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

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Short title

Nutritional biomarkers in honey bee haemolymph

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Abstract

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

Keywords

haemolymph proteins/apolipophorin/vitellogenin/nutritional status/*V. destructor* control

Introduction

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

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

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

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

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

Materials and Methods

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

Study design, sample collection and colony level traits

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

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

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

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

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_2, held between forceps by the thorax while a glass disposable
162 graduated microcapillary (125 mm length, accuracy ± 0.30%, reproducibility ± 0.6%,
163 Blaubrand®) 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

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^{TM}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 form 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

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 ±40 ppm, MS/MS error tolerance ±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

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

Results

Colony level traits

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

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

Total protein and Zn concentrations

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

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

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

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

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

Protein separation by SDS-PAGE

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

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

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).

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

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

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. Apolipophorins 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

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

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

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

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 apolipophorin, 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 furher 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;

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

Conflict of interest

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

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553

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 ± SD (*n*=5).

558

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

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.

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 ± SD (n=5).

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

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) µg/mL (Zn) and
581 reported as mean ± 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 ± SD (n=5). BI,
592 brood interruption; BR-n, nucleus; BR-oc, original colony.

593

Fig. 5 Variations of apolipophorin in honeybee haemolymph groups at different time-points. Data are expressed in (a) mg/mL and (b) percentage and are reported as mean ±SD (n=5). BI brood interruption; BR-n, brood removal nucleus; BR-oc, brood removal original colony.