

1 **Prevalence of *Nosema* microsporidians in commercial bumblebees (*Bombus***
2 ***terrestris*) is not related to the intensity of their use at the landscape scale**

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12 **Abstract** – The use of commercial bumblebees to aid crop pollination may result in
13 overcrowding of agricultural landscapes by pollinators. Consequently, transmission of
14 parasites between pollinators via shared flowers may be substantial. Here we assessed
15 the initial infection status of commercial *Bombus terrestris* colonies, and then explored
16 spatial and seasonal influences on changes in parasite prevalence across a landscape
17 where bumblebee colonies are intensively used to pollinate berry crops in SW Spain.
18 Colonies were placed inside strawberry greenhouse crops and in woodlands adjacent
19 and distant to crops in winter and in spring, as representative periods of high and low
20 use of colonies, respectively. Worker bumblebees were collected from colonies upon
21 arrival from a producer and 30 days after being placed in the field. The abdomen of
22 each bumblebee was morphologically inspected for a range of internal parasites. Upon
23 arrival 71% of the colonies were infected by spores of *Nosema*. Three bumblebees from
24 two colonies harbored *A. bombi* spores at the end of their placement in woodlands
25 adjacent to crops. *Nosema* colony prevalence did not change significantly either among
26 sites or between seasons. We found no evidence for the density of commercial *B.*
27 *terrestris* impacting *Nosema* epidemiology in those commercial colonies, but our results
28 highlight the potential risk for parasites to be transmitted from commercial bumblebees
29 to native pollinators.

30 **agricultural landscape / *Apicystis bombi* / *Fragaria × ananassa* / parasite**

31 **1. INTRODUCTION**

32 In the last half century there has been an increase in the expansion of pollinator-
33 dependent crops (Aizen et al., 2008) that has required a parallel demand for
34 commercially produced bees (Potts et al., 2016). Bumblebees (*Bombus* sp.) started to be
35 commercially produced in Europe in the late 1980s, to replace the costly mechanical-
36 pollination of tomatoes (*Solanum lycopersicum*) (Ravestijn and Sande, 1991; Velthuis
37 and van Doorn, 2006). Quickly, bumblebee breeding techniques advanced and colonies
38 were mass-produced and transported worldwide, where they currently pollinate over 20
39 different pollinator-dependent crops. Over two million bumblebee colonies are
40 produced annually (Graystock, Blane, et al., 2016).

41 The use of commercial pollinators such as bumblebees to aid crop pollination is not free
42 of environmental risks. For instance, queens of commercial bumblebees have become
43 established in many parts of the world (Matsumura et al., 2004; Morales et al., 2013),
44 and there is empirical evidence showing competition for nest sites with other native
45 bumblebee queens in the lab (Ono, 1997) and in the field (Inoue et al., 2008). In
46 addition, commercial bumblebees may compete for food with other native pollinators
47 (Matsumura et al., 2004; Morales et al., 2013), as well as promote the spread of
48 parasites via shared flowers (Colla et al., 2006; Meeus et al., 2011; Schmid-Hempel et
49 al., 2014).

50 Several bee parasite species have been found in commercial bumblebee colonies. In
51 1999, Goka et al. (2000) found for the first time the presence of a parasite, *Locustacarus*
52 *buchneri*, in commercially produced *Bombus terrestris* colonies upon arrival in Japan
53 from an overseas supplier. The presence of this parasite has been linked to shorter

