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Studies on the behavioral responses of *Apis mellifera* to the pathogen *Nosema ceranae* and the parasite *Varroa destructor*, and on the predatory wasp *Vespula germanica* in apiary

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Index

Abstract

Declining honey bee health has been a major concern worldwide and the responses of bees to biotic factors has not been fully understood yet. This thesis is divided into three parts: the first two chapters concern the use of propolis, as an extract or as a raw propolis, in adult bees experimentally treated with the microsporidium *Nosema ceranae* (first chapter) and the parasitic mite *Varroa destructor* (second chapter); and the third chapter relates to the impact of the predatory wasp *Vespula germanica* on bee hives. The aim of this three-year study was to investigate the behavioral responses of *Apis mellifera* to the parasite and the pathogen in laboratory, and observe the behavior of the wasp in an experimental apiary. Our results showed that the lifespan of bees artificially infected with *N. ceranae* or parasitized by *V. destructor* increased when bees were treated with propolis compared to untreated bees. In particular, laboratory experiments evidenced that raw propolis had a narcoleptic effect on *V. destructor* mites, by contact, and propolis extract caused a decrease in spore load of *N. ceranae*. In the study on the feeding strategies of *V. germanica* in apiary, our results showed the major role of the wasp as a scavenger, because we found that its diet is based mostly on bee carrions. In order to improve the protection measures of honey bees, it would be important to conduct further studies focusing on impact and control of biotic stress factors of this species.

Key words: *Apis mellifera*; *Varroa destructor*; *Nosema ceranae*; Propolis; Mite; Fungi.

General Introduction

In the social organization of honey bees, the effective cooperation between individuals of the same colony potentially reduces the risk and incidence of diseases in the hive. Many studies showed that this behavior was associated with immune responses or defensive strategies involving the whole society (Traniello et al. 2002). Social insects are more exposed to pathogens and, consequently, to the risk of epidemics (Brockmann 1984), mainly due to the high population density. Sociality, the continuous interactions between nestmates and the low genetic variability within the same colony make individuals more susceptible to infections (Schmid-Hempel 1998). In honey bees society, social and individual immunity can be distinguished.

Social immunity means a close collaboration and cooperation between individuals of the same colony, with the aim of reducing the risks of transmission and propagation of pathogens and parasites (Cremer et al. 2007, 2018). The bees developed a model based on collective defenses that benefit the colony (Schmid-Hempel 1998). Coordinated preventive and curative defense includes a continuous exchange of information within the colony through, for example, trophallaxis, which creates a dense network of interactions within the group. It also consists of the exchange of not only food but also chemical signals, e. g. pheromones, microRNAs and hormones (LeBoeuf et al. 2016).

Individual immunity of insects has different mechanisms available to oppose the development of pathogens and parasites. They are protected externally by antimicrobial secretions and internally by a specific membrane adverse to the development of pathogens (Evans et al. 2006). In most cases, when pathogens overcome these barriers, the peritrophic membrane is sufficient to block their passage. In addition, there are other defenses called humoral immune systems, such as antimicrobial peptides secretion, phagocytosis, melanization and pathogen enzymatic degradation (Hoffmann 2003; Hultmark 2003).

All members of the group are involved in the identification and defensive responses against diseases caused by pathogens or parasites, because of the interactions among all individuals (Cremer and Sixt 2009). The individual physical and physiological defenses are integrated by the collective ones (Wilson-Rich et al. 2009). In presence of parasites or pathogens (e.g., mites, fungi, bacteria and other arthropods), the colony normally responds by adding the collective defenses to the individual ones. Some aspects of the relationships between *Apis mellifera* Linnaeus 1758 and pathogens are already known (Evans and Spivak 2010). Recently, a study conducted on the collective defenses of bee colonies evidenced the importance of propolis collection against infection by *Varroa destructor* Anderson & Trueman 2000 (Pusceddu et al. 2019).

The health of the honey bee has been a global emergency in recent decades. Although many scientific discussions and publications have focused on honey bee health for decades, many issues have not been solved yet (Neumann and Carreck 2010). For example, the microsporidium *Nosema ceranae* (Fries et al. 1996) (Microsporidia: Nosematidae) and the mite *V. destructor* (Arachnida: Parasitidae) still are among the main causes of decline in bee colonies in several countries, and the impact of the predatory wasp *Vespula germanica* (Fabricius 1793) (Hymenoptera: Vespoidea) on bees has not been fully understood yet.

This three-year study is divided into three specific parts concerning the response of *Apis mellifera* to a pathogen, the microsporidium *N. ceranae* (Fries et al. 1996) (Microsporidia: Nosematidae), and to a parasite, the mite *V. destructor* (Arachnida: Parasitidae), in laboratory, and the impact of the predatory wasp *V. germanica* (Hymenoptera: Vespoidea) in apiary.

Nosemosis is a very important disease caused by the microsporidium *N. ceranae*, which develops in the midgut of adult bees (Higes et al. 2005, 2006, 2019). In the ventriculus of the bees, the spores of *N. ceranae* extrude a polar filament through which the sporoplasm is transferred into the epithelial cells of the host. Once the parasite develops and multiplies within the host-cell cytoplasm, the spores move into the gut lumen, where they may be excreted or infect other epithelial cells. The intracellular proliferation is completed when the epithelial cell is totally destroyed (Higes et. al 2007). The transmission between individuals occurs mainly through spores evacuated in the feces (Fries 1988), floral contamination (Purkiss and Lach 2019), spore dispersion into the air (Sulborska et al. 2019) and mechanical vectors (Valera et al. 2017). *N. ceranae* is transmitted horizontally (Fries 2010; Graystock et al. 2015), but sexual transmission can also occur (Roberts et al. 2015). Several studies have shown that the infection intensity can vary among infected bees within the same colony. Indeed, it can happen that some individuals do not show any symptoms, even in the presence of spores, while others show signs and have a variable concentration of spores (Mulholland et al. 2012; Smart and Sheppard 2012). For this reason, the analysis of the health status of a colony can be easily distorted (Traver and Fell 2011; Botías et al. 2012). In order to develop new treatment strategies against nosemosis, it would be important to understand the exact dynamics and the effects of infection at individual and colony level.

The mite *V. destructor* is an obligate parasite that develops inside the nest, feeding on the adults and preimaginal stages of bees (Rosenkranz et al. 2010; Nazzi and Le Conte 2016; Ramsey et al. 2019). It is widespread in all continents but is not found in Australia (Rosenkranz et al. 2010). Damage caused by *V. destructor* can be direct, such as behavior disorder during flight and learning disorder (Kralj and Fuchs 2006; Kralj et al. 2007), or indirect, i.e. closely related to the transmission of the viruses (Chen and Siede 2007). A typical behavior in *A. mellifera* colony inside the

hive is the use of propolis as an antimicrobial protective substance against pathogens. Normally, the bees use propolis not only as a preventive defense, but also as an additional defense tool that is activated when parasites or pathogens penetrate inside the hive (Simone-Finstrom and Spivak 2012; Pusceddu et al. 2017). A recent study showed that colonies of *A. mellifera* increased resin foraging when infestation by *V. destructor* increased (Pusceddu et al. 2017).

The predatory wasp *V. germanica* is widespread throughout the world (Archer 1998). Probably, because this wasp can readily adapt to different food sources in different environments, it is able to adopt various feeding strategies (Lozada and D'Adamo al. 2006). Its behavior is also influenced by abiotic factors, such as rain, low temperatures and brightness (Kasper et al. 2008). In Sardinia, it is present looking foor food near the hive from September until the end of October (Pusceddu et al. 2017), but its behavior and its impact in this context is not clear.

With the objective of understanding if the bees infected with either *V. destructor* or *N. cereanae* benefit from propolis in terms of lifespan, laboratory bioassays were performed. Our findings clearly demonstrated the lifespan of infected bees was positively influenced by propolis (used as raw propolis or extract) compared to those not treated with propolis. In addition, the raw propolis used in the *Varroa*-bioassay had a narcoleptic effect on the mite, and propolis extract used in the *N. cereanae*-bioassay reduces spore loads. To evaluate the specific role of *V. germanica* in bee hives, a trial was set up in the experimental apiary located in the farm of the Dipartimento di Agraria of the University of Sassari, located in Ottava (an hamlet in the outskirt of Sassari city, Northern Sardinia). Direct observations carried out at soil level allowed us to evidence the predation and necrophagy behavior of this species near the beehives. The wasp played mainly the role of "scavenger", preferring dead or very weak adult bees. When food was fresh, an increase in intraspecific competition was observed.

It would be very important to conduct further studies on these topics, especially on the behavioral responses of *Apis mellifera* infected with pathogens or parasites, with the main goal of achieving an improvement in honey bee health.

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CHAPTER 1

Title:

"Propolis consumption reduces *Nosema ceranae* infection of European honey bees (*Apis mellifera*)"

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Ready for submission

Propolis consumption reduces *Nosema ceranae* infection of European honey bees (*Apis mellifera*)

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Short title: Propolis and *Nosema ceranae*-infected bees

Abstract

Nosema ceranae is a widespread obligate intracellular parasite of the ventriculus of many honey bees (*Apis* spp.) species, including the Western honey bee *Apis mellifera*, in which it may lead to colony death. It can be controlled in *A. mellifera* by feeding the antibiotic fumagillin to a colony, though this product is toxic to humans and its use has now been banned in many counties. In beekeeping, there exists a need for alternative and safe products effective against *N. ceranae*. Honeybees produce propolis from resinous substances collected from plants and use it to protect their nest from parasites and pathogens; propolis is thought to decrease the microbial load of the hive. We hypothesized that propolis might also reduce *N. ceranae* infection of individual bees and that they might consume propolis as a form of self-medication. To test this hypothesis, we evaluated the effects of an ethanolic extract of propolis administered orally on the longevity and spore load of experimentally *N. ceranae*-infected worker bees and also tested whether infected bees were more attracted to, and consume a greater proportion of, a diet containing propolis in comparison to uninfected bees. Propolis extracts and ethanol (solvent control) increased the lifespan of *N. ceranae*-infected bees, but only propolis extract significantly reduced spore load. Our propolis extract primarily contained derivatives of caffeic acid, ferulic acid, ellagic acid and quercetin. Choice, scan sampling and food consumption tests did not reveal any preference of *N. ceranae*-infected bees for commercial candy containing propolis. Our research supports the hypothesis that propolis represents an effective and safe product to control *N. ceranae* but worker bees seem not to use it to self-medicate when infected with this pathogen.

Key words: Ethanol extract; Honey bee health; Infection; Microsporidia; Nosemosis, Self-medication.

Introduction

The microsporidian *Nosema ceranae*, first isolated in *Apis cerana* (Fries et al. 1996), is an obligate gut parasite of several *Apis* (honey bee) species (Goblirsch 2018). It was first identified as an infective agent of *Apis mellifera* in Spain (Higes et al. 2006), but the analysis of preserved specimens of *A. mellifera* suggest its presence in Europe as early as 1993 (Ferroglio et al. 2013). It nowadays has a worldwide distribution (Klee et al. 2007; Chen et al. 2008). In Italy, this parasite seems to have completely replaced the congeneric parasite *N. apis*, which had historically been the only *Nosema* species present (Porrini et al. 2011), possibly through its competitive superiority in warmer climates (Natsopoulou et al. 2015). *Nosema ceranae* infection can cause many physiological and behavioural changes at the level of the individual honey bee (Kralj and Fuchs 2010; Goblirsch et al. 2013; Mayack et al. 2015; Li et al. 2013, 2018). It can also cause pathology at the colony level (Botías et al. 2013) and it has been associated with colony collapse (Higes et al. 2008, 2009; Bromenshenk 2010). For beekeeping, effective control treatments against *Nosema* infections are needed. In the last decades, fumagillin has been used to treat colonies infected by *N. apis* (van den Heever et al. 2014). However, several studies have suggested that this antibiotic may be ineffective against *N. ceranae* (Huang et al. 2013; Giacobino et al. 2016; Mendoza et al. 2017). In addition, the toxicity of fumagillin to humans represents another restriction in its application in beekeeping (van den Heever et al. 2014). These concerns led the European Union to ban the use of fumagillin in agriculture in 2010 (MRL, Commission regulation, EU, 2010, n.37/2010). This heightens demand for new and safe products that are effective against *N. ceranae*. Though many substances have been assayed in laboratory or field conditions for their efficacy in reducing *N. ceranae* infections, with encouraging results (Maistrello et al. 2008; Porrini et al., 2016), further studies are needed to develop alternatives to combat *N. ceranae* infections (Burnham 2019). Among the various promising substances to control *N. ceranae* infection, orally administered organic extracts and natural supplements deserve particular attention due to their putative low toxicity and beneficial effects in increasing bee longevity and in decreasing *Nosema* spore loads (Maistrello et al. 2008; Costa et al. 2010; Damiani et al. 2014; Porrini et al. 2016, 2017; Bravo et al. 2017). Propolis is a resinous mixture of substances with antimicrobial properties that is collected from plants by several Hymenoptera species and used by them to protect their nests from parasites and pathogens (Seeley and Morse 1976; Castella et al. 2008; Leonhardt and Bluthgen 2009; Simone-Finstrom and Spivak 2010). In feral honey bee colonies, a thin layer of propolis covers the entire nest's internal walls, whereas in commercial hives a more irregular distribution of propolis is observed. This is possibly because the smoothness of the inner walls of commercial hives does not elicit propolis deposition behaviour. In both feral and managed colonies, propolis is also used for covering holes and crevices in the nest and to limit access to the hive (Seeley and Morse 1976;

Ghisalberti 1979; Simone-Finstrom and Spivak 2010). In commercial hives enriched with propolis, their microbial load was reduced, resulting in a significant down-regulation of immune function (Simone et al. 2009). This decreased energetic investment in the bee immune system can positively influence colony health and productivity (Cotter et al. 2004; Nicodemo et al. 2013; Borba et al. 2015; Pusceddu et al. 2018). Moreover, propolis can have a direct effect against some hive pathogens, such as *Paenibacillus larvae*, the causative agent of the American foulbrood (Antúnez et al. 2008; Borba and Spivak 2017), and *Ascosphaera apis*, the causative agent of chalkbrood infection (Simone-Finstron and Spivak 2012). Interestingly, an increase in resin collection was observed in honey bees after infections with the fungus *A. apis* (Simone-Finstrom and Spivak 2012) and after an increase in colony infestation by *Varroa destructor* (Pusceddu et al. 2019), suggesting a therapeutic use of propolis in the hive that operates at the colony level. However, such a response does not appear to be associated with infection by the American foulbrood agent *P. larvae* (Simone-Finstrom and Spivak 2012). Although it is not known whether honey bees consume propolis, Turcatto et al. (2018) demonstrated that adding propolis to the diet of bees injected with *Escherichia coli* caused a significant up-regulation of antimicrobial gene expression (defensin-1, abaecin, hymenoptaecin, and apidaecin) compared to that observed in infected bees fed a similar diet without propolis. This increase did not occur in uninfected bees fed with propolis, suggesting that propolis may enhance a bee's response when challenged by pathogens. Yet bees fed with propolis in sucrose syrup showed elevated expression of three CYP6AS cytochrome P450 genes involved in pesticide detoxification (Johnson et al. 2012), suggesting that propolis may have a mildly toxic effect on individual bees. We therefore hypothesise that bees should consume propolis only when they are stressed, such as when challenged by a pathogen. Recently, the toxic properties of propolis extracts against *N. ceranae* were assayed in the Asiatic honey bee species *Apis florea* (Suwannapong et al. 2011, 2018), *Apis cerana* (Yemor et al. 2016) and the European subspecies of *A. mellifera* (Arismendi et al. 2018). In the Asiatic *Apis* species, propolis used in the bioassays was obtained from the stingless bee *Trigona apicalis*, whereas in *A. mellifera* the propolis tested was produced by honey bees themselves. In all these studies, an improvement in bee survival and a decrease in the *N. ceranae* spore load were observed in experimentally infected bees (Suwannapong et al. 2011, 2018; Yemor et al. 2016; Arismendi et al. 2018). For the Asiatic *Apis* species, these results leave open the question of whether the honey bee's own propolis has a similar beneficial effect in reducing *Nosema* pathogenesis. For *A. mellifera*, an open question remains as to whether propolis *per se* or the solvent used to dissolve and administer the propolis, or both, caused an improvement in bee health. To resolve these open questions, we here tested the effects of propolis produced by honey bees (*A. mellifera*) in the Mediterranean on the longevity of *A. mellifera* experimentally infected with *N. ceranae* and on their microsporidian

spore load. Secondly we ascertained whether infected bees were more attracted to a diet containing propolis and consumed a greater proportion of this diet in comparison to uninfected bees. Considering that the biological properties of propolis have mainly been associated with its phenolic components (da Silva et al. 2006; Mihai et al. 2012; Siripatrawan et al. 2013; Pusceddu et al. 2018), we also quantified the polyphenol and flavonoid content, together with the main phenolic compounds, of the propolis used in our bioassays.

