Park *et al.* 2004), or for plants producing resins with specific antimicrobial properties (Wilson *et al.* 2013), were reported. Besides, how bees may benefit from different resin sources was also observed (Drescher *et al.* 2014). Accordingly, Popova *et al.* (2014) demonstrated that the percentage of bioactive compounds (caffeic acid and pentenyl caffeates) was higher in *Varroa* tolerant colonies compared to non-tolerant ones. All these findings suggest that honeybees are able to follow a chemical "trace" leading toward a resin source and to evaluate its quality (Simone-Finstrom & Spivak 2010). In order to verify our hypotheses, comparative experiments involving hives with different mite infestation levels were conducted over a two year period (2014 and 2015), assessing the amount of resin collected and propolis quality in the hive. For this purpose observations on resin foraging dynamics in the hive were conducted along with chemical analyses on propolis samples to quantify the total polyphenol and total flavonoids content.

3.2. MATERIALS AND METHODS

3.2.1. Experimental apiary

The experimental apiary was set-up in the North-West of Sardinia (Lat 40°46’23”, Long 8°29’34”) during March 2014 and consisted of 18 hives, prepared with queens of *Apis mellifera ligustica* breed and with a homogeneous genetic profile (sisters) as provided by a local specialist breeder. Colonies were maintained in new Dadan-Blatt hives containing 10 frames of nest comb checked every two weeks to verify the presence of the queen, to provide pollen and nectar, to evaluate the sanitary status (possible symptoms of viral, fungal, and/or bacterial infections), and, when necessary, to match for population size (about 25000 – 30000 adult bees) through frame removal from stronger families. Each nest entrance was featured by a different color pattern to reduce drifting (Free & Spencer-Booth 1961).

3.2.2. Experiments

This study was based on different experiments conducted over a two-year period employing the same colonies (18 in 2014 and 12 in 2015) from the apiary.

A first experiment was conducted in July 2014 (experiment 1) on 18 colonies that did not receive any previous management intervention (e.g., equalization of colony strength, supplementary feeding, etc.), including no chemical or biological treatments against parasites and/or pathogens. Colony inspection, routinely conducted on a biweekly basis, did not report any symptoms of the main honeybee diseases (bacterial, viral and/or nosemosis). In total 22.5 h of observations were conducted to assess the number of resin and pollen foragers and the number of removed adults in hives with different adult infestation levels (from 2.4 to 8.7 %) and different colony strength (from 11242 to 31171 adult workers + sealed brood cells). Following the outcome of observations conducted in 2014 on colonies with varying mite infestation levels, the approach of experiments carried out in 2015 involved the manipulation of infestation levels using acaricidal treatments and strenght equalization among different colonies through frame removal from stronger families, two months before starting experiments. Observations were therefore conducted on two experimental hive groups: 1) *Varroa* free group, where *Varroa* infestation was maintained close to zero with acaricidal treatments, and 2) *Varroa* infested group, where no treatments were applied and the mite population could naturally increase. Treatments were based on

Apivar® (a.i. amitraz) application, a strip-based commercial formulation with long term action, suitable for acaricidal treatments in presence of sealed brood. Preliminarily, the possible effects of acaricidal treatments on resin and pollen collection behavior of honeybee foragers were verified. For this purpose, specific observations (experiment 2) were conducted in July 2015 on two hive groups (treated and control) having equivalent strength and a low mite infestation level (1.0 ± 0.7 % and 1.2 ± 0.5 %, respectively). During this experiment, no acaricidal treatments were applied to the control group, while in the treated group, Apivar® applications were performed three days after video-recording started. In total 15 h were recorded during the three days before treatments (pre-treatment) and further 15 h in the three days after treatment (post-treatment). Both experimental groups initially included six colonies, but two colonies in the treated group were excluded from data analysis as they were orphaned during the experimental period.

Although ascertaining that amitraz treatments did not produce significant effects on resin collection, the following experiments were conducted ensuring that no strips were present inside treated hives (*Varroa* free group). For this purpose, strips were removed a week before video-recording operations started and were put back in place afterwards. Two additional experiments, using the same 6 colonies for each group, were conducted in August (experiment 3) and September (experiment 4), when *Varroa* infestation percentages in the infested group increased from 2.8 ± 0.4 to 6.7 ± 1.0, respectively. In total 36 h video-recording was conducted in each of these experiments.

A final experiment (experiment 5) was conducted under the same conditions and with analogous observation time in October after the average mite infestation level in the *Varroa infested* group was reduced to the same level as the *Varroa* free group, through Apivar® treatment during four weeks. This experiment was conducted in order to exclude the possible influence of other pathogens carried by *Varroa* in the observed behavior. Also in this case, each experimental group initially involved six colonies, but just one in the *Varroa* free group was excluded from data analysis because orphaned during experiments. A colony in the *ex-Varroa* infested group was also excluded because its infestation level was still too high (3.1%).

3.2.3. Resin foragers detection and chemical analysis of propolis

The number of resin foragers returning to the hive was determined using "*all occurrences sampling*" method (Altmann, 1979; Simone-Finstrom and Spivak, 2012). To measure the total foraging force of hives used in the experiments, the number of pollen foragers was also determined. In order to compare the use of propolis to other social defense mechanisms potentially implicated in parasite management, the number of adult bees (dead or dying) removed from the hive was counted; larvae were not considered as their removal was only sporadically observed (3 times in 160.5 hours). Observations were based on video-recording employing an HD camera (Canon LEGRIA HF R506) placed at around 20 cm from the hive entrance. Following preliminary observations, 15 minutes (min) was established as the standard duration of each video slot, as it allowed to count an adequate number of resin foragers. For each experiment, video-recording sessions were repeated within the same time slot (10:30-15:30) during consecutive days (5-6 depending on weather). Each colony, within a group, was filmed daily according to a random pattern. In 2014, each hive was video recorded for 15 min per day, while in 2015, two slots of 15 min each were dedicated to each hive, so as to double observation time. Three days after starting video-recording, mite infestation level in adult bees (Pappas and Thrasyvoulou, 1988) and colony strength, considering an estimation of the total sealed brood extension and the amount of adult bees in the hive, were assessed (Marchetti, 1985). For this purpose, one-sixth of a Dadant-Blatt frame (188 cm^2) was used as a unit of measure converted in the tables of the results section in number of sealed cells and adult bees obtained by multiplying the number of sixth of each matrix for 780 and 254, respectively (Marchetti, 1985). After these surveys in the hive, 2-3 additional days of video-recording followed. Within the same experiment, each colony received an equal number of observation hours, and video-recording activities were simultaneously conducted in different experimental groups. All recorded videos (in total 160.5 h) were observed in slow motion by a single operator, who did not know the hive infestation level (blind experimental plan).

The number of resin foragers and of removed adult bees were recorded throughout the whole 15 min interval. Being significantly more frequent, the number of pollen foragers were recorded only during the first 5 min of each video.

Newly produced propolis was sampled in between recording periods using specific collection nets placed above nest-combs (Bankova *et al*., 2016). In October 2014, twelve propolis samples were collected from colonies with different colony strength and mite infestation level. In 2015, propolis was sampled twice (August and September) from twelve hives divided into two groups (*Varroa* free and *Varroa* infested) including six colonies each. In the *Varroa* free group, average infestation in both sampling was 0.1 ± 0.1 %, while in the *Varroa* infested group it ranged between 2.8 ± 0.4 % in August and 4.9 ± 0.8 % in September. To collect a sufficient amount of propolis for chemical analyses, collection nets were maintained in the hives for 7-10 days in both years for each sampling period. Similarly to the behavioral experiments, amitraz strips were removed from the hives before placing propolis collection nets. After collection, propolis was prepared for chemical analysis as reported by Gómez-Caravaca *et al.* (2006) with the following modifications: after being ground to a fine powder with liquid nitrogen, about 50 mg of raw propolis was extracted with 2.5 ml of 80% ethanol for 24 h at room temperature and in the dark. The samples were then centrifuged for 10 min at 3900 rpm and the supernatant was stored at 4°C until use for chemical determinations. The total amount of polyphenols (Tot P) in propolis samples was determined using the Folin Ciocalteu method (Singleton & Rossi 1965) with modifications (Piluzza & Bullitta 2010). Results were expressed as g gallic acid equivalent kg^{-1} dry weight of propolis material (g GAE kg^{-1}DW). Total flavonoids (Tot F) were determined by $AlCl_3$ method (Kim *et al.* 2003) with adaptations (Piluzza & Bullitta 2011). Results were expressed as g catechin equivalent kg^{-1} dry weight of propolis material (g CE kg^{-1}DW).

3.3. STATISTICAL ANALYSIS

In all experiments performed during 2015, we used Mann-Whitney *U* test to compare the *Varroa* infestation rate (%) and the colony strength among the different experimental hive groups.

For experiments 1-5 we performed generalized linear mixed models (GLMMs) with Poisson error structure. For experiment 1 (2014) GLMMs was used to study the effects of *Varroa* infestation level, colony strength and their interaction on the number of resin and pollen foragers and of removed adults. For experiment 2 GLMMs were used to study the effects of time (pre vs post) and group treatment (Apivar®) vs control (untreated) on the number of resin and pollen foragers. We used a GLMM model for experiments 3, 4 and 5 to study the

effects of *Varroa* infestation level, on the number of resin, pollen foragers and number of removed adults. For this model, month was used as a random effect factor to account for temporal autocorrelation. For all GLMMs day of observation nested within each hive was treated as a random effect factor.
We used a general linear model (LM) to analyse the effects of *Varroa* infestation level on the total amount of polyphenols and flavonoids found in propolis samples collected in 2014. We used a linear mixed model (LMM),to study the effects of *Varroa* infestation level (*Varroa* free vs *Varroa* infested) and sampling time (August and September) on the total amount of polyphenols and flavonoids found in propolis samples collected in 2015, including hive as a random effect factor to account for pseudo-replication.
We used automated model selection based on the Akaike Information Criterion (AICc), when models included several factors and their interactions (R package MuMIn; Barton, 2015). All mixed models were performed using the package lme4 (Bates *et al.* 2015). All model (GLMM, LMM and LM) assumptions were checked visually. For GLMMs, if over-dispersion was detected we used a negative binomial model (Zuur *et al.* 2009) implemented using the package glmmADMB (Fournier *et al.* 2012). To analyse single parameters and interactions we used a likelihood ratio test. We compared the goodness-of-fit between each model by setting up the model so that parameter can be dropped followed the examples in Zurr *et al.* (2009). We further analyzed mixed effect models to test differences between treatments with Bonferroni corrected post hoc tests. Post hoc tests were performed using the package multcomp (Hothor *et al.* 2008). All analysis was performed in R statistical software (R Core Team 2013).

3.4. RESULTS

3.4.1. Experiments

In the experiment 1, the best model explaining variability in the number of resin foragers included only the level of *Varroa* infestation. However, the relationship was not significant (GLMM poisson: Z = 1.487, P = 0.137, R^2=0.52) (Figure 1a). For the number of pollen foragers, the best model included both the level of *Varroa* infestation and colony strength. However, only colony strength (GLMM negative binomial: Z = 6.58, P = 4.8e-11, R^2=0.31) and not the level of *Varroa* infestation (GLMM negative binomial: Z = -1.140, Z= 6.58, P = 0.250, R^2=0.31) affected the number of pollen foragers (Figure 1b and 1c). None of the two

factors (level of *Varroa* infestation and colony strength) and their interaction explained variability in the number of workers removed from each colony.

