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Biomarkers of nutritional status in honeybee haemolymph: effects of different biotechnical approaches for Varroa destructor treatment and wintering phase

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22 **Biomarkers of nutritional status in honey bee haemolymph: effects of different**  
23 **biotechnical approaches for *Varroa destructor* treatment and wintering phase**

24

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34 **Short title**

35 Nutritional biomarkers in honey bee haemolymph

36

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44 **Abstract**

45 Oxalic acid achieves its maximum efficacy during the active season only when coupled  
46 with brood manipulation techniques like brood interruption and brood removal. This  
47 study aimed to assess the impact of these manipulations on the colony nutritional status  
48 and the subsequent wintering phase, focusing on selected haemolymph biomarkers: total  
49 proteins (TP), zinc (Zn), vitellogenin (VG), and apolipoprotein (APO). Twenty-five days  
50 after the manipulations (T1) colonies that underwent brood interruption (BI) stored more  
51 TP and VG than colonies in the brood removal groups (BR), with a lower APO  
52 percentage, suggesting a lower metabolic effort in summer. In winter, honey bee colonies  
53 of all groups reached similar concentrations of the abovementioned parameters, but  
54 colonies in the BI group showed a higher population. TP, VG and APO are shown to be  
55 promising biomarkers of nutritional status of the colony. Basing on the results obtained  
56 we suggest brood interruption coupled with oxalic acid as the preferred organic method  
57 for the control of *Varroa destructor* in summer.

58

59 **Keywords**

60 **haemolymph proteins/apolipoprotein/vitellogenin/nutritional status/*V. destructor***  
61 **control**

62

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63 **Introduction**

64 Biomarkers are a common tool to evaluate health, diagnose disease, monitor therapies  
65 and predict outcomes both in human and veterinary medicine (Myers et al., 2017). Few  
66 biomarkers have been proposed in apidology and none of them made the way to clinical  
67 practice. The activity of some enzymes has been described in literature as biomarker of  
68 xenobiotic exposure: AChE activity increases during imidacloprid or clothianidin  
69 intoxication (Boily et al., 2013), while CaEs, GST, CAT and ALP activity is modified  
70 in bees exposed to thiamethoxam (Badiou-Bénéteau et al., 2012). Regarding nutritional  
71 status, haemolymph protein concentration is a good candidate as it relates with the  
72 quality of food proteins (De Jong, 2009). Moreover, being vitellogenin the main protein  
73 of the haemolymph in adult bees (Amdam et al., 2003), its concentration is also a  
74 valuable tool to evaluate nutritional status due to its relation with quality (Cremonez et  
75 al., 1998) and quantity (Basualdo et al., 2013; Bitondi and Simões, 1996) of proteins in  
76 the diet.

77 The nutritional status is of capital importance in the wintering phase to allow colony  
78 survival across several months, relying on carbohydrates only. In fact, in temperate  
79 climates, honey bees exhibit two different phenotypes: short lived bees, reared from late  
80 spring to summer and long lived bees, derived from the generation produced in late  
81 summer and autumn (Fluri et al., 1982; Maurizio, 1950). The lifespan of the first type is  
82 about 25-35 days, while the lifespan of the latter can exceed 6 months (Free and  
83 Spencer-Booth, 1959). The longer lifespan of winter bees is related to a higher  
84 haemolymph protein concentration, mainly due to the accumulation of vitellogenin  
85 (Amdam et al., 2004a; Fluri et al., 1982, 1977). The long-lived phenotype can be also

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86 obtained, regardless of the season, by manipulating the colony into interrupting brood  
87 rearing, for example by caging the queen and thus preventing oviposition (Amdam et  
88 al., 2004a; Fluri et al., 1982, 1977; Maurizio, 1950). The absence of brood is a  
89 prerequisite to achieve maximum efficacy in summer of oxalic acid, of one of the most  
90 widely adopted organic acaricides; in fact, this compound is active only on phoretic  
91 mites (Nanetti et al. 2003).

92 Gregorc et al. (2017) recently studied the efficacy of the two most popular solutions  
93 proposed by beekeepers: brood interruption by queen caging and brood removal. Less is  
94 known about the impact of these two techniques on the nutritional status of the colony, in  
95 the short term and, more importantly, in the wintering phase.

96 Considering the expected effects of brood manipulations on honeybee physiology, the  
97 aim of this paper is to study these modifications in the context of the treatment against *V.*  
98 *destructor*, using a panel of haemolymph biomarkers: total proteins, vitellogenin and  
99 apolipophorin and Zn.

100

## 101 **Materials and Methods**

102 All the colonies were initially housed at the CREA-AA apiary in Bologna, Italy. All the  
103 analyses were performed at the Department of Veterinary Medical Sciences, Alma Mater  
104 Studiorum - University of Bologna. All reagents were purchased from Thermo Fisher  
105 Scientific unless otherwise specified.