54 lifespan (Otterstatter and Whidden, 2004) and changes in behavior of bumblebees
55 (Otterstatter et al., 2005). Further studies have reported that commercial bumblebee
56 colonies frequently have a range of bumblebee parasites (Graystock et al., 2013a;
57 Murray et al., 2013) and even honeybee parasites (Graystock et al., 2013a), with the
58 latter probably via the consumption of honeybee pollen by reared bumblebees (Goulson
59 and Hughes, 2015). Importantly, the use of commercial pollinators in crops produces
60 high densities of pollinators not only in the agricultural fields or greenhouses, but in
61 adjacent natural areas as well (Ishii et al., 2008; González-Varo and Vilà, 2017; Trillo et
62 al., 2018). Presumably, in those areas, the rate of parasite transmission among
63 pollinators will rise, because high densities of hosts provide ideal conditions for the
64 spread of parasites (Arneberg et al., 1998). In fact, several studies have shown, through
65 the collection of free-flying bumblebees, high prevalence of parasites in sites adjacent to
66 greenhouses where commercial bumblebees are used compared with sites distant to
67 those greenhouses (Colla et al., 2006; Murray et al., 2013) or in greenhouses absent of
68 such commercial bumblebees (Graystock et al., 2014), although there is also evidence
69 against this (Whitehorn et al., 2013).

70 To partially reduce the impact of commercial bumblebees on native pollinator
71 populations and because healthy bumblebees may perform better, as is seen with
72 honeybees (Geslin et al., 2017), producers are under pressure to produce parasite-free
73 bumblebee colonies. In this study, we first examined whether commercially produced *B.*
74 *terrestris* colonies, used to pollinate berry crops in Huelva (SW Spain), carried parasites
75 upon arrival from a producer. We morphologically searched for five common internal
76 bee parasites: larvae of the family Conopidae and Braconidae, *L. buchneri*, *Apicystis*
77 *bombi* and parasites of the genus *Nosema*, which all potentially affect bumblebee health.

78 For instance, larvae of parasitic flies lead to bee death (Schmid-Hempel and Schmid-
79 Hempel, 1996), *L. buchneri* likely reduces lifespan of individual infected host
80 (Otterstatter and Whidden, 2004), *A. bombi* is linked to deterioration of the fat body
81 (Graystock, Meeus, et al., 2016; Macfarlane et al., 1995) and most *Nosema* species
82 reduce worker survival and colony size (Otti and Schmid-Hempel, 2007; Rutrecht and
83 Brown, 2009; Graystock et al., 2013a). We then experimentally tested spatial and
84 seasonal influences on changes in the prevalence of these parasites across a landscape
85 where bumblebee colonies are intensively used. Importantly, *L. buchneri*, *A. bombi* and
86 *Nosema* are likely to be transmitted among pollinators via shared flowers (Durrer and
87 Schmid-Hempel, 1994; Goka et al., 2006; Graystock et al., 2015). Colonies were placed
88 inside strawberry crops and in woodlands adjacent and distant to those crops in January
89 (winter) and again in April (spring), as representative periods of high and low use of
90 bumblebee colonies in berry crops, respectively. We expected parasite prevalence to be
91 highest with high densities of commercial bumblebees in the landscape, that is, 1)
92 higher levels of prevalence at sites inside and adjacent to greenhouse crops than distant,
93 and 2) higher levels in winter than in spring because of the greater use of colonies in
94 winter.

95 2. MATERIALS AND METHODS

96 2.1. Study system

97 The study was conducted in the Guadalquivir Valley in the province of Huelva (SW
98 Spain). In this region there are large intensively cultivated areas of berries (9,500 ha),
99 especially strawberries (~70% of the total area devoted to berry crops) (Freshuelva,
100 2015). Strawberries are cultivated in semi-open polytunnel greenhouses with open sides
101 from November to May. In order to aid crop pollination, farmers use commercial
102 bumblebees (*Bombus terrestris*). Notably, bumblebee colonies are especially used at the
103 beginning of the flowering period (i.e. in winter; personal observations) due to major
104 revenues and worse weather conditions than in spring. The most common remaining
105 natural habitat patches across berry crops are woodlands composed of a rich flora of
106 entomophilous Mediterranean shrubs and herbs, which provide flowers throughout the
107 strawberry cultivation period (Herrera, 1988).