Data of the second experiment, performed to assess possible effects of Amitraz[@] treatment on the number of resin and pollen foragers, are shown in figure 2. Both treatments had equal levels of colony strength and *Varroa* infestation level at the beginning of our experiment (for colony strength: Mann-Whitney U test: U = 9.0, N1 =4, N2 =6, P = 0.609; for *Varroa* infestation level: Mann-Whitney U test: U = 12.0, N1 = 4, N2 = 6, P = 0.751) (Table 1). The best model explaining variation in the number of resin foragers included only time (pre and post treatment) and not treatment (treated group vs control group). There was a significant decrease in resin foragers in response to time (pre vs post) irrespective of treatment group (GLMM poisson: Z = 3.356, P = 0.0007, R^2=0.46) (Table S1). Similarly, time (pre vs post, GLMM poisson: Z = 3.949, P = 0.0008 R^2=0.90) and not treatment (GLMM poisson: Z = 1.562, P = 0.118, R^2=0.90) was the main predictor for the observed variability in the number of pollen foragers (Table S1).

In the experiment 3 (August 2015), we did not find any significant differences in the number of resin and pollen foragers, and removed workers (Bonferroni post hoc test: Z = 1.244, P = 0.640; Z = 0.734, P = 1.000 and Z = 0.411, P = 1.000, respectively) between *Varroa* free and *Varroa* infested colonies (Figure 3, 4 and 5). However, the average number of resin foragers in the *Varroa* infested group almost doubled in respect to the *Varroa* free group.

In the experiment 4 (September 2015), we found a significantly higher number of resin foragers and removed workers (Bonferroni post hoc test: Z = 3.166, P = 0.004 and Z = 2.458, P = 0.042, respectively) in the *Varroa* infested compared to the *Varroa* free group (Figure 3 and 5). No significant differences were found between the two groups considering the number of pollen foragers (Bonferroni post hoc test: Z = 0.093, P = 1.000) (Figure 4).

Finally, in the experiment 5 (October 2015), we did not find any significant differences between the *Varroa* free and the *ex-Varroa* infested colonies in the number of resin, pollen foragers and removed workers (Bonferroni post hoc test: Z = 0.149, P = 1.000; Z = 0.375, P = 1.000 and Z = 1.167, P = 0.729, respectively) (Figure 3, 4 and 5). All groups had equal levels of colony strength across the course of all our experiments (for August: Mann-Whitney U test: U = 18.0, N1 =N2 = 6, P = 0.999; for September: Mann-Whitney U test: U = 16.0, N1 = N2 = 6, P = 0.818; for October: Mann-Whitney U test: U = 13.0, N1 = N2 = 5, P = 0.999) (Table 2). Furthermore, in experiments 3 and 4 there was a significant difference

in infestation level between *Varroa* free vs *Varroa* infested colonies (Mann-Whitney *U* test: $U = 0$, N1 = N2 = 6, $P = 0.002$; U = 0, N1 = N2 = 6, $P = 0.002$, respectively) (Table 2). While, in experiment 5 there was no difference in infestation level between *Varroa* free vs *ex-Varroa* infested colonies (Mann-Whitney *U* test: $U = 5$, N1 = N2 = 5, $P = 0.166$) (Table 2).

3.4.2. Chemical Analyses

In propolis collected in 2014, total phenolic and flavonoid content ranged from 130.3 g GAE Kg^{-1} DW (infestation level 4.1%) to 474.7 g GAE Kg^{-1} DW (infestation level 2.5%) and from 30.7 g CE Kg^{-1} DW (infestation level 4.1%) to 104.6 g CE Kg^{-1} DW (infestation level 0.3%), respectively. The amount of these compounds was not influenced by the mite infestation level (polyphenols: LM; t = -0.736, $P = 0.478$, R^2=0.05; flavonoids: LM; t = -1.263, P = 0.478, R^2=0.13) (Figure 6a and 6b).

In 2015 we did not find any significant differences between *Varroa* infested and *Varroa* free colonies in the total amount of polyphenols (Bonferroni post hoc test; $Z = 0.995$, $P = 1.000$; Table 3) and flavonoids (Bonferroni post hoc test; $Z = 1.186$, $P = 1.000$; Table 3) in propolis collected in August. Differently, in September, we found decreased polyphenol contents (415.3 g GAE Kg^{-1} DW) in the *Varroa* infested group compared to the *Varroa* free group (618.7 g GAE Kg^{-1} DW) (Bonferroni post hoc test; $Z = 2.909$, $P = 0.021$) (Table 3). No significant differences were observed between the two groups in the total amount of flavonoids (Bonferroni post hoc test; $Z = 1.805$, $P = 0.426$) (Table 3).

3.5. DISCUSSION AND CONCLUSIONS

This study demonstrates that when *A. mellifera* colonies are under stress conditions because of *Varroa* infestation, an increase in the number of resin foragers is recorded, even if a general intensification of the foraging activity is not observed. Similarly, Drescher et al. (2017) have recently found a positive correlation between *Varroa* infestation and resin collection. However, such results, obtained using propolis traps, are not directly comparable with our experiments based on the quantification of the resin foragers.

We also found an increase in the rate of adult removal in infested colonies, possibly affected by the virus titer (Baracchi et al., 2012).

The increase in resin foragers is in line with the results of experiments with the fungus *A. apis* (Simone-Finstrom & Spivak 2012) and apparently meets the first adaptive behavior criterion defined by Clayton and Wolfe (1993), according to which the use or the incremented use of the therapeutic substance should be associated with a health impairment caused by parasites and/or pathogens (de Roode *et al.* 2013).

The hypothesis that non-parasitized bee workers can change their behavior in favor of an infested colony that increases the number of resin foragers as a social immunity response, is really fascinating. The results of experiment 5, showing that differences in the number of resin foragers and removed workers were not anymore detectable after reducing mite infestation in the *Varroa* infested group to the same level as the *Varroa* free group (close to zero) through Apivar® applications, support the hypothesis that behavioral changes must be somehow closely related to the presence of *Varroa*. In fact, this acaricide is specific to mites and is not supposed to inhibit viruses, bacteria or fungi. According to the results of studies on honeybee viruses associated with vorroosis, DWV was shown to become undetectable in the sealed brood of colonies treated with pyrethroids (flumethrin and fluvalinate), paralleling the rate of mite loss after treatment (Martin et al. 2010; Locke et al. 2012). The titre of sac brood virus (SBV) and black queen cell virus (BQCV) was instead variably affected by these acaricidal applications and did not show any direct relationship with mite infestation (Drescher 2017; Locke *et al*. 2012). Al Naggar *et al.* (2015) demonstrated that acaricidal applications of amitraz (Apivar@) do not affect the percentage of hives infected by DWV and IAPV compared with untreated control. Accordingly, and based on the results of our experiments involving antivarroa treatments with amitraz, we can assume that a resin collection increase can be a direct result of the mite presence. In a study conducted by Drescher et al (2017) by artificially adding and removing natural propolis in colonies where *Varroa* population could naturally increase, significant effects on DWV titer, but not on mite infestation, were noticed. However, no information on the dynamic of the artificially added propolis in the hive were provided by these experiments, in which propolis could have been re-used by bees within the hive, thus affecting the overall resin collection behavior. Consequently, knowledge in this field remains limited and the actual relationship between *Varroa* and resin collection still need to be elucidated.

Further support to a mite infestation-resin collection correlation is given by the fulfillment of the other criteria defining a self-medication behavior. In fact, based on the second criterion

of Clayton and Wolfe (1993), the medicinal substance should negatively affect the parasite and/or pathogen. Accordingly, the acaricidal properties of ethanolic extracts of propolis are well documented (Damiani *et al.* 2010; Garedew *et al.* 2002). Besides, a reduction in the number of mature mite females per cell was obtained through treatments with propolis extracts inside the beehive (Simone-Finstrom & Spivak 2010). However, because the main bioactive compounds were found in the resinous fraction of propolis and are only soluble in alcohol (Medana *et al.* 2008), it still need to be clarified how crude propolis might directly or indirectly affect *Varroa* biological cycle, and how it might prevent the development of secondary infections, including the possibility that chemical-physical conditions inside the hive may help the release of bioactive substances (DeGrandi-Hoffman & Chen, 2015). Besides, in a laboratory experiment, no effects of volatile compounds possibly released by propolis were detected on mite survival (Drescher *et al*. 2017). Nicodemo *et al.* (2013) investigated whether propolis collection behavior is associated with resistance to the parasitic bee mite *V. destructor*, but no significant correlation between these two traits was found. However, this study was conducted employing Africanized honeybees that are *per se* more resistant to the mite, and considered relatively low infestation levels (mean infestation rate of sealed brood varying from 1.0 to 2.6%). For these reasons, this aspect deserves further investigation. On the other side, the incorporation of a high propolis amount inside the nest was found to cause a relative decrease in the microbial titer and in the expression level of immune-related genes of single bees (Simone *et al.* 2009). Since high individual immunity activation may correspond to significant fitness costs for the colony (Evans & Pettis 2005), traits that reduce chronic elevation of an individual's immune response may benefit colony-level productivity (Cotter *et al.* 2004). Accordingly, a positive correlation between propolis and honey production have been reported (Manrique & Soares 2002). For all these reasons, also the third adaptive behavior criterion of Clayton and Wolfe (1993) appears to be fulfilled. With regard to the criterion proposed by Singer *et al.* (2009), an augmented fitness cost for uninfected individuals would translate into a higher energy investment at the expense of resin in respect to pollen foragers (Nakamura & Seeley 2006; Simone-Finstrom & Spivak 2010). Indeed, time and energy consumed to collect resin from the outside environment and to handle it inside the hive, represent a cost that does not apparently reward the individual forager, that more obviously would receive a direct food recompense when collecting nectar

or pollen. It is remarkable to note that similarly to Simone-Finstrom and Spivak (2012), we observed this behavior within the host environment: the hive.

A higher expression of the adult removal behavior as a social immunity mechanism we observed in infested hives, suggests that the model describing the colony response against *Varroa* infestation is complex and includes different social defense behaviors that may work with pharmacophory.

Our study also revealed some effects on the quality of honeybee produced propolis in consequence of *Varroa* infestations. More in detail, the total polyphenolic content was reduced in propolis produced in infested hives in comparison with the *Varroa* free group. This preliminary finding encourages further investigation to understand if the observed propolis differences derive from similar differences in resins collected by foragers or from their dissimilar manipulation inside the hive. Considering that different propolis types differ in their inhibition properties against a variety of honey bee pathogens in vitro (Wilson *et al.* 2013), it would be very important to study the effects against *Varroa* of the two diverse types of propolis: from *Varroa* free and from *Varroa* infested hives.