106

107 *Study design, sample collection and colony level traits*

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108 Ten homogeneous *Apis mellifera ligustica* derived colonies, naturally infested with *V.*  
109 *destructor* and with similar anamnesis were enrolled in the present study; all of them  
110 consisted of nine frames covered with bees and seven frames with brood of variable age,  
111 honey and pollen stores were sufficient and normal for the season. Five colonies were  
112 randomly assigned to group BI that underwent 25 days of brood interruption by queen  
113 caging, while the remaining five colonies were assigned to group BR that underwent  
114 brood removal. Since in our climatic conditions colonies left untreated for mites usually  
115 collapse before winter (Rosenkranz et al., 2010), no control group was used as the death  
116 of these colonies would have prevented a complete and useful collection of data.  
117 Moreover, colonies with uncontrolled mite infestation can result in increased risk of mite  
118 exchange with treated colonies.

119 All the brood frames of each BR colony were separated from the original colony and  
120 used to establish a single nucleus; a small number of bees (ca. 700 workers per comb,  
121 approximately 3 portions of frame) was left on these combs for brood tending. Care was  
122 taken during nucleus formation to guarantee that a sufficient amount of honey was present  
123 on the brood frames in order to satisfy colony needs. Therefore, from BR group derived  
124 two sub-groups: original colony (BR-oc), consisting of adult bees, the queen and frames  
125 of stores; and nucleus (BR-n), consisting of a small number of adults and the frames with  
126 brood and stores. The BR-oc colonies were immediately transferred to another apiary in  
127 order to prevent robbery and mite exchange between them (just treated) and the untreated  
128 colonies of the other two groups. The second apiary was distant about three kilometres  
129 from the first one and was selected for similarity of micro-climatic conditions and trophic  
130 sources.

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131 Each treatment was administered to the colonies in absence of brood by trickling a  
132 solution of sucrose and API-Bioxal (Chemicals Laif) according to the manufacturer  
133 instructions. The BR-oc colonies were treated after 5 days instead of at day 0 to allow  
134 them to build new combs and recover the internal dynamics. After 5 days no receptive  
135 brood was present so full treatment efficacy could be achieved. The BR-n and BI hives  
136 were treated after 25 days when all the brood was hatched. To ensure the absence of  
137 receptive brood in BR-n colonies, natural queen cells were removed on day 7 and replaced  
138 with artificial ones at day 13. Samplings were performed at three critical time-points: pre-  
139 manipulation (T0, end of July), post-manipulation (T1, 25<sup>th</sup> day, August) and winter (T2,  
140 148<sup>th</sup> day, December). At T0 and T2, colony-level traits (areas covered with bees, brood,  
141 sealed brood and pollen) were estimated. A modified Liebefelder standard method  
142 (Delaplane et al., 2013) was used: during the inspection, every face of the frame was  
143 divided into six portions and the number of portions covered with each matrix recorded.  
144 The number of mites killed by the treatments was used to estimate the *V. destructor*  
145 population size, as suggested by (Branco et al., 2006). The mites were counted carefully  
146 on sticky boards, placed under mesh floor equipped hives, from the beginning of the trial  
147 until the 8<sup>th</sup> day after the treatment.

148 The evaluation of mite fall started at day 0 for the colonies of all groups and continued  
149 until day 33 for the colonies of BI and BR-n groups (25 days of natural mite fall, until  
150 colonies were broodless, and 8 days of treatment effect) and until day 13 for the colonies  
151 of BR-oc group (5 days of natural mite fall + 8 days of treatment effect). The estimation  
152 of the *V. destructor* population of the colonies of BR group resulted from the sum of the  
153 respective BR-n and BR-oc colonies.

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154 The graphic timeline of the field trial is reported in Figure 1.

155

156 *Haemolymph collection*

157 For each colony, 30 worker bees were collected between the last brood frame and the  
158 stores as suggested by van der Steen, et al., (2016). At T1 when no brood was present in  
159 BR-n and BI colonies, bees were collected between a store frame and the adjacent frame  
160 where brood is expected to be in presence of a fertile queen. Worker honeybees were  
161 narcotized with gaseous CO<sub>2</sub>, held between forceps by the thorax while a glass disposable  
162 graduated microcapillary (125 mm length, accuracy  $\pm$  0.30%, reproducibility  $\pm$  0.6%,  
163 Blaubrand<sup>®</sup>) was inserted between fourth and fifth tergite. Two microliters of transparent  
164 uncontaminated haemolymph were collected from each bee. Haemolymph samples  
165 collected from 30 specimens were pooled together, added with glutathione to a final  
166 concentration of 0.1% p/v (L-glutathione reduced, Sigma) centrifuged at 3000 g for 15  
167 min in order to separate the cells and stored at -80°C for subsequent analysis.

168

169 *Total protein and Zn analysis*

170 Total protein (TP) concentration was measured by the Bradford method (Coomassie  
171 protein assay) following manufacturer instruction.

172 The quantification of Zn was conducted by graphite furnace atomic absorption  
173 spectroscopy (GFAAS) (Varian Spectra AA 20 Plus) using palladium, magnesium and  
174 EDTA matrix modification with high temperature pyrolysis (Stevens et al. 2017). The  
175 haemolymph samples were centrifuged into small tubes at 3000 g for 15 min to avoid

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176 possible solid material and 2.5 µl were dissolved in 1000 µl 0.1% Pd/Mg/EDTA matrix  
177 modifier for the estimation of Zn concentration.

