Resin foraging dynamics in Varroa destructor-infested hives: a case of medication of kin?

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

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Abstract

Social insects have evolved colony behavioral, physiological and organizational adaptations (social immunity) to reduce the risks of parasitization and/or disease transmission. The collection of resin from various plants and its use in the hive as propolis, is a clear example of behavioral defense. For *Apis mellifera*, an increased propolis content in the hive may correspond to variations in the microbial load of the colony and to a down-regulation of an individual bee's immune response. However, many aspects of such antimicrobial mechanism still need to be clarified. Assuming that bacterial and fungal infection mechanisms differ from the action of a parasite, we studied the resin collection dynamics in *Varroa destructor* infested honeybee colonies. Comparative experiments involving hives with different mite infestation levels were conducted in order to assess the amount of resin collected and propolis quality within the hive, over a two year period (2014 and 2015). Our study demonstrates that when *A. mellifera* colonies are under stress because of *Varroa* infestation, an increase in the number of resin foragers is recorded, even if a general intensification of the foraging activity is not observed. A reduction in the total polyphenolic content in propolis produced in infested vs uninfested hives was also noticed. Considering that different propolis types show varying levels of inhibition against a variety of honey bee pathogens in vitro, it would be very important to study the effects against *Varroa* of two diverse types of propolis: from *Varroa* free and from *Varroa* infested hives.

Introduction

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

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

Mostly studied in higher vertebrates (Gompper & Hoylman, 1993; Gwinner *et al.* 2000; Wimberger, 1984; Wrangham & Nishida 1983), self-medication was also observed on a variety of solitary insects, such as *Grammia incorrupta* (Singer *et al.* 2009; Smilanich *et al.*

2011) and *Drosophila melanogaster* (Milan *et al.* 2012). In eusocial insects, it is necessary to distinguish between self-medication and medication of kin, which extends the self-medication concept to the colony level (Abbott, 2014). In fact, eusocial insects add to their immunological individual defenses against pathogens and parasites (Schmid-Hempel, 2005), several evolutionary behavioral and organizational adaptations within the colony (Cotter & Kilner 2010). Some of these defense mechanisms generally prevent or limit disease transmission, while others are induced by the presence of either parasites or pathogens. This "social immunity" system results from single member cooperation toward reducing the disease transmission risks typically associated with social life (Cremer *et al.* 2007). A higher exposure to pathogens and parasites is indeed expected as a consequence of high population density, frequent physical interactions among colony members, and the continuous use of the same nesting sites with microclimatic conditions (i.e., temperature and relative humidity) favoring the development of microorganisms (Schmid-Hempel, 1998). The reduced number of immune-related genes in *Apis mellifera* in comparison with other insect species, is in line with observations on other Hymenopteran species (Barribeau *et al.*, 2015). Different social immunity behaviors have been observed on the honeybee. These include social fever (Starks *et al.* 2000), hygienic behavior (Ibrahim & Spivak, 2005), allogrooming (Pettis & Pankiw, 1998), and self-medication through ingestion (Gherman *et al.* 2014). An interesting and scarcely studied self-medication behavior (by contact or proximity) involves the collection and use of resins in the hive (Simone-Finstrom & Spivak 2012). These viscous and complex substances are normally secreted by plants that exploit their bioactive properties to protect against parasites and pathogens (Langenheim, 2013; Simone *et al.* 2009; Simone-Finstrom & Spivak 2010). After being collected from diverse plant species, resins are carried to the colony where they are mixed with wax and incorporated into the hive structure as propolis (Simone-Finstrom & Spivak 2010). The colony mechanisms regulating resin collection have

not been clarified. Besides, how workers communicate the need of collecting resins to other colony members is still under investigation (Nakamura & Seeley, 2006). It was demonstrated that an increased propolis content in the hive may correspond to a decrease in its microbial load (Simone *et al.* 2009), even if such effect was not observed by Borba *et al.* (2015). On the other side, a significant down regulation of individual immune-related genes was reported (Borba *et al.* 2015; Simone *et al.* 2009). Moreover, an increase in resin collection after infections of the fungus *Ascosphaera apis* was observed, suggesting a therapeutic use of propolis in the hive (Simone-Finstrom & Spivak 2012). Nevertheless, such response does not appear to be associated with the action of the American foulbrood agent, *Paenibacillus larvae* (Simone-Finstrom & Spivak 2012).

Assuming that bacterial and fungal infection mechanisms can be different from the action of a parasite, the objective of this study was to verify if the amount of resin collected and propolis quality within the hives infested by *Varroa destructor* were different from non-infested ones. We propose two hypotheses to explain the behavior of resin foragers in response to *Varroa* parasitism: 1) an increase in the usually collected amount of resins (quantitative hypothesis); and 2) an increase in the bioactive substance content (i.e., polyphenols and flavonoids) in propolis (qualitative hypothesis). The quantitative hypothesis is based on the antiparasitic, antimicrobial and antioxidant properties of propolis (Dresher *et al.*, 2017; Huang *et al.* 2014; Marcucci, 1995), mostly associated with its polyphenolic and flavonoid content (da Silva *et al.* 2006; Siripatrawan *et al.* 2013). Acaricidal effects of propolis extracts against *V. destructor* have been reported (Damiani *et al.* 2010; Garedew *et al.* 2002). The qualitative hypothesis is based on the ability of *A. mellifera* to select different kinds of resins (Erler & Moritz 2015; Isidorov *et al.* 2016; Loenhardt *et al.,* 2009). For instance, a preference for *Baccharis dracunculifolia* (alecrim plant, Asteraceae) females versus males (Teixeira *et al.* 2005), for buds and younger leaves (Park *et al.* 2004), or for

plants producing resins with specific antimicrobial properties (Wilson *et al.* 2013), were reported. Besides, how bees may benefit from different resin sources was also observed (Drescher *et al.* 2014). Accordingly, Popova *et al.* (2014) demonstrated that the percentage of bioactive compounds (caffeic acid and pentenyl caffeates) was higher in *Varroa* tolerant colonies compared to non-tolerant ones. All these findings suggest that honeybees are able to follow a chemical "trace" leading toward a resin source and to evaluate its quality (Simone-Finstrom & Spivak 2010). In order to verify our hypotheses, comparative experiments involving hives with different mite infestation levels were conducted over a two year period (2014 and 2015), assessing the amount of resin collected and propolis quality in the hive. For this purpose observations on resin foraging dynamics in the hive were conducted along with chemical analyses on propolis samples to quantify the total polyphenol and total flavonoids content.