Most studies on the acaricidal properties of propolis were conducted employing the total ethanolic extract (balsamic components), which includes both polyphenols and other compounds that despite not being considered in our study, might possibly be implicated in the toxic action against *Varroa* (Damiani *et al.* 2010; Garedew *et al.* 2002). Whilst propolis is usually considered of high quality when having a high flavonoid content (Bonvehi & Coll 1994; Park *et al.* 1998), the current literature on its biological properties proves the involvement of other components. For instance, substances with non-phenolic origin isolated from propolis samples collected in Brazil showed significant antimicrobial activity (Bankova *et al.* 1996). More in general, the biological activity of propolis derives from its high resin content, which is essentially (but not exclusively) associated with phenolic compounds, mostly flavonoids (Bankova, *et al.* 1983). Despite a growing interest in the potential of propolis against hive pathogens and parasites, only few studies investigated the relationship between colony health and propolis composition. In a recent study (Popova *et al.* 2014), the chemical composition of propolis from *Varroa*-tolerant colonies was analyzed and compared to non-tolerant colonies from the same apiary. A lower resin content was found in tolerant colonies that were also characterized by a higher percentage of the biologically active compounds, caffeic acid and pentenyl caffeates, thus highlighting a

significant relationship between *Varroa* infestation and propolis quality in the hive (Popova *et al.* 2014).

In conclusion, according to the results of our study and to previous knowledge in the field, resin foraging activities in *A. mellifera* have to be considered both as a constitutive and as an inducible behavior, thus representing a response influenced by an infection/infestation status. However, many other aspects still need to be investigated to definitely consider this behavior as a case of medication of kin against *Varroa* and its intimately associated virus.

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3.7. TABLES AND FIGURES

Table 1. Adult infestation level and strength of colony (mean ± SE) in the hive groups used in the experiment 2 to test the effect of Amitraz@ treatment on resin collection (2015).

	Adult bees infestation level (%)	Strength of colony (n)**	Colonies (N)
Treated (Amitraz)	1.0 ± 0.7 a*	29 106 ± 2 795 a	4
Untreated (Control)	1.2 ± 0.5 a	34 294 ± 2 341 a	6

* Different letters in the same column indicate significant differences (Mann-Whitney *U* test, $P < 0.05$)
** Colony strength was calculated adding the number of sealed brood cells to the number of adult bees.

Table 2. Adult infestation level and strength of colony (mean ± SE) in the hive groups used in the experiment 3, 4, 5 to test for differences on resin collection between two groups *Varroa* free and *Varroa* infested (2015).

		Adult bees infestation level (%)	Strength of colony (n)**	Colonies (N)
Experiment 3	Varroa free	0.1 ± 0.1 a*	26 220 ± 2 908 a	6
	Varroa infested	2.8 ± 0.4 b	26 679 ± 2 805 a	6
Experiment 4	Varroa free	0.2 ± 0.1 a	26 129 ± 1 262 a	6
	Varroa infested	6.7 ± 1.0 b	26 808 ± 1 379 a	6
Experiment 5	Varroa free	0 a	27 133 ± 1 612 a	5
	Ex Varroa infested	0.5 ± 0.2 a	27 363 ± 2 224 a	5

* Different letters in the same column indicate significant differences (Mann-Whitney *U* test, $P < 0.05$)
**Colony strength was calculated adding the number of sealed brood cells to the number of adult bees.

Table 3. Total polyphenols (Tot P) and total flavonoids (Tot F) (mean ± SE) of propolis samples collected in 2015.

Period		Adult bees infestation level (%)	colonies (N)	Tot P (g GAE Kg^{-1} DW) §	Tot F (g CE Kg^{-1} DW) §§
August	Varroa free	0.1 ± 0.1 a*	6	527.1 ± 66.3 a	67.1 ± 9.9 a
	Varroa infested	2.8 ± 0.4 b	6	596.7 ± 29.4 a	78.5 ± 2.8 a
September	Varroa free	0.1 ± 0.1 a	6	618.7 ± 55.6 a	76.6 ± 7.5 a
	Varroa infested	4.9 ± 0.8 b	6	415.3 ± 37.9 b	59.2 ± 4.7 a

* Different letters in the same column indicate significant differences (Mann-Whitney *U* test, $P < 0.05$; Bonferroni post hoc test $P < 0.05$)
§ GAE=gallic acid equivalent
§§CE=catechin equivalent

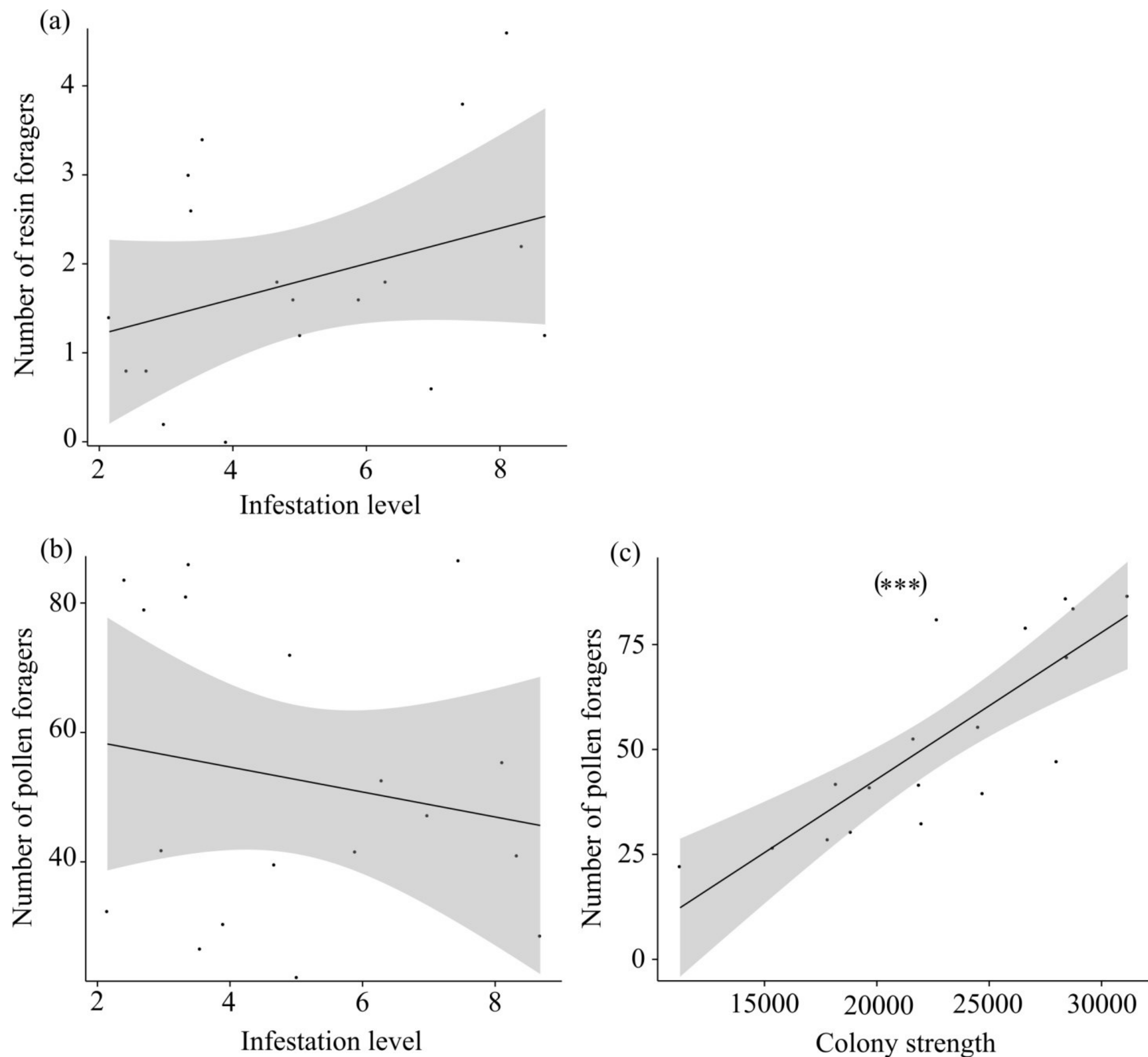

Figure 1. Relationships between the (a) number of resin foragers in 15 minutes and *Varroa* infestation level (%) (b) number of pollen foragers in 5 min and *Varroa* infestation level (%) and (c) number of pollen foragers and colony strength. Plotted lines show predicted relationship and the shaded areas indicate the 95% confidence intervals: ***, $P < 0.001$. (Experiment 1, July 2014).

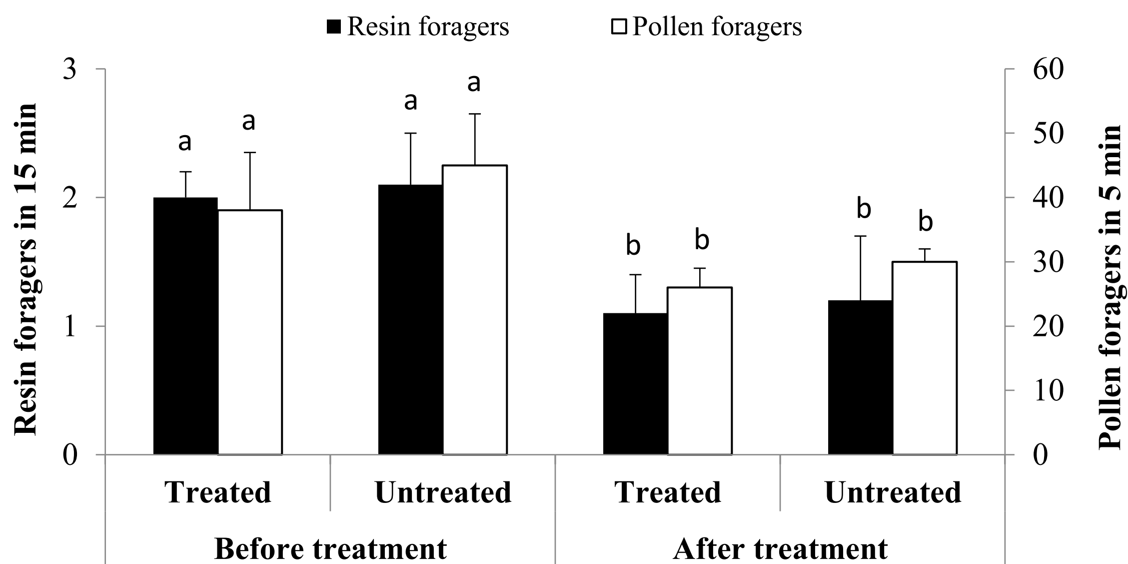

Figure 2. Effect of Apivar@ treatment on the number of resin and pollen foragers (mean ± SE). Both groups were homogeneous for colony strength and *Varroa* infestation level. For each variable, different letters above bars indicate significant differences between groups before and after treatment $P < 0.05$) (Experiment 2, July 2015).