178

#### 179 *SDS-PAGE*

180 For each sample, three µg of proteins were loaded and analysed with the protocol that  
181 assured the best protein separation in our experimental conditions (4–12% gradient gels,  
182 in MOPS buffer; NuPAGE, Invitrogen). The gels were stained with Coomassie G250  
183 compatible with mass spectrometry analysis, digitalized by ChemiDoc<sup>TM</sup>MP (BioRad)  
184 and the pherograms obtained using ImageLab 5.2.1 software (BioRad). The software  
185 determines the volume of each protein band through the analysis of the pixel values in  
186 the digital image, meaning as volume the sum of all the pixel intensities within the band  
187 boundaries. The band volumes were subsequently compared to the entire volume of the  
188 lane and the relative abundance of each protein band was reported as percentage,  
189 representing therefore the respective relative abundance as a percentage of the total  
190 proteins. The concentration of vitellogenin and apolipophorin expressed as mg/mL was  
191 obtained by multiplying the total protein concentration by the corresponding relative  
192 abundance in percentage.

193

#### 194 *Protein identification by mass spectrometry*

195 The bands which molecular weight corresponded to vitellogenin (180 kDa) and  
196 apolipophorins (250 kDa) were cut from the gels, digested in-gel with trypsin and finally  
197 analysed by a Nano LC-CHIP-MS system (ESI-Q-TOF 6520; Agilent Technologies) as  
198 previously described (Bellei et al. 2013). Raw mass spectrometry data were processed to

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199 obtain the Mascot generic files for database searching. Since the honeybee haemolymph  
200 protein database is not well annotated, a broader taxonomy was selected for identification  
201 to be based on sequence homology. Protein-identification peak lists were generated using  
202 the Mascot search engine (<http://mascot.cigs.unimo.it/mascot>) against the UniProt  
203 database (UniProt.org) specifying the following parameters: all entries, parent ion  
204 tolerance  $\pm 40$  ppm, MS/MS error tolerance  $\pm 0.12$  Da, alkylated cysteine as fixed  
205 modification and oxidized methionine as variable modification, and two potential missed  
206 trypsin cleavages. Proteins with a score  $>80$  or identified with at least two or more  
207 significant sequences were selected. The significant threshold in Mascot searches was set  
208 to obtain a false discovery rate  $<5\%$  (5% probability of false match for each protein with  
209 a score above 80).

210

### 211 *Statistical analysis*

212 Statistical analysis was performed with R software (3.2.5). Data are reported with mean  
213 (M) and standard deviation (SD). Normal distribution was tested by Shapiro-Wilk  
214 normality test. Correlation between laboratory (TP, Zn, VG, APO) and colony-level traits  
215 (*Bees*, *Total brood*, *Sealed brood*, *Pollen*, *V. destructor* population size) was performed  
216 by a Pearson's correlation test. Two-way ANOVA for repeated measures was performed  
217 to evaluate the effect of the interaction of the factors group and time on the laboratory  
218 parameters. One-way ANOVA with Tukey's post-hoc test for pairwise comparison was  
219 performed to evaluate differences among groups (BI, BR-oc, BR-n) within time-points  
220 (T0, T1, T2) for colony-level traits and laboratory parameter or among time-points within  
221 groups for laboratory parameters. Paired *t*-test was performed to evaluate differences

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222 between time-points (T0 and T2) within groups for colony-level traits and to evaluate  
223 differences between the groups BI and BR at T0. Values of  $P < 0.05$  were considered  
224 statistically significant.

225

## 226 **Results**

### 227 *Colony level traits*

228 Data on *Bees*, *Total brood*, *Sealed brood* and *Pollen* are reported in Table I. At T0 there  
229 were no statistically significant differences between BI and BR. Also *V. destructor*  
230 population size did not show significant differences between BI (M = 2691, SD = 2070)  
231 and BR (M = 2823, SD = 1202). At T2 only the parameter *Bees* differed significantly  
232 between groups (F [2,12] = 7.35, P=0.008); portions of the frame covered with bees was  
233 significantly higher in the colonies of BI group (M = 45.9, SD = 14.08) than those from  
234 BR-oc group (M = 20.8, SD = 4.42).

235 Regarding differences between T0 and T2, all the parameters showed a significant  
236 decrease in the wintering phase (T2).

237

### 238 *Total protein and Zn concentrations*

239 Total protein and Zn concentrations in honeybee haemolymph are reported in Figure 2  
240 and Table II. TP ranged from 12.4 at T0 to 33.7 mg/mL at T2, while Zn from 2.7 at T0  
241 to 4.8 µg/mL at T2.

242 A two way analysis of variance showed that the effect of the interaction between time and  
243 group on TP was significant (F[2,11] = 4.302, P = .041670).

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244 As regards significant differences among groups, at T0 and T2 there were no significant  
245 differences. At T1, TP concentration differed significantly between groups ( $F [2,12] =$   
246  $33.38, P= 1.25e-05$ ); protein concentration was significantly higher in the colonies of BI  
247 group ( $M = 31.72, SD = 4.55$ ) than in those from BR-oc group ( $M = 14.44, SD = 3.09$ )  
248 and BR-n group ( $M = 19.84, SD = 2.22$ ).

