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1 **Resin foraging dynamics in *Varroa destructor* infested hives. A case of medication of**
2 **kin?**
3

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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).

45 Whilst the conditions defining this adaptive behavior have over time been refined, three
46 classic criteria were provided by Clayton and Wolfe (1993): 1) The substance in question
47 must be deliberately contacted; 2) The substance must be detrimental to one or more
48 parasites; 3) The detrimental effect on parasites must lead to increased host fitness. The
49 second and the third criteria are rather self-evident: a substance that does not reduce parasite
50 fitness or does not increase host fitness can hardly be considered medicinal. According to de
51 Roode *et al.* (2013) it is not essential to meet the second criterion, because medication
52 behavior may enhance host fitness by increasing tolerance to infection (allowing the host to
53 maintain fitness despite being infected) without reducing parasite fitness (Lars *et al.* 2007).
54 The first criterion however, is of fundamental importance as it assumes that the use or the
55 incremented use of the medicinal substance would be a direct consequence of a parasitic
56 and/or pathogenic action (de Roode *et al.* 2013). Singer *et al.* (2009) see self-medication as a
57 type of adaptive plasticity resulting from behavioral changes induced by the outside
58 environment and improving the animal survival and reproduction prospect. In agreement with
59 these authors, because of its fitness cost, self-medication is observed only in the presence of a
60 disease or a parasite. On this basis, an additional criterion to define self-medication was
61 described: 4) self-medication behavior decreases fitness in uninfected animals, having a
62 detrimental effect or a major cost for the host in the absence of parasites or diseases (Singer
63 *et al*. 2009). Finally, de Roode *et al.* (2013) suggested that to be considered an adaptive form
64 of medication, self-medication has to be relevant in the natural environment of the host. It
65 follows that experiments using artificial diets to investigate medication mechanisms, are not
66 sufficient to demonstrate their relevance in nature.
67 Mostly studied in higher vertebrates (Gompper & Hoylman, 1993; Gwinner *et al.* 2000;
68 Wimberger, 1984; Wrangham & Nishida 1983), self-medication was also observed on a
69 variety of solitary insects, such as *Grammia incorrupta* (Singer *et al.* 2009; Smilanich *et al.*

70 2011) and *Drosophila melanogaster* (Milan *et al.* 2012). In eusocial insects, it is necessary to
71 distinguish between self-medication and medication of kin, which extends the self-
72 medication concept to the colony level (Abbott, 2014). In fact, eusocial insects add to their
73 immunological individual defenses against pathogens and parasites (Schmid-Hempel, 2005),
74 several evolutionary behavioral and organizational adaptations within the colony (Cotter &
75 Kilner 2010). Some of these defense mechanisms generally prevent or limit disease
76 transmission, while others are induced by the presence of either parasites or pathogens. This
77 "social immunity" system results from single member cooperation toward reducing the
78 disease transmission risks typically associated with social life (Cremer *et al.* 2007). A higher
79 exposure to pathogens and parasites is indeed expected as a consequence of high population
80 density, frequent physical interactions among colony members, and the continuous use of the
81 same nesting sites with microclimatic conditions (i.e., temperature and relative humidity)
82 favoring the development of microorganisms (Schmid-Hempel, 1998). The reduced number
83 of immune-related genes in *Apis mellifera* in comparison with other insect species, is in line
84 with observations on other Hymenopteran species (Barribeau *et al.,* 2015). Different social
85 immunity behaviors have been observed on the honeybee. These include social fever (Starks
86 *et al.* 2000), hygienic behavior (Ibrahim & Spivak, 2005), allogrooming (Pettis & Pankiw,
87 1998), and self-medication through ingestion (Gherman *et al.* 2014). An interesting and
88 scarcely studied self-medication behavior (by contact or proximity) involves the collection
89 and use of resins in the hive (Simone-Finstrom & Spivak 2012). These viscous and complex
90 substances are normally secreted by plants that exploit their bioactive properties to protect
91 against parasites and pathogens (Langenheim, 2013; Simone *et al.* 2009; Simone-Finstrom &
92 Spivak 2010). After being collected from diverse plant species, resins are carried to the
93 colony where they are mixed with wax and incorporated into the hive structure as propolis
94 (Simone-Finstrom & Spivak 2010). The colony mechanisms regulating resin collection have

95 not been clarified. Besides, how workers communicate the need of collecting resins to other
96 colony members is still under investigation (Nakamura & Seeley, 2006). It was demonstrated
97 that an increased propolis content in the hive may correspond to a decrease in its microbial
98 load (Simone *et al.* 2009), even if such effect was not observed by Borba *et al.* (2015). On the
99 other side, a significant down regulation of individual immune-related genes was reported
100 (Borba *et al.* 2015; Simone *et al.* 2009). Moreover, an increase in resin collection after
101 infections of the fungus *Ascosphaera apis* was observed, suggesting a therapeutic use of
102 propolis in the hive (Simone-Finstrom & Spivak 2012). Nevertheless, such response does not
103 appear to be associated with the action of the American foulbrood agent, *Paenibacillus larvae*
104 (Simone-Finstrom & Spivak 2012).
105 Assuming that bacterial and fungal infection mechanisms can be different from the action of
106 a parasite, the objective of this study was to verify if the amount of resin collected and
107 propolis quality within the hives infested by *Varroa destructor* were different from non-
108 infested ones. We propose two hypotheses to explain the behavior of resin foragers in
109 response to *Varroa* parasitism: 1) an increase in the usually collected amount of resins
110 (quantitative hypothesis); and 2) an increase in the bioactive substance content (i.e.,
111 polyphenols and flavonoids) in propolis (qualitative hypothesis). The quantitative hypothesis
112 is based on the antiparasitic, antimicrobial and antioxidant properties of propolis (Dresher *et*
113 *al.*, 2017; Huang *et al.* 2014; Marcucci, 1995), mostly associated with its polyphenolic and
114 flavonoid content (da Silva *et al.* 2006; Siripatrawan *et al.* 2013). Acaricidal effects of
115 propolis extracts against *V. destructor* have been reported (Damiani *et al.* 2010; Garedew *et*
116 *al.* 2002). The qualitative hypothesis is based on the ability of *A. mellifera* to select different
117 kinds of resins (Erler & Moritz 2015; Isidorov *et al.* 2016; Loenhardt *et al.,* 2009). For
118 instance, a preference for *Baccharis dracunculifolia* (alecrim plant, Asteraceae) females
119 versus males (Teixeira *et al.* 2005), for buds and younger leaves (Park *et al.* 2004), or for