Materials and methods

Experimental apiary

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

Experiments

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

A first experiment was conducted in July 2014 (experiment 1) on 18 colonies that did not receive any previous management intervention (e.g., equalization of colony strength, supplementary feeding, etc.), including no chemical or biological treatments against parasites and/or pathogens. Colony inspection, routinely conducted on a biweekly basis, did not report any symptoms of the main honeybee diseases (bacterial, viral and/or nosemosis). In total 22.5 h of observations were conducted to assess the number of resin and pollen foragers and the number of removed adults in hives with different adult infestation levels (from 2.4 to 8.7 %) and different colony strength (from 11242 to 31171 adult workers + sealed brood cells). Following the outcome of observations conducted in 2014 on colonies with varying mite infestation levels, the approach of experiments carried out in 2015 involved the manipulation of infestation levels using acaricidal treatments and strength equalization among different colonies through frame removal from stronger families, two months before starting experiments. Observations were therefore conducted on two experimental hive groups: 1) *Varroa* free group, where *Varroa* infestation was maintained close to zero with acaricidal treatments, and 2) *Varroa* infested group, where no treatments were applied and the mite population could naturally increase. Treatments were based on Apivar® (a.i. amitraz) application, a strip-based commercial formulation with long term action, suitable for acaricidal treatments in presence of sealed brood. Preliminarily, the possible effects of acaricidal treatments on resin and pollen collection behavior of honeybee foragers were verified. For this purpose, specific observations (experiment 2) were conducted in July 2015 on two hive groups (treated and control) having equivalent strength and a low mite infestation level (1.0 ± 0.7 % and 1.2 ± 0.5 %, respectively). During this experiment, no acaricidal

170 treatments were applied to the control group, while in the treated group, Apivar® applications
171 were performed three days after video-recording started. In total 15 h were recorded during
172 the three days before treatments (pre-treatment) and further 15 h in the three days after
173 treatment (post-treatment). Both experimental groups initially included six colonies, but two
174 colonies in the treated group were excluded from data analysis as they were orphaned during
175 the experimental period.
176 Although ascertaining that amitraz treatments did not produce significant effects on resin
177 collection, the following experiments were conducted ensuring that no strips were present
178 inside treated hives (*Varroa* free group). For this purpose, strips were removed a week before
179 video-recording operations started and were put back in place afterwards. Two additional
180 experiments, using the same 6 colonies for each group, were conducted in August
181 (experiment 3) and September (experiment 4), when *Varroa* infestation percentages in the
182 infested group increased from 2.8 ± 0.4 to 6.7 ± 1.0, respectively. In total 36 h video-
183 recording was conducted in each of these experiments.
184 A final experiment (experiment 5) was conducted under the same conditions and with
185 analogous observation time in October after the average mite infestation level in the *Varroa*
186 *infested* group was reduced to the same level as the *Varroa* free group, through Apivar®
187 treatment during four weeks. This experiment was conducted in order to exclude the possible
188 influence of other pathogens carried by *Varroa* in the observed behavior. Also in this case,
189 each experimental group initially involved six colonies, but just one in the *Varroa* free group
190 was excluded from data analysis because orphaned during experiments. A colony in the *ex-*
191 *Varroa* infested group was also excluded because its infestation level was still too high
192 (3.1%).
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194

195 *Resin foragers detection*

196 The number of resin foragers returning to the hive was determined using "*all occurrences*
197 *sampling*" method (Altmann, 1979; Simone-Finstrom and Spivak, 2012). To measure the
198 total foraging force of hives used in the experiments, the number of pollen foragers was also
199 determined. In order to compare the use of propolis to other social defense mechanisms
200 potentially implicated in parasite management, the number of adult bees (dead or dying)
201 removed from the hive was counted; larvae were not considered as their removal was only
202 sporadically observed (3 times in 160.5 hours). Observations were based on video-recording
203 employing an HD camera (Canon LEGRIA HF R506) placed at around 20 cm from the hive
204 entrance. Following preliminary observations, 15 minutes (min) was established as the
205 standard duration of each video slot, as it allowed to count an adequate number of resin
206 foragers. For each experiment, video-recording sessions were repeated within the same time
207 slot (10:30-15:30) during consecutive days (5-6 depending on weather). Each colony, within
208 a group, was filmed daily according to a random pattern. In 2014, each hive was video
209 recorded for 15 min per day, while in 2015, two slots of 15 min each were dedicated to each
210 hive, so as to double observation time. Three days after starting video-recording, mite
211 infestation level in adult bees (Pappas and Thrasyvoulou, 1988) and colony strength,
212 considering an estimation of the total sealed brood extension and the amount of adult bees in
213 the hive, were assessed (Marchetti, 1985). For this purpose, one-sixth of a Dadant-Blatt frame
214 (188 cm^2) was used as a unit of measure converted in the tables of the results section in
215 number of sealed cells and adult bees obtained by multiplying the number of sixth of each
216 matrix for 780 and 254, respectively (Marchetti, 1985). After these surveys in the hive, 2-3
217 additional days of video-recording followed. Within the same experiment, each colony
218 received an equal number of observation hours, and video-recording activities were
219 simultaneously conducted in different experimental groups. All recorded videos (in total

160.5 h) were observed in slow motion by a single operator, who did not know the hive infestation level (blind experimental plan).

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

Chemical analysis of propolis

Newly produced propolis was sampled in between recording periods using specific collection nets placed above nest-combs (Bankova *et al.*, 2016). In October 2014, twelve propolis samples were collected from colonies with different colony strength and mite infestation level. In 2015, propolis was sampled twice (August and September) from twelve hives divided into two groups (*Varroa* free and *Varroa* infested) including six colonies each. In the *Varroa* free group, average infestation in both sampling was 0.1 ± 0.1 %, while in the *Varroa* infested group it ranged between 2.8 ± 0.4 % in August and 4.9 ± 0.8 % in September.