249 An analysis of variance showed also that the effect of time on TP was significant for  
250 group BI ( $F[2,11] = 8.71, P = .0054$ ), BR-n ( $F[1,7]= 26.06 P = .000108$ ) and BR-oc,  
251 ( $F[2,11] = 20.67 P = .000188$ ). Protein concentration was significantly higher at T1 ( $M$   
252  $= 31.72, SD = 4.55$ ) and T2 ( $M = 33.70, SD = 11.03$ ) than at T0 ( $M = 14.90, SD = 1.72$ )  
253 for BI colonies and significantly higher at T2 ( $M = 36.25, SD = 7.75$ ) than T1( $M = 19.84,$   
254  $SD = 2.22$ ) and T0 ( $M = 13.60, SD = 2.02$ ) for BR-n. Same as BR-n can be said about  
255 BR-oc with T2 ( $M = 33.40, SD = 8.21$ ) higher than T0 ( $M = 13.60, SD = 2.019901$ ) and  
256 T1 ( $M = 14.44, SD = 3.086746$ ).

257 Regarding Zinc, there were no significant differences between groups or between time  
258 points.

259

### 260 *Protein separation by SDS-PAGE*

261 The optimized protocol resulted in the separation of different protein bands in samples of  
262 worker honeybee haemolymph. All samples presented a similar pattern characterized by  
263 the presence of five most abundant protein bands (Fig. 3). The two most consistent bands  
264 were identified by mass spectrometry: the band at an apparent molecular weight  $>200$   
265 kDa was unambiguously identified as apolipoprotein (APO), while the band at 180 kDa  
266 was identified as vitellogenin. Other important protein bands were present at 72, 70 and

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267 67 kDa in all the analysed samples. Less abundant bands were present in particular at  
268 molecular weights less than 60 kDa.

269

#### 270 *Percentage and concentration of VG and APO*

271 Data on VG and APO are reported in Figures 4 and 5, respectively and in Table II. VG  
272 concentration in haemolymph ranged from 2.8 to 17 mg/mL accounting for 43-46 % of  
273 haemolymph TP at the wintering phase (T2). On the contrary, APO concentration showed  
274 minor variations ranging from 3.6 to 5.8 mg/mL and at T2 this protein represented only  
275 11-13%. When evaluating the differences among BI, BR-n and BR-oc within each time-  
276 point, at T0 and T2, there were no significant differences.

277 A two way analysis of variance showed that the effect of the interaction between time and  
278 group was significant on VG percentage ( $F[2,11] = 14.55$ ,  $P = .000813$ ) and APO  
279 percentage ( $F[2,11] = 19.61$ ,  $P = .000236$ ) but not on VG concentration and APO  
280 concentration.

281 At T1, after the manipulations, there were statistically significant differences among  
282 groups regarding VG percentage ( $F [2,12] = 13.54$ ,  $P = .000837$ ) and VG concentration  
283 ( $F [2, 12] = 30.94$ ,  $P = 1.84e-05$ ). VG concentration in colonies of the group BR-oc ( $M =$   
284  $26.98$ ,  $SD = 5.84$ ) was significantly lower than that of groups BR-n ( $M = 35.08$ ,  $SD =$   
285  $1.79$ ) and BI ( $M = 39.48$ ,  $SD = 2.68$ ). On the other hand, VG concentration differed  
286 among all the groups with the highest value in BI group ( $M = 12.55$ ,  $SD = 2.18$ ) followed  
287 by BR-n group ( $M = 6.99$ ,  $SD = 1.08$ ) and BR-oc ( $M = 4.03$ ,  $SD = 1.78$ ). A similar pattern  
288 could be observed regarding APO percentage ( $F [2,12]$ ,  $P = 6.51e-05$ ), with highest value

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289 in BI group (M = 5.83, SD = 0.53) followed by BR-n group (M = 4.60, SD = 0.45) and  
290 BR-oc (M = 3.50, SD = 0.60).

291 APO percentage showed also significant differences among groups (F [2,12] = 17.87, P  
292 = .000252). In this case, the value for the BI group (M = 18.50, SD = 1.27) was  
293 significantly lower than those from BR-n (M = 23.32, SD = 2.30) and BR-oc (M = 24.46,  
294 SD = 1.22).

295 Regarding variations among time-points, there were significant differences in VG  
296 percentage for BI group (F [2,11] = 22.1, P = .00014), BR-n (F [2,11] = 36.41, P = 2.57e-  
297 05) and BR-oc (F [2,11] = 20.46, P = .000196). In BI group the percentage was  
298 significantly higher at T1 (M = 39.48, SD = 2.68) and T2 (M = 43.68, SD = 3.97) than at  
299 T0 (M = 23.63, SD = 7.03). In BR-n group all the time points differed significantly from  
300 each other with, in ascending order, T0 (M = 23.60, SD = 5.33), T1 (M = 35.08, SD =  
301 1.79) and T2 (M = 46.43, SD = 3.87). A totally different pattern can be observed in BR-  
302 oc group with only the percentage at T2 (M = 44.12, SD = 4.62) significantly higher than  
303 those at T0 (M = 23.60, SD = 5.33) and T1 (M = 26.98, SD = 5.84).