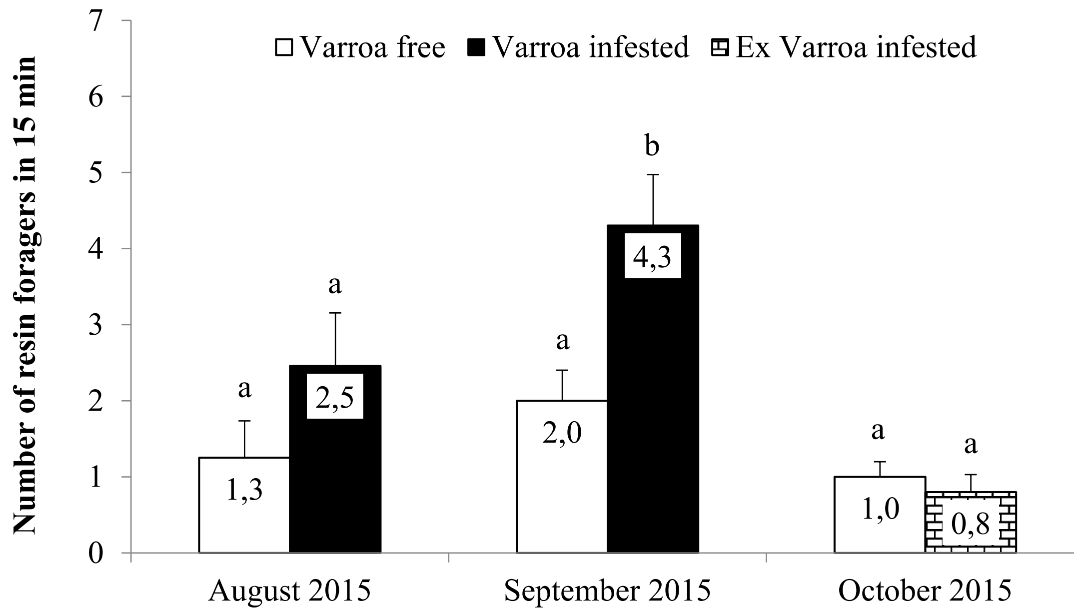

Figure 3. Effect of different infestation level of *Varroa destructor* (2.8 ± 0.4 % vs 0.1 ± 0.1 % in August; 6.7 ± 1.0 % vs 0.2 ± 0.1 % in September; 0.5 ± 0.1 vs 0 in October) on the number of resin foragers (mean ± SE). In the ex *Varroa* infested group the infestation level was reduced to the same level as the *Varroa* free group through miticide treatment. In each date, the two experimental groups were homogeneous for colony strength. Different letters above bars, within each experiment, indicate significant differences between groups (Bonferroni post hoc test $P < 0.05$).

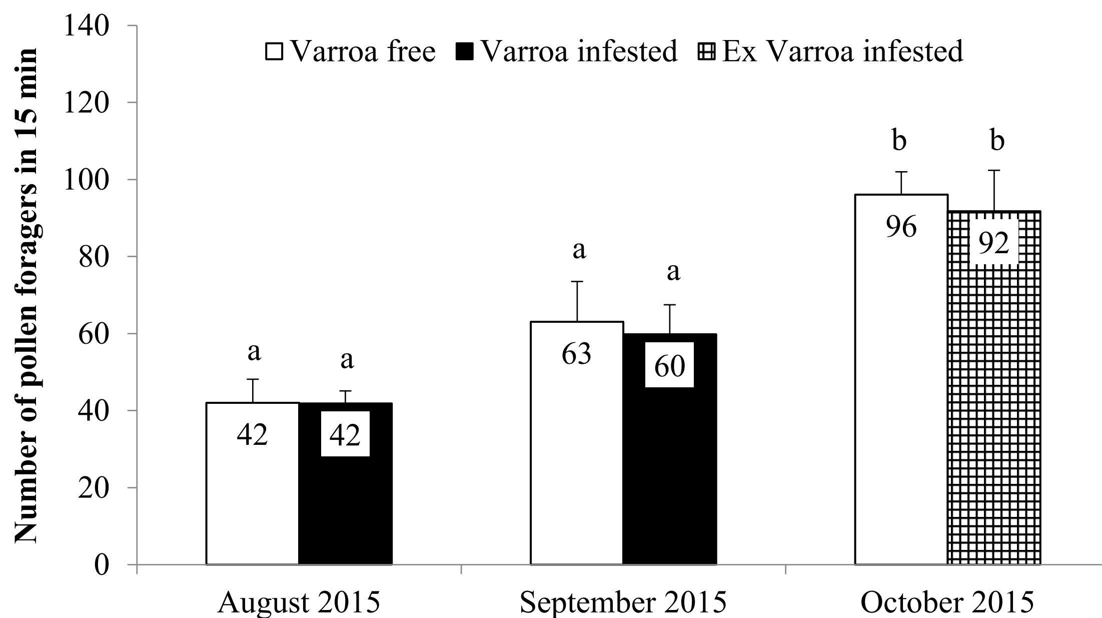

Figure 4. Effect of different infestation level of *Varroa destructor* (2.8 ± 0.4 % vs 0.1 ± 0.1 % in August; 6.7 ± 1.0 % vs 0.2 ± 0.1 % in September; 0.5 ± 0.1 vs 0 in October) on the number of pollen foragers (mean ± SE). In the ex *Varroa* infested group the infestation level was reduced to the same level as the *Varroa* free group through miticide treatment. In each date, the two experimental groups were homogeneous for colony strength. Different letters above bars, within each experiment, indicate significant differences between groups (Bonferroni post hoc test $P < 0.05$).

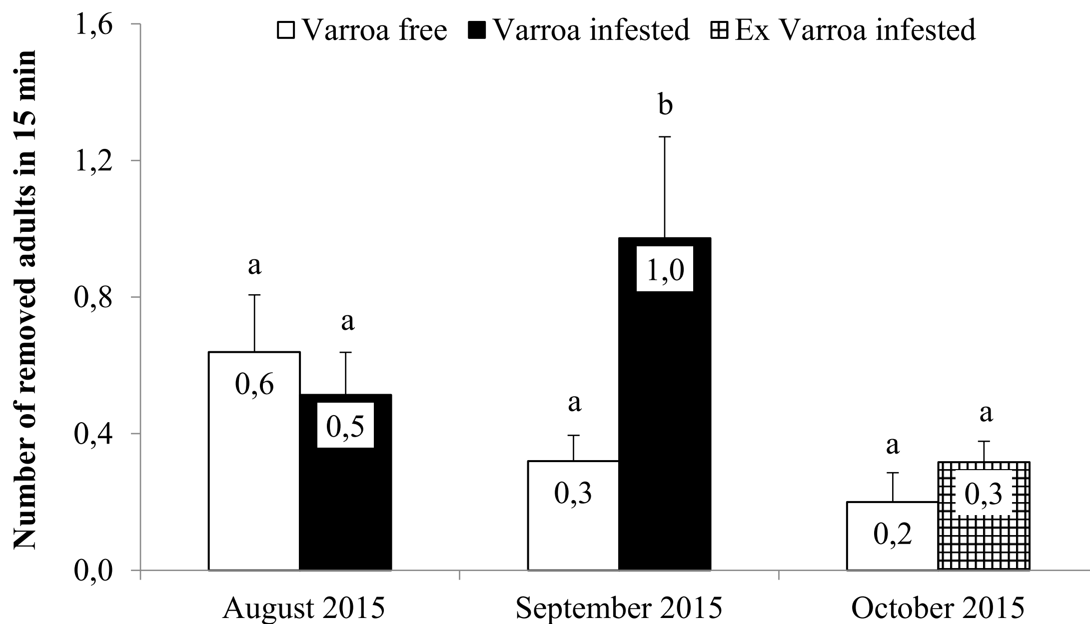

Figure 5. Effect of different infestation level of *Varroa destructor* (2.8 ± 0.4 % vs 0.1 ± 0.1 % in August; 6.7 ± 1.0 % vs 0.2 ± 0.1 % in September; 0.5 ± 0.1 vs 0 in October) on the number of removed adults (mean ± SE). In the ex *Varroa* infested group the infestation level was reduced to the same level as the *Varroa* free group through miticide treatment. In each date, the two experimental groups were homogeneous for colony strength. Different letters above bars, within each experiment, indicate significant differences between groups (Bonferroni post hoc test $P < 0.05$).

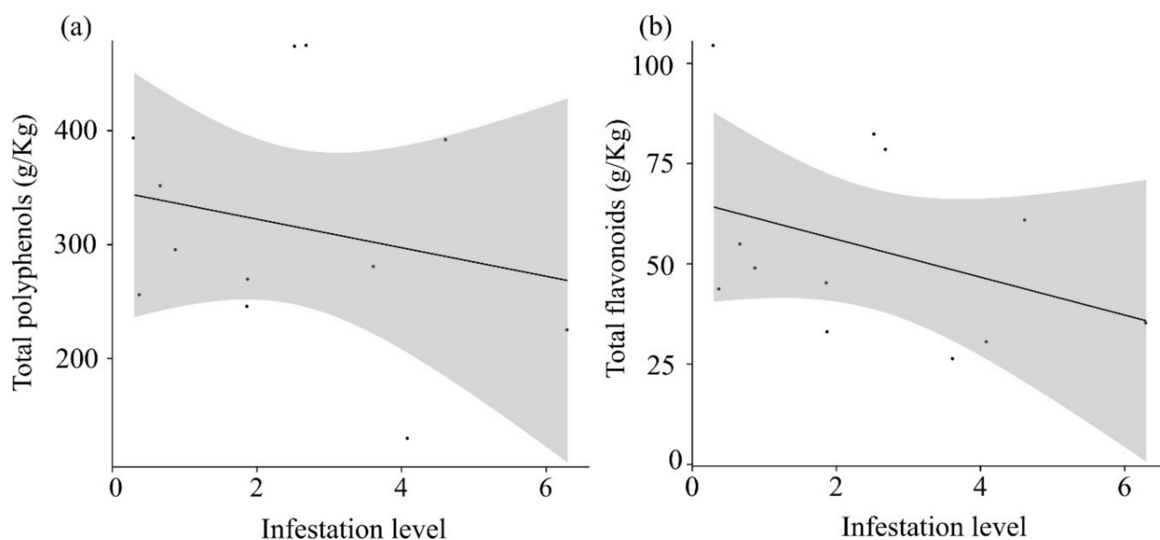

Figure 6. The effects of infestation level (%) on (a) the total polyphenols and (b) total flavonoids found in propolis for the 2014 experiment. Total polyphenols are expressed in g GAE Kg^{-1} DW= g Gallic Acid Equivalent Kg^{-1} Dry Weight of plant material. Total flavonoids are expressed in g CE Kg^{-1} DW= g Catechin equivalent Kg^{-1} Dry Weight of plant material. Plotted lines show predicted relationship and the shaded areas indicate the 95% confidence intervals.

CHAPTER 4

Quantitative variations in the core bacterial community associated with honeybees from *Varroa*-infested colonies.