To collect a sufficient amount of propolis for chemical analyses, collection nets were maintained in the hives for 7-10 days in both years for each sampling period. Similarly to the behavioral experiments, amitraz strips were removed from the hives before placing propolis collection nets. After collection, propolis was prepared for chemical analysis as reported by Gómez-Caravaca *et al.* (2006) with the following modifications: after being ground to a fine powder with liquid nitrogen, about 50 mg of raw propolis was extracted with 2.5 ml of 80% ethanol for 24 h at room temperature and in the dark. The samples were then centrifuged for 10 min at 3900 rpm and the supernatant was stored at 4°C until use for chemical determinations. The total amount of polyphenols (Tot P) in propolis samples was determined using the Folin Ciocalteu method (Singleton & Rossi 1965) with modifications (Piluzza & Bullitta 2010). Results were expressed as g gallic acid equivalent kg^{-1} dry weight of propolis

material (g GAE kg^{-1}DW). Total flavonoids (Tot F) were determined by $AlCl_3$ method (Kim *et al.* 2003) with adaptations (Piluzza & Bullitta 2011). Results were expressed as g catechin equivalent kg^{-1} dry weight of propolis material (g CE kg^{-1}DW).

Statistical analysis

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

For experiments 1-5 we performed generalized linear mixed models (GLMMs) with Poisson error structure. For experiment 1 (2014) GLMMs was used to study the effects of *Varroa* infestation level, colony strength and their interaction on the number of resin and pollen foragers and of removed adults. For experiment 2 GLMMs were used to study the effects of time (pre vs post) and group treatment (Apivar®) vs control (untreated) on the number of resin and pollen foragers. For experiment 2, we used two approaches to evaluate the statistical power with which differences in resin and pollen foragers between Apivar® treated and control colonies could be detected. Firstly, given our sample sizes and the variation we observed in our dataset, we increased the difference between the two treatments and then tested for significance of differences in number of foragers. Secondly, we estimated the power of our current analysis (ß) and the sample size of colonies necessary to increase statistical power so as to be able to reject the null hypothesis of no difference in the number of foragers between control and Apivar® treatment using the R package 'simr' (Green & MacLeod 2016).

We used a GLMM model for experiments 3, 4 and 5 to study the effects of *Varroa* infestation level, on the number of resin, pollen foragers and number of removed adults. For this model, month was used as a random effect factor to account for temporal autocorrelation. To

describe change in the number of resin foragers, pollen foragers and adult removal behavior due to *Varroa* infestation (experiments 3 and 4), the difference between the number of foragers and removed workers in August and September was calculated for each colony and treatment group (sum in September-sum in August). Data were then analysed using a linear mixed model (LMM) with treatment as fixed effect factor. For all GLMMs and LMMs day of observation nested within each hive was treated as a random effect factor.

We used a general linear model (LM) to analyse the effects of *Varroa* infestation level on the total amount of polyphenols and flavonoids found in propolis samples collected in 2014. We used a LMM, to study the effects of *Varroa* infestation level (*Varroa* free vs *Varroa* infested) and sampling time (August and September) on the total amount of polyphenols and flavonoids found in propolis samples collected in 2015, including hive as a random effect factor to account for pseudo-replication.

We used automated model selection based on the Akaike Information Criterion (AICc), when models included several factors and their interactions (R package MuMIn; Barton, 2015). All mixed models were performed using the package lme4 (Bates *et al.* 2015). All model (GLMM, LMM and LM) assumptions were checked visually. For GLMMs, if over-dispersion was detected we used a negative binomial model (Zuur *et al.* 2009) implemented using the package glmmADMB (Fournier *et al.* 2012). To analyse single parameters and interactions we used a likelihood ratio test. We compared the goodness-of-fit between each model by setting up the model so that parameter can be dropped followed the examples in Zurr *et al.* (2009). We further analyzed mixed effect models to test differences between treatments with Bonferroni corrected post hoc tests. Post hoc tests were performed using the package multcomp (Hothor *et al.* 2008). All analysis was performed in R statistical software (R Core Team 2013).

Results

Experiments

In the experiment 1, the best model explaining variability in the number of resin foragers included only the level of *Varroa* infestation. However, the relationship was not significant (GLMM poisson: $Z = 1.487$, $P = 0.137$, R^2=0.52; Fig. 1a, Table S1). For the number of pollen foragers, the best model included both the level of *Varroa* infestation and colony strength. However, only colony strength (GLMM negative binomial: $Z = 6.58$, P = 4.8e-11, R^2=0.31; Table S1) and not the level of *Varroa* infestation (GLMM negative binomial: Z = -1.140, Z= 6.58, $P = 0.250$, R^2=0.31; Table S1) affected the number of pollen foragers (Fig. 1b and 1c). None of the two factors (level of *Varroa* infestation and colony strength) and their interaction explained variability in the number of workers removed from each colony.

Data of the second experiment, performed to assess possible effects of Amitraz[@] treatment on the number of resin and pollen foragers, are shown in figure 2. Both treatments had equal levels of colony strength and *Varroa* infestation level at the beginning of our experiment (for colony strength: Mann-Whitney *U* test: $U = 9.0$, N1 =4, N2 =6, $P = 0.609$; for *Varroa* infestation level: Mann-Whitney *U* test: $U = 12.0$, N1 = 4, N2 = 6, $P = 0.751$; Table 1). The best model explaining variation in the number of resin foragers included only time (pre and post treatment) and not treatment (treated group vs control group). There was a significant decrease in resin foragers in response to time (pre vs post) irrespective of treatment group (GLMM poisson: $Z = 3.356$, $P = 0.0007$, R^2=0.46; Table S1). Similarly, time (pre vs post, GLMM poisson: $Z = 3.949$, $P = 0.0008$ R^2=0.90; Table S1) and not treatment (GLMM poisson: $Z = 1.562$, $P = 0.118$, R^2=0.90; Table S1) was the main predictor for the observed variability in the number of pollen foragers. Our power analysis showed that, given our samples sizes and the variance detected in our dataset, if differences in mean number of resin and pollen foragers of the two treatments had been over 200% and 35%, respectively, the