120 plants producing resins with specific antimicrobial properties (Wilson *et al.* 2013), were
121 reported. Besides, how bees may benefit from different resin sources was also observed
122 (Drescher *et al.* 2014). Accordingly, Popova *et al.* (2014) demonstrated that the percentage of
123 bioactive compounds (caffeic acid and pentenyl caffeates) was higher in *Varroa* tolerant
124 colonies compared to non-tolerant ones. All these findings suggest that honeybees are able to
125 follow a chemical "trace" leading toward a resin source and to evaluate its quality (Simone-
126 Finstrom & Spivak 2010). In order to verify our hypotheses, comparative experiments
127 involving hives with different mite infestation levels were conducted over a two year period
128 (2014 and 2015), assessing the amount of resin collected and propolis quality in the hive. For
129 this purpose observations on resin foraging dynamics in the hive were conducted along with
130 chemical analyses on propolis samples to quantify the total polyphenol and total flavonoids
131 content.
132
133 **Materials and methods**
134
135 *Experimental apiary*
136 The experimental apiary was set-up in the North-West of Sardinia (Lat 40°46'23", Long
137 8°29'34") during March 2014 and consisted of 18 hives, prepared with queens of *Apis*
138 *mellifera ligustica* breed and with a homogeneous genetic profile (sisters) as provided by a
139 local specialist breeder. Colonies were maintained in new Dadan-Blatt hives containing 10
140 frames of nest comb checked every two weeks to verify the presence of the queen, to provide
141 pollen and nectar, to evaluate the sanitary status (possible symptoms of viral, fungal, and/or
142 bacterial infections), and, when necessary, to match for population size (about 25000 – 30000
143 adult bees) through frame removal from stronger families. Each nest entrance was featured by
144 a different color pattern to reduce drifting (Free & Spencer-Booth 1961).

145 *Experiments*

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

148 A first experiment was conducted in July 2014 (experiment 1) on 18 colonies that did not
149 receive any previous management intervention (e.g., equalization of colony strength,
150 supplementary feeding, etc.), including no chemical or biological treatments against parasites
151 and/or pathogens. Colony inspection, routinely conducted on a biweekly basis, did not report
152 any symptoms of the main honeybee diseases (bacterial, viral and/or nosemosis). In total 22.5
153 h of observations were conducted to assess the number of resin and pollen foragers and the
154 number of removed adults in hives with different adult infestation levels (from 2.4 to 8.7 %)
155 and different colony strength (from 11242 to 31171 adult workers + sealed brood cells).
156 Following the outcome of observations conducted in 2014 on colonies with varying mite
157 infestation levels, the approach of experiments carried out in 2015 involved the manipulation
158 of infestation levels using acaricidal treatments and strength equalization among different
159 colonies through frame removal from stronger families, two months before starting
160 experiments. Observations were therefore conducted on two experimental hive groups: 1)
161 *Varroa* free group, where *Varroa* infestation was maintained close to zero with acaricidal
162 treatments, and 2) *Varroa* infested group, where no treatments were applied and the mite
163 population could naturally increase. Treatments were based on Apivar® (a.i. amitraz)
164 application, a strip-based commercial formulation with long term action, suitable for
165 acaricidal treatments in presence of sealed brood. Preliminarily, the possible effects of
166 acaricidal treatments on resin and pollen collection behavior of honeybee foragers were
167 verified. For this purpose, specific observations (experiment 2) were conducted in July 2015
168 on two hive groups (treated and control) having equivalent strength and a low mite infestation
169 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%).
193
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

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

248

249 *Statistical analysis*

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

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

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

270 describe change in the number of resin foragers, pollen foragers and adult removal behavior
271 due to *Varroa* infestation (experiments 3 and 4), the difference between the number of
272 foragers and removed workers in August and September was calculated for each colony and
273 treatment group (sum in September-sum in August). Data were then analysed using a linear
274 mixed model (LMM) with treatment as fixed effect factor. For all GLMMs and LMMs day of
275 observation nested within each hive was treated as a random effect factor.
276 We used a general linear model (LM) to analyse the effects of *Varroa* infestation level on the
277 total amount of polyphenols and flavonoids found in propolis samples collected in 2014. We
278 used a LMM, to study the effects of *Varroa* infestation level (*Varroa* free vs *Varroa* infested)
279 and sampling time (August and September) on the total amount of polyphenols and
280 flavonoids found in propolis samples collected in 2015, including hive as a random effect
281 factor to account for pseudo-replication.
282 We used automated model selection based on the Akaike Information Criterion (AICc), when
283 models included several factors and their interactions (R package MuMIn; Barton, 2015). All
284 mixed models were performed using the package lme4 (Bates *et al.* 2015). All model
285 (GLMM, LMM and LM) assumptions were checked visually. For GLMMs, if over-
286 dispersion was detected we used a negative binomial model (Zuur *et al.* 2009) implemented
287 using the package glmmADMB (Fournier *et al.* 2012). To analyse single parameters and
288 interactions we used a likelihood ratio test. We compared the goodness-of-fit between each
289 model by setting up the model so that parameter can be dropped followed the examples in
290 Zurr *et al.* (2009). We further analyzed mixed effect models to test differences between
291 treatments with Bonferroni corrected post hoc tests. Post hoc tests were performed using the
292 package multcomp (Hothor *et al.* 2008). All analysis was performed in R statistical software
293 (R Core Team 2013).
294