Materials and methods

Honey bees and propolis sources

Honey bees and propolis used in this study were collected from the hives of the experimental apiary of the Dipartimento di Agraria of the University of Sassari, located in Ottava, an hamlet in the outskirt of Sassari city, Sardinia, Italy (latitude 40°46'23" N, longitude 8°29'34" E). The apiary consisted of 15 colonies of *Apis mellifera ligustica* Spinola maintained in Dadant-Blatt hives each containing 10 combs. During the experimental period (from May to November 2018), colonies were checked regularly to verify the presence of the queen and the supply of pollen and nectar, and to check the health of the bees and that they were devoid of *Nosema*. Propolis samples used in the bioassays were collected from within twelve hives from May to July 2018 by fine mesh nylon traps placed above the combs. After scraping propolis from the mesh, each sample was cleaned of impurities, weighed and stored in a freezer at − 18 °C. Before starting bioassays, all frozen samples of propolis were milled using a coffee mill (GS Arendo, Germany) and mixed thoroughly.

Propolis extract preparation

To obtain the propolis extract, 2.4 g of crude propolis were dissolved in 4 mL of 70% ethanol, and the solution obtained was maintained in a chamber at 31 °C in the dark for 24 h (Suwannapong et al. 2011). Then, the propolis extract was filtered by suction to clean it from wax and other impurities (Damiani et. al. 2010). Based on previous studies (Suwannapong et al. 2011; Yemor et al. 2016), a concentration of 50% of this extract in distilled water (v/v) was prepared for the experiments.

Pathogen preparation

Nosema ceranae spores used in bioassays were first propagated in the laboratory through mass feeding of caged honey bees with *N. ceranae* spores originating from infected bees provided by the Institute Crea – Consiglio per la Ricerca in

Agricoltura e l'Analisi dell'Economia agraria – Agricoltura e Ambiente (Bologna, Italy). Inocula were prepared freshly on the day of experimental bee infection by crushing the ventriculus of infected honey bees in distilled water and purifying using Percoll ® following standard procedures (Fries et al. 2013). Spore numbers were counted with a Neubauer haemocytometer under a light microscope (×400) and diluted to obtain the required concentration (10^5 per µL) in 50% (w/v) sucrose solution. For the control treatment, an extract from the ventriculus of uninfected caged honey bees was obtained as above. Before starting the propagation, *N. ceranae* identification was confirmed by PCR (Fries et al. 2013) and the absence of spores in the control bees was checked under a light microscope (×400).

Survival bioassay set up

To obtain adult workers and perform the laboratory bioassays, frames of honey bee brood ready to emerge were collected from three *N. ceranae*-free colonies and kept for 14 h in an incubator at 35 °C and 70% relative humidity (RH). Each freshly emerged bee was individually fed with 2 µl of one of the following six treatments obtained by mixing at a 1:1 ratio a 50% (w/v) sucrose solution with: 1) aqueous homogenate of healthy bee gut homogenate (Control); 2) 35% ethanol solvent (Control + Ethanol); 3) 50% propolis extract in ethanol (Control + Propolis); 4) gut homogenate with 10^5 *N. ceranae* spores per bee (Nosema), 5) 35% ethanol solution with 10^5 *N. ceranae* spores per bee (Nosema + Ethanol), and 6) 50% propolis extract with 10^5 *N. ceranae* spores per bee (Nosema + Propolis). After feeding, each bee was placed inside a perforated microfuge tube for 30 min to check if the inoculum had been eaten. Bees that did not eat all the food supplied or did not appear healthy were eliminated from the bioassay. A spore concentration used (10^5 per µL) was chosen to ensure infection of every bee (Malone and Stefanovic 1999; Forsgren and Fries 2010). We performed the bioassay using three replicates of 21 bees each (7 bees from three colonies, equally mixed in a cage to eliminate colony effects) per treatment. Each group was placed in a metal cage (10 cm × 10 cm × 5 cm) with perforated walls. Bees were kept in an incubator at 31 °C and fed *ad libitum* with 50% (w/v) sucrose solution administered using a syringe (Sterile Siring PIC, 5 mL) (Williams et al. 2013). Dead bees were counted daily and removed from the cages. All experimental treatments were performed at the same time. The bioassay ended on the 30^{th} day of observation.

Food choice test

Adult bees used in the choice test were obtained as described in the survival experiment. Freshly emerged bees were split into two groups of 60 individuals each. One group (infected group) was mass fed with 2 mL of a 50% (w/v) sucrose solution containing 10^5 *N. ceranae* spores per bee, sufficient to guarantee 100% infection. When the food

containing the spores was completely consumed, the bees were fed with sucrose solution (50% w/v) administered *ad libitum* until the start of the experiment (4 days after infection). The control group received only sucrose solution (50% w/v) administered *ad libitum* until the beginning of the experiment. The attractiveness of a commercial protein candy (Chemicals LAIF s.p.a, Vigonza (PD), Italy) and to which we had added propolis was compared with that of the same candy without propolis in infected and healthy bees, tested individually in a Y-shaped olfactometer. Two different concentrations (2% and 5%) of crude propolis were compared with the candy without propolis in two separate experiments. In each test, candy devoid of propolis was given in one arm and candy with propolis at a concentration of 2% or 5% was offered in the other arm. The candy was placed on squares (4 × 4 cm) of filter paper of 67 g/mq (APTACA SRL, Canelli, Italy) in the olfactometer. The bioassays were conducted under artificial light at 27 ± 1 °C using a Y-shaped olfactometer with a transparent plexiglass cover (main arm: 25 cm length, 20 cm width, 10 cm height; each side-arm: 20 cm length, 20 cm width, 10 cm height). Starting from the fourth day after infection, 60 bees from the *Nosema*-infected or control groups were tested within the subsequent three days. Each bee was placed carefully inside the main arm through a circular hole (1.5 cm diameter) using soft tweezers and then allowed to walk freely in all three arms. Every trial was terminated when a bee arrived at one of the two arm ends, containing either control or propolis-laced candy, and extended its proboscis. Bees that did not choose either of the two candies within 10 min ("no choice" subjects) were excluded from statistical analysis. Infected and control bees were tested alternately and, for each bee belonging to the same treatment group, we exchanged the position of the two types of candy. Each bee was tested only once.

Scan bees sampling and food consumption test

In a metal cage containing 1 g of two different candies (one with and one without propolis), we placed 10 to 15 bees belonging to the *Nosema*-infected or control groups. After each hour for 8 consecutive hours, the number of bees eating the two types of candy (control and either 2% propolis or 5% propolis candy) was recorded. To estimate the total food consumed, each candy was weighed at the beginning and the end of the bioassay. The proportion of bees feeding on the two types of candy and the relative amount of propolis consumed (calculated as the propolis-candy consumed/total candy consumed) were compared between the infected and the control groups. The bioassays were conducted from the 4^{th} day post infection (dpi) and finished after three consecutive days (i.e. 7 dpi). We carried out bioassays to test candy at two crude propolis concentrations (2% and 5%) and each treatment was replicated using 3 independent cages.

Quantifying *N. ceranae* infection: DNA extraction and qPCR

In the survival bioassay, we sampled one bee per treatment from each cage after 7 and 14 days post infection (36 bees in total) to quantify *N. ceranae* genome equivalents. The level of infection was determined using whole bee DNA extracts. Bees were individually crushed in 500 µL DEPC-H_2O, and then 100 µL were used for genomic DNA extraction using a DNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's instructions. To quantify *N. ceranae* infection, we used the following qPCR primers, previously described by vanEngelsdorp et al. (2009) and with a modification to the reverse primer by Natsoupolou et al. (2015):

Forward (3'-5'): CAATATTTTATTATTTTGAGAGA

Reverse (3'-5'): TATATCTATTGTATTGCGCGTGCA

giving an amplicon length of 232 bp for *N.ceranae*. PCR reactions were performed in a Bio-Rad C1000 Thermal Cycler (Bio-Rad) using 2x SensiMixTM SYBR and Fluorescein (Bioline), 0.2 µM of each primer and 1 µL of template in a final volume of 10 µL. A negative control without template was included in each run. Each reaction was performed in duplicate and the average quantification cycle (*Cq*) value was taken (accepting a maximum *Cq* difference of 1 between duplicates). Amplification was performed using the thermal profile described in vanEngelsdorp et al. (2009) with an optimal annealing temperature of 54 °C. Post amplification melt curve analysis was used to check for non-specific amplification (50 °C to 95 °C with an increase of 0.5 °C per second). Standard curves were included in each run for absolute quantification of DNA copy number (genome equivalents) of *N. ceranae*; we accepted PCR efficiencies between 90% and 100%. In the choice and scan sampling bioassays of food consumption, we ascertained the presence of *N. ceranae* in infected group and its absence in the control group by sampling one bee per cage for the control group on days 4 and 7 post treatment and two bees per cage on days 4 and 7 from each propolis treatment (2% and 5%). DNA was extracted from individual bees and a standard PCR for presence/absence was performed using the same protocol as described above for qPCR (Fries et al. 2013). PCR products were visualised on 1.5% agarose gels after staining with EZ-Vision two® (Amresco, Germany).

Chemical analyses of propolis

To determine the chemical composition of our propolis, an HPLC 1100 system equipped with a DAD detector G1315A, an autosampler G1313A, a pump G1311A, and a column oven G1316 (Agilent Technologies, Milan, Italy) were used. The system was controlled by the HP CHEMSTATION for LC software. The wavelengths monitored were 280, 360, and 520 nm. The column was a Varian Polaris C18 (5 µm, 300 A, 250 mm x 4.6 mm). The solvents used were 0.22 M

phosphoric acid (A) and acetonitrile/methanol (1/1, v/v) (B). The gradient used for separation and analysis was the following: T = 0 A 96% A; T = 40 50% A; T = 45 40% A; T = 60 0% A, hold for 10 min; at the end of the analysis the column was reconditioned at the initial conditions for 15 min. The flow was 1 mL/min. Identification of the compounds was made using certified analytical standards supplied by Sigma Aldrich. Quantification was made plotting area versus concentration of compounds in the sample versus five-point calibration curves made with authentical standards.The total polyphenol content of propolis was determined by the Folin-Ciocalteu method (Singleton and Rossi 1965). The solutions for analysis were prepared by reacting 100 µL of the propolis solution or standard with 500 µL of Folin-Ciocalteau reagent for 5 min and then adding 3 mL of 10% (w/v) sodium carbonate solution and ultrapure water up to a final volume of 10 mL. After a 90 min incubation at room temperature, the samples were read at λ = 725 nm against a blank using 1 cm quartz cuvettes. The quantitative analysis was carried out using the external standard method (gallic acid) correlating absorbance (Abs) with concentration (400 − 8000 mg/kg). The results were expressed in mg/kg of gallic acid.

Statistical methods

We analysed survival using a Cox proportional hazard mixed model, with experimental group (i.e. Control, Control + Propolis, Control + Ethanol, *Nosema*, *Nosema* + Propolis, *Nosema* + Ethanol) as a fixed factor and cage as a random factor. The analysis was performed using the R package *coxme* (Therneau 2018). We used the R packages *survival* (Therneau and Grambsch 2000) and *survminer* (Kassambara and Kosinski 2018) to plot survival curves and the function *termplot* from the *stats* R package to plot hazard ratios. Statistical significance of differences in hazard ratios were calculated using post-hoc pairwise contrasts adjusted for multiple comparisons with the Benjamini-Hochberg method to control the false discovery rate (FDR) using the R package *multcomp* (Hothorn et al. 2008). We used a linear mixed (LMM) model to test for differences among treatments in log-transformed *Nosema* genome equivalents per bee and post-hoc comparison of means adjusted for multiple comparisons using the FDR method. We used a generalised linear mixed effect model (GLMM) with binomial error structure to examine the effect of treatment (control vs. *Nosema*-infected) on choice of food (candy with or without propolis) in our Y maze experiment. Treatment (control vs. *Nosema*-infected), candy type (2% propolis or 5% propolis) and their interaction were used as fixed factors and the colony of origin of tested bees was used as a random factor. We also used a GLMM with binomial error structure to investigate the effects of treatment (control vs. *Nosema*-infected) on the proportion of bees feeding on the candy with or without propolis. Treatment (control vs. *Nosema*-infected), candy type (2% propolis or 5% propolis) and their interaction were used as fixed factors and cage was used as a random factor. We furthermore used a generalised linear

model (GLM) to investigate the effects of treatment (control vs. *Nosema*-infected) on the proportion of propolis-candy bees consumed in their diet. All mixed effect models were performed using the *lme4* package in R (Bates et al. 2015). All model assumptions were checked visually and were found to conform to expectations (residuals normally distributed, homogeneity of variance, linearity). All statistical analyses were conducted using R version 3.5.2 (R Core Team 2018).

Results

Survival bioassay

We found a strong effect of the experimental treatment on bee survival (coxme; $\chi^2 = 58.094$, $P < 0.001$; Fig. 1). The *Nosema* treatment caused the fastest mortality, faster than any control treatment and with 50% of bees dead within 13 days post infection (Figs. 1 and 2). Survival was significantly lengthened in the *Nosema* + Propolis and *Nosema* + Ethanol treatments compared to *Nosema* alone (post-hoc test adjusted for multiple comparisons with the FDR method; Z = -3.825, $P = 0.001$; Z = -3.644, $P = 0.004$; respectively; Figs. 1 and 2, Supplementary Table S1), with 50% of dead individuals recorded within 17 days and 18 days post infection, respectively. The *Nosema* + Propolis and *Nosema* + Ethanol treatments were not significantly different from Control + Propolis (post-hoc test adjusted for multiple comparisons with the FDR method; $P > 0.05$; Figs. 1 and 2, Supplementary Table S1). Differences were not significant between Control and Control + Ethanol or between Control and Control + Propolis treatments (post-hoc test adjusted for multiple comparisons with the FDR method; $P > 0.05$; Figs. 1 and 2, Supplementary Table S1). A post-hoc qRT-PCR screening of a subsample of bees collected on the 7th day and 14th day post infection showed that *Nosema* infection levels differed between the treatments tested (LMM; $\chi^2 = 67.997$, $P < 0.001$; Fig. 3). The *Nosema* and *Nosema* + Ethanol experimental groups had higher *Nosema* spore loads, evaluated as genome equivalents, compared to all other experimental groups (post-hoc test adjusted for multiple comparisons with the FDR method; $P < 0.05$; Fig. 3). The *Nosema* + Propolis group did not differ from the three control treatments (Control, Control + Ethanol and Control + Propolis) (post-hoc test adjusted for multiple comparisons with the FDR method; $P > 0.05$; Fig. 3).