304 There were significant differences in VG concentration for BI group (F [2,11] = 9.85, P  
305 = .00353), BR-n (F [2,11] = 36.41, P = 2.57e-05) and BR-oc (F [2,11] = 20.46, P =  
306 .000196). In BI group the percentage was significantly higher at T1 (M = 12.55, SD =  
307 2.18) and T2 (M = 15.03, SD = 6.14) than at T0 (M = 3.58, SD = 1.31). In BR-n group  
308 only T2 (M = 36.25, SD = 7.75) differed significantly from T0 (M = 13.60, SD = 2.02)  
309 and T1 (M = 19.84, SD = 2.22) while in BR-oc group only the percentage at T2 (M =  
310 15.03, SD = 5.62) significantly higher than those at T0 (M = 3.23, SD = 1.04) and T1 (M  
311 = 4.03, SD = 1.78).

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312 APO percentage showed also significant differences among time points in BI group (F  
313 [2,11] = 25.72,  $P = 7.12e-05$ ), BR-n (F [2,11] = 30.61,  $P = 5.46e-05$ ) and BR-oc (F [2,11]  
314 = 48.87,  $P = 3.37e-06$ ). In BI group the percentages at T1 (M = 18.50, SD = 1.27) and T2  
315 (M = 13.12, SD = 2.09) were significantly lower than that at T0 (M = 28.25, SD = 5.36).  
316 In BR-n group only the percentage at T2 (M = 11.30, SD = 1.89) was significantly lower  
317 than those at T0 (M = 28.88, SD = 4.97) and T1 (M = 23.320, SD = 2.30).  
318 The same pattern can be observed for BR-oc group where only the percentage at T2 (M  
319 = 11.08, SD = 1.57) was significantly lower than those at T0 (M = 28.88, SD = 4.97) and  
320 T1 (M = 24.460, SD = 1.22).  
321 Correlations were studied using all the values in the dataset, independently from treatment  
322 groups or time of sampling. There were significant positive correlations between TP and  
323 VG concentration ( $r=0.991$ ,  $P<0.01$ ), TP and VG percentage ( $r =0.89$ ,  $P<0.01$ ), TP and  
324 Zn concentration ( $r =0.533$   $P<0.05$ ), Zn and VG concentration ( $r =0.55$ ,  $P<0.01$ ), Zn and  
325 VG percentage ( $r =0.526$ ,  $P<0.05$ ). There were significant negative correlations between  
326 APO percentage and TP ( $r =-0.839$ ,  $p<0.01$ ), Zn ( $r =-0.498$ ,  $P<0.05$ ), VG concentration  
327 ( $r =-0.864$ ,  $P<0.01$ ) and VG percentage ( $r =-0.929$ ,  $P<0.01$ ). Regarding the correlation  
328 between field and laboratory data, *Total brood* was negatively correlated with TP ( $r=-$   
329  $0.75$ ,  $P<0.01$ ), VG concentration ( $r =-0.77$ ,  $P<0.01$ ) and VG percentage ( $r =-0.72$ ,  
330  $P<0.01$ ) and positively correlated with APO percentage ( $r =0.867$ ,  $P<0.01$ ). Similarly,  
331 *Sealed brood* was negatively correlated with TP ( $r =-0.77$ ,  $P<0.01$ ), VG concentration ( $r$   
332  $=-0.79$ ,  $P<0.01$ ) and VG percentage ( $r =-0.73$ ,  $P<0.01$ ) and positively with APO  
333 percentage ( $r =0.874$ ,  $P<0.01$ ).  
334

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335 **Discussion**

336 The aim of the present paper was to assess the impact of the manipulations needed for the  
337 summer *V. destructor* treatment (T1) and the subsequent wintering phase (T2) on the  
338 colony nutritional status, using an innovative panel of biomarkers.

339 At T1 the adopted manipulations determined a different impact on colony nutritional  
340 status: hives from the BI group showed significantly higher TP concentration, VG  
341 concentration and percentage than the other groups. The consistency among the above-  
342 mentioned biomarkers is not surprising since vitellogenin is the most abundant protein  
343 in honey bee haemolymph (Amdam et al., 2003). In insects, this protein plays an  
344 important role in lipid transport, immune function, longevity and production of royal  
345 jelly (Blacklock and Ryan 1994; Amdam et al. 2004a; Havukainen et al. 2013).

346 Therefore, the accumulation of vitellogenin in haemolymph of workers subjected to  
347 gradual reduction of brood caring is predictable. In fact, colonies of BI and BR-n  
348 groups, where oviposition was interrupted, showed significantly higher concentration  
349 and percentage of vitellogenin, than colonies of BR-oc group where oviposition  
350 continued throughout summer.

351 Notably, only colonies of BI group reached in summer TP and VG concentrations  
352 comparable to those found in winter. This confirms previous findings regarding the  
353 possibility of obtaining winter bees in summer by controlling brood rearing (Amdam et  
354 al., 2004a; Fluri et al., 1982, 1977; Maurizio, 1950). This phenomenon probably  
355 resembles what physiologically happens in temperate zones when winter is approaching:  
356 during autumn, the unfavourable environmental conditions drive the colony to cease  
357 brood rearing and store protein in their fat bodies (Fluri et al., 1982). A further evidence

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358 of the relationship between a specific nutritional status and the presence of brood is  
359 provided by the correlations found in the present study between brood amount (total and  
360 sealed) and TP, VG concentration and VG%.