295 **Results**

296 *Experiments*

297 In the experiment 1, the best model explaining variability in the number of resin foragers
298 included only the level of *Varroa* infestation. However, the relationship was not significant
299 (GLMM poisson: $Z = 1.487$, $P = 0.137$, R^2=0.52; Fig. 1a, Table S1). For the number of pollen
300 foragers, the best model included both the level of *Varroa* infestation and colony strength.
301 However, only colony strength (GLMM negative binomial: $Z = 6.58$, $P = 4.8e\text{-}11$, R^2=0.31;
302 Table S1) and not the level of *Varroa* infestation (GLMM negative binomial: $Z = -1.140$, Z=
303 6.58, $P = 0.250$, R^2=0.31; Table S1) affected the number of pollen foragers (Fig. 1b and 1c).
304 None of the two factors (level of *Varroa* infestation and colony strength) and their interaction
305 explained variability in the number of workers removed from each colony.

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

320 differences would have been significant. Differences in resin and pollen foragers that we
321 observed were clearly less than 200% and 35%. Indeed, the statistical power (ß) of our
322 analyses given the observed differences was found to be very low, 9.00% (95%CI=7.30-
323 10.95) for resin and 28.60% (95%CI=25.82-31.51) for pollen foragers, indicating that small
324 differences, as we found, need a great number of colony replicates in order to detect a
325 difference as statistically significant (estimated sample size, >8000 colonies per treatment for
326 detecting differences in resin and >35 colonies per treatment for detecting differences in the
327 number of pollen foragers). Overall, our power analyses suggest that the observed differences
328 in numbers of resin and pollen foragers between Apivar treated and control colonies were
329 minimal in our experimental paradigm.
330 In the experiment 3 (August 2015), we did not find any significant differences in the number
331 of resin and pollen foragers, and removed workers (Bonferroni post hoc test: Z = 1.244, P =
332 0.640; Z = 0.734, P = 1.000 and Z = 0.411, P = 1.000, respectively; Table S2) between
333 *Varroa* free and *Varroa* infested colonies (Fig. 3, 4 and 5). In the experiment 4 (September
334 2015), we found a significantly higher number of resin foragers and removed workers
335 (Bonferroni post hoc test: Z = 3.166, P = 0.004 and Z = 2.458, P = 0.042, respectively; Table
336 S2) in the *Varroa* infested compared to the *Varroa* free group (Fig. 3 and 5). No significant
337 differences were found between the two groups considering the number of pollen foragers
338 (Bonferroni post hoc test: Z = 0.093, P = 1.000; Fig. 4; Table S2). The mean difference in the
339 number of resin foragers (sum in September-sum in August), was 1.85±0.35 for the *Varroa*
340 infested colonies and 0.75±0.27 for the *Varroa* free colonies. Furthermore, the mean
341 difference in the number of removed workers and number of pollen foragers was 0.46±0.17
342 and 25.4±3.8 for the *Varroa* infested colonies and -0.32±0.15 and 29.8±4.1 for the *Varroa*
343 free colonies, respectively. Our LMM analysis showed a significant increase in the number of
344 resin foragers (LMM: χ^2 = 6.874, P = 0.008; Fig. 6) and removed workers (LMM: χ^2 =

345 11.425, P = 0.0007; Fig. 6) due to *Varroa* infestation. We did not find any difference in
346 regards to the number of pollen foragers (LMM: χ^2 = 0.778, P = 0.377; Fig. 6).
347 Finally, in the experiment 5 (October 2015), we did not find any significant differences
348 between the *Varroa* free and the *ex-Varroa* infested colonies in the number of resin, pollen
349 foragers and removed workers (Bonferroni post hoc test: Z = 0.149, P = 1.000; Z = 0.375, P =
350 1.000 and Z = 1.167, P = 0.729, respectively; Fig. 3, 4 and 5; Table S2). All groups had equal
351 levels of colony strength across the course of all our experiments (for August: Mann-Whitney
352 U test: U = 18.0, N1 =N2 = 6, P = 0.999; for September: Mann-Whitney U test: U = 16.0, N1
353 = N2 = 6, P = 0.818; for October: Mann-Whitney U test: U = 13.0, N1 = N2 = 5, P = 0.999;
354 Table 2). Furthermore, in experiments 3 and 4 there was a significant difference in infestation
355 level between *Varroa* free vs *Varroa* infested colonies (Mann-Whitney U test: U = 0, N1 =
356 N2 = 6, P = 0.002; U = 0, N1 = N2 = 6, P = 0.002, respectively; Table 2). While, in
357 experiment 5 there was no difference in infestation level between *Varroa* free vs *ex-Varroa*
358 infested colonies (Mann-Whitney U test: U = 5, N1 = N2 = 5, P = 0.166; Table 2).
359
360 *Chemical Analyses*
361 In propolis collected in 2014, total phenolic and flavonoid content ranged from 130.3 g GAE
362 Kg^{-1} DW (infestation level 4.1%) to 474.7 g GAE Kg^{-1} DW (infestation level 2.5%) and from
363 30.7 g CE Kg^{-1} DW (infestation level 4.1%) to 104.6 g CE Kg^{-1} DW (infestation level 0.3%),
364 respectively. The amount of these compounds was not influenced by the mite infestation level
365 (polyphenols: LM; t = -0.736, P = 0.478, R^2=0.05; flavonoids: LM; t = -1.263, P = 0.478,
366 R^2=0.13; Fig. 7a and 7b).
367 In 2015 we did not find any significant differences between *Varroa* infested and *Varroa* free
368 colonies in the total amount of polyphenols (Bonferroni post hoc test; Z = 0.995, P = 1.000;
369 Table 3) and flavonoids (Bonferroni post hoc test; Z = 1.186, P = 1.000; Table 3) in propolis