Food choice test

In the food choice test, the addition of propolis to candy at 2% or 5% did not significantly increase its attractiveness to *Nosema*-infected or control workers (Fig. 4). In fact, the proportion of bees choosing the propolis-candy rather than the candy devoid of propolis did not differ between *Nosema*-infected and control bees (GLMM; $\chi^2 = 1.915$, $P = 0.166$, Fig.

4), candy type (with 2% or 5% propolis; GLMM; $\chi^2 = 1.187$, $P = 0.275$, Fig. 4) and neither their interaction (GLMM; $\chi^2 = 0.136$, $P = 0.711$; Fig. 4). In each bioassay, a PCR confirmed the presence of *N. ceranae* in the infected group and its absence in the control group, both on days 4 and 7 post infection.

Scan sampling and food consumption

In the scan and food consumption tests, the addition of propolis to candy at 2% or 5% did not significantly increase the number of *Nosema*-infected or control workers that fed on it (Fig. 5). In fact, the proportion of bees feeding on the propolis-candy did not differ between *Nosema*-infected and control bees (GLMM; $\chi^2 = 1.188$, $P = 0.664$; Fig. 5), candy type (with 2% or 5% propolis; GLMM; $\chi^2 = 0.070$, $P = 0.791$; Fig. 5) and their interaction (GLMM; $\chi^2 = 0.141$, $P = 0.706$; Fig. 5). No significant differences were found in the proportion of propolis-candy consumed by *Nosema*-treated bees and control bees (GLM; $P > 0.05$), regardless of the concentration of propolis (2% or 5%) of the treated candy (Fig. 6). In each bioassay, a PCR confirmed the presence of *N. ceranae* in the infected group and its absence in the control group, both on days 4 and 7.

Chemical analysis of propolis

HPLC analysis revealed the presence of almost 50 compounds belonging to the family of phenols, in particular flavones, flavonols, and simple phenols, like caffeic and ferulic acid, in the propolis extract. The most abundant were derivatives of quercetin, caffeic acid, ferulic acid, and ellagic acid, while only small amounts of kaempherol, and derivatives of cinnamic acid, rosmarinic acid and narigin were detected. The degradation pathway of larger phenols usually leads to smaller phenols (Sridevi et al., 2012); in fact, it was possible to detect high levels of caffeic, ellagic and ferulic acid derivatives. However, most phenol compounds were present in low amounts, therefore, it was very difficult to identify them unambiguously, especially because standards are not available for many of them. Nevertheless, the compounds here reported represent almost 95% of those present in the propolis extract (Table 1). Total phenols in the propolis extract amounted to almost 120 mg/g. Differences with the total amounts obtained with the HPLC analysis (Table 1) has two possible explanations, the first related to the fact that in spectrophotometry phenols are expressed as gallic acid, and in HPLC using authentical standards, and the second that phenols react differently to different wavelengths. Therefore, no comparison can be made between the two datasets.

Discussion

In this study, we evaluated the effects of propolis extracts on the survival and intensity of infection of *Apis mellifera* workers experimentally inoculated with *N.ceranae*. Our results showed that both propolis extracts and ethanol (solvent control) have a positive effect on the lifespan of *N. ceranae* infected bees. However, only propolis caused a significant reduction in *Nosema* spore load, evaluated as genome equivalents. Experiments similar to ours have been conducted on the red dwarf honey bee, *A. florea* (Suwannapong et al. 2010), and on the Asian hive honey bee, *A. cerana* (Yemor et al. 2016), using propolis produced by the stingless bee *Trigona apicalis*. In these studies, only the propolis extract and not ethanol showed positive effects on *Nosema*-infected bees, causing an increase in survival and a decrease in spore load. Similar effects of propolis on workers of *A. mellifera* infected by *Nosema* have also been reported by Arismendi et al. (2018), but a different solvent was used to extract the active ingredients from propolis (methanol instead of ethanol) and the study lacked a positive control (infected bees treated with the solvent alone). In our experiments, a single administration of 2 µL of a sugar solution containing 17.5% ethanol did not cause any increase in mortality of uninfected bees nor did it change *Nosema* load in experimentally infected bees. Ptaszynska et al. (2013) studied the impact of the prolonged administration (10 consecutive days) of ethanol on *Nosema*-infected bees, based on the fact that some beekeepers add ethanol to sucrose solution (fed in autumn to honey bees) to prevent *Nosema* infection and to cure already infested colonies. Under these conditions, they observed that the administration of sucrose syrup with 5% ethanol promoted the development of nosemosis, whereas ethanol at 10% concentration exerted severe toxic effects on uninfected bees. However, no side effects using ethanol at 2.5% concentration were observed. The acute toxicity of ethanol on honey bees was also studied by Maze et al. (2006), who administered 9 µL of a 1.0 M sucrose solution containing 0, 5, 10, 25, 50, or 75% ethanol in a single dose. Maze et al. (2006) observed time and dose-dependent effects of ethanol on locomotor behaviour (walking, stopping or walking upside down; grooming and flying behaviour), but only honey bees given the highest doses (50% and 75%) showed a significant increase in mortality. Moreover, behavioural recovery occurred between 12 and 24 h post-ingestion for low doses and at 24 to 48 h for higher doses. The total amount of ethanol supplied to each honey bee in our study (equivalent to 0.35 µL of 100% ethanol) was lower than that supplied by Maze et al. (2006) at their lowest dose (equivalent to 0.45 µL of 100% ethanol). Therefore, no sub-lethal side-effects were expected in our experimental paradigm. The positive effect of ethanol on the survival of *Nosema*-infected bees observed in our study could be due to the broad-spectrum antimicrobial activity of ethanol, which includes impacts on bacteria, viruses and fungi (McDonnell and Russell 1999). The antifungal properties of ethanol on honey bees have been recently highlighted although, at low ethanol concentrations (30%), *Nosema* spore germination

was only partially reduced (Sequeira et al. 2017). In our study, the slight, although not significant, reduction in spore loads, evaluated as genome equivalents, in the *Nosema*-infected bees treated with ethanol (Nosema + Ethanol treatment) compared to the Nosema treatment might have also led to their greater survival. Because low concentrations of ethanol can enhance the activity of other biocides (McDonnell and Russell 1999), we also hypothesize that ethanol strengthened our observed, very marked effect of propolis against *Nosema* in the Nosema + Propolis treatment. The propolis used in our bioassays effectively halted the proliferation of *N. ceranae* spores in the bee gut, as previously reported by Suwannapong et al. (2011, 2018), Yemor et al. (2016) and Arismendi et al. (2018). This is an important finding because resin chemical composition can vary with geographical area of origin, and consequently so can its antimicrobial activity (Lindenfelser 1967; Wilson et al. 2015). In general, the antimicrobial property of propolis derives from its high resin content, which is essentially associated with the content of phenolic compounds, mostly flavonoids and organic acid esters (Bankova et al. 1983). Some components isolated from Bulgarian propolis, including pinocembrin, pinobanksin-3-acetate and caffeic acid ester mixtures, are well known to be effective against the honeybee pathogens *P. larvae* and *A. apis* (Bilikova et al. 2012; Voight and Rademacher 2015). Wilson et al. (2017) isolated eleven dihydro-flavonols from propolis collected in Fallon (Nevada, USA) and found that those with longer acyl groups had increased activity against *P. larvae*, whereas shorter acyl groups had increased activity against *A. apis*. To our knowledge, similar studies on *N. ceranae* are lacking. Nevertheless, several ethanolic plant extracts have shown significant anti-*Nosema* activity (Kim et al. 2016; Arismendi et al. 2018), probably due to their polyphenolic compound content (Mocan et al. 2014; Arismendi et al. 2018). Arismendi et al. (2018), who chemically characterised two types of propolis that enhanced the survival of *N. ceranae*-infected bees, found many flavonol compounds (rutin, myricetin, querecetin, kaempferol and galangin) as well as phenolic acids, such as apigenin, pinocembrin and caffeic acid phenethyl ester in their propolis. Many of these compounds show broad-spectrum antimicrobial activity (Cushnie and Lamb 2005). In contrast, chemical analysis of the propolis extract used in our study revealed the presence of mainly quercetin derivatives as well as ellagic acid, ferulic acid and caffeic acid derivatives. These differences can be explained by the environmental conditions affecting plants from which honey bees collect resins. This suggests that the anti-*Nosema* properties of propolis in all these studies are probably due to a synergistic action of various components rather than the action of a single component. In relation to the mechanism of action of propolis, Turcatto et al (2018) reported that propolis consumption may enhance the immune response of bees when infected with *E. coli*. Nozevit, a natural herbal preparation produced as a water solution of plant polyphenols and sold on the market as a "partner for *Nosema* disease repression", induces the production and secretion of mucous from the epithelial layer of bees treated with the product, and coats the peritrophic

membrane to form a firm and resilient envelope, thus ensuring protection against new invasions of *Nosema* sp. (Tlak et al. 2011). The effect of propolis administration on reducing *Nosema* proliferation that we detected may be due to the enhanced immune response of bees induced by propolis or due to propolis also altering ventricular mucous secretion. We evaluated for the first time if *Nosema*-infected bees are more attracted to a diet containing propolis, and if they consumed more of this diet in comparison to uninfected bees. The lack of significant differences observed between infected and uninfected honey bees in our choice, scan and food consumption tests contradict the hypothesis that bees therapeutically use propolis in self-medication, as proposed by Simone-Finstrom and Spivak (2012) and Pusceddu et al. (2019). However, those two studies addressed the role of propolis against two hive enemies (*A. apis* and *V. destructor*) and were conducted in an apiary rather than the laboratory. As a consequence, both studies dealt with cases of social medication, in which the benefit derived from the use of a therapeutic substance is evaluated at the colony level rather than at the individual level (Spivak et al. 2019). In contrast, our study dealt with self-medication by bees for the benefit of the individual bee. Our study does not support the idea that *Nosema*-infected honey bees consume propolis as a form of self-medication. Nevertheless, in tests similar to those we carried out with *Nosema* in the present study, we observed that honey bee workers parasitized by *Varroa* consumed more food enriched with propolis in comparison with non-infested bees (Pusceddu et al., personal communication). Based on these findings, we think that propolis self-medication deserves further investigation. For instance, it would now be important to test diets containing propolis at different concentrations from those used in our study in protecting worker honey bees from *N. ceranae* infection.

Conclusions

The reduced proliferation of *N. ceranae* spores in the honey bees treated with propolis that we observed strengthens the view that it would be preferable and feasible to use natural compounds as an alternative to synthetic chemicals in the management of diseases of the honey bee and the hive, especially considering that consumers demand high-quality food products. In this context, active ingredients obtained from natural substances such as propolis seem to have the potential to control many important parasites of the hive in a safety manner. It is now necessary to conduct field studies to confirm the results observed under controlled experimental settings in order to develop a new method for the control of *Nosema* infection which would avoid the use of synthetic antibiotics in honey bee colonies.

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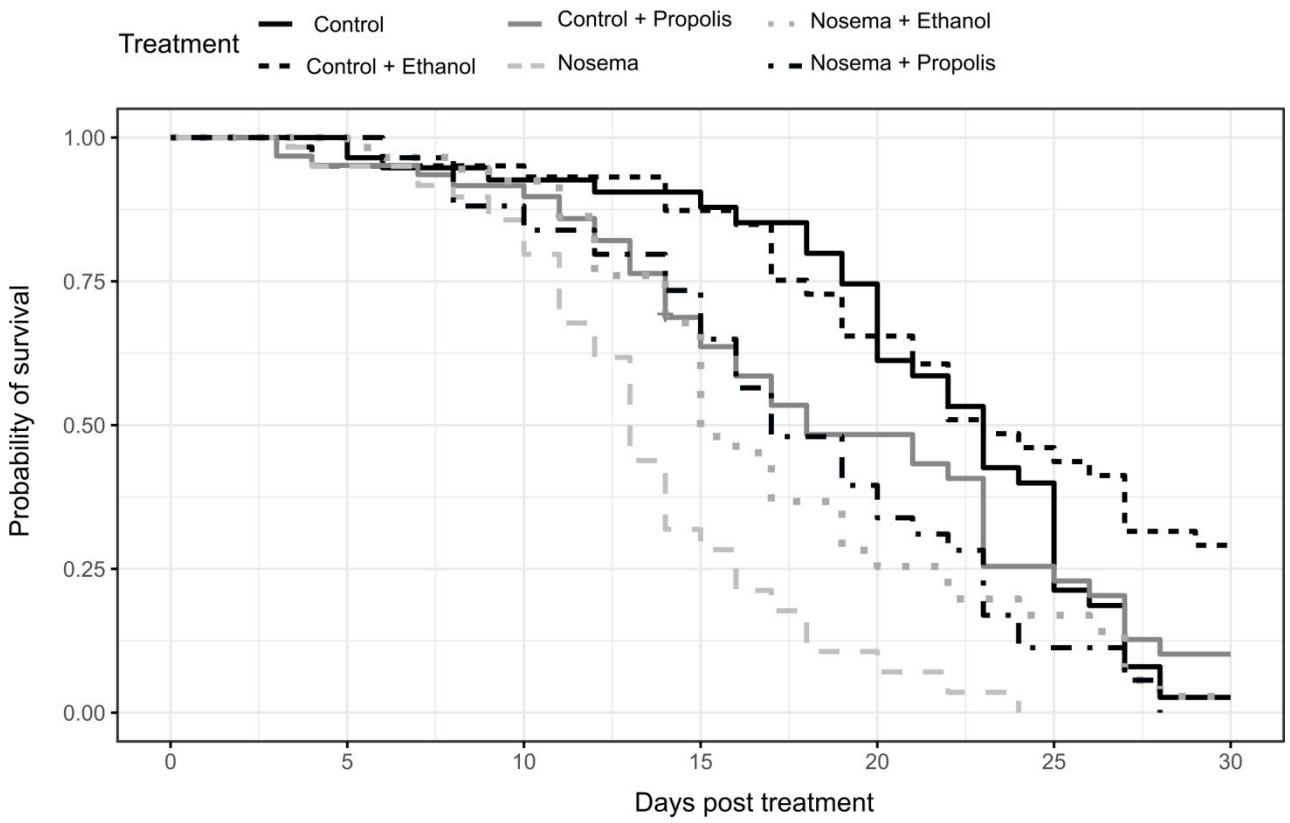

Fig. 1

Kaplan-Meyer survival curve of caged and experimentally treated honey bees *(Apis mellifera*): Control, Control + Ethanol, Control + Propolis, Nosema, Nosema + Ethanol and Nosema + Propolis