differences would have been significant. Differences in resin and pollen foragers that we observed were clearly less than 200% and 35%. Indeed, the statistical power (ß) of our analyses given the observed differences was found to be very low, 9.00% (95%CI=7.30-10.95) for resin and 28.60% (95%CI=25.82-31.51) for pollen foragers, indicating that small differences, as we found, need a great number of colony replicates in order to detect a difference as statistically significant (estimated sample size, >8000 colonies per treatment for detecting differences in resin and >35 colonies per treatment for detecting differences in the number of pollen foragers). Overall, our power analyses suggest that the observed differences in numbers of resin and pollen foragers between Apivar treated and control colonies were minimal in our experimental paradigm.

In the experiment 3 (August 2015), we did not find any significant differences in the number of resin and pollen foragers, and removed workers (Bonferroni post hoc test: $Z = 1.244$, $P = 0.640$; $Z = 0.734$, $P = 1.000$ and $Z = 0.411$, $P = 1.000$, respectively; Table S2) between *Varroa* free and *Varroa* infested colonies (Fig. 3, 4 and 5). In the experiment 4 (September 2015), we found a significantly higher number of resin foragers and removed workers (Bonferroni post hoc test: $Z = 3.166$, $P = 0.004$ and $Z = 2.458$, $P = 0.042$, respectively; Table S2) in the *Varroa* infested compared to the *Varroa* free group (Fig. 3 and 5). No significant differences were found between the two groups considering the number of pollen foragers (Bonferroni post hoc test: $Z = 0.093$, $P = 1.000$; Fig. 4; Table S2). The mean difference in the number of resin foragers (sum in September-sum in August), was 1.85±0.35 for the *Varroa* infested colonies and 0.75±0.27 for the *Varroa* free colonies. Furthermore, the mean difference in the number of removed workers and number of pollen foragers was 0.46±0.17 and 25.4±3.8 for the *Varroa* infested colonies and -0.32±0.15 and 29.8±4.1 for the *Varroa* free colonies, respectively. Our LMM analysis showed a significant increase in the number of resin foragers (LMM: $\chi^2 = 6.874$, $P = 0.008$; Fig. 6) and removed workers (LMM: $\chi^2 =$

11.425, P = 0.0007; Fig. 6) due to *Varroa* infestation. We did not find any difference in regards to the number of pollen foragers (LMM: χ^2 = 0.778, P = 0.377; Fig. 6).

Finally, in the experiment 5 (October 2015), we did not find any significant differences between the *Varroa* free and the *ex-Varroa* infested colonies in the number of resin, pollen foragers and removed workers (Bonferroni post hoc test: Z = 0.149, P = 1.000; Z = 0.375, P = 1.000 and Z = 1.167, P = 0.729, respectively; Fig. 3, 4 and 5; Table S2). All groups had equal levels of colony strength across the course of all our experiments (for August: Mann-Whitney U test: U = 18.0, N1 =N2 = 6, P = 0.999; for September: Mann-Whitney U test: U = 16.0, N1 = N2 = 6, P = 0.818; for October: Mann-Whitney U test: U = 13.0, N1 = N2 = 5, P = 0.999; Table 2). Furthermore, in experiments 3 and 4 there was a significant difference in infestation level between *Varroa* free vs *Varroa* infested colonies (Mann-Whitney U test: U = 0, N1 = N2 = 6, P = 0.002; U = 0, N1 = N2 = 6, P = 0.002, respectively; Table 2). While, in experiment 5 there was no difference in infestation level between *Varroa* free vs *ex-Varroa* infested colonies (Mann-Whitney U test: U = 5, N1 = N2 = 5, P = 0.166; Table 2).

Chemical Analyses

In propolis collected in 2014, total phenolic and flavonoid content ranged from 130.3 g GAE Kg^{-1} DW (infestation level 4.1%) to 474.7 g GAE Kg^{-1} DW (infestation level 2.5%) and from 30.7 g CE Kg^{-1} DW (infestation level 4.1%) to 104.6 g CE Kg^{-1} DW (infestation level 0.3%), respectively. The amount of these compounds was not influenced by the mite infestation level (polyphenols: LM; t = -0.736, P = 0.478, R^2=0.05; flavonoids: LM; t = -1.263, P = 0.478, R^2=0.13; Fig. 7a and 7b).

In 2015 we did not find any significant differences between *Varroa* infested and *Varroa* free colonies in the total amount of polyphenols (Bonferroni post hoc test; Z = 0.995, P = 1.000; Table 3) and flavonoids (Bonferroni post hoc test; Z = 1.186, P = 1.000; Table 3) in propolis

collected in August. Differently, in September, we found decreased polyphenol contents (415.3 g GAE Kg^{-1} DW) in the *Varroa* infested group compared to the *Varroa* free group (618.7 g GAE Kg^{-1} DW) (Bonferroni post hoc test; $Z = 2.909$, $P = 0.021$; Table 3). No significant differences were observed between the two groups in the total amount of flavonoids (Bonferroni post hoc test; $Z = 1.805$, $P = 0.426$; Table 3).