108 2.2. Experimental design

109 In 2015, we purchased 48 *B. t. terrestris* colonies from Koppert Biological Systems, one
110 of the main producers in Europe and specifically in this region. Each colony consisted
111 of a plastic box within a cardboard container, with syrup solution provided *ad libitum*.
112 Each colony included a queen and ~100 workers.

113 First, to quantify colony parasite prevalence, at the arrival of the colonies (period
114 ‘before’), we collected 10 workers from each colony. Each worker was frozen in an
115 individual clean vial at -20 °C for later analyses.

116 Second, to investigate changes in colony parasite prevalence across the landscape, we
117 placed two colonies each in four strawberry crops ('inside') and in eight woodlands,
118 four adjacent to the selected strawberry crops (~50 m; 'adjacent') and four without
119 berry crops in the surrounding 2 km radius landscape ('distant') (Fig. 1). We chose a 2
120 km buffer radius because most bumblebee foraging flights do not exceed this distance
121 (Osborne et al., 2008). The surrounding landscape for inside and adjacent plots had a
122 high berry crop cover (overall mean \pm SE = $48 \pm 5.6\%$; see Table S1). Inside/adjacent
123 plots and distant plots are representative of contrasting landscapes in terms of
124 commercial bumblebee colony density. The density is high and absent in those
125 landscapes, respectively. In fact, commercial bumblebees are frequently observed in
126 landscapes with berry crop cover, rather than when berry crop cover is absent in the
127 landscapes (Trillo et al., 2019). The average (\pm SE) distance between adjacent and
128 distant woodland plots was 5903 ± 1038 m (range = 3.1–11.4 km). This distance meets
129 independence criteria to avoid spatial pseudoreplication between non-paired plots.

130 Third, to investigate seasonal change effects, the experiment was conducted in January
131 ('winter') and repeated in April ('spring'), as representative periods of high and low use
132 of bumblebee colonies in strawberry crops, respectively. Here, the climate is typically
133 Mediterranean with mild winters and warm springs (AEMET, 2015). These two seasons
134 also differ in wild floral resources. The flowering peak is in spring when the floral
135 richness and density are almost triple that in winter (Trillo et al., 2019). Wild pollinator
136 species occur as flowering plant species thrive (Herrera, 1988).

137 In each season, we placed two colonies of bumblebees in the center of each plot.
138 Bumblebees were allowed to forage for 30 days. In strawberry crops the two colonies
139 were hung between four separate greenhouses. The distance between the two colonies

140 within a plot was ~20 m. In woodland plots the two colonies were hidden in wooden
141 boxes to avoid predation. At the end of the experiment, we collected 10 bumblebee
142 workers per colony returning to it (period ‘after’) over two days using aerial nets.
143 Bumblebees were kept in individual clean vials with ice until arrival at the lab where
144 they were stored at -20 °C.

145 **2.3. Parasite screening**

146 The abdomen of each bumblebee was dissected and inspected under a magnifying lens
147 for larvae of parasitic conopid flies (Conopidae, Diptera) and braconid wasps of the
148 genus *Syntretus* (Braconidae, Hymenoptera), and the air sacs were specifically inspected
149 for the tracheal mite *Locustacarus buchneri* (Podapolipidae) (Yoneda et al., 2008).
150 Then, a piece (0.2 cm × 0.2 cm, approx.) of the fat body was dissected out from each
151 bumblebee and mounted on a slide (note that the gut was not included for these
152 analyses). By screening only the fat body, we were able to confirm that we were
153 detecting true infections, not just passage through the gut by vectored spores. We
154 completely screened each slide at ×400 magnification for the presence of spores of the
155 neogregarine *Apicystis bombi* (Lipotrophidae) and microsporidians of the genus *Nosema*
156 (*Nosematidae*). We estimated parasite prevalence (presence or absence) instead of
157 individual infection levels (abundance) because the latter is influenced by many
158 confounding factors that drive infection intensity (Rutrecht and Brown, 2009).