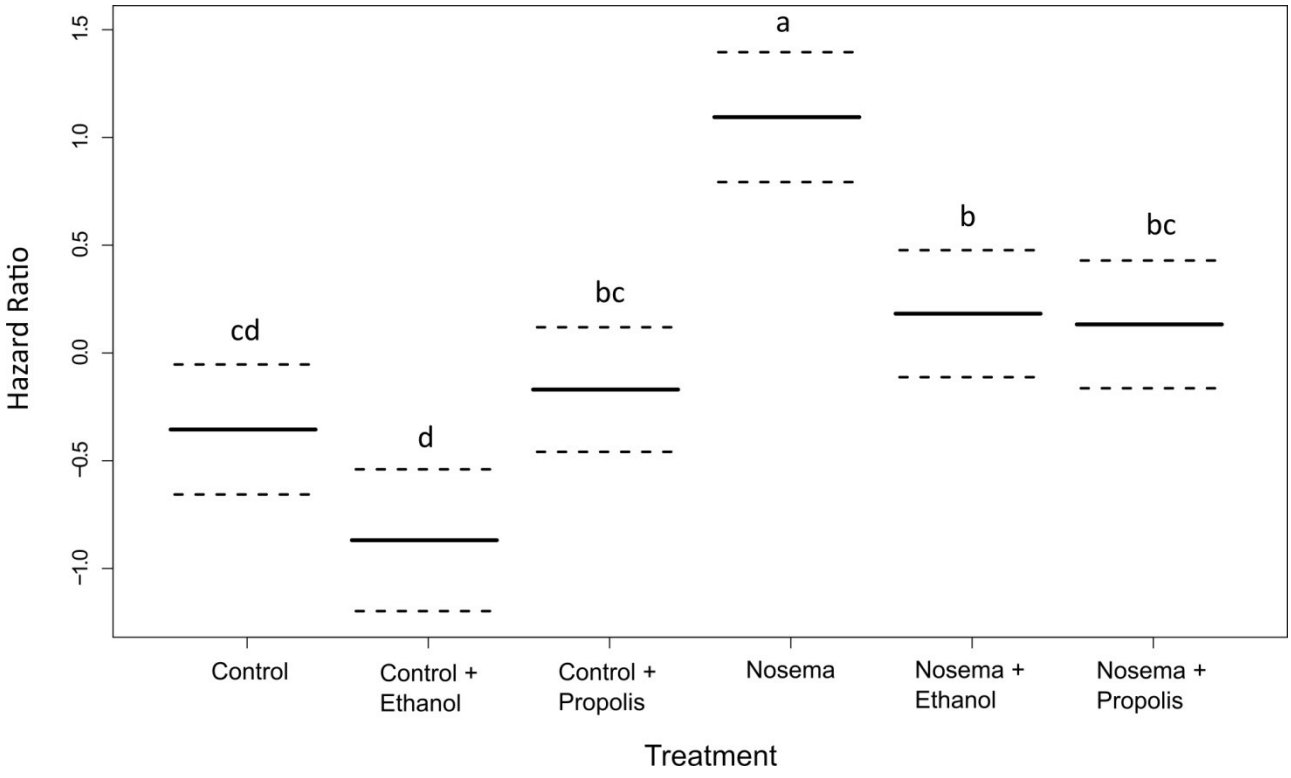

Fig. 2

Instantaneous risk of death (hazard ratio, ± 95% CI) for adult honey bees (*Apis mellifera*) in each experimental treatment compared with the model average of 0. Different letters correspond to significant differences between treatments at $P < 0.05$ (coxme and post-hoc pairwise contrasts adjusted for multiple comparisons with the Benjamini-Hochberg method to control the false discovery rate)

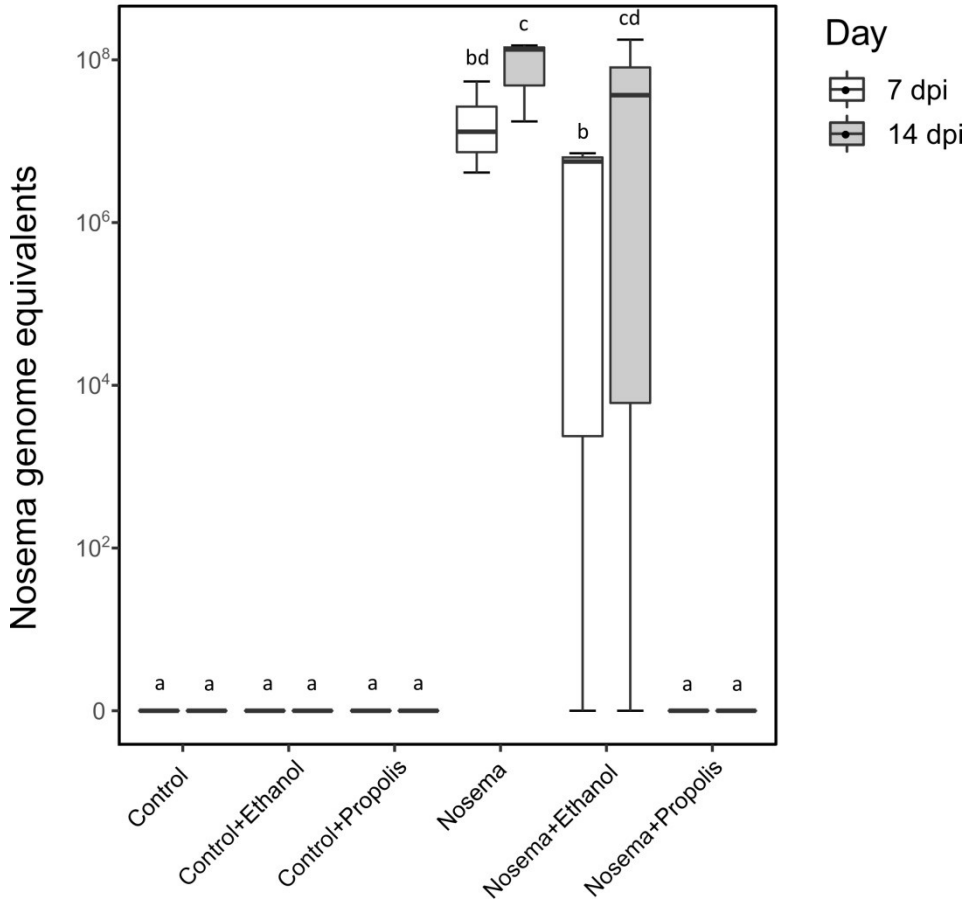

Fig. 3

Nosema ceranae genome equivalents per honey bee (*Apis mellifera*) in treatments: Control, Control + Ethanol, Control + Propolis, Nosema, Nosema + Ethanol, and Nosema + Propolis at 7 and 14 days post experimental infection (dpi). Different letters correspond to significant differences between treatments at $P < 0.05$ (LMM and post-hoc pairwise contrasts adjusted for multiple comparisons with the Benjamini-Hochberg method to control the false discovery rate)

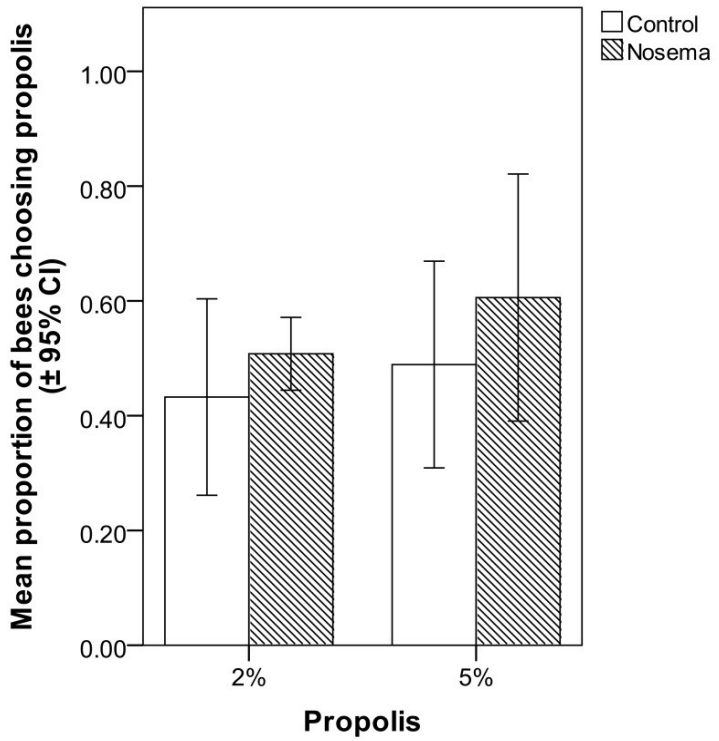

Fig. 4

Mean proportion (± 95% CI) of honey bees (*Apis mellifera*) in *Nosema ceranae*-infected and control groups that chose candy with 2% or 5% propolis in comparison with candy without propolis in the choice test; differences between treatments were not significant (GLMM, $P > 0.05$)

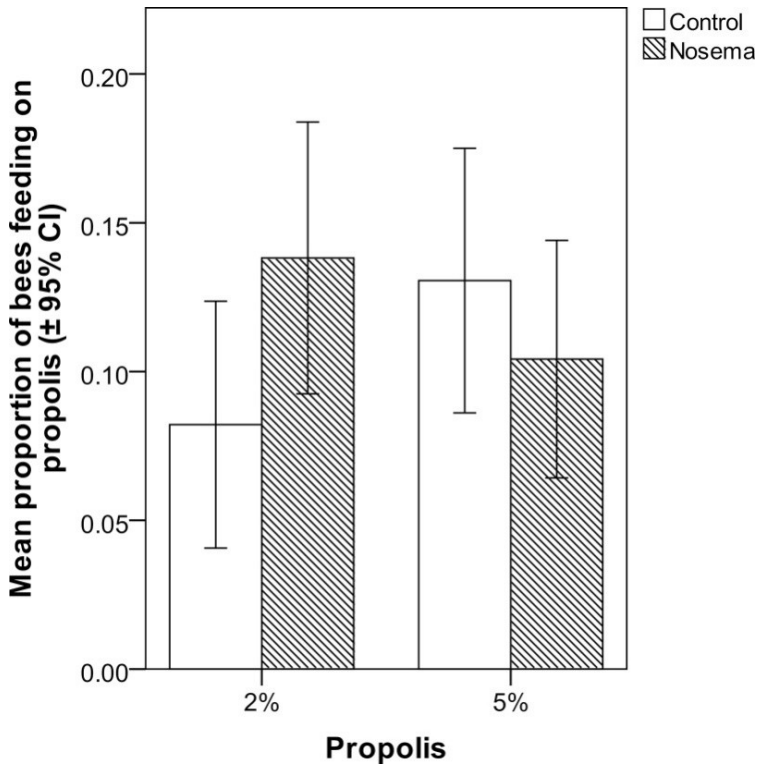

Fig. 5

Mean proportion (± 95% CI) of honey bees (*Apis mellifera*) in *Nosema ceranae*-infected and control groups that chose to feed on the candy with 2% or 5% propolis in comparison with candy without propolis in the scan test; differences between treatments were not significant (GLMM, $P > 0.05$)

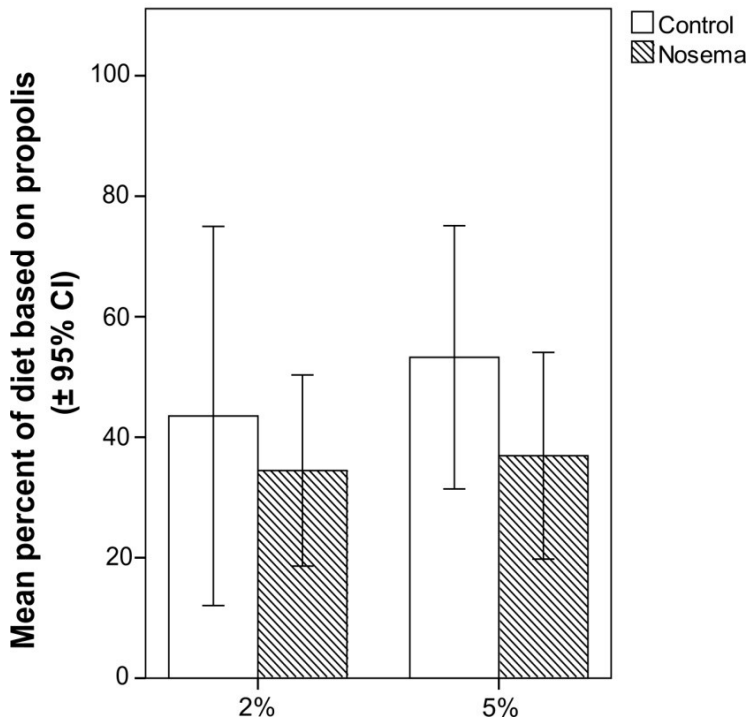

Fig. 6

Percentage (± 95% CI) of candy with 2% or 5% propolis in the total diet consumed in *Nosema ceranae*-infected and control honey bees (*Apis mellifera*); differences between treatments were not significant (GLM, $P > 0.05$)

Table 1

Total Phenols (spettrofotomethric) and main phenolic compounds (HPLC-DAD) in the propolis used in the bioassay

Compound	**mg/g ± RSD%**
Caffeic acid [y]	31.04 ± 5.01
Caffeic acid derivatives [z]	113.17 ± 12.35
Cinnamic acid derivatives [z]	82.04 ± 10.78
Ellagic acid derivatives [z]	125.44 ± 4.65
Ferulic acid [y]	7.62 ± 5.25
Ferulic acid derivatives [z]	122.70 ± 14.68
Galangina [y]	15.05 ± 2.48
Kaempherol [y]	34.01 ± 5.78
Luteolin [y]	3.00 ± 5.68
Naringin [y]	59.01 ± 10.45
Naringin derivatives [z]	21.99 ± 1.56
Narirutin [y]	1.37 ± 6.51
OH-Flavone derivatives [z]	17.21 ± 2.56
Paracumaric acid [y]	7.12 ± 3.68
Quercetin [y]	10.83 ± 3.45
Quercetin derivatives [z]	238.75 ± 6.59
Quinic acid [y]	1.39 ± 8.95
Quinic acid derivatives [z]	4.01 ± 6.01
Rosmarinic acid derivatives [z]	44.84 ± 13.98
Total Phenols *	119.77 ± 16.05

* expressed as gallic acid

[y] identification and quantification has been made using authentic analytical standards

[z] tentatively identification has been made using DAD spectra similarities, quantifications were expressed as the parent compound

Supplementary Table S1

Resutls of post-hoc tests adjusted for multiple comparisons with the FDR method, for the hazard ratio of each treatment in the survival bioassay

Treatment comparisons			**Estimate**	**Std. Error**	**z value**	***P* value**
Control+Ethanol	vs.	Control	-0.51323	0.25129	-2.042	0.056
Control+Propolis	vs.	Control	0.18859	0.23400	0.806	0.450
Nosema	vs.	Control	1.46227	0.24079	6.073	< 0.001 ***
Nosema+Ethanol	vs.	Control	0.54122	0.23595	2.294	0.036 *
Nosema+Propolis	vs.	Control	0.49223	0.23562	2.089	0.055
Control+Propolis	vs.	Control+Ethanol	0.70182	0.24689	2.843	0.008 **
Nosema	vs.	Control+Ethanol	1.97550	0.25856	7.640	< 0.001 ***
Nosema+Ethanol	vs.	Control+Ethanol	1.05445	0.25057	4.208	< 0.001 ***
Nosema+Propolis	vs.	Control+Ethanol	1.00547	0.25154	3.997	< 0.001 ***
Nosema	vs.	Control+Propolis	1.27368	0.23719	5.370	< 0.001 ***
Nosema+Ethanol	vs.	Control+Propolis	0.35263	0.23271	1.515	0.162
Nosema+Propolis	vs.	Control+Propolis	0.30364	0.23306	1.303	0.222
Nosema+Ethanol	vs.	Nosema	-0.92105	0.23387	-3.938	< 0.001 ***
Nosema+Propolis	vs.	Nosema	-0.97004	0.23410	0.23410	< 0.001 ***
Nosema+Propolis	vs.	Nosema+Ethanol	-0.04898	0.23360	-0.210	0.833

*** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$

CHAPTER 2

Title:

"**The effects of raw propolis on *Varroa*-infested honey bee (*Apis mellifera*) workers**"

Authors:

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ORIGINAL PAPER

The effects of raw propolis on *Varroa*-infested honey bee (*Apis mellifera*) workers

Michelina Pusceddu[1] · Ignazio Floris[1] · Alessandra Mura[1] · Panagiotis Theodorou[2] · Giorgia Cirotto[3] · Giovanna Piluzza[4] · Simonetta Bullitta[4] · Alberto Angioni[5] · Alberto Satta[1]

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Abstract
Self-medication plays a major role in the behavioral defense against pathogens and parasites that animals have developed during evolution. The conditions defining this adaptive behavior are: (1) contact with the substance in question must be deliberate; (2) the substance must be detrimental to one or more parasites; (3) the detrimental effect on parasites must lead to increased host fitness. Recent studies have shown that *A. mellifera* colonies are able to increase resin foraging rates when infested by *V. destructor*, whereas further investigations are needed for evidence of parasite and host fitness. In order to understand whether *Varroa*-infested colonies could benefit from increasing levels of resin, we carried out laboratory bioassays to investigate the effects of propolis on the fitness of infested workers. The longevity and energetic stress of adult bees kept in experimental cages and artificially infested with the mite were thus monitored over time. At the same time, in vitro experiments were performed to study the contact effects of crude propolis on *Varroa* mites. Our results clearly demonstrate the positive effects of raw propolis on the lifespan of *Varroa*-infested adult bees. A low narcoleptic effect (19–22%) of raw propolis on phoretic mites after 5 h was also observed. In terms of energetic stress, we found no differences between *Varroa*-free and *Varroa*-infested bees in terms of the daily sucrose solution demand. Our findings seem to confirm the hypothesis that resin collection and propolis use in the hive represent an example of self-medication behavior in social insects.

Keywords Self-medication · Energetic stress · Bee longevity · Narcoleptic power · Polyphenols

Introduction

The health of the honey bee (*Apis mellifera* Linnaeus 1758) is one of the main global environmental emergencies of this century (Neumann and Carreck 2010). The high annual losses among honeybee colonies have a huge socioeconomic and ecological impact on apiculture, agriculture, and the environment (Neumann and Carreck 2010; Potts et al. 2010). Although many pathogens and parasites of honeybees and their effects on the colony population have been characterized, strategies for their control are still unsatisfactory (Martin 2001; Cox-Foster et al. 2007; Nazzi et al. 2012; Higes et al. 2013). Of these, the parasitic mite *Varroa destructor* Anderson and Trueman 2000, has the most severe economic/sanitary impacts (Rosenkranz et al. 2010). In the past decade, the control of this parasite has mainly been carried out using synthetic acaricides which often show different side effects on the honeybee and residues on hive products, and could also lead to *Varroa* resistance phenomena (Rosenkranz et al. 2010). Only recently have new interesting control methods based on natural hive products (e.g., propolis) been proposed (Simone-Finstrom et al. 2017).

Another new challenge is understanding how the natural immune responses (individual immunity) and collective behavioral defenses (social immunity) of honeybees can support bee health and reduce stressors without human intervention.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00436-018-6050-0) contains supplementary material, which is available to authorized users.

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The social immunity system refers to all collective defense mechanisms that honeybees and other social insects have evolved to combat the increased risk of disease transmission that arises from both social interactions and group living (Cremer et al. 2007). Some of these defense systems are preventative, keeping parasites and pathogens away from the nest, whereas others are activated as needed, when pathogens and/or pests have already penetrated the hive (Cremer et al. 2007, 2018).

One form of social immunity in *A. mellifera* is the formation of a propolis envelope within the nest which acts as a protective antimicrobial layer against pathogens. Currently, there is a scientific hypothesis regarding the possibility that honey bees use propolis not only as a preventive defense, but also as another type of defense that is activated when parasites or pathogens penetrate inside the nest (self-medication) (Simone-Finstrom and Spivak 2012; Pusceddu et al. 2017).

Self-medication plays a major role among behavioral defense mechanisms against pathogens and parasites which animals have developed during evolution (Lozano 1998). The conditions defining this adaptive behavior have been refined over time (Singer et al. 2009; de Roode et al. 2013). However, according to Clayton and Wolfe (1993), self-medication must fulfill at least three criteria: (1) the intake or contact with the substance in question must increase during the infestation or infection, (2) the substance must have a negative effect on the fitness of the parasite/pathogen, and (3) the substance must have a positive effect on the host fitness. In addition, according to Singer et al. (2009), self-medication behavior decreases fitness in uninfected animals, since it has a detrimental effect or is a major cost for the host in the absence of parasites or diseases. Recent studies have shown that *A. mellifera* colonies increase resin foraging rates when infested by *V. destructor* (Pusceddu et al. 2017) and in response to deforming wing virus titer (Drescher et al. 2017), and further investigations are needed for evidence of parasite and host fitness. 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 with respect to pollen foragers (Nakamura and Seeley 2006; Simone-Finstrom and Spivak 2010). Indeed, the 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, which would be in a better position if compensated with food when collecting nectar or pollen.

The narcoleptic effect, as a temporary inactivity of the mites, and the acaricidal properties of bioactive compounds, recovered from propolis alcoholic extracts, have been well documented against *V. destructor* (Garedew et al. 2002, 2003; Damiani et al. 2010). However, no unambiguous data have been collected on the direct or indirect effect of the crude propolis on the biological cycle of *V. destructor* (Erler and Moritz 2016). In the laboratory, Drescher et al. (2017) found no evidence of any effect of volatile compounds released from crude propolis on mite survival. However, a possible effect of the different chemical composition of propolis on *V. destructor* was suggested by Popova et al. (2014) who found propolis with a higher percentage of some biological active components (caffeic acid and pentenyl caffeates) in *Varroa*-resistant bee colonies, and a significantly lower resin content, compared with propolis samples from susceptible colonies. Moreover, no results have been collected on the effects of the chemical–physical conditions inside the hive on the release of bioactive substances from the resinous fraction of propolis.

Concerning the third criterion (i.e., the substance must have a positive effect on the host fitness), it is known that propolis inside the nest has an impact on a bee's immune responsiveness, decreasing the baseline expression of antimicrobial peptide-related genes in individual bees (Simone et al. 2009; Borba et al. 2015). Evans and Pettis (2005) showed that a high level of immunity activation on the part of the individual can be costly in terms of colony productivity. In Africanized honeybees, there also seems to be a positive correlation between propolis production and stored pollen and honey (Manrique and Soares 2002; Padilha et al. 2013). For the same subspecies, Nicodemo et al. (2013, 2014) reported that colonies selected for high propolis production had a lower brood rate loss, a longer worker lifespan, and more pollen and honey stored in the nest. In addition, colonies that had collected more propolis showed a greater aptitude for hygienic behavior (Garcia et al. 2013; Nicodemo et al. 2013; Padilha et al. 2013).

Borba et al. (2015) found that unchallenged colonies with a natural propolis envelope showed an increased colony strength and vitellogenin levels after overwintering. In fact, the protein status, and in particular, the vitellogenin titer in the bee's hemolymph play an important role in determining its lifespan (Amdam and Omholt 2002) and overwintering colony survival (Alaux et al. 2017; Smart et al. 2016).

However, little information is available regarding the benefits of propolis in colonies challenged by pathogens or parasites. Simone-Finstrom and Spivak (2012) reported that colonies with an experimentally applied propolis envelope showed significantly lower clinical signs of disease when challenged with *Ascosphaera apis* (Masenex ex Claussen) L. S. Olive and Spiltoir, 1955. Borba and Spivak (2017) demonstrated that the larval food collected from field colonies with a propolis envelope, challenged with *Paenibacillus larvae* White 1906 (the causative agent of American foulbrood disease, AFB), had a significantly higher inhibitory activity against *P. larvae* compared to larval food from challenged colonies without a propolis envelope. The presence of a propolis envelope resulted in a significant reduction in the number of larvae with clinical signs of AFB 2 months after *P. larvae* infection. In addition, Drescher et al. (2017) found a lower increase in deformed wing virus titers after *V. destructor* infestation in colonies provided with additional propolis, thus suggesting that it may help to block virus transmission.

In order to understand whether *Varroa*-infested colonies could benefit from increasing levels of resin, laboratory bioassays were carried out to investigate the effects of propolis on infested worker fitness. The longevity and energetic stress of adult bees kept in experimental cages and artificially infested with the mite were monitored over time. At the same time, in vitro experiments were performed to evaluate the effects on *Varroa* mites of coming into contact with crude propolis.

Since biological activity and a wide range of pharmacological properties of propolis have been intensively investigated and mainly associated with its phenolic component (da Silva et al. 2006; Mihai et al. 2012; Siripatrawan et al. 2013), specific analyses were carried out to define the total content of polyphenols and flavonoids, together with the main individual phenolic compounds of the propolis used in the bioassays.

Materials and methods

Experimental apiary

The study was performed from May 2017 to November 2017 in an experimental apiary in northwestern Sardinia, at the experimental farm of the University of Sassari, Department of Agricultural Science (latitude 40°46′23″, longitude 8°29′34″). The apiary consisted of 21 colonies of *Apis mellifera ligustica* Spinola 1806 maintained in Dadan-Blatt hives containing 10 combs each. During the experimental period, the colonies were checked every 2 weeks to check the presence of the queen, as well as the pollen and nectar supplies, and to evaluate the sanitary status (disease symptoms and varroosis). Before each experiment, a sample of about 300 adult bees from at least three different frames per each colony was collected and placed inside a container with a hydroalcoholic solution to facilitate the *Varroa* separation, and the percentage of infestation was calculated (Pappas and Thrasyvoulou 1988). The infested colonies were used as sources of *V. destructor* mites, while the non-infested ones (infestation level < 1%) were used as sources of honey bee brood that were ready to emerge.

Propolis collection, preparation, and analyses

Propolis specimens used in the bioassays were collected in two separate periods (August and September 2015) from seven hives in our experimental apiary using specific collection nets placed above nest combs. After net scraping, each propolis sample was inspected to remove visible impurities, then pre-weighed and stored in a freezer at − 18 °C.

Before starting the experiments, frozen samples were homogenized using a coffee mill (GS Arendo, Germany). The determination of total phenolic and total flavonoid content was carried out using the same methods described in Pusceddu et al. (2017) and they were characterized using an HPLC DAD (Diod Array Detector) according to Pellati et al. (2011) with some modifications. Analyses of phenolic compounds were carried out using an Agilent 1260 series HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump, degasser, column thermostat, auto-sampler, and diode array detector. Identification and peak assignment of polyphenolic compounds were based on a comparison of their retention times and spectra with the analytically pure standards. The concentrations of 12 standards (caffeic acid, p-coumaric acid, ferulic acid, quercetin, isorhamnetin, cinnamic acid, apigenin, kaempferol, chrysin, pinocembrin, and caffeic acid phenethyl ester, galangin) were calculated according to the external standard method curve (four known concentrations for each standard in duplicate, $R^2 = 0.99$) and expressed in mg per g of dry weight. Data were processed using the Agilent OpenLAB CDS ChemStation (edition 2012).

To verify that the propolis samples had not been contaminated by acaricide residues, the samples were analyzed by gas chromatography-ion trap-mass spectrometry (GC-ITMS analysis). An aliquot of 1 g of propolis was dissolved in 2 mL of acetone (for pesticide analysis, 99.5% purity). The solution was stirred for 5 min in a vortex (Falck, instrument, Milan, Italy) and centrifuged for 10 min at 10 °C and 4000 rpm. The organic phase was recovered and injected into the analytical system.

A gas-chromatograph Varian 3800 (Agilent Technologies, Milan, Italy) was used, coupled with a mass spectrometry Ion Trap system 2000 ITMS and an autosampler Varian 7800. Helium was the carrier gas at 1 mL/min. The injector temperature was set at 150 °C. The column was a Varian VF17 for the GC/MS analysis, and the oven temperature program was as follows: $T = 0$ 50 °C (1 min), till 300 °C (10 °C/min). The ITMS was set in EI mode at 70 eV, with the SCAN range from 50 to 650 amu. Quantitative analyses were carried out in SIS mode using the following m/z rates: tau-fluvalinate m/z 250, 181, coumaphos m/z 362, 226, fenpyroximate m/z 213, 198, amitraz m/z 293, 162, bromopropylate m/z 341, 339, tetradifon m/z 356, 159, and cymiazole m/z 218, 185.

Host and parasite collection

To obtain adult workers and perform the laboratory bioassays, honeybee brood that were ready to emerge were collected from three *Varroa*-free colonies and kept for 14 h in an incubator at + 35 °C.

Female mites were sampled from *Varroa*-infested colonies using an inert dust "powdered sugar" (Paneangeli, Cameo) because up to 48 h, there is no difference in mite survival recovered from broods or with powdered sugar (Macedo et al. 2002). During the time required to set up the experiment (about 2 h), the mites used to test how they were affected by the propolis were kept in Petri dishes together with fresh bee pupae to prevent starvation. Conversely, the mites used to evaluate the propolis effects on infested worker fitness were kept in Petri

Alessandra Mura
Studies on the behavioral responses of *Apis mellifera* to the pathogen *Nosema ceranae* and the parasite *Varroa destructor*, and on the predatory wasp *Vespula germanica* in apiary
Tesi di Dottorato in "Scienze Agrarie"
Curriculum "Monitoraggio e Controllo dei Sistemi Agrari e Forestali in Ambiente Mediterraneo"
Università degli Studi di Sassari

dishes without bee pupae. Abnormal mites (faded in color, small size, slowly moving) were excluded from the experiment.

Host bioassays

In order to test the effects of propolis on *Varroa*-infested worker fitness, 30 newly-emerged bees collected from three different colonies with a low level of *Varroa* infestation (< 1%) were mixed in order to prevent any genotypic effects (10 bees from each colony) and placed in a metal cage (10 cm × 10 cm × 5 cm) whose two inner sides were covered with two sheets of bee wax comb, on which 30 mites had already been placed.

To verify the infestation success in each cage, we observed that the mites flew above the bees. The cages were then kept in an incubator at + 31 °C with ad libitum 50% (*w*/*v*) sucrose solution administered with a graduated syringe (Williams et al. 2013). Dead bees were counted and removed from the cages every day (at the same time), to monitor the bees' longevity. In addition, the bees' energetic stress in terms of nutritional demand was also monitored by recording the amount of sucrose solution 50% (*w*/*v*) consumed daily in each cage (Martín-Hernández et al. 2011). Experiments finished when all the bees were dead.