361 Brood interruption is often considered with concern by beekeepers, due to the loss of 25  
362 days of queen oviposition. However, considering that Amdam et al (2005a) found a  
363 positive correlation between vitellogenin concentration and worker longevity and that  
364 longevity is a key factor for colonies (DeGrandi-Hoffman and Curry 2004), it is possible  
365 that the loss of oviposition is at least partially compensated by the increase in honey bee  
366 longevity.

367 BI differed significantly from the others groups also regarding APO %: the percentage at  
368 T1 was significantly lower than values found in the other groups, and from the percentage  
369 found at T0, approaching the winter value determined at T2. Apolipoporphins are the major  
370 lipoproteins in insects and their presence in haemolymph is closely related to lipid  
371 mobilisation (Robbs et al. 1985). According to Arrese and Soulages (2010), the  
372 mobilisation of lipids in insects can be related to several causes, including embryogenesis,  
373 immune response, and starvation. In the present study, the cause of the higher lipid  
374 mobilisation observed in BR-n and BR-oc is probably related to nutritional stress. In fact,  
375 subgroup BR-n underwent depletion of most of adult bees with their endogenous  
376 reserves, whose function is to produce royal jelly to feed the young larvae (Haydak 1970),  
377 and of part of the pollen reserves, contained mainly in the frames left in the original  
378 colony. At the same time, the subgroup BR-oc is deprived of part of honey stores  
379 contained in the removed brood frames and of drawn combs, therefore it is forced to build  
380 new combs, an energetically expensive task. Schulz, Huang & Robinson (1998) showed

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381 how carbohydrate starvation causes a precocious transition from house bees to foragers.  
382 Moreover, Huang and Robinson (1996) demonstrated that wax deprivation, obtained by  
383 removing drawn combs, produced the same effect. Interestingly this physiological  
384 transition has been correlated to a decrease in the vitellogenin endogenous reserves and  
385 with a precocious senescence (Amdam et al. 2003). It is possible that the manipulation  
386 elicited an effect on the nutritional status both directly and indirectly via an alteration of  
387 the division of labor in the colony. In addition, the recovery of the nutritional stress is  
388 probably impaired by the late summer condition of the zone where the experiment took  
389 place, characterised by dry climate with scarce flowering.

390 In honey bee colonies, there is a strict relation between nutritional status and immunity  
391 (Münch et al. 2013). Basualdo et al. (2014) showed that the use of "beebread" (pollen  
392 supplemented with enzymes and bacteria of the salivary bee glands, fermented and stored  
393 in honey cells) promotes a significant increase of haemolymph total proteins and a higher  
394 survival in bees subjected to artificial infection by *Nosema ceranae* compared to  
395 honeybee fed with a protein substitute. This supports the hypothesis that bee resilience to  
396 pathogens is related to the protein content in haemolymph. In this context the infestation  
397 by *V. destructor* could play an important role. In particular, a negative effect of this  
398 parasite on the ability of bees in accumulating vitellogenin has been reported (Amdam et  
399 al. 2004b). Differently, in the present paper, *V. destructor* infestation did not correlate  
400 with vitellogenin. The discrepancy could be ascribed to the fact that Amdam et al.  
401 (2004b) evaluated the effect of the parasite on single bees individually, while in our study  
402 the evaluation was performed on the entire colony. Moreover, the relatively low level of

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403 infestation in the colonies, that was also not different among groups, could have been not  
404 sufficient to influence the concentration of vitellogenin in haemolymph.

405 Vitellogenin is the main haemolymph zinc binding molecule and a relationship between  
406 this protein, haemolymph zinc concentration and cellular immunity has been suggested  
407 (Amdam et al., 2004a). Our study adds more evidence of the positive relation between  
408 vitellogenin and circulating zinc.

409 Strict relation between zinc and immune function in insects has been demonstrated also  
410 in *Manduca sexta* (Willott and Tran, 2002). The levels found in our study are similar to  
411 those reported by Amdam et al. (2004b) in foragers and lower than the levels found in  
412 hive bees and winter bees.

413 At winter time (T2), no significant differences in total proteins, vitellogenin and  
414 apolipophorinVG and VG concentration among the three groups were measured,  
415 suggesting that bees of all the colonies were able to store similar protein reserves. The  
416 data obtained in the present study are in accord with those reported for winter bees by  
417 other authors (van der Steen et al. 2016). Interestingly, hive population was the only  
418 parameter showing significant differences among groups at T2 . While BI and BR-n  
419 groups showed a population in line with the expectations for *Apis mellifera ligustica*  
420 colonies, colonies of the BR-oc group overwintered with a mean population of only  
421 approximately 5000 adult bees, roughly corresponding to three Dadant-Blatt frames  
422 covered with bees. Such small cluster is very similar to that studied by Harbo (1983)  
423 (approximately 4400 bees) which is reported to be less capable in thermoregulation and  
424 less efficient in the use of reserves; indeed the single bee consumption of honey is  
425 inversely correlated with the number of individuals in the cluster itself. Despite the low

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426 population, all the colonies of BR-oc group survived until next spring, thanks to the  
427 relatively warm winter of 2015.