159 **2.4. Statistical analyses**

160 Only *Nosema* infections (Table S2) were statistically analysed, because the remaining
161 parasites showed no or very low prevalence in the colonies (see results). *Nosema*
162 prevalence was calculated estimating the percentage of bumblebees infected taking into

163 account the 10 individuals collected per colony. A linear mixed model (LMM; Gaussian
164 error distribution based on homogeneity in the residuals) was used to analyse whether
165 changes in *Nosema* prevalence were related to our experimental setting. The difference
166 in *Nosema* prevalence in the colonies before and after being placed in the field was used
167 as the response variable. Season (winter/spring), plot type (inside, adjacent and distant),
168 and their interaction were included as fixed factors in the model, while study plot was
169 included as a random factor to account for the paired design between inside and
170 adjacent plots and the re-sampled plots in winter and in spring (see Table S3 for the R
171 code). All statistical analyses were conducted in R (v.3.1.3, R Core Team, 2014). We
172 used the package *lmerTest* (Kuznetsova et al., 2013) for the LMM and Satterthwaite's
173 approximations for F- and p- values.

174

175 **3. RESULTS**

176 In total, over the two seasons we screened 919 bumblebee workers. We missed one
177 colony and several individuals from other colonies due to low colony activity. On
178 average (\pm SE) we collected 19.55 ± 0.13 (range = 16–20) bumblebees per colony.
179 None of the bumblebees were infected by larvae of parasitic conopid flies (Conopidae,
180 Diptera), braconid wasps of the genus *Syntretus* (Braconidae, Hymenoptera), or the
181 tracheal mite, *Locustacarus buchneri*. The prevalence of *Apicystis bombi* was extremely
182 low; only three bumblebees harbored spores in their fat body, and these were collected
183 from two colonies at the end of their placement in adjacent woodlands.

184 In contrast, spores of *Nosema* were found in 58.3% (14 out of 24 colonies) of colonies
185 in winter and in 83.3% (20 out of 24) in spring at the start of each experimental block,
186 that is, upon arrival from the producer prior to their placement in the field. The average
187 *Nosema* prevalence per colony in the before period was $14.0 \pm 3.4\%$ (mean \pm SE,
188 hereafter) in winter, and $19.7 \pm 3.2\%$ in spring. The average *Nosema* prevalence in the
189 after period was $10.2 \pm 2.3\%$ in winter and $26.4 \pm 6.6\%$ in spring. Neither the season ($F_{1,35} = 2.88$, $p < 0.10$) nor the distance ($F_{2,19} = 0.25$, $p < 0.79$) or their interaction ($F_{2,35} = 0.50$,
190 $p < 0.61$) had a significant effect on changes in *Nosema* colony prevalence between
191 periods (Fig. 2A and 2B).
192

193

194 **4. DISCUSSION**

195 The use of commercial bumblebees has been linked to the decline of several native
196 pollinator species (Cameron et al., 2011; Morales et al., 2013; Schmid-Hempel et al.,
197 2014). Among the mechanisms behind this decline, parasite spillover from commercial
198 to native pollinator populations may play a substantial role (Meeus et al., 2011). Mass
199 commercial breeding programs may facilitate the probability of parasite transmission
200 among hosts, as companies usually handle high densities of bumblebees in their
201 facilities. In parallel, the provision of *ad libitum* food may facilitate the reproduction of
202 infected hosts (Brown et al., 2000). Furthermore, even in the case that commercial
203 bumblebees are parasite-free, they may act as reservoirs for parasites in the field,
204 through a spill-back mechanism, leading to an increase in parasite prevalence (Stout and
205 Morales, 2009; Meeus et al., 2011).