The following experimental groups were compared: (1) infested bees with raw propolis, (2) infested bees without propolis, (3) non-infested bees with raw propolis, and (4) non-infested bees without raw propolis. In the propolis treatments, 0.5 g of raw powdered propolis was placed inside the cage over the sheets of wax comb. The two propolis samples collected in August and September 2015 were used in two separate experiments performed in May and October 2017, respectively. In each experiment, all treatments were replicated with three independent cages and were set up on the same day.

Parasite bioassays

The effect of raw propolis on *Varroa* activity was investigated according to Garedew et al. (2002) and Damiani et al. (2010) with various modifications; briefly, 100 μL of distilled water was applied on 3 cm × 3 cm absorbent paper 67 g/mq (APTACA SRL, Canelli, Italy) in a Petri dish and adding 0.25 g of powdered propolis. The propolis thus adhered to the absorbent paper creating a viscous film that prevented the mechanical effect of the powder on the mite. Distilled water was used as a control treatment. Subsequently, six mites were put in each Petri dish corresponding to 36 mites per treatment (six replicates). Mite activity was observed under a stereo microscope every 15 min for the first hour, every 20 min for the second hour, and every 30 min for the next 3 h. The two propolis samples collected in August and September 2015 were used in two distinct experiments performed in July and October 2017, respectively. Mites were considered inactive when they showed no response to the stimulus (Milani 1995). All experiments were performed at ambient temperature (31 °C).

Statistical analysis

All statistical analyses were performed using R statistical software v 3.3.2 (R Core Team 2017).

Survival analysis was performed in R using a mixed effects Cox proportional hazard model (R package 'survival' (Therneau and Grambsch 2000), and the R package 'coxme' (Therneau 2015), with experimental groups (control vs. control + propolis vs. *Varroa* infested vs. *Varroa* infested + propolis) and month (May and October) of experiment as fixed factors and cage (replicate) as a random effect factor. A further post hoc analysis was performed using the R package 'multcomp' (Hothorn et al. 2008), applying a Bonferroni correction for multiple testing. We used linear mixed-effect modeling (LMMs) for repeated measures to investigate the effects of treatment (control vs. control + propolis vs. *Varroa* infested vs. *Varroa* infested + propolis) and date (May and October) on the cumulative sucrose consumption per bee. Cage (replicate) was again used as a random effect factor.

We also used LMMs for repeated measures to investigate the effects of propolis and date of the experiment (July and October) on *Varroa* inactivity. Petri dish was used as a random effect factor. A further post hoc analysis was performed using 'multcomp', applying a Bonferroni correction for multiple testing. We used LMMs to investigate the differences in the chemical composition of the two samples (August vs. September) of propolis used in our bioassays. Colony was used as a random effect factor.

When models included several factors, we used automated model selection based on the Akaike information criterion (AIC) using the 'dredge' function (R package 'MuMIn' (Bartoń 2018). All mixed models were performed using the R package 'lme4' (Bates et al. 2015) and all model assumptions were checked visually.

Results

Propolis chemical analysis

The two propolis samples used in the separate bioassay showed significant differences in their total polyphenol (LMM: $\chi^2 = 56.517$, $P < 0.001$, Table 1) and total flavonoid content (LMM: $\chi^2 = 42.961$, $P < 0.001$, Table 1) with the highest content in the propolis collected in the first period (16/8/2015 vs. 11/09/2015).

On average, the content of individual polyphenolic compounds was 50% higher in the propolis collected in the first period (August) than that collected in the second period

Alessandra Mura
Studies on the behavioral responses of *Apis mellifera* to the pathogen *Nosema ceranae* and the parasite *Varroa destructor*, and on the predatory wasp *Vespula germanica* in apiary
Tesi di Dottorato in "Scienze Agrarie"
Curriculum "Monitoraggio e Controllo dei Sistemi Agrari e Forestali in Ambiente Mediterraneo"
Università degli Studi di Sassari

Table 1 Total phenolics, total flavonoids, and polyphenolic compounds in propolis used in our bioassays and collected from seven untreated colonies in two different periods

Chemical components	Sample 1 (August 2015)	Sample 2 (September 2015)
Total polyphenols[a]	570.2 ± 20.5	413.5 ± 18.8
Total flavonoids[b]	74.8 ± 2.6	57.7 ± 2.4
Caffeic acid[c]	4.95 ± 0.2	3.0 ± 0.1
p-coumaric acid[c]	0.74 ± 0.04	0.37 ± 0.02
Ferulic acid[c]	1.39 ± 0.07	0.96 ± 0.05
Quercetin[c]	1.21 ± 0.08	0.46 ± 0.03
Isorhamnetin[c]	1.73 ± 0.1	0.87 ± 0.04
Cinnamic acid[c]	2.12 ± 0.1	1.46 ± 0.1
Apigenin[c]	2.97 ± 0.1	1.45 ± 0.09
Kaempferol[c]	2.83 ± 0.1	1.18 ± 0.09
Chrysin[c]	35.09 ± 1.7	23.0 ± 1.0
Pinocembrin[c]	19.2 ± 1.1	9.8 ± 0.5
Galangin[c]	22.59 ± 1.0	13.1 ± 0.7
CAPE[c]	9.75 ± 0.69	6.7 ± 0.2

[a] Total polyphenols are expressed in g GAE/Kg DW = g gallic acid equivalent/kg dry weight of plant material

[b] Total flavonoids are expressed in g CE/kg DW = g catechin/kg dry weight of plant material

[c] The individual components are expressed in mg/g

(September). Chrysin was the main component in both propolis samples with values of 35.1 and 23.0 mg g^{-1}, respectively. Myricetin, acacetin, and rosmarinic acid were not detected in our study.

No acaricide residues were detected in either of the propolis samples.

Host bioassays

Experimental treatment ($\chi^2 = 322.577$, $P < 0.001$, Fig. 1), month ($\chi^2 = 87.672$, $P < 0.001$, Fig. 1), and their interaction ($\chi^2 = 103.445$, $P < 0.001$, Fig. 1) were significant predictors of bee survival. The treatment of *Varroa*-infested bees with propolis significantly decreased mortality in both May (hazard ratio: $z = -8.996$, $P < 0.001$, Fig. 1a, c) and October (hazard ratio: $z = -8.876$, $P < 0.001$, Fig. 1b, d) compared to the respective *Varroa*-infested groups without propolis. *Varroa*-infested May and October bees did not differ in their mortality (hazard ratio: $z = 1.071$, $P = 1.00$). Similarly *Varroa*-infested May and October bees treated with propolis did not differ in their mortality (hazard ratio: $z = 0.378$, $P = 1.00$). Additionally, there was no difference in mortality between *Varroa*-infested May bees treated with propolis and control bees (hazard ratio: $z = 0.302$, $P = 1.00$, Fig. 1a, c) and *Varroa*-infested May bees treated with propolis and control bees treated with propolis (hazard ratio: $z = 1.503$, $P = 1.00$, Fig. 1a, c). However, the *Varroa*-infested October bees treated with propolis had a higher mortality both compared to control bees (hazard ratio: $z = 9.574$, $P < 0.001$, Fig. 1b, d) and compared to control bees treated with propolis (hazard ratio: $z = 10.425$, $P < 0.001$, Fig. 1b, d).

The experimental group was not a good predictor of bee sucrose consumption (repeated measures LMMs: $\chi^2 = 3.664$, $P = 0.300$, Fig. S1). *Varroa*-infested bees consumed a similar amount of sucrose compared to control bees (May: *Varroa*-infested vs. control; $z = -0.396$, $P = 1.000$; October: $z = -1.396$, $P = 1.000$). The amount consumed by the bees significantly increased over time ($\chi^2 = 6160.100$, $P < 0.0001$, Fig. S1) and bees sampled in May consumed a significantly higher amount of sucrose compared with bees sampled in October ($\chi^2 = 353.590$, $P < 0.001$, Fig. S1).

Parasite bioassays

The activity of *Varroa* mites was significantly negatively affected by the propolis treatment across both experiments (July and October) (LMMs: $\chi^2 = 40.015$, $P < 0.001$, Fig. 2a, b). Additionally, we found a significant three-way interaction between the treatment course of the experiment and the month (LMMs: $\chi^2 = 4.149$, $P = 0.041$, Fig. 2a, b). The number of inactive mites did not differ between July and October control treatments (LMMs: $z = 0.178$, $P = 0.859$). However, the number of inactive mites in the propolis treatment was higher in July compared to October experiments (LMMs: $z = 3.906$, $P < 0.001$).

Discussion and conclusion

In a previous study, we demonstrated that *A. mellifera* colonies under stress conditions due to *Varroa* infestation increase the number of resin foragers, although we found no evidence of a general intensification of foraging activity (Pusceddu et al. 2017). This result was in line with the first criterion which defines self-medication behavior (Clayton and Wolfe 1993).

The present paper clearly demonstrates the positive effects of raw propolis on the lifespan of *Varroa*-infested adult bees. In fact, in both experiments, the infested bees reared with propolis showed a twice median survival time compared with infested bees reared without propolis. This result is in line with the third criterion which defines the self-medication behavior (Clayton and Wolfe 1993). The longer lifespan observed in the bee workers belonging to *Varroa*-free groups sampled in October compared to those sampled in May is likely due to the gradual increase in the proportion of winter bees (Mattila et al. 2001). Another factor that may have influenced the survival rate of bees in the two experiments may have been the seasonal variation of viroses incidence (Tentcheva et al. 2004). Interestingly, in the first bioassay (May 2017), which was performed using the propolis with the highest content of polyphenols, the survival curve of the *Varroa*-infested bees

Alessandra Mura
Studies on the behavioral responses of *Apis mellifera* to the pathogen *Nosema ceranae* and the parasite *Varroa destructor*, and on the predatory wasp *Vespula germanica* in apiary
Tesi di Dottorato in "Scienze Agrarie"
Curriculum "Monitoraggio e Controllo dei Sistemi Agrari e Forestali in Ambiente Mediterraneo"
Università degli Studi di Sassari

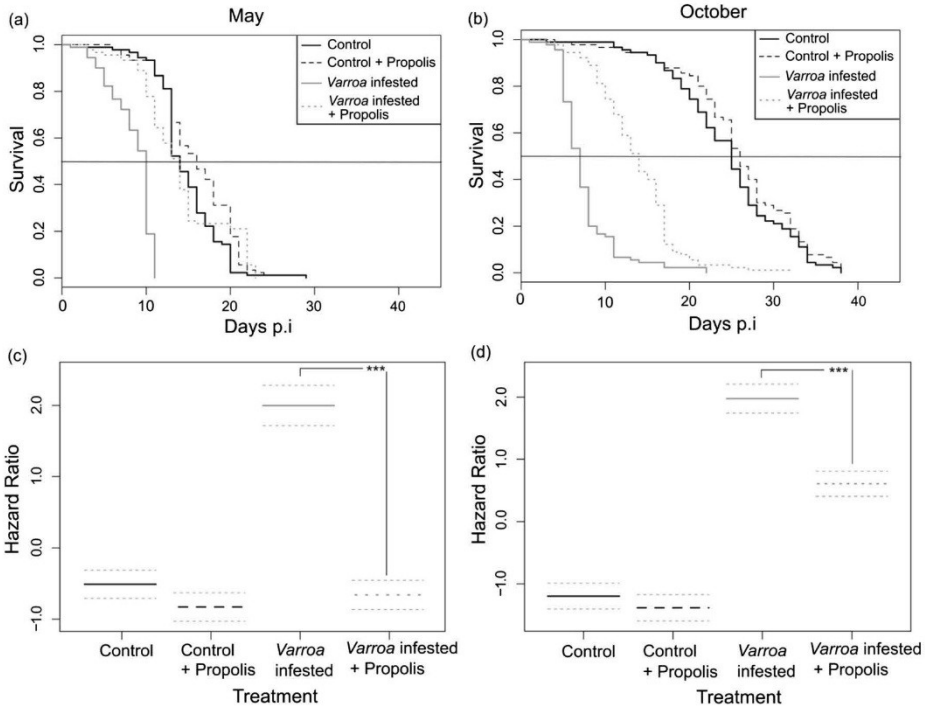

Fig. 1 Kaplan-Meier survivorship curves of *A. mellifera* workers infested by *V. destructor* in comparison with non-infested bees (control groups), both maintained with and without propolis (0.5 g of propolis per cage). Instantaneous risk of death (hazard ratio, ± 95% CI) for honeybees in each experimental treatment and month compared with the model average of 0

treated with propolis was similar to that observed in the control group with and without propolis.

It is still not clear how the propolis influences the adult bee's fitness. We can speculate an effect on the parasite, on the host or on both assuming that one does not exclude the other. Our study suggests a direct effect on the parasite in line with the second criterion of Clayton and Wolfe (1993). In fact, we found evidence of a narcoleptic effect of raw propolis on phoretic mites after a contact lasting 5 h. However, the effects shown by raw propolis are very low compared with those observed using ethanolic extracts (Garedew et al. 2002, 2003; Damiani et al. 2010). In fact, the percentage of inactivated mites varied only between 19 and 22%. The highest number of inactive mites was observed with the propolis with the highest polyphenol content.

However, Simone-Finstrom and Spivak (2010) suggested that a narcoleptic effect could also negatively affect the success of the mites thereby leading to a lower heat production and metabolic rate. Moreover, Drescher et al. (2017) found no acaricide or narcoleptic effects of volatile compounds released from crude propolis on *Varroa* mites. On the other hand, unlike in Drescher's experiment, in our bioassays, the direct contact between *Varroa* and propolis was not prevented. Hence, the contact time as well as the propolis composition and origin may have played an important role in affecting propolis activity in relation to the parasite.

Through its narcoleptic effect, propolis can interfere with the *Varroa's* ability to feed itself from the host, thus reducing the transmission of the viruses associated with it. It has also been demonstrated that the deformed wing virus (DWV) usually associated with *V. destructor* infestation (Ball and Allen 1988; Martin 2001) can also be transmitted horizontally through feeding and trophallaxis since it has been found to be infectious in larval food, pollen, and honey inside the hive (Yue and Genersch 2005; Yue et al. 2007; Mazzei et al. 2014).

In line with the above studies and considering that propolis is known to help combat human viruses (Marcucci 1995), the hypothesis that propolis may also have a direct action on the horizontal virus transmission should be considered. Drescher et al. (2017) observed that DWV titers increased to a significantly lower extent in colonies with added propolis than in propolis-removed colonies, whereas SBV titers were similar. This thus supports the hypothesis that propolis may interfere only with the dynamics of *V. destructor*-transmitted viruses.