428 In conclusion, the innovative panel of biomarkers presented in this study, including also  
429 for the first time apolipoprotein, proved to be useful in assessing the impact of brood  
430 manipulation on the colonies. Our data suggest that the duplication of the colonies by  
431 brood removal could determine a greater risk of colony losses facing the winter, due to  
432 the impact on endogenous stores in summer and on hive population in winter.  
433 Supplemental feeding might be an option to mitigate the negative effects of brood  
434 removal even if scientific consensus on pollen substitutes is lacking. The better nutritional  
435 status obtained with brood interruption in summer should be further investigated in order  
436 to confirm the supposed benefits on bee immunity and resilience to pathogens.  
437 For the above-mentioned reasons, we suggest the brood interruption protocol as an  
438 organic, effective and less stressful method to control *V. destructor* in summer.

439

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446

#### 447 **Authors contribution**

448 RC, EF, AN, GA, RG, GI conceived the experiment; RC performed the experiment;

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449 RC, EF, EM and GA performed the analyses; RC, EF, GA, RG, GI wrote the paper and  
450 participated in the revision of it. All authors read and approved the final manuscript.

451

452 **Conflict of interest**

453 None of the authors of this paper has any financial or personal relationship that could  
454 inappropriately influence or bias the content of the paper.

455

456

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554 **Tables**

555 **Table I.** *Bees, Total brood, Sealed brood and Pollen* evaluated at T0 and T2 following a  
 556 modified Liebefeld method (Delaplane et al., 2013). Data are reported in portions of  
 557 frame and as mean  $\pm$  SD ( $n=5$ ).

558

<i>Bees (portions of frame)</i>	<b>Group\Time</b>	<b>T0</b>	<b>T2</b>
	<b>BI</b>	100.8 $\pm$ 26.4 A a	45.9 $\pm$ 12.6 A b
	<b>BR-n</b>	112.6 $\pm$ 34.8 A a	33.6 $\pm$ 9.1 AB b
	<b>BR-oc</b>	112.6 $\pm$ 34.8 A a †	20.8 $\pm$ 4.0 B b
<i>Total Brood (portions of frame)</i>	<b>Group\Time</b>	<b>T0</b>	<b>T2</b>
	<b>BI</b>	49.5 $\pm$ 7.6 A a	4.9 $\pm$ 4.4 A b
	<b>BR-n</b>	60.7 $\pm$ 10.9 A a	3.8 $\pm$ 4.2 A b
	<b>BR-oc</b>	60.7 $\pm$ 10.9 A a †	0.4 $\pm$ 0.8 A b
<i>Sealed Brood (portions of frame)</i>	<b>Group\Time</b>	<b>T0</b>	<b>T2</b>
	<b>BI</b>	34.8 $\pm$ 5.1 A a	4.1 $\pm$ 3.8 A b
	<b>BR-n</b>	39.5 $\pm$ 6.9 A a	3.0 $\pm$ 3.3 A b
	<b>BR-oc</b>	39.5 $\pm$ 6.9 A a †	0.4 $\pm$ 0.8 A b
<i>Pollen (portions of frame)</i>	<b>Group\Time</b>	<b>T0</b>	<b>T2</b>
	<b>BI</b>	5.7 $\pm$ 4.6 A a	0.6 $\pm$ 0.5 A b
	<b>BR-n</b>	7.0 $\pm$ 3.2 A a	0.2 $\pm$ 0.2 A b
	<b>BR-oc</b>	7.0 $\pm$ 3.2 A a †	0.1 $\pm$ 0.2 A b

559

560 Different lower case letters within rows indicate significant differences ( $P<0.05$ ) among time-points within groups. Different capital

561 letters in the column indicate significant differences ( $P<0.05$ ) among groups within time-points. BI, brood interruption; BR, brood

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562 removal; BR-n, nucleus; BR-oc, original colony. † since BR-n derived from BR-oc after the sampling at T0, the values of the analytes  
563 at T0 are the same and refer to the BR colonies before the removal of the brood frames.

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564 **Table II.** Total proteins (TP), Zn, vitellogenin (VG) and apolipophorin (APO) determined  
 565 in honeybee haemolymph at different time-points. Data are reported as mean  $\pm$  SD (n=5).