206 Upon arrival, we found no evidence for the presence of larvae of parasitic conopid flies
207 (Conopidae, Diptera) and braconid wasps of the genus *Syntretus* (Braconidae,
208 Hymenoptera), or the tracheal mite, *Locustacarus buchneri*, in the screened *Bombus*
209 *terrestris* colonies. Although the presence of larvae of parasitic insects has never been
210 reported in commercial bumblebees, the tracheal mite, *L. buchneri*, was highly
211 prevalent at the end of the 20th century (Goka et al., 2000) spilling over to native
212 bumblebees (Goka et al., 2006). However, it seems that producers have largely
213 eliminated this parasite from commercial bumblebee colonies (Goka et al., 2006;
214 Murray et al., 2013; although see Sachman-Ruiz et al., 2015). In addition, neither these
215 parasitoids nor the tracheal mite, *L. buchneri*, were observed in bumblebees from the
216 colonies after being placed in the field for a month. One explanation for this is that
217 parasitoids of bumblebees might be at low abundance in our study sites, because native

218 bumblebees (*B. terrestris lusitanicus*) are rare (Magrach et al., 2017; Trillo et al., 2019),
219 as they are at the limit of their distributional range (Goulson, 2010). In fact, in this
220 region, the density of commercial bumblebees is around four times greater than that of
221 native bumblebees (Trillo et al., 2019). Another possible and complementary
222 explanation for this low prevalence might be that when bumblebees are parasitized, they
223 desert their colony (Schmid-Hempel and Müller, 1991). In addition, even though *L.*
224 *buchneri* may be present in native bumblebees (although we note that there is no
225 information in Spain; Jabal-Uriel *et al.* 2017) it might be very difficult to detect parasite
226 spillover from native to managed bumblebees because native bumblebees are at very
227 low abundance, as described above.

228 Similarly, there was no evidence for the presence of the neogregarine *Apicystis bombi*
229 (Lipotrophidae) in the screened colonies upon arrival. However, three bumblebees were
230 found to be infected after having been placed in the field. In other regions, the parasite
231 *A. bombi* has been detected infecting commercial bumblebee colonies, although in a low
232 number of colonies (Graystock et al., 2013b; Murray et al., 2013; although again see
233 Sachman-Ruiz et al., 2015). Native bumblebees can host *A. bombi* (Jabal-Uriel et al.,
234 2017), but, as noted above, they are rare in our study region (Magrach et al., 2017;
235 Trillo et al., 2019). In contrast, thousands of commercial colonies from at least three
236 producers (Koppert, Biobest and Agrobio, personal observation) are used on an annual
237 basis. Therefore, it is more likely that other commercial bumblebees infected by *A.*
238 *bombi* transmitted the parasite to the bumblebee colonies we screened, rather than
239 native bumblebees, or, more parsimoniously, our initial screen failed to detect it in
240 arriving colonies.

241 In contrast, we found commercially produced bumblebee colonies to be heavily infected
242 with parasites of the genus *Nosema* upon arrival from the producer. Other studies have
243 also reported similar levels of prevalence with around three quarters of commercial
244 colonies infected (Graystock et al., 2013a; Murray et al., 2013). Unfortunately, our
245 methodology did not allow us to distinguish between the bumblebee parasite *N. bombi*
246 and the honeybee parasite *N. ceranae*. Both can infect bumblebees (Graystock et al.,
247 2013a; Fürst et al., 2014).

248 Unexpectedly, our results showed no significant variation in *Nosema* infection rate at a
249 colony level over time, as in a previous study that monitored wild bumblebees (Goulson
250 et al., 2018), even in landscapes where commercial bumblebees were intensively used to
251 pollinate crops. Even in parasite-free landscapes, one would expect that if commercial
252 colonies are infected by a parasite, it spreads within the colony across time due to the
253 high density of hosts and low genetic variability (Schmid-Hempel, 1998). We propose
254 two potential explanations. On the one hand, bumblebees, in line with other social
255 insects, have evolved social immune systems that combine prophylactic and activated
256 responses to avoid, control or eliminate parasite infections (reviewed by Cremer et al.,
257 2007). Both colony and individual (i.e. immunocompetence, reviewed by Schmid-
258 Hempel 2005) defense mechanisms might be involved in maintaining roughly constant
259 *Nosema* prevalence over time. On the other hand, it has been experimentally
260 demonstrated that *Nosema*, specifically *N. bombi*, relies more on transmission through
261 the larval stage than through transmission among adults (Rutrecht et al., 2007). If we
262 consider that colonies were placed in the field for a month period and that the total
263 development of a bumblebee from larvae to adult is about 4-5 weeks (Alford, 1975),
264 this could explain why we failed to detect an increase in prevalence. Imhoof and