Maria Giovanna Marche[a], Alberto Satta[a], Ignazio Floris[a], Michelina Pusceddu[a], Franco Buffa[a], Luca Ruiu[a]

[a]Dipartimento di Agraria, Sezione di Patologia Vegetale ed Entomologia, Università degli Studi di Sassari, Viale Italia 39, 07100, Sassari

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4.1. INTRODUCTION

Health preservation of the western honey bee *Apis mellifera* is a global issue to ensure efficient crop pollination services and wild plant biodiversity, and to support a range of bee-associated products like honey, royal jelly, bee bread, propolis, venom, and wax. Several efforts have been devoted to understanding the mechanisms contributing to the regulation of colony decline phenomena (i.e., colony collapse disorder or CCD), often associated with diverse pathogens, parasites and, more in general, stress factors (VanEngelsdorp et al., 2009; Hedtke, Jensen, Jensen, & Genersch, 2011; Dainat, Evans, Chen, Gauthier, & Neumanna, 2012; Nazzi et al., 2012). Among them, infestations of the ectoparasitic mite *Varroa destructor* are cause of significant colony weakening, due to both host-feeding and virus transmission effects (Le Conte, Ellis, & Ritter, 2010). Although a variety of viruses can be transmitted by *Varroa* to *A. mellifera*, the deformed wing virus (DWV) is one of the most widespread species whose prevalence and persistence in symptomatic or asymptomatic colonies is strictly dependent on the presence of the mite (Martin, Ball, & Carreck, 2010). *Varroa* life cycle includes a phoretic phase in which mites spread out riding on adult workers or drones, and a reproductive phase that starts when mites invade uncapped brood cells to feed on honeybee immature stages and to reproduce, after the cell has been sealed (Rosenkranz, Aumeier, & Ziegelmann, 2010). The natural mechanisms of defense against the mite include the ‘‘grooming behavior” and the ‘‘removal of parasitized brood cells” (Guzman-Novoa, Emsen, Unger, Espinosa-Montaño, & Petukhova, 2012). An additional hygienic behavior of the honey bee is the collection of resin that is incorporated into the nest as propolis, exhibiting broad-spectrum antimicrobial properties (Simone-Finstrom & Spivak, 2010). Innate mechanisms of defense, normally acting at individual level, are also involved in the colony response to varroosis. These include the production of antimicrobial peptides, melanization, phagocytosis and the enzymatic degradation of pathogens, in response to the combined action of the mite and DWV, which normally causes immunosuppressive effects on different insect stages (Di Prisco et al., 2016). An additional contribution to insect innate mechanisms of defense might be given by the beneficial microbial community inhabiting their body (Engel & Moran, 2013). The core bacterial community of honeybees resides in the intestine and include the two proteobacterial species *Snodgrassella alvi* (Neisseriaceae) and *Gilliamella apicola* (Orbaceae) in the midgut, and the Gram-positive bacteria *Lactobacillus spp.* and *Bifidobacterium spp.* in the rectum, as the

most abundant (Kwong & Moran, 2016). The contribution of these bacterial phylotypes to the honeybee immune system and their balance in diseased or parasitized honey bees are important aspects that need to be more thoroughly investigated (Alberoni, Gaggìa, Baffoni, & Di Gioia, 2016). Other bacterial species like Pasteurellaceae family members are involved in critical metabolic functions (i.e., digestion) and, accordingly, are thought to indirectly contribute to honeybee health conditions and susceptibility to infections (Anderson, Sheehan, Eckholm, Mott, & DeGrandi-Hoffman, 2011). The role of further bacterial residents of the bee body may become significantly important to counteract against certain pathogens. This is the case of the endospore former *Brevibacillus laterosporus* (Ruiu, 2013; Marche, Mura, & Ruiu, 2016), whose antimicrobial properties and the inhibitory effects on the honeybee pathogen *Paenibacillus larvae* have been documented (Alippi & Reynaldi, 2006; Hamdi & Daffonchio, 2011).

Whilst the beneficial role of a good microbial balance for honeybee colony health is well recognized (Budge et al., 2016), available information on the dynamics of core bacterial species in honeybees parasitized by DWV-carrying *Varroa* is scarce. Besides, most data on honeybee response to this pathogen and parasite combination derive from laboratory studies (Di Prisco et al., 2016), therefore, investigations on the actual scenario at the colony level are needed.

The present study describes the variations in the immune-related gene expression levels and in the relative abundance of representative bacterial phylotypes of the core honeybee microbiota in colonies infested by DWV carrying-*Varroa* mites in comparison with honeybees from non-infested colonies. The aim of the present investigation was to test the hypothesis that significant changes in the beneficial microbial community occur in immune-suppressed individuals from *Varroa*-infested colonies.

4.2. MATERIALS AND METHODS

4.2.1. Sample collection and processing

Honey bee samples were collected in 2014 from an experimental apiary consisting of 10 beehives located in Nuoro (Central Sardinia, Italy), an area characterized by common *Varroa* infestations. During the experimental period, a group of five beehives (non-infested) underwent routine antivarroa treatments based on applications of amitraz (Apivar®) and thymol (Apiguard®) in summer. A second group of five beehives (infested) did not receive

any acaricidal treatment in order to facilitate a natural mite population development. *Varroa* management in the first group was suspended a month before honeybee sampling activities began (October). At this date, *Varroa* infestation level in each beehive was evaluated, determining the percentage of infested workers (Pappas & Thrasyvoulou, 1988). On the same date, colony strength was determined through an estimation of the sealed brood extent and of the number of adult bees. For this purpose, one-sixth of a Dadant-Blatt frame (188 cm^2) was used as a unit of measured, according to the method described by Marchetti (1985). At the same time, three replicates of the following insect samples were collected from each infested or non-infested hive: 1) honeybee workers, 2) emerging adults, 3) pupae, and 4) larvae (5-6 day old). Additional samples of emerging adults were collected from infested hives distinguishing between insects bearing (Varroa attached) or not (without Varroa) a sucking mite on their body. After collection, each individual was preliminarily surface-sterilized with sodium hypochlorite (0.2 %) (Genersch et al., 2013) and rinsed in sterile and cold phosphate-buffered saline (PBS), before being pooled (n = 10) and homogenized in PBS using sterile plastic pestles. The homogenate was filtered through sterile gauze to remove any debris, and the remaining suspension was centrifuged at 15,000 x g for 15 min at 4 °C. After discarding the supernatant, aliquots of the remaining pellet were used for either DNA or RNA extraction.

4.2.2. RNA extraction, retro-transcription and relative quantification of immune-related genes

Total RNA extraction from homogenized pools was routinely conducted with TRIzol® Reagent (Life Technologies) according to manufacturer's instructions (Chomczynski & Sacchi, 1987). All RNA samples, after being quantified and purity checked with NanoDrop ND-1000 Spectrophotemeter (Thermo Scientific), were treated with RQ1 RNase-Free DNase (Promega). An aliquot (2 µg) of each sample was used for first-strand cDNA synthesis using SuperScript® II Reverse Transcriptase (Life Technologies), oligo dT (Promega), and RNaseOUT™ Recombinant Ribonuclease Inhibitor (Life Technologies) according to the manufacturers' protocol. *Power* SYBR® Green PCR Master Mix (Life Technologies) was used for quantitative PCR experiments on an Applied Biosystems 7900HT Fast Real-Time PCR System, with the following cycle conditions: denaturation at

95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 60 °C for 1 min.
A list of genes and primer pairs used in qPCR reactions is provided in Table 1. Forward and reverse primers were selected from previous reports or newly designed on gene sequences deposited in GenBank (National Center for Biotechnology Information, NCBI) using Primer3web (version 4.0.0) (Untergasser et al*.,* 2012). Preliminarily, each primer set was tested for PCR efficiency by standard curve and dissociation curve analyses (Pfaffl, 2001).
Additional quantitative reverse transcription PCR (RT-qPCR) analyses were conducted for a relative quantification of the deformed wing virus (DWV).
Each analysis was performed in three technical replicates and included at least three biological replicates.

4.2.3. DNA extraction and relative quantification of the core bacterial community

Homogenized honeybee pools were resuspended in a lysis buffer containing 50 mM Tris-HCl (pH 8), 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100. Then, total DNA extraction was routinely conducted as described in Evans et al. (2013). After DNA quantification with NanoDrop ND-1000 Spectrophotemeter (Thermo Scientific), samples were normalized for qPCR analyses.
For a relative quantification of the core bacterial community in different honeybee stages either from infested or non-infested hives, normalized DNA samples were used in Quantitative Real-Time PCR (qPCR) reactions employing *Power* SYBR® Green PCR Master Mix (Life Technologies) and primer pairs targeting specific regions of 16S rRNA gene of the following representative phylotypes, including honeybee core bacterial species: Pasteurellaceae, Neisseriaceae, *Lactobacillus spp*., *Bifidobacterium spp*. Universal bacterial primers were also used to assess total bacterial numbers (Table 1). For relative quantification of *B. laterosporus* in honeybee, species-specific primer pair *BlQuant* (Bioecopest, Italy) were used (Marche *et al*., 2016).
Relative q-PCR reactions were conducted in an Applied Biosystems 7900HT Fast Real-Time PCR System with the following cycle conditions: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 60 °C for 1 min.

Primers efficiency was preliminarily tested by standard curve and dissociation curve analyses (Pfaffl, 2009). Each sample was run in technical triplicates and at least three biological replicates were involved in analyses.
Additional PCR analyses were conducted on all samples to verify the possible presence of the honeybee pathogens *Paenibacillus larvae* and *Nosema ceranae*, employing primer pairs shown in Table 1.
Additional PCR analyses were conducted on all samples to verify the possible presence of the honeybee pathogens *Paenibacillus larvae* and *Nosema ceranae* employing primer pairs shown in Table 1.

4.3. STATISTICAL ANALYSIS

Statistical analyses were performed with SAS software (version 9.1) with significance level set at $\alpha = 0.05$ (SAS, 2004).
Data on *Varroa* infestation level and colony strength were compared between hive groups using Wilcoxon rank-sum test.
The relative abundance of the target genes was analyzed using the comparative $2^{-\Delta\Delta Ct}$ method, using *5S rRNA* and *β-actin* as endogenous reference genes (Livak & Schmittgen, 2001). Fold changes in immune-related gene expression, DWV and bacterial abundance in honeybees from different hives were subjected to one-way ANOVA, followed by multiple comparison of means (adjust = Bonferroni).

4.4. RESULTS

4.4.1. Varroa infestation and colony strength

Colony strength level, determined using one-sixth of a Dadant-Blatt frame (188 cm^2) as a unit of measure (Marchetti, 1985), and the percentage of Varroa infestation in the infested and non-infested hive groups of the experimental apiary, are shown in Table 2. Comparing the two groups, no significant differences emerged for colony strength, expressed in terms of number of sealed cells ($W = 20$, $P = 0.1508$) and of adult bees ($W = 21$, $P = 0.0952$), while a significantly higher (more than a hundred-fold) *Varroa* infestation level ($W = 0$, $P = 0.0079$) was detected in the infested compared with the non-infested group.

4.4.2. Relative abundance of the Deformed Wing Virus (DWV) and of the overall bacterial community

The relative abundance of the Deformed Wing Virus in different honeybee stages from infested compared with non-infested hives, including emerging adults bearing mites on their body, is shown in Figure 1. DVW was detected in all samples from infested and non-infested hives with a generally increased virus load in samples from infested hives ($F_{4,40} = 14.56$, $P < 0.0001$). The virus load was significantly increased in larvae ($F_{1,16} = 17.44$, $P = 0.0007$), pupae ($F_{1,16} = 5.21$, $P = 0.0456$), emerging adults ($F_{1,16} = 9.32$, $P = 0.0076$), and workers ($F_{1,16} = 25.11$, $P = 0.0002$). A significantly higher load was found in *Varroa*-bearing emerging adults ($F_{1,16} = 14.60$, $P = 0.0015$).

Although DWV was detected in both *Varroa*-infested and non-infested hives, no signs or symptoms associated with the disease it causes were observed.

The variations in the relative abundance of the overall bacterial number in honeybee samples from infested in respect to non-infested hives are shown in Figure 2. While differences were not detected for the diverse insect stages analyzed (larvae: $F_{1,16} = 1.03$, $P = 0.3245$; pupae: $F_{1,16} = 0.37$, $P = 0.5523$; emerging adults: $F_{1,16} = 1.33$, $P = 0.2654$; workers: $F_{1,16} = 2.66$, $P = 0.1222$), a significant increase was found in emerging adults bearing sucking *Varroa* mites $F_{1,16} = 6.74$, $P = 0.0195$.