TP mg/mL	Group\Time	T0	T1	T2
	<b>BI</b>	13.6 $\pm$ 2.9 A a	31.7 $\pm$ 4.1 A b	33.7 $\pm$ 9.9 A b
	<b>BR-n</b>	12.4 $\pm$ 2.8 A a	19.8 $\pm$ 2.0 B a	30.7 $\pm$ 12.6 A b
	<b>BR-oc</b>	12.4 $\pm$ 2.8 A a †	14.4 $\pm$ 2.8 B a	33.4 $\pm$ 7.3 A b
Zn $\mu$ g/mL	Group\Time	T0	T1	T2
	<b>BI</b>	2.7 $\pm$ 0.7 A a	2.9 $\pm$ 0.7 A a	4.8 $\pm$ 1.8 A a
	<b>BR-n</b>	2.9 $\pm$ 1.1 A a	2.9 $\pm$ 1.9 A a	4.4 $\pm$ 1.2 A a
	<b>BR-oc</b>	2.9 $\pm$ 1.1 A a †	1.6 $\pm$ 0.7 A a	4.2 $\pm$ 0.5 A a
VG mg/mL	Group\Time	T0	T1	T2
	<b>BI</b>	3.1 $\pm$ 1.4 A a	12.6 $\pm$ 1.9 A b	15.0 $\pm$ 5.5 A b
	<b>BR-n</b>	2.8 $\pm$ 1.2 A a	7.0 $\pm$ 1.0 B a	17.0 $\pm$ 4.3 A b
	<b>BR-oc</b>	2.8 $\pm$ 1.2 A a †	4.0 $\pm$ 1.6 C a	15.0 $\pm$ 5.0 A b
APO mg/mL	Group\Time	T0	T1	T2
	<b>BI</b>	3.9 $\pm$ 0.7 A a	5.8 $\pm$ 0.5 A b	4.3 $\pm$ 0.8 A a
	<b>BR-n</b>	3.7 $\pm$ 0.9 A a	4.6 $\pm$ 0.4 B a	4.0 $\pm$ 0.8 A a
	<b>BR-oc</b>	3.7 $\pm$ 0.9 A a †	3.5 $\pm$ 0.5 C a	3.6 $\pm$ 0.2 A a
VG %	Group\Time	T0	T1	T2
	<b>BI</b>	21.5 $\pm$ 6.9 A a	39.5 $\pm$ 2.4 A b	43.7 $\pm$ 3.5 A b
	<b>BR-n</b>	21.5 $\pm$ 5.9 A a	35.1 $\pm$ 1.6 A ab	46.4 $\pm$ 3.3 A b
	<b>BR-oc</b>	21.5 $\pm$ 5.9 A a †	27.0 $\pm$ 5.2 B a	44.1 $\pm$ 4.1 A b
APO %	Group\Time	T0	T1	T2
	<b>BI</b>	29.7 $\pm$ 5.1 A a	18.5 $\pm$ 1.1 A b	13.1 $\pm$ 1.9 A b
	<b>BR-n</b>	29.7 $\pm$ 4.2 A a	23.3 $\pm$ 2.1 B a	11.3 $\pm$ 1.6 A b
	<b>BR-oc</b>	29.7 $\pm$ 4.2 A a †	24.5 $\pm$ 1.1 B a	11.1 $\pm$ 1.4 A b

566

567 Different lower case letters within rows indicate significant differences ( $P < 0.05$ ) among time-points within groups. Different capital

568 letters in the column indicate significant differences ( $P < 0.05$ ) among groups within time-points. BI brood interruption; BR-n, brood

569 removal nucleus; BR-oc, brood removal original colony. † since BR-n derived from BR-oc after the sampling at T0, the values of the

570 analytes at T0 are the same and refer to the BR colonies before the removal of the brood frames.

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571 **Figure legends**

572 **Fig. 1** Graphical timeline of the field trial. At T0 (July) colony-level traits inspection and  
573 haemolymph collection, queen caging on BI, brood removal on BR establishing BR-n  
574 and BR-oc, chemical treatment on BR-oc were performed. At T1 (August) haemolymph  
575 collection, queen releasing, chemical treatment on BI and BR-n were performed. At T2  
576 (December) colony-level traits inspection and haemolymph collection were performed.  
577 BI, brood interruption; BR, brood removal; BR-n, nucleus; BR-oc, original colony.

578

579 **Fig. 2** Variations of total protein and Zn concentrations in honeybee haemolymph at  
580 different time-points. Data are expressed in (a) mg/mL (TP) and (b)  $\mu\text{g/mL}$  (Zn) and  
581 reported as mean  $\pm$  SD (n=5). BI, brood interruption; BR-n, nucleus; BR-oc, original  
582 colony.

583

584 **Fig. 3** Representative SDS-PAGE gel and pherograms of honeybee haemolymph. (a)  
585 SDS-PAGE: lanes 1, 2 haemolymph samples from BR group at T0; lanes 3, haemolymph  
586 sample from BI group at T0; lanes 4,5,6 haemolymph samples from BR-n, BR-oc and BI,  
587 respectively, at T2. (b) pherogram of lane 3 (BI, T0); (c) pherogram of lane 6 (BI, T2).  
588 BI, brood interruption; BR, brood removal; BR-n, nucleus; BR-oc, original colony.

589

590 **Fig. 4** Variations of vitellogenin in honeybee haemolymph at different time-points. Data  
591 are expressed in (a) mg/mL and (b) percentage and are reported as mean  $\pm$  SD (n=5). BI,  
592 brood interruption; BR-n, nucleus; BR-oc, original colony.

593

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594 **Fig. 5** Variations of apolipoprotein in honeybee haemolymph groups at different time-  
595 points. Data are expressed in (a) mg/mL and (b) percentage and are reported as mean  $\pm$ SD  
596 (n=5). BI brood interruption; BR-n, brood removal nucleus; BR-oc, brood removal  
597 original colony.

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