370 collected in August. Differently, in September, we found decreased polyphenol contents
371 (415.3 g GAE Kg^{-1} DW) in the *Varroa* infested group compared to the *Varroa* free group
372 (618.7 g GAE Kg^{-1} DW) (Bonferroni post hoc test; $Z = 2.909$, $P = 0.021$; Table 3). No
373 significant differences were observed between the two groups in the total amount of
374 flavonoids (Bonferroni post hoc test; $Z = 1.805$, $P = 0.426$; Table 3).
375
376 **Discussion and conclusion**
377 This study demonstrates that when *A. mellifera* colonies are under stress conditions because
378 of *Varroa* infestation, an increase in the number of resin foragers is recorded, even if a
379 general intensification of the foraging activity is not observed. Similarly, Drescher et al.
380 (2017) have recently found a positive correlation between *Varroa* infestation and resin
381 collection. However, such results, obtained using propolis traps, are not directly comparable
382 with our experiments based on the quantification of the resin foragers.
383 We also found an increase in the rate of adult removal in infested colonies, likely affected by
384 the virus titer (Baracchi et al., 2012).
385 The increase in resin foragers is in line with the results of experiments with the fungus *A. apis*
386 (Simone-Finstrom & Spivak 2012) and apparently meets the first adaptive behavior criterion
387 defined by Clayton and Wolfe (1993), according to which the use or the incremented use of
388 the therapeutic substance should be associated with a health impairment caused by parasites
389 and/or pathogens (de Roode *et al.* 2013).
390 The hypothesis that non-parasitized bee workers can change their behavior in favor of an
391 infested colony that increases the number of resin foragers as a social immunity response, is
392 really fascinating. The results of experiment 5, showing that differences in the number of
393 resin foragers and removed workers were not anymore detectable after reducing mite
394 infestation in the *Varroa* infested group to the same level as the *Varroa* free group (close to

395 zero) through Apivar® applications, support the hypothesis that behavioral changes must be
396 somehow closely related to the presence of *Varroa*. In fact, this acaricide is specific to mites
397 and is not supposed to inhibit viruses, bacteria or fungi. According to the results of studies on
398 honeybee viruses associated with vorroosis, DWV was shown to become undetectable in the
399 sealed brood of colonies treated with pyrethroids (flumethrin and fluvalinate), paralleling the
400 rate of mite loss after treatment (Martin et al. 2010; Locke et al. 2012). The titre of sac brood
401 virus (SBV) and black queen cell virus (BQCV) was instead variably affected by these
402 acaricidal applications and did not show any direct relationship with mite infestation
403 (Drescher 2017; Locke *et al*. 2012). Al Naggar *et al.* (2015) demonstrated that acaricidal
404 applications of amitraz (Apivar[@]) do not affect the percentage of hives infected by DWV and
405 IAPV compared with untreated control. Accordingly, and based on the results of our
406 experiments involving antivarroa treatments with amitraz, we can assume that a resin
407 collection increase can be a direct result of the mite presence. In a study conducted by
408 Drescher et al (2017) by artificially adding and removing natural propolis in colonies where
409 *Varroa* population could naturally increase, significant effects on DWV titer, but not on mite
410 infestation, were noticed. However, no information on the dynamic of the artificially added
411 propolis in the hive were provided by these experiments, in which propolis could have been
412 re-used by bees within the hive, thus affecting the overall resin collection behavior.
413 Consequently, knowledge in this field remains limited and the actual relationship between
414 *Varroa* and resin collection still need to be elucidated.
415 Further support to a mite infestation-resin collection correlation is given by the fulfillment of
416 the other criteria defining a self-medication behavior. In fact, based on the second criterion of
417 Clayton and Wolfe (1993), the medicinal substance should negatively affect the parasite
418 and/or pathogen. Accordingly, the acaricidal properties of ethanolic extracts of propolis are
419 well documented (Damiani *et al.* 2010; Garedew *et al.* 2002). Besides, a reduction in the

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

445 Indeed, time and energy consumed to collect resin from the outside environment and to
446 handle it inside the hive, represent a cost that does not apparently reward the individual
447 forager, that more obviously would receive a direct food recompense when collecting nectar
448 or pollen. It is remarkable to note that similarly to Simone-Finstrom and Spivak (2012), we
449 observed this behavior within the host environment: the hive.
450 A higher expression of the adult removal behavior as a social immunity mechanism we
451 observed in infested hives, suggests that the model describing the colony response against
452 *Varroa* infestation is complex and includes different social defense behaviors that may work
453 with pharmacophory.
454 Our study also revealed some effects on the quality of honeybee produced propolis in
455 consequence of *Varroa* infestations. More in detail, the total polyphenolic content was
456 reduced in propolis produced in infested hives in comparison with the *Varroa* free group.
457 This preliminary finding encourages further investigation to understand if the observed
458 propolis differences derive from similar differences in resins collected by foragers or from
459 their dissimilar manipulation inside the hive. Considering that different propolis types differ
460 in their inhibition properties against a variety of honey bee pathogens in vitro (Wilson *et al.*
461 2013), it would be very important to study the effects against *Varroa* of the two diverse types
462 of propolis: from *Varroa* free and from *Varroa* infested hives.
463 Most studies on the acaricidal properties of propolis were conducted employing the total
464 ethanolic extract (balsamic components), which includes both polyphenols and other
465 compounds that despite not being considered in our study, might possibly be implicated in
466 the toxic action against *Varroa* (Damiani *et al.* 2010; Garedew *et al.* 2002). Whilst propolis is
467 usually considered of high quality when having a high flavonoid content (Bonvehi & Coll
468 1994; Park *et al.* 1998), the current literature on its biological properties proves the
469 involvement of other components. For instance, substances with non-phenolic origin isolated