Discussion and conclusion

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

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

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

The hypothesis that non-parasitized bee workers can change their behavior in favor of an infested colony that increases the number of resin foragers as a social immunity response, is really fascinating. The results of experiment 5, showing that differences in the number of resin foragers and removed workers were not anymore detectable after reducing mite infestation in the *Varroa* infested group to the same level as the *Varroa* free group (close to

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

Further support to a mite infestation-resin collection correlation is given by the fulfillment of the other criteria defining a self-medication behavior. In fact, based on the second criterion of Clayton and Wolfe (1993), the medicinal substance should negatively affect the parasite and/or pathogen. Accordingly, the acaricidal properties of ethanolic extracts of propolis are well documented (Damiani *et al.* 2010; Garedew *et al.* 2002). Besides, a reduction in the

number of mature mite females per cell was obtained through treatments with propolis extracts inside the beehive (Simone-Finstrom & Spivak 2010). However, because the main bioactive compounds were found in the resinous fraction of propolis and are only soluble in alcohol (Medana *et al.* 2008), it still need to be clarified how crude propolis might directly or indirectly affect *Varroa* biological cycle, and how it might prevent the development of secondary infections, including the possibility that chemical-physical conditions inside the hive may help the release of bioactive substances (DeGrandi-Hoffman & Chen, 2015). Besides, in a laboratory experiment, no effects of volatile compounds possibly released by propolis were detected on mite survival (Drescher *et al*. 2017). Nicodemo *et al.* (2013) investigated whether propolis collection behavior is associated with resistance to the parasitic bee mite *V. destructor*, but no significant correlation between these two traits was found. However, this study was conducted employing Africanized honeybees that are *per se* more resistant to the mite, and considered relatively low infestation levels (mean infestation rate of sealed brood varying from 1.0 to 2.6%). For these reasons, this aspect deserves further investigation. On the other side, the incorporation of a high propolis amount inside the nest was found to cause a relative decrease in the microbial titer and in the expression level of immune-related genes of single bees (Simone *et al.* 2009). Since high individual immunity activation may correspond to significant fitness costs for the colony (Evans & Pettis 2005), traits that reduce chronic elevation of an individual's immune response may benefit colony-level productivity (Cotter *et al.* 2004). Accordingly, a positive correlation between propolis and honey production have been reported (Manrique & Soares 2002). For all these reasons, also the third adaptive behavior criterion of Clayton and Wolfe (1993) appears to be fulfilled. With regard to the criterion proposed by Singer *et al.* (2009), an augmented fitness cost for uninfected individuals would translate into a higher energy investment at the expense of resin in respect to pollen foragers (Nakamura & Seeley 2006; Simone-Finstrom & Spivak 2010).

Indeed, time and energy consumed to collect resin from the outside environment and to handle it inside the hive, represent a cost that does not apparently reward the individual forager, that more obviously would receive a direct food recompense when collecting nectar or pollen. It is remarkable to note that similarly to Simone-Finstrom and Spivak (2012), we observed this behavior within the host environment: the hive.

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

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

Most studies on the acaricidal properties of propolis were conducted employing the total ethanolic extract (balsamic components), which includes both polyphenols and other compounds that despite not being considered in our study, might possibly be implicated in the toxic action against *Varroa* (Damiani *et al.* 2010; Garedew *et al.* 2002). Whilst propolis is usually considered of high quality when having a high flavonoid content (Bonvehi & Coll 1994; Park *et al.* 1998), the current literature on its biological properties proves the involvement of other components. For instance, substances with non-phenolic origin isolated

from propolis samples collected in Brazil showed significant antimicrobial activity (Bankova *et al.* 1996). More in general, the biological activity of propolis derives from its high resin content, which is essentially (but not exclusively) associated with phenolic compounds, mostly flavonoids (Bankova, *et al.* 1983). Despite a growing interest in the potential of propolis against hive pathogens and parasites, only few studies investigated the relationship between colony health and propolis composition. In a recent study (Popova *et al.* 2014), the chemical composition of propolis from *Varroa*-tolerant colonies was analyzed and compared to non-tolerant colonies from the same apiary. A lower resin content was found in tolerant colonies that were also characterized by a higher percentage of the biologically active compounds, caffeic acid and pentenyl caffeates, thus highlighting a significant relationship between *Varroa* infestation and propolis quality in the hive (Popova *et al.* 2014).

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

Acknowledgements

This study was financially supported by the Italian Ministry of Education, University and Research (MIUR; Research Project PRIN 2012), 'Social immunity in honeybee: behavioural, chemical and microbiological aspects'. In addition, we are grateful to National Institute of Social providence (INPS, Italy) for the PhD scholarship granted to Michelina Pusceddu.

Disclosure

All authors are without conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject of this manuscript.