No symptoms related to the two common honeybee pathogens *Paenibacillus larvae* and *Nosema ceranae* were observed in the colony, and these species were never molecularly detected in samples used in this study.

4.4.3. Relative expression of immune-related genes in honeybees from infested and non-infested colonies

Relative expression of *Hymenoptaecin* (Hym), *Defensin* 2 (Def 2), *Apidaecin* (Apid), *PGRP-S1*, and *Nimrod-C2* genes in different honeybee stages from hives infested by *Varroa* compared with non-infested hives are shown in Figure 3.

A general decrease in the transcript level of these immune-related genes was observed in larvae, pupae and emerging adults from infested hives. However, differences between infested and non-infested hives were not significant for *Hymenoptaecin* ($F_{1,16} = 3.17$, $P = 0.0939$), *Defensin* 2 ($F_{1,16} = 3.76$, $P = 0.0705$), *Apidaecin* ($F_{1,16} = 0.04$, $P = 0.8468$), *PGRP-S1* ($F_{1,16} = 3.02$, $P = 0.1014$), and *Nimrod-C2* ($F_{1,16} = 4.42$, $P = 0.0516$) in larvae. A

significant decrease in the expression of *Apidaecin* ($F_{1,16}$ = 4.60, P = 0.0477) in infested hives was noticed on pupae, while for *Hymenoptaecin* ($F_{1,16}$ = 1.52, P = 0.2359)*, Defensin* 2 ($F_{1,16}$ = 4.15, P = 0.0596), *PGRP-S1* ($F_{1,16}$ = 2.86, P = 0.1103), and *Nimrod-C2* ($F_{1,16}$ = 0.97, P = 0.3389), differences were not significant. In the case of emerging adults, a significant immunosuppression was instead observed for each of the target genes: *Hymenoptaecin* ($F_{1,16}$ = 7.93, P = 0.0146)*, Defensin* 2 ($F_{1,16}$ = 15.36, P = 0.0012), *Apidaecin* ($F_{1,16}$ = 14.52, P = 0.0015), *PGRP-S1* ($F_{1,16}$ = 5.01, P = 0.0398), and *Nimrod-C2* ($F_{1,16}$ = 28.76, P < 0.0001). No significant fold changes for *Hymenoptaecin* ($F_{1,16}$ = 0.83, P = 0.3862)*, Defensin* 2 ($F_{1,16}$ = 2.32, P = 0.1482), *Apidaecin* ($F_{1,16}$ = 0.58, P = 0.4613), *PGRP-S1* ($F_{1,16}$ = 0.83, P = 0.3758), and *Nimrod-C2* ($F_{1,16}$ = 1.13, P = 0.3039) were associated with workers.

4.4.4. Relative abundance of core bacterial community in honeybees from infested and non-infested colonies

Relative abundance of Pasteurellaceae, Neisseriaceae, *Lactobacillus spp*., *Bifidobacterium spp*., and *B. laterosporus* in different honeybee stages from *Varroa*-infested compared with non-infested hives are shown in Figure 4. A high variability, but a significant reduction in the relative abundance of *Lactobacillus spp.* ($F_{1,16}$ = 5.79, P = 0.0428) was observed in larvae from infested hives, while non significant were differences for Neisseriaceae ($F_{1,16}$ = 3.98, P = 0.0634), Pasteurellaceae ($F_{1,16}$ = 1.59, P = 0.2263), *Bifidobacterium spp*. ($F_{1,16}$= 0.23, P = 0.6360), and *B. laterosporus* ($F_{1,16}$ = 0.05, P = 0.8260). In the case of pupae, a significant decrease was detected for most of the target bacterial groups including Pasteurellaceae ($F_{1,16}$ = 6.55, P = 0.0238), Neisseriaceae ($F_{1,16}$ = 8.28, P = 0.0165), and *Bifidobacterium spp*. ($F_{1,16}$ = 6.67, P = 0.0228), whereas differences were not significant for *Lactobacillus spp.* ($F_{1,16}$ = 0.55, P = 0.5008) and *B. laterosporus* ($F_{1,16}$ = 1.78, P = 0.2045). A high variability and non significant differences between infested and non infested hives were observed on emerging adults for Pasteurellaceae ($F_{1,16}$ = 0.06, P = 0.8124), Neisseriaceae ($F_{1,16}$ = 0.30, P = 0.5904), *Lactobacillus spp*. ($F_{1,16}$ = 0.73, P = 0.4304), *Bifidobacterium spp*. ($F_{1,16}$ = 2.63, P = 0.1290), with the sole exception of *B. laterosporus* for which a slight but significant decrease was detected ($F_{1,16}$ = 6.93, P = 0.0181). In workers from infested hives, a significant reduction was found for Pasteurellaceae ($F_{1,16}$ = 10.26, P = 0.0055), whereas differences between infested and non-infested hives were highly variable and non significant for Neisseriaceae

($F_{1,16}$ = 1.63, P = 0.2203), *Lactobacillus spp*. ($F_{1,16}$ = 0.22, P = 0.6471), *Bifidobacterium spp*. ($F_{1,16}$ = 2.47, P = 0.1355), and *B. laterosporus* ($F_{1,16}$ = 1.28, P = 0.2739).

4.4.5. Relative expression of immune-related genes in emerging adults bearing sucking mites.

Relative expression of immune-related gene in emerging adults with *Varroa* mites attached in respect to those without *Varroa* from the same infested hive are shown in Figure 5.
In general, honeybees with *Varroa* mites attached to their body showed a more significant decrease in the relative expression level of these target genes. More in detail, a significant decrease in the transcript level of *Hymenoptaecin* ($F_{1,16}$ = 6.53, P = 0.0212), *Defensin* 2 ($F_{1,16}$ = 26.00, P = 0.0001), *Apidaecin* ($F_{1,16}$ = 98.70, P < 0.0001), *PGRP-S1* ($F_{1,16}$ = 33.43, P < 0.0001), and *Nimrod-C2* ($F_{1,16}$ = 28.30, P < 0.0001), was observed.

4.4.6. Relative abundance of core bacterial community in emerging adults bearing sucking mites

Relative abundance of bacterial phylotypes in emerging adults from infested hives comparing honeybees with and without *Varroa* attached are shown in Figure 6. A significantly increased relative abundance of Pasteurellaceae ($F_{1,16}$ = 10.17, P = 0.0078), Neisseriaceae ($F_{1,16}$ = 7.32, P = 0.0156), *Lactobacillus spp*. ($F_{1,16}$ = 9.99, P = 0.0251), *Bifidobacterium spp*. ($F_{1,16}$ = 10.45, P = 0.0072), and *Brevibacillus laterosporus* ($F_{1,16}$ = 8.90, P = 0.0088) was detected in emerging adults with attached mites compared with adults without mites.

4.5. DISCUSSION AND CONCLUSIONS

The impact of *V. destructor* on the honeybee is not limited to the direct consequences of its sucking activities, but a significant contribution to colony weakening phenomena derives from the pathogenic action of mite-carried viruses, among which DWV plays a major role (Wilfert *et al.,* 2016). As a result of the intimate relationship between this virus and *Varroa*, it is difficult to differentiate between specific pathogenic and parasitic effects on individual bees (Nazzi *et al*., 2012). Furthermore, a higher complexity characterizes the hive ecosystem (Nazzi & Pennachio, 2014). It is generally accepted that the combined *Varroa*-virus action causes a general honeybee immunosuppression (Di Prisco *et al*., 2016) and these effects are

significantly correlated with the virus titer in the host (Kuster, Boncristiani, & Rueppell, 2014).

Accordingly, we observed a general downregulation of immune-related genes in most honeybee samples from *Varroa*-infested compared with non-infested hives. However, a higher variability was observed on larvae and pupae and no significant immunosuppression was detected in workers. A clear immune-impairing effect was instead associated with emerging adults, and such effect was even more significant in adults bearing mites on their body. As expected, a higher DWV load was detected in samples from infested compared with non infested hives, and a significantly higher virus titer was found in *Varroa*-bearing emerging adults, which confirms that *Varroa* and virus actions are tightly correlated (Di Prisco *et al*., 2016).

The quantitative variation in the overall bacterial community in different honeybee stages from infested in respect to non-infested hives, was not significant, while a significant reduction in the abundance of specific bacterial phylotypes was observed in different stages. It is known that the bacterial balance in the honeybee body is an indicator of the colony health conditions (Kwong & Moran, 2016). Because no significant changes were detected for the total bacterial number, we could speculate that the reduction in specific bacterial groups are balanced by the increase in other species groups. Accordingly, the significant decrease in *Lactobacillus* spp. we observed in larvae was associated with a considerable average increase in the other target phylotypes including Pasteurellaceae, Neisseriaceae, and *Bifidobacterium* spp. On the contrary, the same species groups showed a substantial reduction in pupae, for which an increase in the *Lactobacillus* spp. load was recorded. Taken together, these results suggests that observed *Lactobacillus* spp. fold changes might be the result of an altered colony health status (Budge *et al.,* 2016). By contrast, a relevant though not significant increase in this genus members was detected in emerging adults and in workers. Lactobacilli and bifidobacteria include lactic acid bacterial (LAB) symbionts of *A. mellifera*, that play an important role in honeybee nutrition and antimicrobial response against pathogens (Vasquez *et al.,* 2012)*, in analogy with their ability to modulate* the host immune response in humans and animals (Servin, 2004).

The relative changes in the abundance of *B. laterosporus* generally followed the same trend as the main bacterial community, which aligns this honeybee body resident to the core

bacterial community, and supports its possible involvement in the response against pathogens (Hamdi & Daffonchio, 2011).

A significant decrease in Pasteurellaceae was found in workers. This bacterial family includes member species that are involved in the carbohydrate metabolism (Anderson *et al*., 2011). In addition, these species harbour genes encoding pectin-degrading enzymes involved in the breakdown of pollen walls (Engel, Martinson, & Moran, 2012). Impairment of such metabolic functions may consequently result from a reduction in bacterial populations engaged to carry out these tasks in workers. Analogously, slight quantitative variations were associated with members of the family Neisseriaceae, that includes *Snodgrassella* alvi, a species involved in the formation of biofilm-like layers on the ileum epithelium (Engel *et al.,* 2012). This betaproteobacterium is a dominant member of the honey bee and bumble bee gut microbiota and appears to be involved in both nutrition and stress response (Kwong, Engel, Koch, & Moran, 2014).

More in general, significant fold changes in the bacterial community composition and in the proportion of represented species in diseased compared with healthy colonies is expected (Hamdi *et al.,* 2011). Hence, the dysbiosis we observed may directly derive from the combined DWV-*Varroa* action. The inconsistency of fold changes observed comparing different honeybee stages is likely to be directly related to the bacterial community diversity characterizing each development stage (Martinson *et al.,* 2012). Accordingly, pupae are expected to lose most of the gut bacterial community acquired during the larval stage. However, our study did not keep into account a direct comparison between insect stages, but the relative changes in *Varroa*-infested in respect to non-infested colonies.

Other differences may derive from the behavior of *Varroa* mites that feed on the host only during a specific period of its developmental life cycle (Rosenkranz *et al.,* 2010). Besides, *Varroa* microbiome may directly influence the resident microbial community composition in a parasitized honeybee (Sandionigi *et al.,* 2015).