470 from propolis samples collected in Brazil showed significant antimicrobial activity (Bankova
471 *et al.* 1996). More in general, the biological activity of propolis derives from its high resin
472 content, which is essentially (but not exclusively) associated with phenolic compounds,
473 mostly flavonoids (Bankova, *et al.* 1983). Despite a growing interest in the potential of
474 propolis against hive pathogens and parasites, only few studies investigated the relationship
475 between colony health and propolis composition. In a recent study (Popova *et al.* 2014), the
476 chemical composition of propolis from *Varroa*-tolerant colonies was analyzed and compared
477 to non-tolerant colonies from the same apiary. A lower resin content was found in tolerant
478 colonies that were also characterized by a higher percentage of the biologically active
479 compounds, caffeic acid and pentenyl caffeates, thus highlighting a significant relationship
480 between *Varroa* infestation and propolis quality in the hive (Popova *et al.* 2014).
481 In conclusion, according to the results of our study and to previous knowledge in the field,
482 resin foraging activities in *A. mellifera* have to be considered both as a constitutive and as an
483 inducible behavior, thus representing a response influenced by an infection/infestation status.
484 However, many other aspects still need to be investigated to definitely consider this behavior
485 as a case of medication of kin against *Varroa* and its intimately associated virus.
486

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492
493
494

495 **Disclosure**

496 All authors are without conflicts of interest, including specific financial interests and
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498

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686 Table 1. Adult infestation level and strength of colony (mean ± SE) in the hive groups used in
687 the experiment 2 to test the effect of Amitraz[@] treatment on resin collection (2015).
688

	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

689
690 [*] Different letters in the same column indicate significant differences (Mann-Whitney *U* test, $P < 0.05$)
691 [**] Colony strength was calculated adding the number of sealed brood cells to the number of adult bees.
692
693
694
695 Table 2. Adult infestation level and strength of colony (mean ± SE) in the hive groups used in
696 the experiment 3, 4, 5 to test for differences on resin collection between two groups *Varroa*
697 free and *Varroa* infested (2015).
698

		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

699
700 [*] Different letters in the same column indicate significant differences (Mann-Whitney *U* test, $P < 0.05$)
701 [**]Colony strength was calculated adding the number of sealed brood cells to the number of adult bees.
702
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704

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

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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

708
709 * Different letters in the same column indicate significant differences (Mann-Whitney *U* test, $P < 0.05$;
710 Bonferroni post hoc test $P < 0.05$)
711 § GAE=gallic acid equivalent
712 §§CE=catechin equivalent
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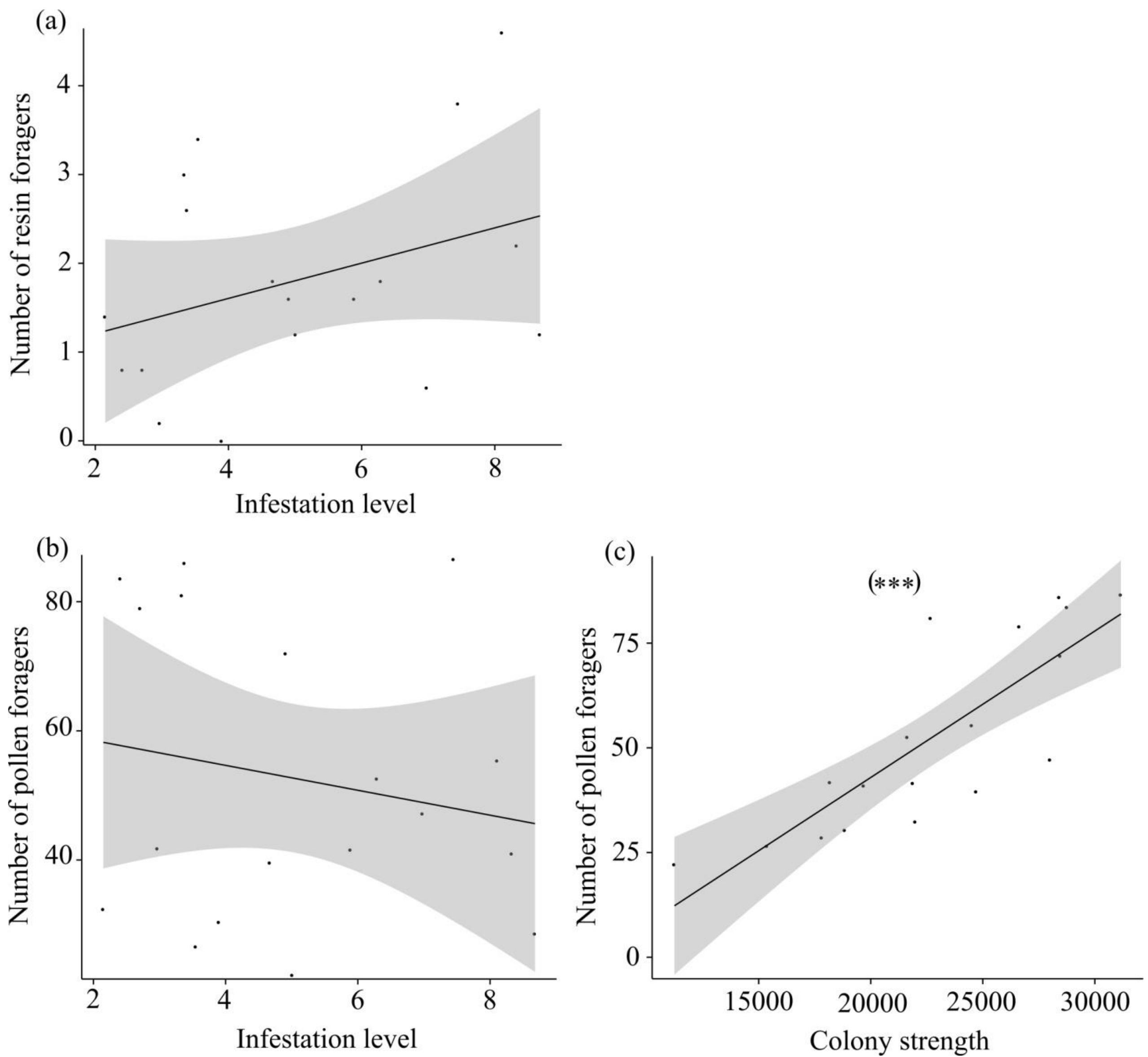