265 Schmid-Hempel (1999) showed an average delay to *Nosema* infection in commercial
266 colonies placed in the field of ~30 days.

267 Our study showed, for the first time in Spain, that commercially produced bumblebee
268 colonies can be infected by *Nosema* parasites prior to their deployment in the field.

269 These parasites may reduce lifespan and have detrimental effects on bumblebee
270 behavior (Otti and Schmid-Hempel, 2007; Rutrecht and Brown, 2009; Graystock et al.,
271 2013a). Because commercial bumblebees placed in semi-open greenhouses frequently
272 forage in natural areas (Foulis and Goulson, 2014), they have the potential to spread the
273 parasites into native pollinator populations (Colla et al., 2006; Murray et al., 2013).

274 Despite the fact that there is some regulation about commercial bee colony health (e.g.
275 for Europe see 92/65/EEC in European Commission 1992), this regulation does not
276 cover all parasites. This implies that commercial colonies can be highly infected by
277 parasites such as *Nosema*, as our study show. Therefore, there is a need for the
278 enforcement of more stringent protocols to preserve the health of commercial and native
279 pollinators.

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293 **AUTHOR CONTRIBUTIONS**

294 AT and MV conceived this research and designed experiments; MJFB participated in
295 the design and interpretation of the data; AT performed experiments, analyses and wrote
296 the first draft of the manuscript; MJFB and MV edited and contributed to the writing of
297 the manuscript. All authors read and approved the final manuscript.

298 **COMPLIANCE WITH ETHICAL STANDARDS**

299 **Conflict of interest.** The authors declare that they have no potential conflict of interest
300 in relation to the study in this paper.

301

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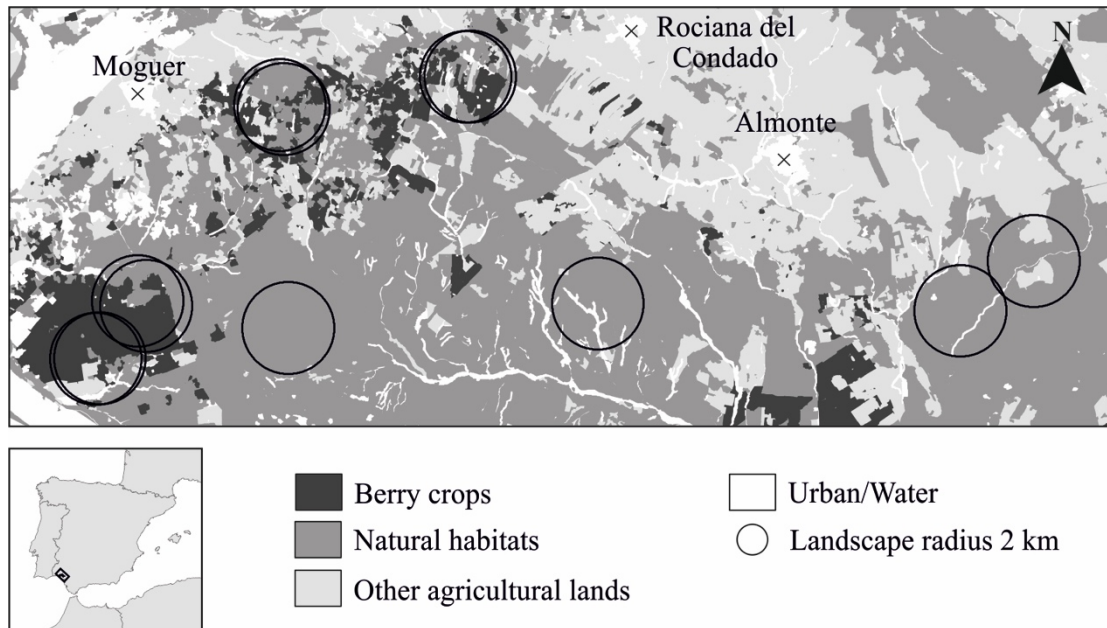
464 **FIGURES CAPTIONS**