The interactions of honeybees between each other and with the hive or the outside environment are other factors affecting the individual and colony microbiota (Powell, Martinson, Urban-Mead, & Moran, 2014). Everything considered, it is difficult to give a conclusive interpretation of the microbiome changes that can be detected in colonies infested by *Varroa*. In the attempt to overcome such complexity, we conducted a direct comparison between emerging adults bearing or lacking *Varroa* mites on their body, collected from the

same infested hives, thus reducing the number of variables affecting the microbiome composition. Adults emerging with a *Varroa* mite attached to their body were characterized by a considerably higher DWV titer and a more important immunosuppression compared with adults without mites from the same hive. In parasitized adults, the abundance of the overall bacterial community and of each beneficial phylotype targeted in this study, increased significantly. A first and intuitive explanation for this very clear trend may relate to the fact that individuals with a lowered immune capacity would reduce their barriers against a variety of bacterial species whose proliferation is consequently favored. However, everything might be under the control of a more sophisticated mechanism. It has been reported that the composition and proportion of resident bacterial species living in the honeybee gut are the result of a co-evolution process (Kwong & Moran, 2015) and that the susceptibility to parasites is affected by microbiota composition (Schwarz, Moran, & Evans, 2016). This implies that they have evolved toward a relationship of mutual convenience, ensured by the establishment of an equilibrium under the control of the insect immune system (Anderson *et al.,* 2011). Other bacterial species have instead developed strategies to overcome insect immune defenses, thus evolving toward a pathogenic interaction (Hornef, Wick, Rhen, & Normark, 2002). It can be inferred that when individual honeybees are immune-impaired, the beneficial inhabitants of their gut go through a natural increase. Although some of these species may show opportunistic behavior, an increased load of beneficial bacteria in diseased honeybees would lead to increase their overall proportion in the colony ecosystem. Such dynamics may constitute a social mechanism of compensation, in response to the combined parasitic and pathogenic action of DWV and *Varroa*. This may also explain why the highly significant immunosuppression effects and the bacterial quantitative variations we observed on individual honeybees (i.e., emerging adults) parasitized by *Varroa* were attenuated at the colony level. Such hypothesis is supported by an evolutionary selection process that led to establish a core bacterial community where the prevailing species are few and beneficial (Kwong & Moran, 2016). Everything considered, the quantitative variations in representative phylotypes of the honeybee core bacterial community support their direct or indirect contribution to the microbial balance of asymptomatic honeybees from colonies infested by DWV-carrying *Varroa* mites. Given the functional diversity within the honeybee microbiome (Engel *et al.,* 2012), further studies are needed to clarify the actual role of each bacterial species.

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4.7. TABLES AND FIGURES

Table 1. Oligonucleotide sequences used for analyses.

Species	Gene[a]	Primer sequence		Reference[b]
		Sense 5'- 3'	Antisense 5'- 3'	
Apis mellifera	*Hymenoptaecin*	5' CTCTTCTGTGCCGTTGCATA3'	5'GCGTCTCCTGTCATTCCATT3'	Evans, 2006
Apis mellifera	*Defensin-2*	5'GGGTAACGTGCGACGTTTTA3'	5'GCAACTACCGCCTTTACGTC3'	NM_001011638.1
Apis mellifera	*Apidaecin*	5'TTTTGCCTTAGCAATTCTTGTTG5'	5'GGATGAGGTGGTCTTGGTT3'	Simone et al., 2013
Apis mellifera	*PGRP-S1*	5'GAGGCTGGTACGACATTGGT3'	5'TTATAACCAGGTGCGTGTGC3'	XM_001121036.4
Apis mellifera	*Nimrod C2*	5'GCGTGGAGGACGGGAAACCG3'	5'ACATCGATGGCAGAGCGGCG3'	Nazzi et al., 2012
Apis mellifera	*5S rRNA*	5' TTAGGAGACAGGCGGTGGA3'	5' TAGACGAACCTTTGGCAGCA3'	XM_006570236
Apis mellifera	*B-actin*	5'AGGAATGGAAGCTTGCGGTA3'	5'AATTTTCATGGTGGATGGTGC3'	Ryabov et al., 2014
Eubacteria	16S rRNA	5'AGAGTTTGATCCTGGCTCAG3'	5'ACGGCTACCTTGTTACGACTT3'	Weisburg et al., 1990
Pasteurellaceae	16S rRNA	5'TTGTTGCCAGCGATTAGG3'	5'ATTCTGATTCACGATTACTAGC3'	Li et al., 2012
Neisseriaceae	16S rRNA	5'AAGCGGTGGATGATGTGG3'	5'TGATGGCAACTAATGACAAGG3'	Li et al., 2012
Lactobacillus spp.	16S rRNA	5'TAACGCATTAAGCACTCC3'	5'GCTGGCAACTAATAATAAGG3'	Li et al., 2012
Bifidobacterium spp.	16S rRNA	5'CAAGCGAGAGTGAGTGTACC3'	5'GCCGATCCACCGTTAAGC3'	Li et al., 2012
Nosema ceranae	*large subunit rRNA*	5'CGGATAAAAGAGTCCGTTACC3'	5'TGAGCAGGGTTCTAGGGAT3'	Nazzi et al., 2012
Deformed Wing Virus	*Polyprotein helicase domain*	5'GCGCTTAGTGGAGGAAATGAA3'	5'GCACCTACGCGATGTAAATCTG3'	Nazzi et al., 2012
Paenibacillus larvae	*gyrA*	5'ATGCGGTCATCCCTATTGAG3'	5'GGTCATCTTCCCGCAAATTA3'	De Graaf et al., 2013

[a]*PGRP-S1* = Peptidoglycan recognition protein S1
[b]In the case of newly designed primers, GenBank accession numbers are shown.

Table 2. Mean (± S.E.) of colony strength and *Varroa destructor* mite infestation percentage in hive groups from the experimental apiary at the sampling time.

Hive group	Colony strength[b]		*Varroa* infestation %
	No. Adults	No. Sealed cells	
Infested	3794.76 ± 901.7a[c]	3728.40 ± 663.0a	9.53 ± 1.45a
Non-infested[a]	6705.60 ± 1135.3a	7363.20 ± 1567.8a	0.06 ± 0.05b

[a] Subjected to antivarroa treatments (suspended 1 month before sampling)

[b] Determined employing one-sixth of a Dadant-Blatt frame (188 cm^2) as a unit of measure (Marchetti, 1985).

[c] Different letters within the same column indicate significantly different means (ANOVA, $p < 0.05$)

Figure 1. Relative abundance (mean ± SE) of the Deformed Wing Virus (DWV) in different honey bees stages sampled from hives infested by *Varroa destructor*. Fold changes were calculated relative to the abundance in honey bees from non infested hives**.** For each mean, asterisk (*) indicates a significant difference with the non-infested group, while different letters indicate significant differences between stages (ANOVA, Bonferroni adjusted P value; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Em. Adults: Emerging Adults; Em. Adults+V: Emerging adults with attached *Varroa* mites.

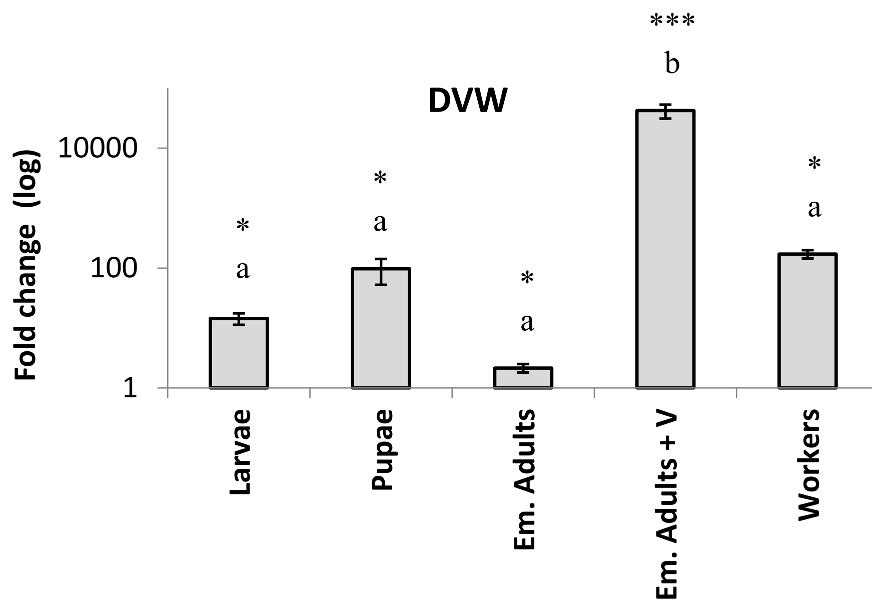


Figure 2. Relative abundance (mean ± SE) **of** the overall eubacterial community in different honey bees stages sampled from hives infested by *Varroa destructor*. Fold changes were calculated relative to the abundance in honey bees from non-infested hives**.** For each mean, asterisk (*) indicates a significant difference with the non-infested group, while different letters indicate significant differences between stages (ANOVA, Bonferroni adjusted P value; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Em. Adults: Emerging Adults; Em. Adults+V: Emerging adults with attached Varroa mites.

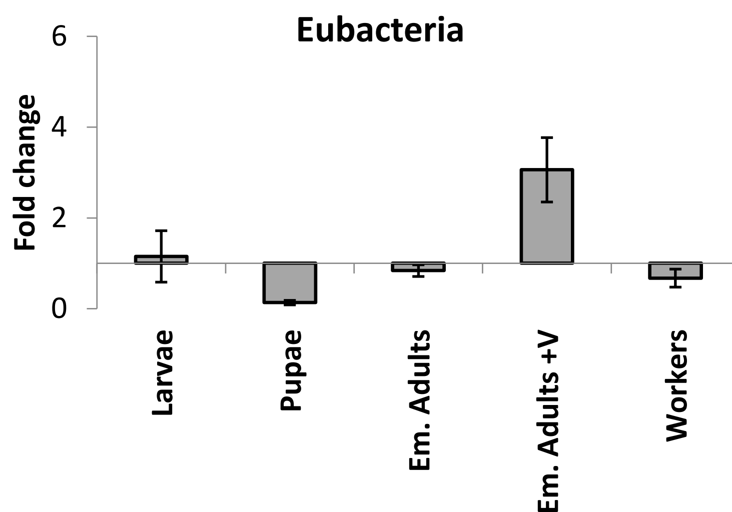


Figure 3. Relative expression fold change (mean ± SE) for different immune-related genes of honey bees stages (A-D) from the *Varroa*-infested hive group. Fold changes were calculated relative to the expression level in honey bees from non infested hives**.** For each mean, asterisk (*) indicates a significant difference with the non-infested group (ANOVA, Bonferroni adjusted P value; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