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724 Figure 1. Relationships between (a) number of resin foragers in 15 minutes and *Varroa*
725 infestation level (%), (b) number of pollen foragers in 5 min and *Varroa* infestation level (%),
726 and (c) number of pollen foragers and colony strength.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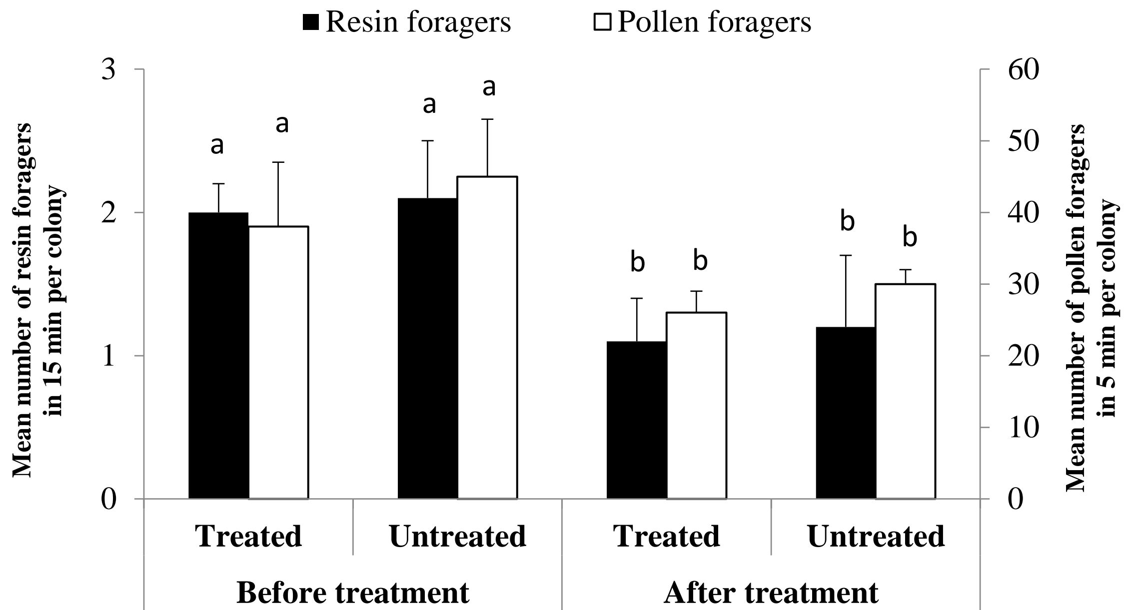

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742 Figure 2. Effect of Apivar@ treatment on the number of resin and pollen foragers (mean ±
743 SE). Both groups were homogeneous for colony strength and *Varroa* infestation level. For
744 each variable, different letters above bars indicate significant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

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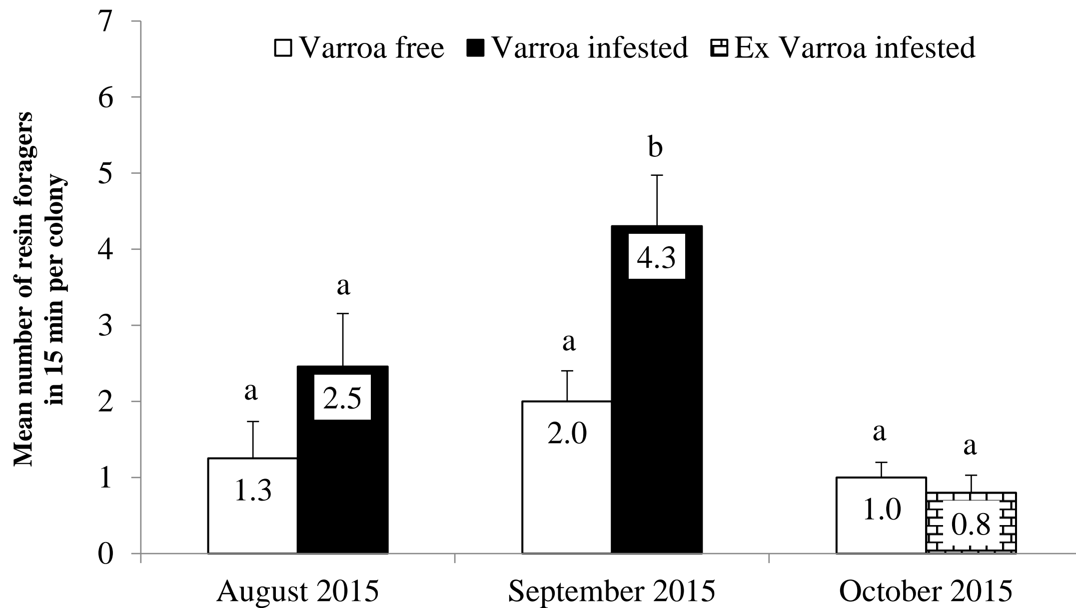