465 **Fig. 1** Geographical distribution of plots inside, adjacent and distant to berry crops
466 located in the province of Huelva (SW Spain). Names denote towns.

467 **Fig. 2** Mean (+SE) change in *Nosema* prevalence in commercially produced bumblebee
468 colonies before and after being placed in plots inside, adjacent (~50 m) and distant (>2
469 km) to berry crops in winter (A) and in spring (B). Differences were not significant

470

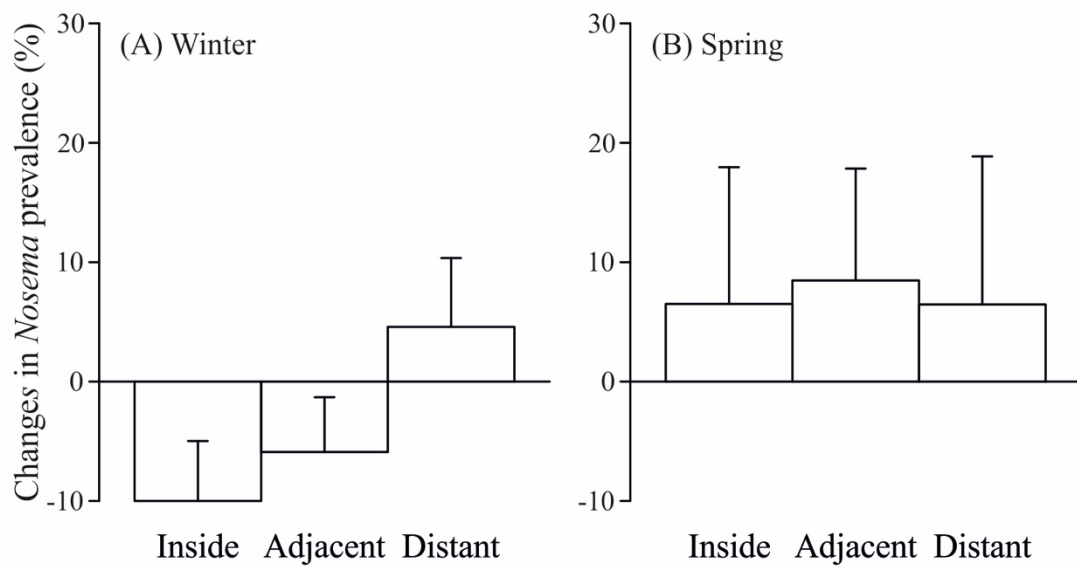
471 **Fig. 1**



473 **Fig. 1** Geographical distribution of plots inside, adjacent and distant to berry crops
474 located in the province of Huelva (SW Spain). In total, 12 plots were selected and two
475 commercial bumblebee colonies were used per plot in winter and again in spring.
476 Names and crosses denote towns.

477

478 **Fig. 2**



479

480 **Fig. 2** Mean (+SE) change in *Nosema* prevalence in commercially produced bumblebee
481 colonies before and after being placed in plots inside, adjacent (~50 m) and distant (>2
482 km) to berry crops in winter (A) and in spring (B). Differences were not significant