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

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

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

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

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

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

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

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

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

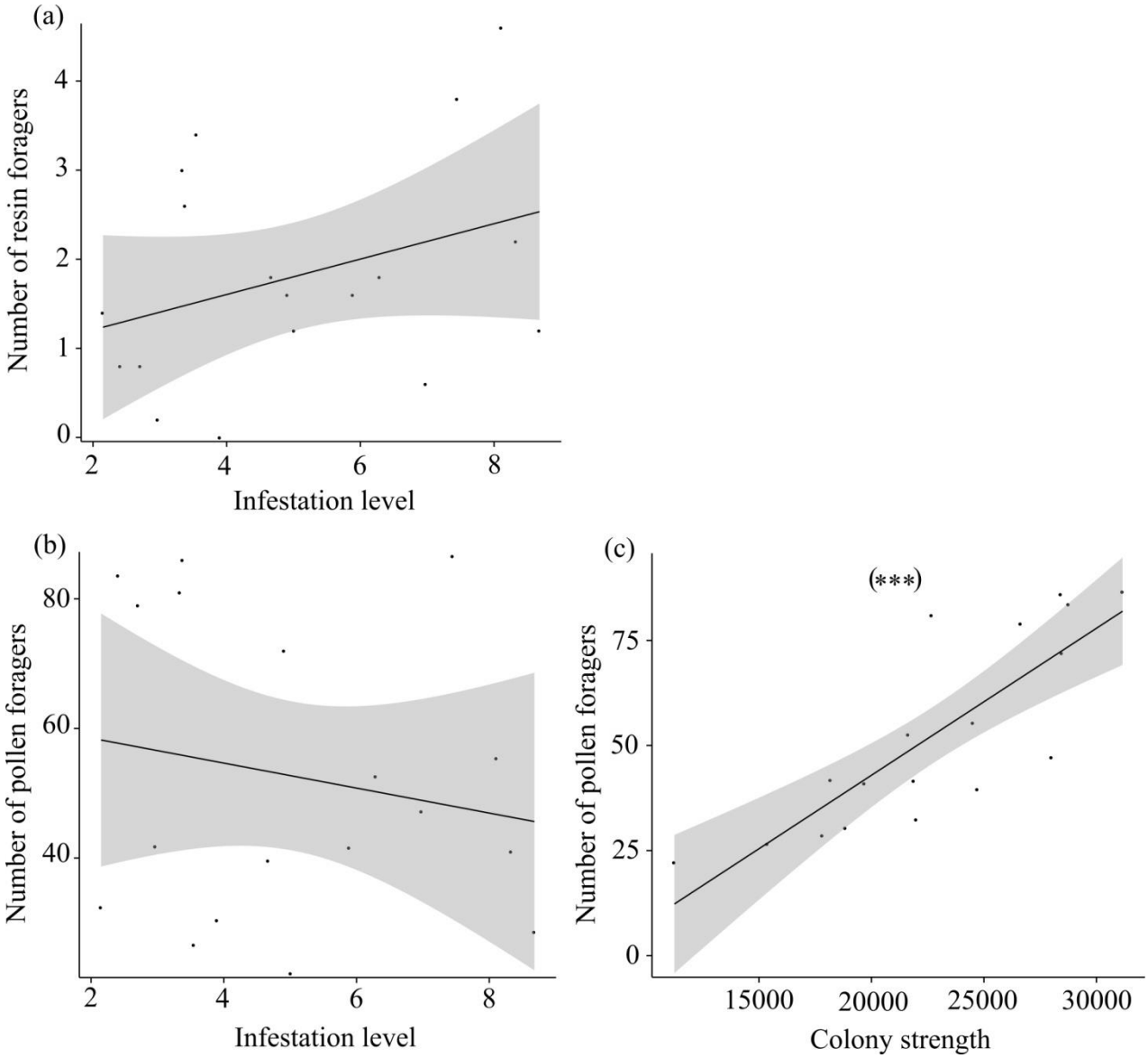

Figure 1. Relationships between (a) number of resin foragers in 15 minutes and *Varroa* infestation level (%), (b) number of pollen foragers in 5 min and *Varroa* infestation level (%), and (c) number of pollen foragers and colony strength. Plotted lines show predicted relationship and the shaded areas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

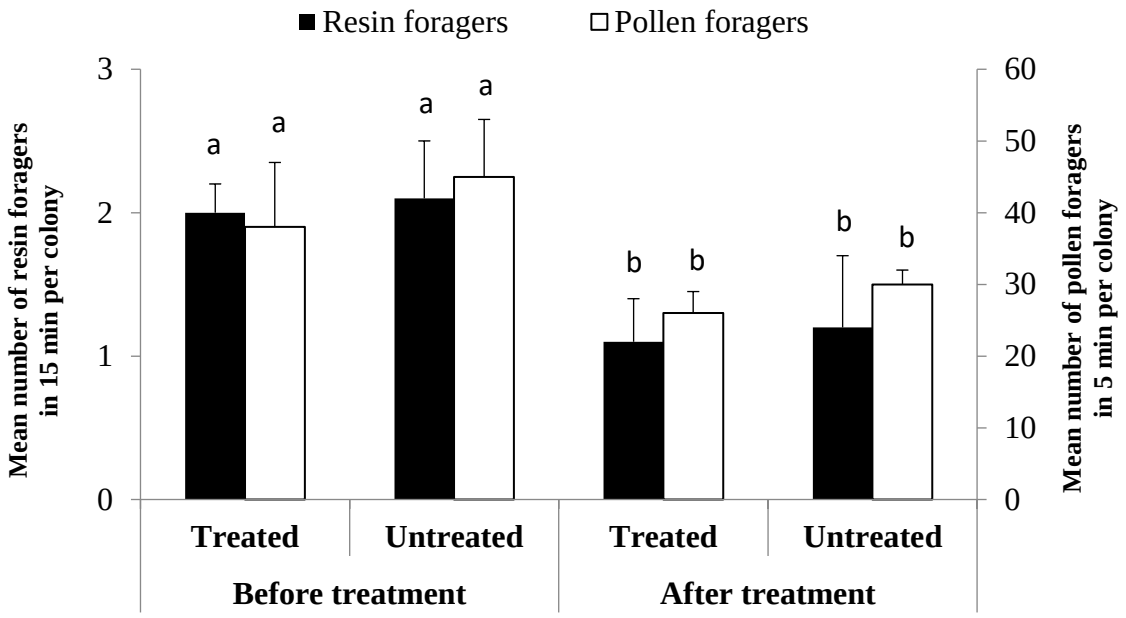

Figure 2. Effect of Apivar[@] treatment on the number of resin and pollen foragers (mean ± SE). Both groups were homogeneous for colony strength and *Varroa* infestation level. For each variable, different letters above bars indicate significant differences between groups before and after treatment $P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