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752 Figure 3. Effect of different infestation level of *Varroa destructor* (2.8 ± 0.4 % vs 0.1 ± 0.1 %
753 in August; 6.7 ± 1.0 % vs 0.2 ± 0.1 % in September; 0.5 ± 0.1 vs 0 in October) on the number
754 of resin foragers (mean ± SE). In the ex *Varroa*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

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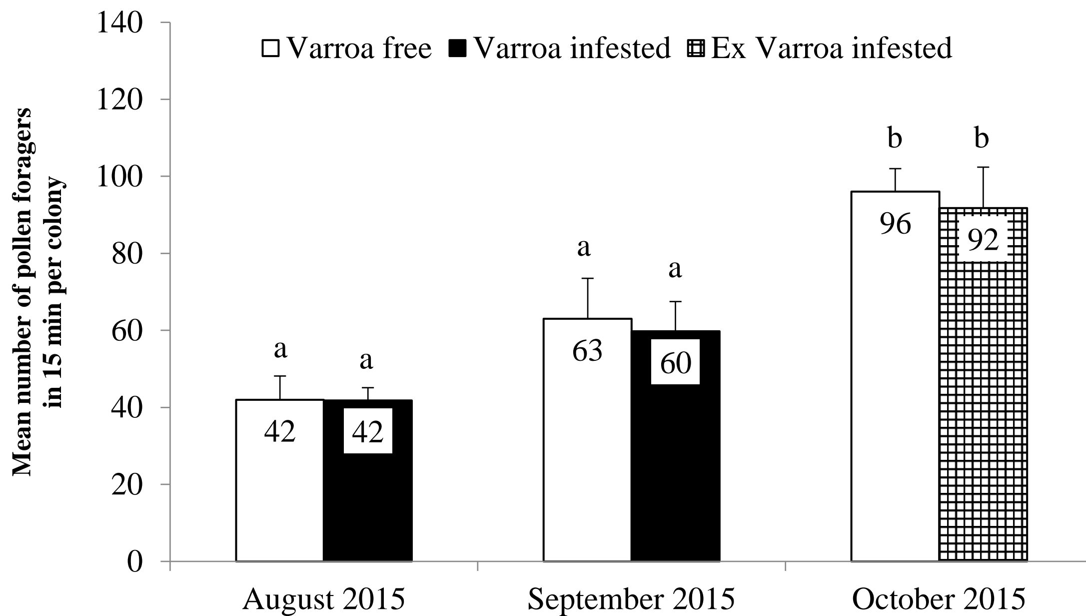

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767
768 Figure 4. Effect of different infestation level of *Varroa destructor* (2.8 ± 0.4 % vs 0.1 ± 0.1 %
769 in August; 6.7 ± 1.0 % vs 0.2 ± 0.1 % in September; 0.5 ± 0.1 vs 0 in October) on the number
770 of pollen foragers (mean ± SE). In the ex *Varroa* infested group the infestation level was
771 reduced to the same level as the *Varroa* free group through miticide treatment. In each date,
772 the two experimental groups were homogeneous for colony strength. Different letters above
773 bars, within each experiment, indicate significant differences between groups (Bonferroni
774 post hoc test $P < 0.05$).

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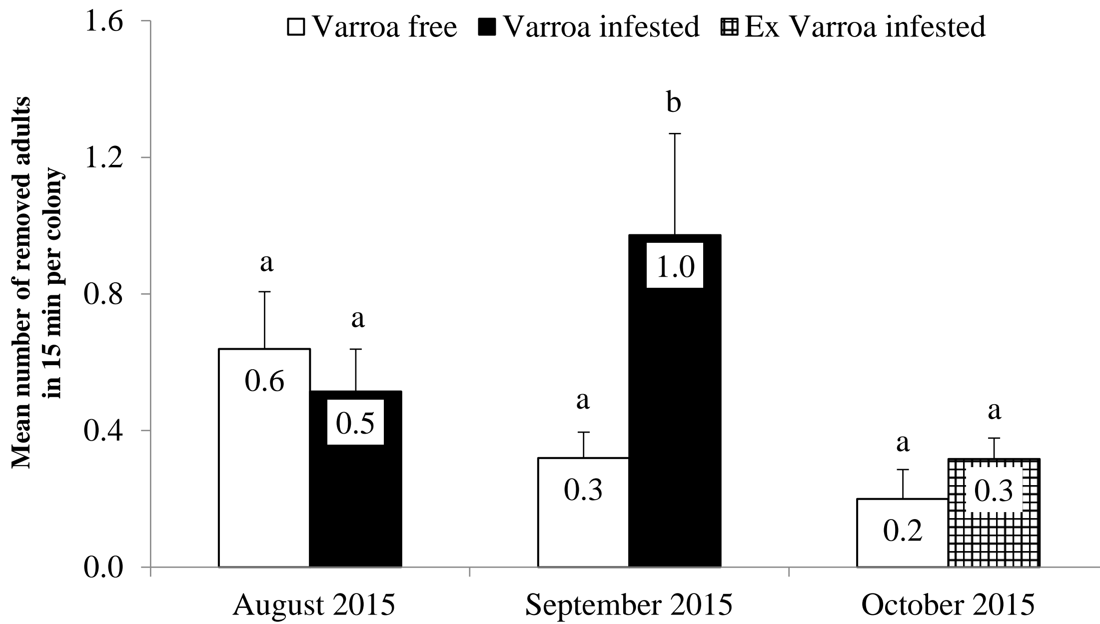