Another hypothesis concerning the modality through which propolis could affect the lifespan of *Varroa*-infested honeybees

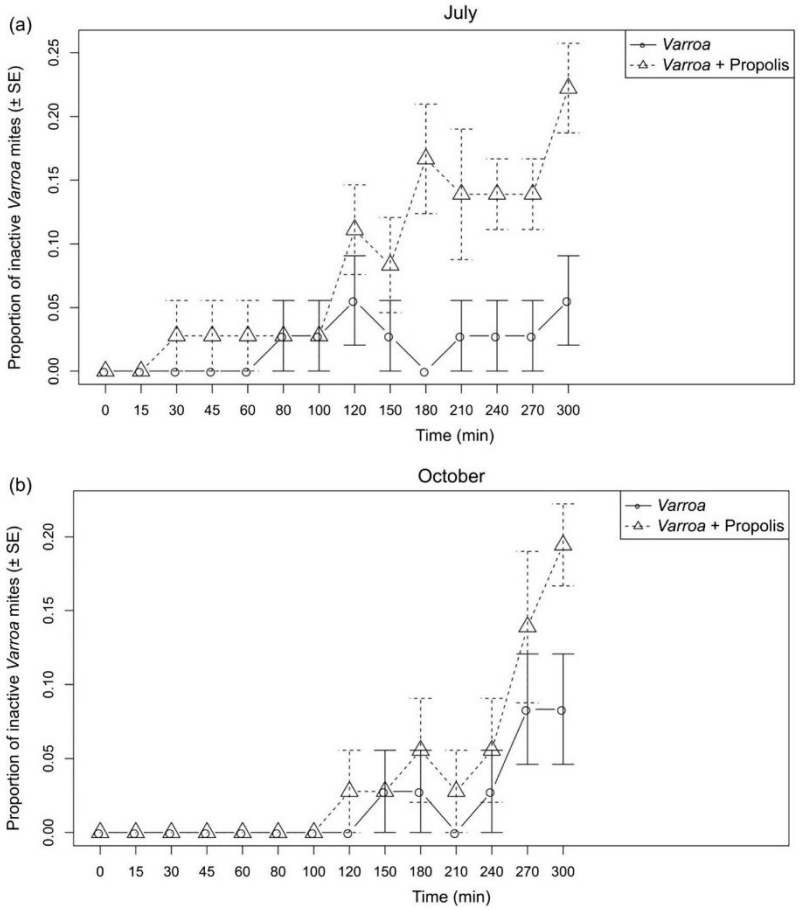

Fig. 2 Proportion of inactive *Varroa* mites treated with raw propolis and in the control. Data represent mean values across cages with standard error bars

is the strengthening of the individual immune system. One of the main functions of propolis in the nest is to regulate the costs of the immune system activity by reducing the investment in immune expression when the colony is *unchallenged* (Simone et al. 2009; Borba et al. 2015; Simone-Finstrom et al. 2017). Since the immune system is one of the greatest physiological costs in animals, a decrease in energetic costs associated with it allows the bees to invest their energy toward vital tasks such as foraging, rearing broods, and maintaining higher protein levels (e.g., vitellogenin) in their hemolymph required for overwintering survival (Borba 2015).

However, Borba (2015) and Borba and Spivak (2017) demonstrated that nurse bees from colonies with a propolis envelope challenged by *P. larvae* showed a higher expression of antimicrobial peptides than colonies without a propolis envelope and higher antimicrobial activity of larval food. It is unclear if the increase in antimicrobial activity of the larval food was due to the presence of antimicrobial peptides produced by adult bees and incorporated into larval food, or to the presence of active compounds from the propolis in the food. In terms of the energetic stress, contrary to findings observed in bees infected by the "gut parasite" *Nosema* spp. (Mayack and Naug 2009; Martín-Hernández et al. 2011), we did not detect differences between *Varroa*-free and *Varroa*-infested bees in terms of the daily demand for sucrose solution.

In conclusion, our findings seem to confirm the hypothesis that the resin collection and propolis used in the hive may represent an example of self-medication behavior in social insects. However, before drawing definitive conclusions, other important aspects merit careful investigation, particularly the effects of propolis on the mite reproductive phase, and the possible existence in the hive of substances (for example derived from the salivary glands) that could promote the release of an active ingredient from the non-water soluble propolis fraction.

Alessandra Mura
Studies on the behavioral responses of *Apis mellifera* to the pathogen *Nosema ceranae* and the parasite *Varroa destructor*, and on the predatory wasp *Vespula germanica* in apiary
Tesi di Dottorato in "Scienze Agrarie"
Curriculum "Monitoraggio e Controllo dei Sistemi Agrari e Forestali in Ambiente Mediterraneo"
Università degli Studi di Sassari

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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CHAPTER 3

Title:

"Feeding strategies and intraspecific competition in German yellowjacket (*Vespula germanica*)"

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Feeding strategies and intraspecific competition in German yellowjacket (*Vespula germanica*)

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Abstract

The German yellowjacket (*Vespula germanica*) is an opportunist predator and a scavenger, whose eclectic diet also includes honey, brood, dead and live honey-bees. There is no evidence in this species of coordinated attacks against bees involving other conspecifics, although intraspecific competition has been already reported between two or more individuals during feeding. Our aim was to gain further knowledge on the feeding behavior of *V. germanica* in order to evaluate its role in an apiary. Sight observations of predation and necrophagy behaviors were carried out at the ground level near hives. We also investigated how intraspecific competition can influence the feeding display in this species. Our results confirm the major role of the German yellowjacket as a scavenger, because its diet is based mostly on bee carrions. Intraspecific competition during feeding was sometimes observed. When these events occurred, the interference of another wasp led to the bee escaping only in three cases. Our study also revealed that intraspecific competition events increase when the resource is fresh (predation *vs* necrophagy), and that the number of competing wasps was significantly higher when the food consisted of pupae and drones, compared to adult bees. When competition involved two individuals (the most frequent case), the winner was frequently the first wasp to reach the resource in both predation and necrophagy events. This suggests that the energy invested in foraging or predating activity and in defence of prey is usually rewarded.

Introduction

Vespula germanica is a social wasp that is widespread in much of the world. Its native distribution includes Europe, northern Africa, northern India, Korea and China [1], however it is also a successful invasive species in Argentina [2] and Australia [3]. The global success of this species is mainly determined by its food plasticity, which makes it easily adaptable to different environments [4,5,6]. In fact, the European wasp is an opportunist predator and a scavenger because its eclectic diet includes small prey, vertebrate and invertebrate carrions, food and garbage from humans, and also carbohydrates from nectar, fruits and honeydew [7,8,9,10]. Coelho and Hoagland [11] reported that in the late summer and early autumn, German

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yellowjackets feed on dead honey-bees found at the ground level near hives. They also steal honey or prey on adult bees and brood [12,13]. Usually the wasps dismember their prey at the cervix and/or petiole level [14], and fly off with selected parts of the dead bee [15]. Aebi and Aebi [16] reported that *Vespula* spp. prefer to sequestrate the abdomen, while according to Duncan [15] and Winston [17] they prefer the thorax, and according to Free [12], they prefer the thorax and abdomen over the head, but specific studies on *V. germanica* are lacking.

Despite this opportunistic feeding behavior, German yellowjacket foragers preserve the memory of their past experience, in fact they return to eat at previously successful foraging sites [9,6,18,19] and show an associative learning between local landmarks (visual, spatial and odor cues) and a certain food source [9,5,20,21,22,23]. The foraging behavior of *V. germanica* may change in relation to the habitat: wasps returned to the original feeding site more frequently in closed habitats than in open ones, probably because closed habitats offer more landmarks to guide the foragers to the food source [2].

Other abiotic factors that affect the daily foraging activity of *V. germanica* include rain, low and high temperatures and low light (e.g. fog) which reduce foraging [24]. Social mechanisms may also contribute to successful finding of alimentary resources. In fact, *V. germanica* foragers are attracted by the presence of conspecifics at food sources by local enhancement processes [25,26] and they can influence other naive foragers to search for an odor sampled inside the nest [27]. Lozada et al. [10] show that there is evidence of social communication for forager recruitment even at a distance from the resource: when foragers return to the nest after the foraging trip, the subsequent number of wasp foragers was approximately four times higher compared to when communication with the nest was not possible.

However, individual foraging and independent hunting typology have been described for this species that explain how several individuals from different colonies can find themselves at the same foraging site and defend their own prey from other conspecifics [12,13]. Intraspecific competition for food as an aggressive interaction between two or more German yellowjackets has also been reported by Parrish and Fowler [28] also when food supplies were not scarce. In areas with a high wasp density but without enough prey, cannibalism has also been observed [3].

In areas where it has been introduced, *V. germanica*, may also have an ecological impact on the natural ecosystem and is considered a pest for certain human activities [29,30,31,32]. In particular, the economic damage caused by this wasp on beekeeping is due to the costs incurred for destroyed or damaged hives (approx. 9% of the total number of hives) and to productivity losses [29], due also to the strong competition for the honeydew resource [33]. Controlling the *V. germanica* population, which is normally based on the use of poison-baits [29,34], also has a financial cost.

Although these studies show that yellowjacket represents a problem in many countries, the biology of this species in native areas has not been studied sufficiently. In a previous work [13] we studied the agonistic interactions between *V. germanica* and *A. mellifera* [13], our aim in the present work was to assess the economic damage of this species for the beekeeping industry through the evaluation of the impact on the bee colonies of the wasp's feeding activity. Through behavioral observations in the field, we investigated the predation and necrophagy behavior, as well as the dismemberment pattern and preferences in the collection of body parts. Finally, the influence of intraspecific competition on the feeding display of this wasp was also assessed.

Materials and methods

Experimental apiary

The study was performed in an experimental apiary in northwest Sardinia in fall 2017, at the experimental farm of the University of Sassari, Department of Agriculture (latitude 40˚46'23",

longitude 8˚29'34"). The apiary, which is located in an area where the predation activity of *V. germanica* on honeybees has been reported since September 2014 [13], comprised 10 *A. mellifera ligustica* colonies maintained in Dadan-Blatt hives containing 10 combs each. During the experimental period, the hives were checked every week to confirm the presence of the queen, as well as the pollen and nectar provision, and to evaluate the sanitary status (symptoms of diseases and varroosis) [35].

The presence of *V. germanica* in our experimental apiary was also monitored from August 2017 using two wasp-traps (a bottle with beer) placed near the hives. After the arrival of the wasps (29/09/2017), the weekly number of *V. germanica* individuals along a transect (0.70 m x 4.25 m) traced in line with the apiary was recorded.

Behavioral observations

The feeding behavior of wasps on bees at the ground level was observed using the "all-occurrences sampling" method [36], by which the frequencies of a series of behavioral events were recorded as set out in the ethogram described below. We focused on the attacks targeted on *isolated bees* and *bees removing other bees* at the ground level (failed attacks and predation), and on dead bees (necrophagy behavior). It was not possible to record blind data because our study involved animals in the field. A total of 99 observation hours were conducted during the period in which the predatory and foraging activity of *V. germanica* is more intense (from late September 2017 to early November 2017) [12]. Two operators simultaneously observed the ground surface under five hives in two sessions per day, each lasting 45 min. These observations were conducted by sight, and the frequency (number of events per unit of time) for each of the observed predation and necrophagy behaviors was annotated. In addition, for each predation and necrophagy events, the degree of dismemberment and the specific body part sequestered by the wasps were reported.

Intraspecific competition between wasps

For each predation or necrophagy event in which intraspecific competition between wasps was observed, the arrival of subsequent wasps after the first was registered as well as the type of resource for which they competed (adult bees, drones, or pupae). When possible, the winner (the wasp that monopolized the resource) was identified in terms of arrival order (first, second etc.).

Ethogram at the ground level

Attack–The wasp grasps the bee and starts biting it (usually on the abdomen or between the head and thorax) [13].

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) [37].

Necrophagy–The wasp consumes the body parts of dead bees that it finds at the ground level [11].

Dismemberment–After predation or during necrophagy, the wasp divides the honeybee into different parts (head, thorax, abdomen; head + thorax and abdomen; head and thorax + abdomen) [12]. If the resource is a dead bee not intact (e.g. without head or without abdomen), the wasp may divide it further (e.g. head and thorax; thorax and abdomen).

Sequestration–After predation or necrophagy and having divided the honeybee into different parts, the wasp flies off with one of the parts, usually the thorax [15,17]. In some cases, the wasp may also carry the whole bee.

Retreat–The wasp escapes when the attack has not been successful and the honeybee defends itself effectively [13].

Killing wasp–Wasps can be killed by a single bee sting or by balling [13].

Intraspecific competition–Aggressive interactions between two or more individuals of *V. germanica* during feeding [28].

Statistical analysis

A chi squared test was used to measure the proportional difference in intraspecific competition, between necrophagy and predation events. The same test was used to measure the proportional difference in dismemberment and in sequestration behavior between competitive and non-competitive events during predation and also during necrophagy. To reduce the chances of a type I error, continuity correction was used for the chi-squared test when the sample size was less than 200 [38]. The Wilcoxon rank sum test (unpaired comparisons) was used to compare the number of wasps that compete in the predation and necrophagy events. To reduce the chances of a type I error in this analysis, we used Bonferroni correction in the case of multiple testing with significance set at $\alpha = 0.05/2 = 0.025$.

The Kruskal Wallis test was used to compare the number of wasps that competed (in the predation + necrophagy events) for different resources: pupae, drones and adult bees. Subsequently Dunn's post-hoc test with Bonferroni correction was used to find the significant differences. We also verified the correlation between the density of wasps under 10 hives and the number of competitive events detected in the following three hours using non-parametric Spearman correlation. All tests were carried out using R v 3.0.2 implemented with library: exactRanktests, coin and asbio.

Raw experimental data are available in supporting materials (S1 Datafile).

Results

Attack behavior

We observed 816 attacks at ground level in 99 hours, representing ~ 8.2 attacks per hour. Specifically, 760 attacks (93%) were targeted at *isolated bees* and 56 (7%) at *bees removing other bees*. The most frequent outcome was the wasp escaping without the prey (450 events, corresponding to 55%), while predation occurred on another 364 occasions (45%). Only in two cases was the wasp killed (0.3%). No significant differences in the success rate of the attacks comparing the two targets: *isolated bees* and *bees removing other bees* were observed (45% vs 34%; *chi-squared* = 2.33, *df* = 1, $P = 0.1269$). The attack data are summarized in Fig 1.

Predation behavior

Of the 364 bee predation events observed, 304 cases (83%) also involved victim dismemberment (Fig 2). The wasp eating its prey on-site was observed 42 times (11%), but more often, after predation and dismemberment, sequestration occurred (322 events, corresponding to 88%) with a preference for the thorax followed by the abdomen and the head + thorax (Fig 3). In 29 cases (9%) the prey was sequestered without dismemberment.

Necrophagy behavior

We observed 775 bee necrophagy events at ground level in 99 hours, corresponding to ~ 7.8 cases per hour. Necrophagy on *integral carrions* was observed 707 times (91%), while, in the remaining cases, we observed the wasp eating *non-integral carrions* (Head+Thorax or Thorax+Abdomen). The pattern of dismemberment observed on *integral carrions* was similar to that observed during

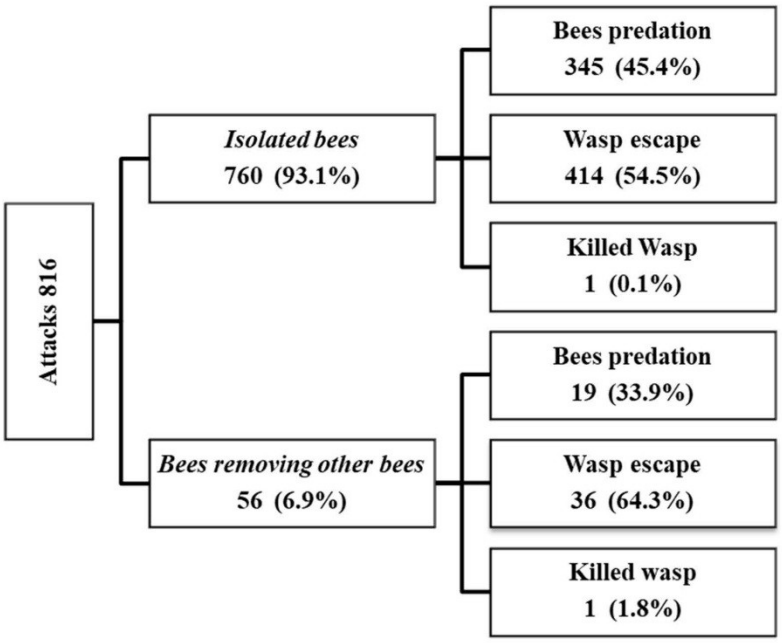

Fig 1. Outcome of attacks conducted at ground level toward *isolated bees* and *bees removing other bees*.