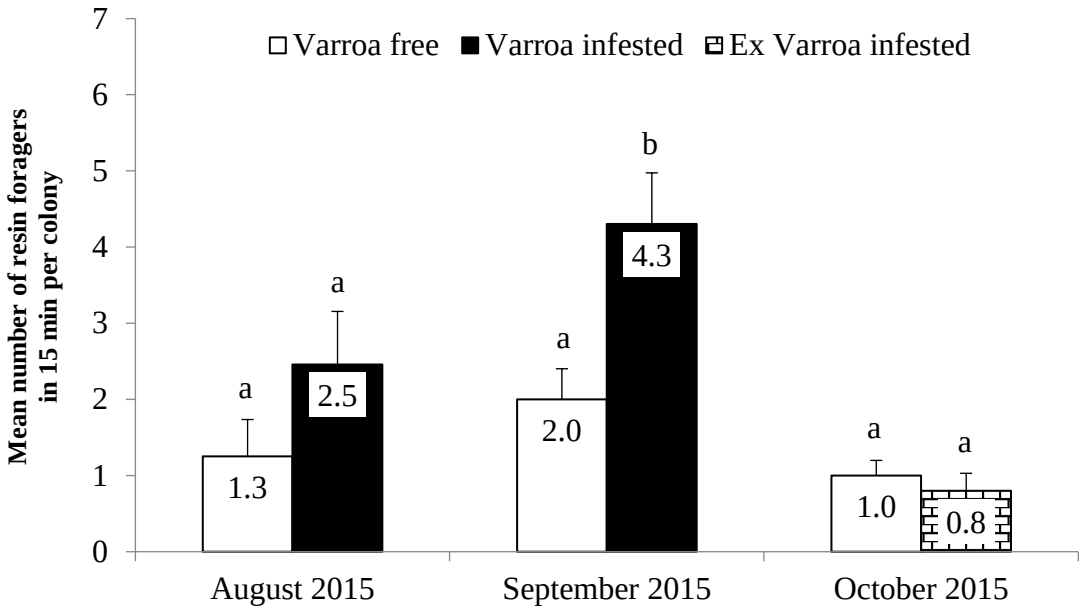

Figure 3. Effect of different infestation level of *Varroa destructor* (2.8 ± 0.4 % vs 0.1 ± 0.1 % in August; 6.7 ± 1.0 % vs 0.2 ± 0.1 % in September; 0.5 ± 0.1 vs 0 in October) on the number of resin foragers (mean ± SE). In the ex *Varroa* infested group the infestation level was reduced to the same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

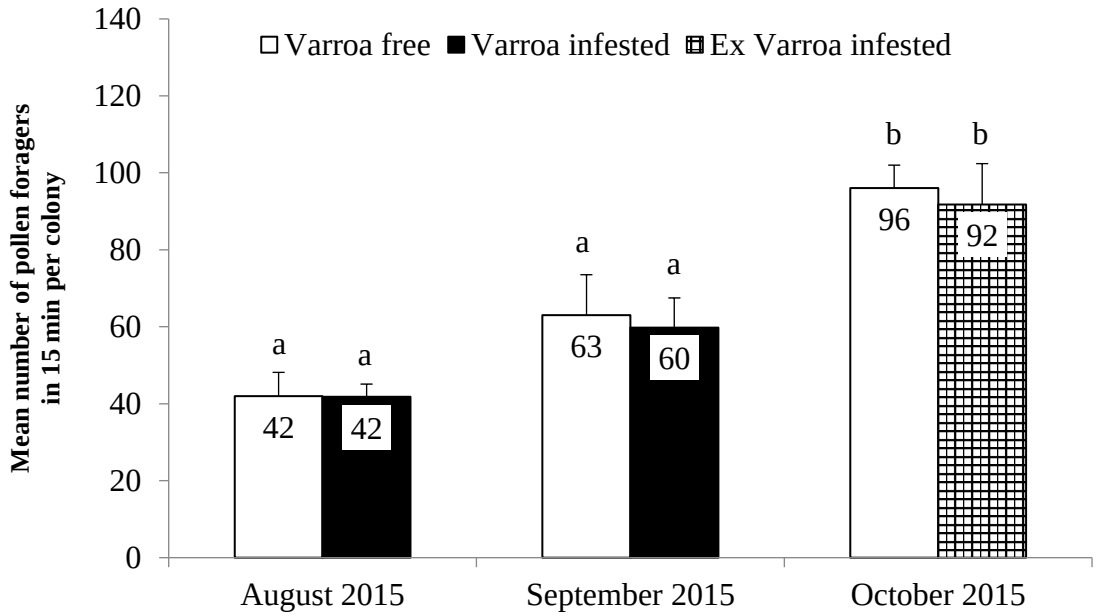

Figure 4. Effect of different infestation level of *Varroa destructor* (2.8 ± 0.4 % vs 0.1 ± 0.1 % in August; 6.7 ± 1.0 % vs 0.2 ± 0.1 % in September; 0.5 ± 0.1 vs 0 in October) on the number of pollen foragers (mean ± SE). In the ex *Varroa* infested group the infestation level was reduced to the same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

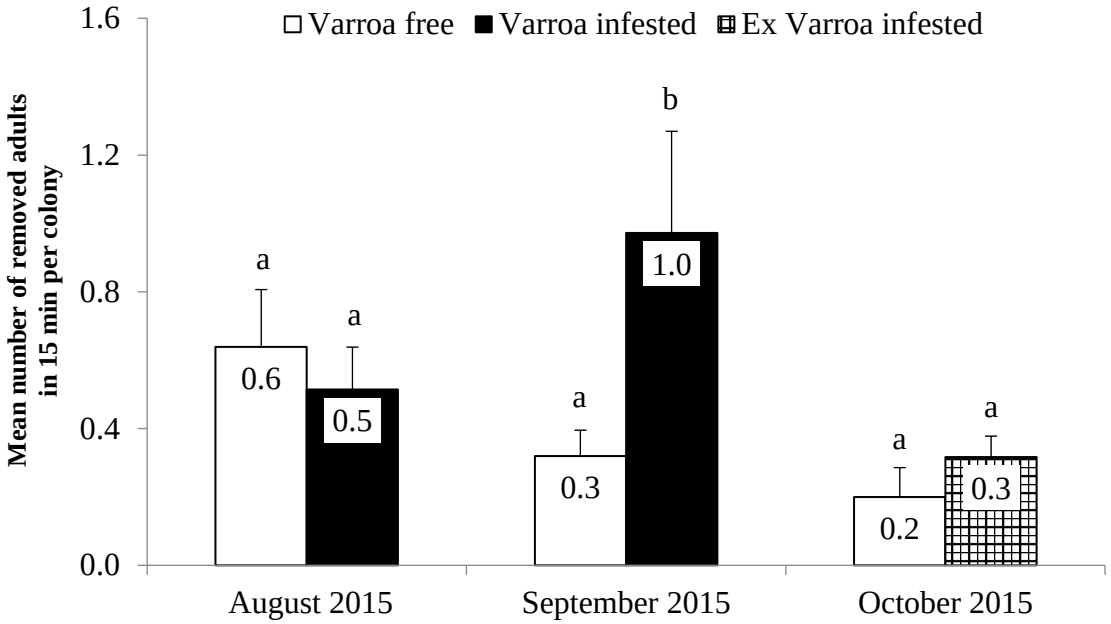

Figure 5. Effect of different infestation level of *Varroa destructor* (2.8 ± 0.4 % vs 0.1 ± 0.1 % in August; 6.7 ± 1.0 % vs 0.2 ± 0.1 % in September; 0.5 ± 0.1 vs 0 in October) on the number of removed adults (mean ± SE). In the ex *Varroa* infested group the infestation level was reduced to the same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