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783 Figure 5. Effect of different infestation level of *Varroa destructor* (2.8 ± 0.4 % vs 0.1 ± 0.1 %
784 in August; 6.7 ± 1.0 % vs 0.2 ± 0.1 % in September; 0.5 ± 0.1 vs 0 in October) on the number
785 of removed adults (mean ± SE). In the ex *Varroa*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

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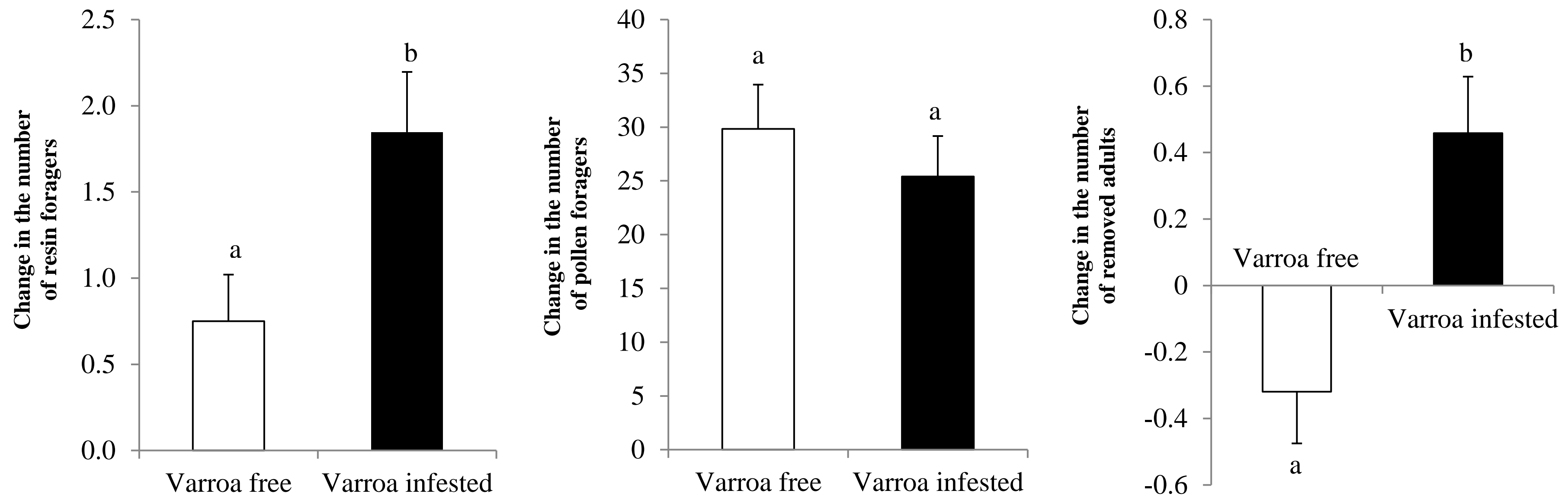

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795 Figure 6. Change in the number of resin and pollen foragers and of removed adult workers (average difference between sum in September and
796 sum in August ± SE) in the *Varroa* free and *Varroa* infested groups. For each variable, different letters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

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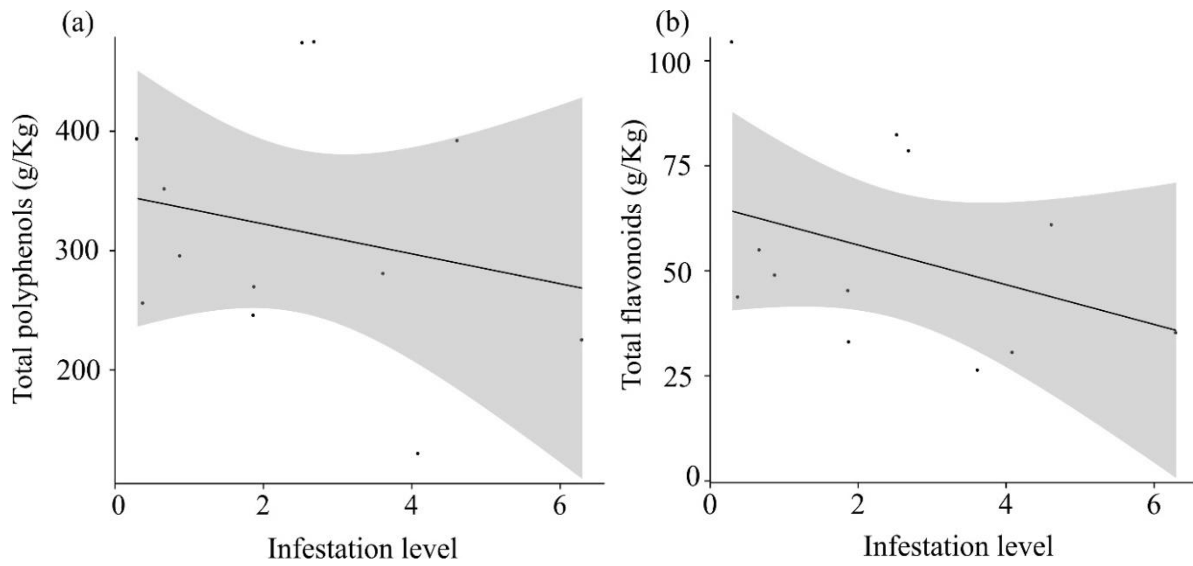

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804 Figure 7. The effects of infestation level (%) on (a) the total polyphenols and (b) total
805 flavonoids found in propolis for the 2014 experiment. Total polyphenols are expressed in g
806 GAE Kg^{-1} DW= g Gallic Acid Equivalent Kg^{-1} Dry Weight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

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