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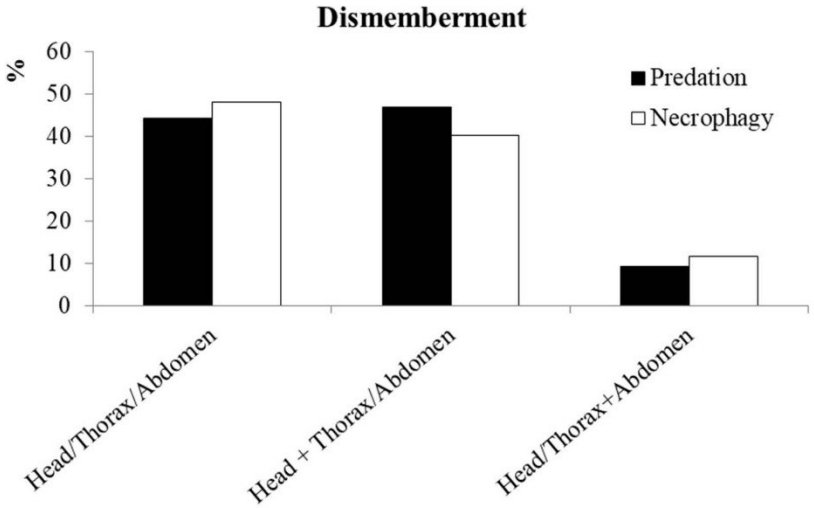

Fig 2. Proportion of different types of dismemberment in predation (n = 304) and necrophagy (n = 241) events.

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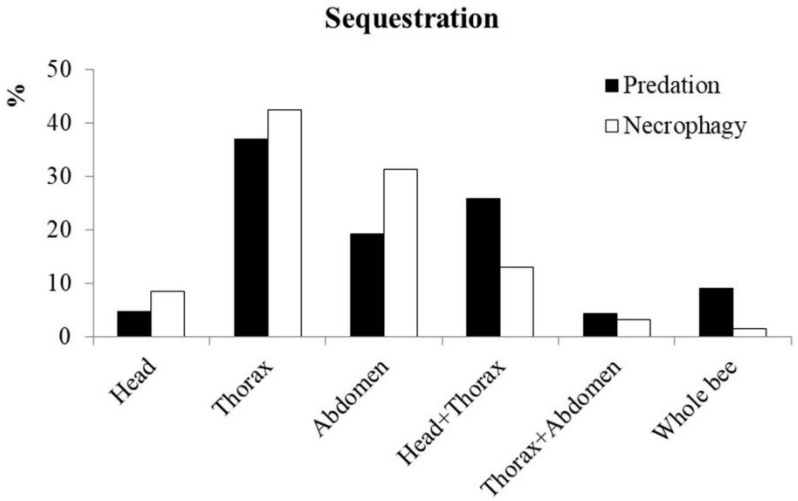

Fig 3. Proportion of different bee parts sequestered in predation (n = 322) and necrophagy (n = 575) events.

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predation (Fig 2), however the frequency was significantly lower (34% vs 83%) (chi-squared = 234.90, df = 1, P < 0.001). Conversely, when the target of necrophagy was *non-integral carrions*, dismemberment was always reported. After necrophagy, sequestration was observed in 575 cases, corresponding to 74%. This frequency was statistically lower than in the case of predation (88%) (chi-squared = 30.13, df = 1, P < 0.001). In the other 200 occasions (26%), the carrion was consumed on-site. The preference of body parts sequestered is summarized in Fig 3. In nine cases (1.6%) the carrions were sequestered without dismemberment.

Intraspecific competition

We observed intraspecific competition in both predation and necrophagy events however, in the first case, the rate at which it occurred was significantly higher (50% vs 23%) (*chi-squared* = 77.34, *df* = 1, *P* < 0.001). Only in three cases (0.7%) did the interference of another wasp lead to the failure of the attack and the bee escaping.

The average number of competing wasps was significantly higher when food consisted of pupae (1.8 ± 1.1) and drones (1.2 ± 0.5) compared to adult bees (2.3 ± 0.1) (Kruskal Wallis *chi-squared* = 58.33, *df* = 2, *P* < 0.001; Dunn's post-hoc test with the Bonferroni correction: pupae-drones = 1; pupae-adult bees = 1e-06; drones-adult bees = 0).

A positive linear correlation between the wasp density in the observation area and the number of competition events was also recorded (S = 10.5928, P = 0.0269, rho = 0.81).

In addition, when competition involved two individuals, frequently the winner was the wasp that first reached the resource in both predation (64%) and necrophagy (66%) events.

Finally, the influence of competition on dismemberment and sequestration behaviors was also observed. The frequency of dismemberment significantly increased when competition occurred, both in predation and necrophagy events as shown in Fig 4 (Predation: chi-squared = 4.29, df = 1, P = 0.038; Necrophagy: chi-squared = 50.24, df = 1, P < 0.001). Similar results were also observed for sequestration (Predation: *chi-squared* = 11.61, *df* = 1, *P* < 0.001; Necrophagy: *chi-squared* = 24.32, *df* = 1, *P* < 0.001 Fig 5).

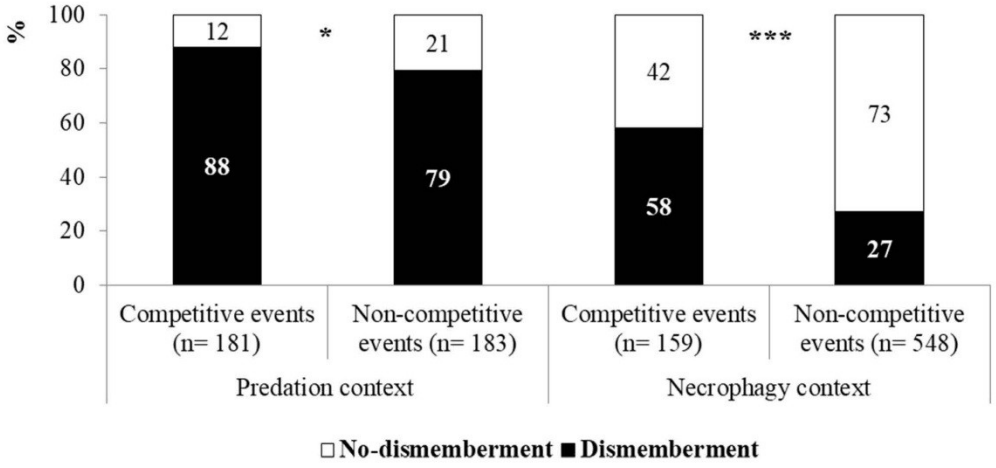

Fig 4. Percentage incidence of the dismemberment activity in competitive and non-competitive events in a predation (n = 181 and n = 183, respectively) and in a necrophagy (n = 159 and n = 548, respectively) context. In the necrophagy context only the events on *integral-carrions* were considered.

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Discussion

In a previous study regarding the agonistic interactions between *A. mellifera* and *V. germanica*, we observed that the wasp attacked the hive entrance infrequently due to the low success rate of this strategy, while preferring a specialized attack aimed at weak or isolated adult honeybees at ground level [13]. The results of the present work showed that the number of attacks on live

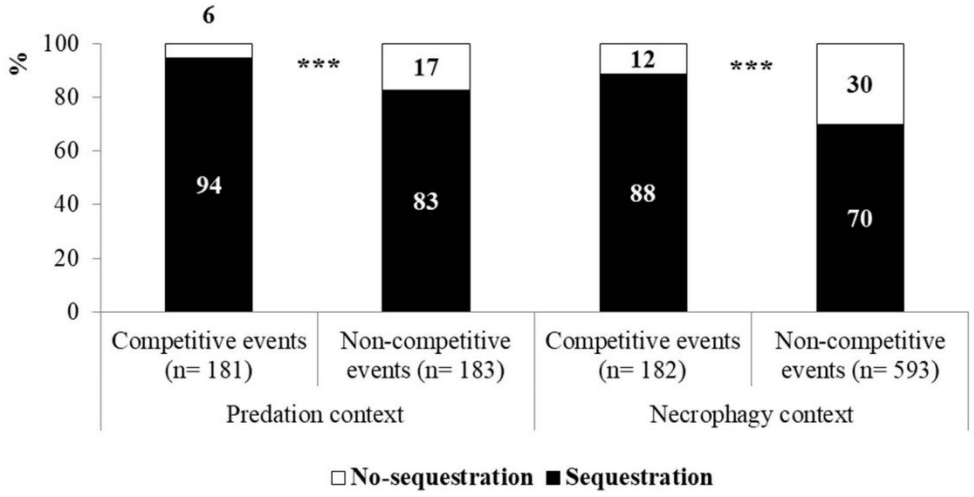

Fig 5. Percentage incidence of the sequestration activity in competitive and non-competitive events in a predation (n = 181 and n = 183, respectively) and in a necrophagy context (n = 182 and n = 593, respectively).

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bees at the ground level was balanced by the number of necrophagy acts. Considering that only half of the attacks on live bees were successful with predation, we can affirm that the diet of *V. germanica* is based mostly on bee carrions. This suggests that in our apiary context the main role of the German yellowjacket was as a scavenger and highlights the highly opportunistic behavior of the wasp which, avoiding any physical fight with the live bees defending themselves from the attack, minimizes the risk of dying or losing prey and obtains a good reward from carrions. The evolution of this low-cost foraging strategy can be explained by the optimal foraging theory, which in particular postulates a trade-off between the foraging behaviour and lifetime fitness [39]. However, considering the large plasticity of the alimentary behaviour of this species [4,5,6], there may be a serious impact on honey bee colonies when favourable environmental conditions occur (wasp nest density, food source availability, weak colonies). However, contrary to the general opinion of beekeepers, our research did not find evidence that the presence of the *V. germanica* represents a threat for the hives in native areas.

Unlike findings described for *Vespa tropica* [40], *Vespa velutina* and *Vespa crabro* [41], we never observed *V. germanica* capturing foragers in flight returning to the hive, despite being easy prey because they are weighed down by nectar or pollen load, and "tired" after the flight activity. *V. germanica* probably does not adopt this attack strategy because it is smaller than the other species of wasps cited above, preferring to attack its prey on the ground. Another attack strategy that we observed at the ground level was aimed at *bees removing other bees*. In this case the attacked bee is less ready to defend itself as it is engaged in another activity. In addition, it is weighed down by the carried bee who, if still alive, fights to avoid its removal from the nest. However, when this attack strategy is successful, the reward that the wasp obtains is represented by the removed bee and not by the bee that is doing the removing.

During the period of observation (from late September 2017 to end of October 2017) and within individual observation sessions, we noted a progressive increase in the number of foraging wasps at the same site. This can be explained by considering that German yellowjacket foragers are attracted by the presence of conspecifics at food sources [25,26] and by the fact that they preserve the memory of their past experience, particularly regarding the reward obtained at a food site [9,6,18,19]. Although this type of social facilitation probably took place during our experiments, competitive interactions between wasps were also observed. It is highly probable that several individuals from different colonies may find themselves at the same foraging site and defend their own prey from other conspecifics [12]. On the other hand, competitive interactions between individuals of *V. germanica* during feeding, even when the food resource was not scarce, have been described by Parrish and Fowler [28].

As in other animal species, a greater density of individuals at the same site favors the intraspecific competition for resources [42,3,43]. In fact, our data showed a positive correlation between the number of wasps present at the ground level and the number of agonistic interactions for food. However, only in three cases (0.7%) of the total intraspecific competition events observed, did the interference of another wasp lead to the failure of the previous attack and the bee escaping.

Our study also revealed that intraspecific competition increases when the resource is fresh (predation *vs* necrophagy). This outcome suggests that necrophagy, compared to predation, may represent the best trade-off between reward and energy costs (in terms of risk, energy investment in foraging on carrion and also in defending food from conspecifics).

We found the highest number of competing wasps when the food consisted of pupae and drones, compared to adult worker bees. In fact, pupae and drones are a larger and "unarmed" source of food compared to workers, and consequently represent a low risk for the wasp. Furthermore, Free [12] reported that the alimentary preference of *V. germanica* and *V. vulgaris* for pupae compared to adult bees, is probably due to the difference in cuticle hardness.

In addition, intraspecific competition led to a significant increase in the rate of the dismemberment and sequestration in both predation and necrophagy events. However, it did not affect the dismemberment pattern and body parts sequestered by the wasp, which was preferentially the thorax, probably due to its higher protein content compared to the head and abdomen [12].

Our study also revealed that many German yellowjacket individuals are able to sequester a whole bee. It has been already reported that *V. germanica* have a higher load-lifting capacity, compared to *V. squamosa* and *V. maculifrons* [11]. The highest theoretical load can be calculated considering different factors, including for example flight muscle mass. However, the wide intraspecific variation in load-carrying capacity, mainly depending on the size of the wasp, can influence the foraging ability of each individual [11]. Individual size and arriving early at the resource are factors that can also play an important role in intraspecific competition [44]. In fact, we found that when competition involved two individuals, the winner was frequently the first wasp that reached the resource in both predation and necrophagy events. This suggests that the energy invested in foraging or predating and in defence of their own prey is usually rewarded.

Finally, in the future it would be interesting to quantify how the individual size influences intraspecific competition in this species, particularly in relation to the competition involving two individuals which was the most frequent event observed in our study.

Supporting information

S1 Datafile.
(XLSX)

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Appendix 1 - Pictures taken during the study on

"Propolis consumption reduces *Nosema ceranae* infection of European honey bees (*Apis mellifera*)"

Figures: 1, Experimental apiary (Ottava, Sassari) **2,** Honey bee brood ready to emerge **3,** Experimental cages in incubator at 31 °C **4,** Bees fed *ad libitum* with 50% (w/v) sucrose solution administered using a syringe **5,** Arena used in the choice test **6,** A bee choosing a candy **7,** Scan test.

Appendix 2 - Pictures taken during the study on

"The effects of raw propolis on *Varroa*-infested honey bee (*Apis mellifer*a) workers"

Figures: 1, Bee colonies in the experimental apiary **2,** *Varroa* sampling from infested colonies using an inert dust "powdered sugar" **3,** Phoretic mites used in the mortality bioassays **4,** Honeybee brood ready to emerge collected from *Varroa*-free colonies **5,** Survival bioassay of adult bees in incubator **6,** Dead bees counted and removed from the cages every day to monitor bee longevity **7,** Bee pupae collection for parasite bioassay **8,** Raw propolis ("Propoli grezza") used in the parasite bioassay.

Appendix 3 - Pictures taken during the study on

"Feeding strategies and intraspecific competition in German yellowjacket (*Vespula germanica*)"

Figures: 1, Observations in apiary using the "all-occurrences sampling" method **2-3,** Attacks of *Vespula germanica* on the hives **4,** Attack of *Vespula germanica* on an isolated bee pupa **5,** Dismemberment and feeding of the bee abdomen.