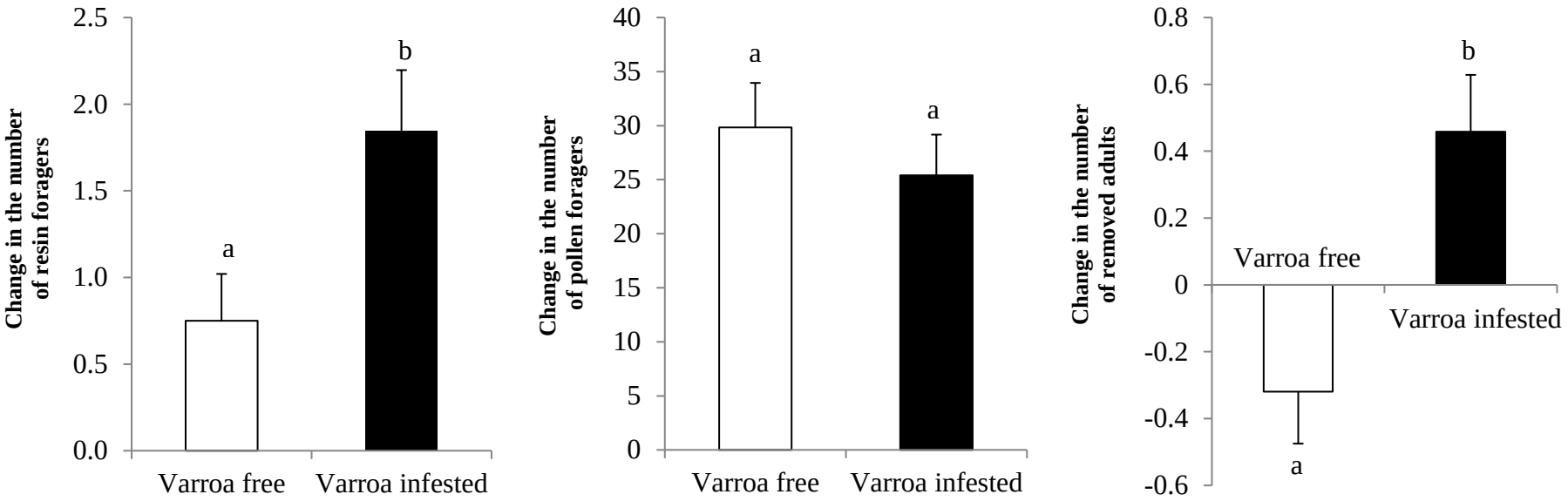

Figure 6. Change in the number of resin and pollen foragers and of removed adult workers (average difference between sum in September and sum in August ± SE) in the *Varroa* free and *Varroa* infested groups. For each variable, different letters above bars indicate significant differences between groups (LMM: χ^2 test; $P < 0.05$).

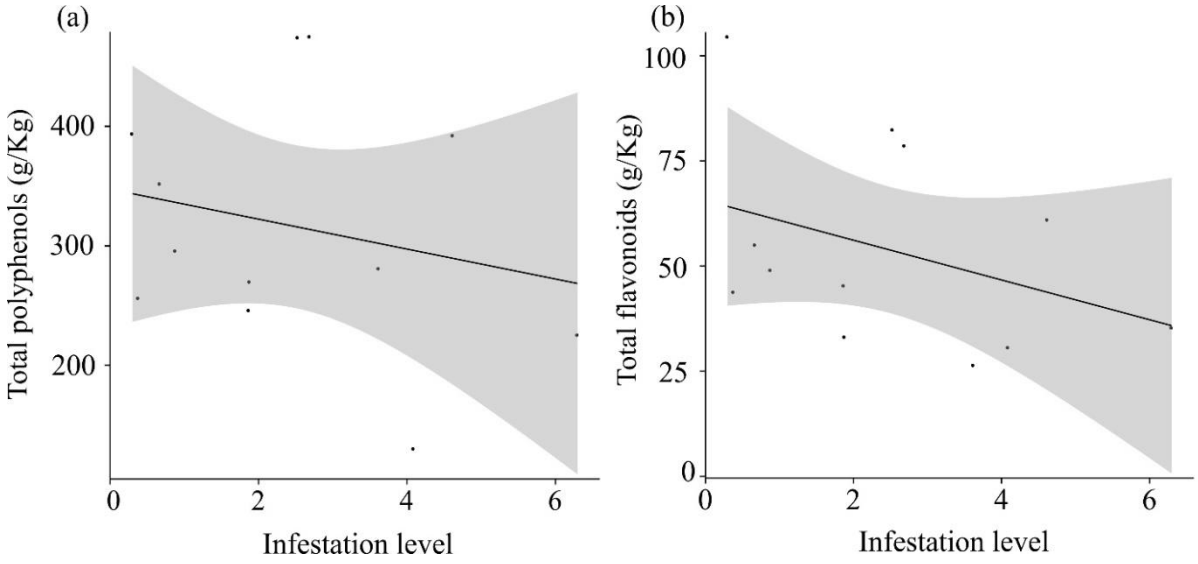

Figure 7. The effects of infestation level (%) on (a) the total polyphenols and (b) total flavonoids found in propolis for the 2014 experiment. Total polyphenols are expressed in g GAE Kg^{-1} DW= g Gallic Acid Equivalent Kg^{-1} Dry Weight of plant material. Total flavonoids are expressed in g CE Kg^{-1} DW= g Catechin equivalent Kg^{-1} Dry Weight of plant material. Plotted lines show predicted relationship and the shaded areas indicate the 95% confidence intervals.