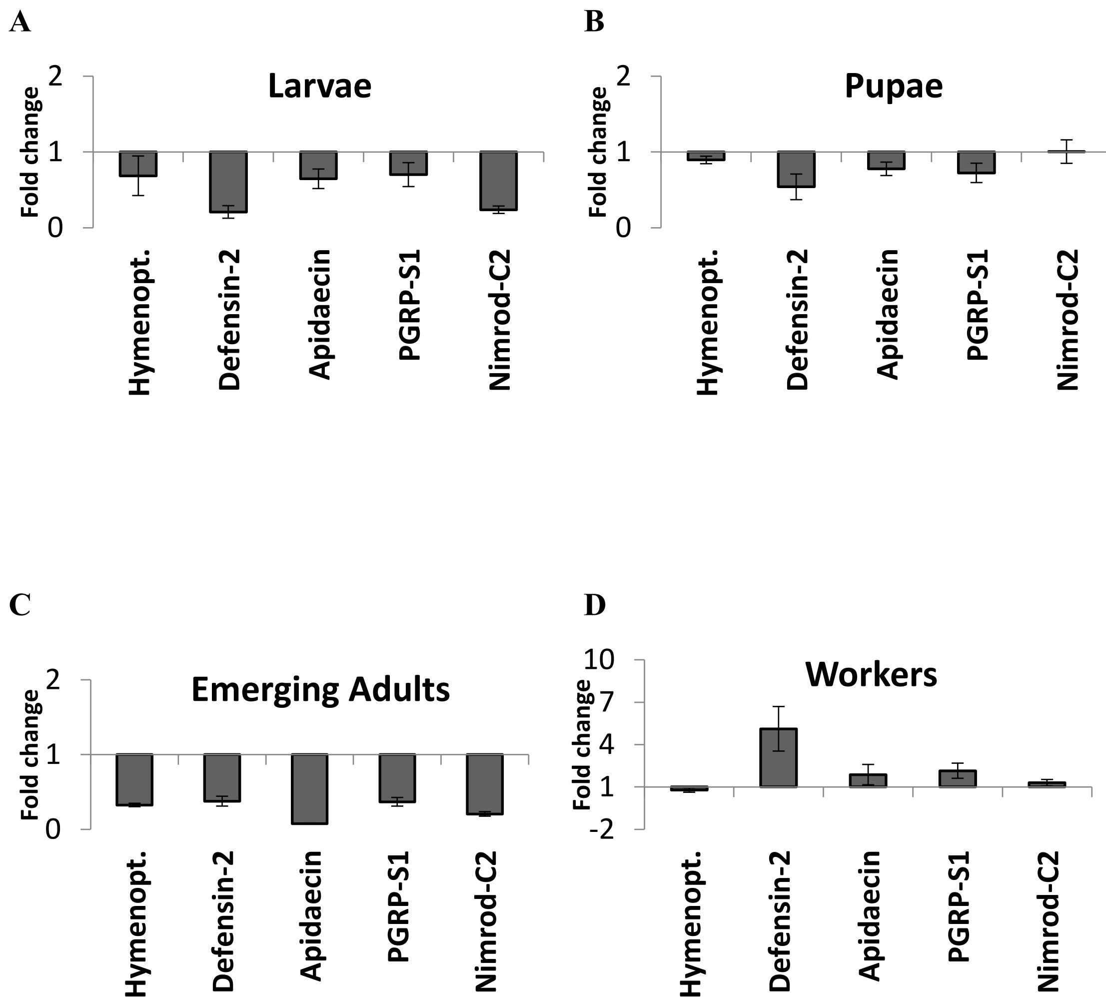


Figure 4. Relative abundance (mean ± SE) of selected group species representing the core bacterial community of different honey bee stages (A-D) from the *Varroa*-infested hive group. Fold changes were calculated relative to the relative abundance in honey bees from non infested hives**.** For each mean**,** asterisk (*) indicates a significant difference with the non-infested group (ANOVA, Bonferroni adjusted P value; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

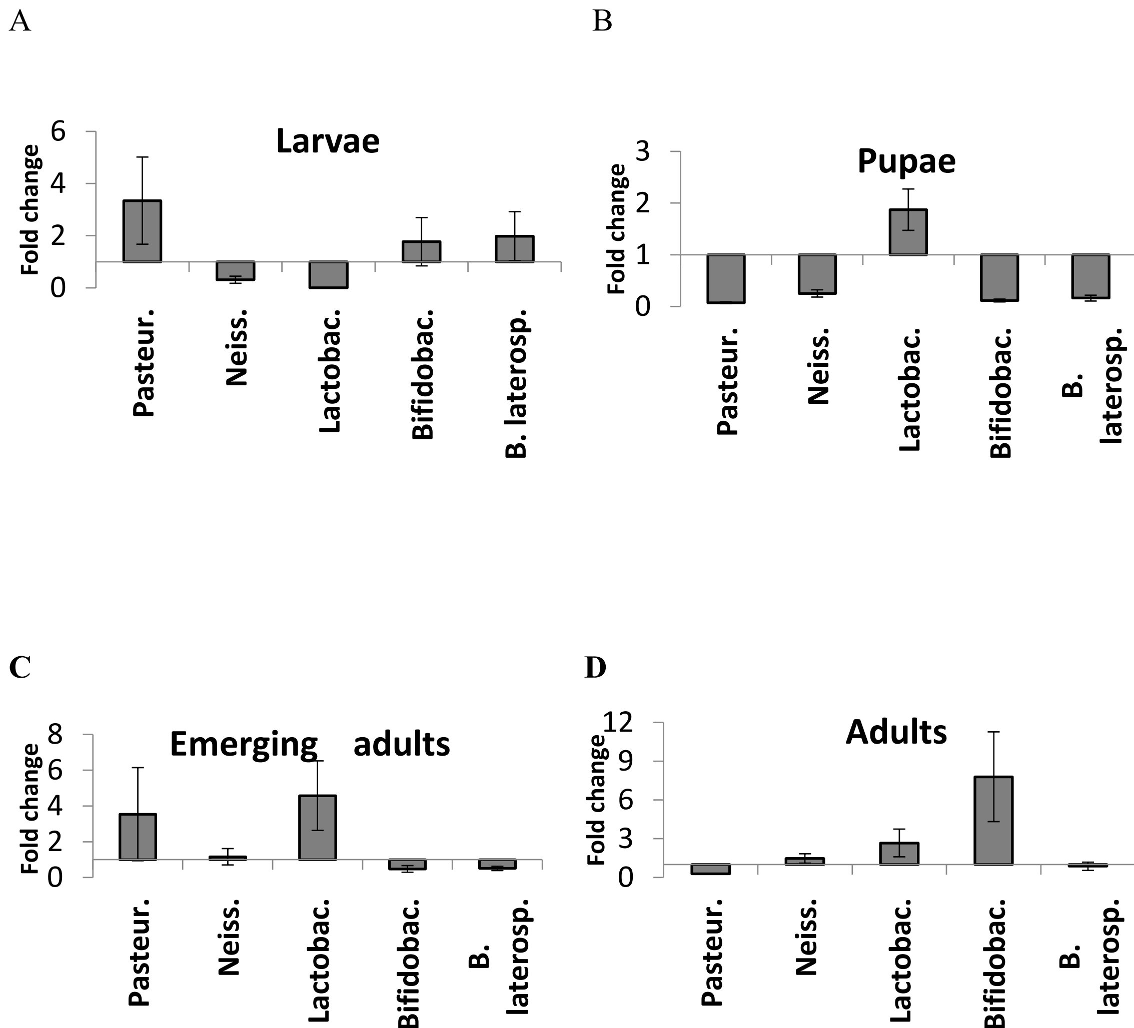


Figure 5. Comparison of relative expression fold change (mean ± SE) for different immune-related genes of honey bee emerging adults from the *Varroa*-infested hive group, bearing (*Varroa* attached) or lacking (No *Varroa*) mites on their body. Fold changes were calculated relative to the expression level in honey bees from non-infested hives**.** Asterisks (*) indicate significant differences between samples with and without attached mites (ANOVA, Bonferroni adjusted P value; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

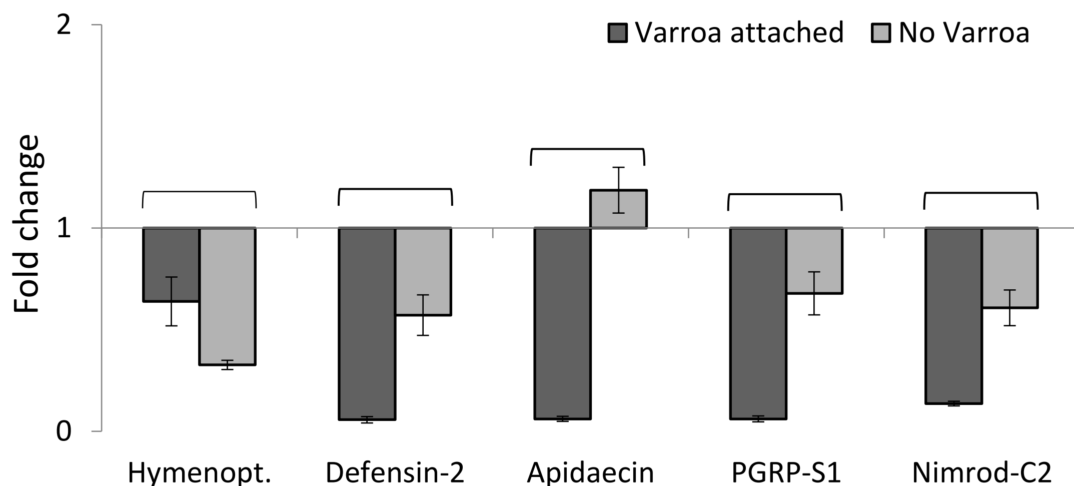
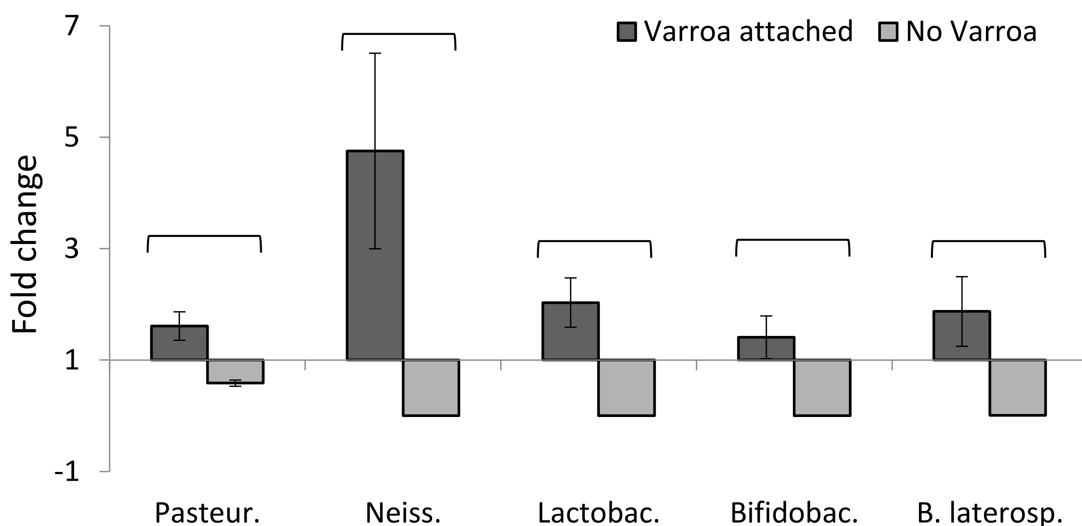


Figure 6. Comparison of relative abundance (mean ± SE) of selected group species representing the core bacterial community of honey bee emerging adults from the *Varroa*-infested hive group, bearing (*Varroa* attached) or lacking (No *Varroa*) mites on their body. Fold changes were calculated relative to the relative abundance in honey bees from non-infested hives. Asterisks (*) indicate significant differences between samples with and without attached mites (ANOVA, Bonferroni adjusted P value; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).



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