Artificial diets modulate infection rates by *Nosema ceranae* in bumblebees

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Abstract

Parasites alter the physiology and behaviour of their hosts. In domestic honey bees, the microsporidia *Nosema ceranae* induces an energetic stress and impairs the behaviour of foragers, potentially leading to colony collapse. Whether this emerging parasite similarly affects wild pollinators is little understood because of the low success rates of experimental infection protocols. Here we established a new apporach for infecting bumblebees (*Bombus terrestris*) with controlled amounts of *N. ceranae*, by briefly exposing individual bumblebees to a sucrose solution containing parasite spores, before feeding them with artificial diets. We validated our protocol by testing the effect of two spore dosages and two diets varying in their protein to carbohydrate ratio, on the prevalence of the parasite (proportion of infected bees), the intensity of infection (spore count in the gut), and the survival of bumblebees. Insects fed a low-protein high-carbohydrate diet showed highest parasite prevalence (up to 70%) but lived longest, suggesting that immunity and survival of bumblebees are maximised at different protein to carbohydrate ratios. Spore dosage had no effect on parasite infection rate and host survival. The identification of experimental conditions for successfully infecting bumblebees with *N. ceranae* in the lab will facilitate investigations of the sub-lethal effects of this parasite on the behaviour and cognition of wild pollinators.

Keywords: *Bombus terrestris*, *Nosema ceranae*, nutrition, experimental infection, survival analyses, PCR.

39 # 1. Introduction

40 Bees face a high diversity of parasites and pathogens that negatively affect their
41 metabolism (Li et al., 2018; Mayack and Naug, 2009), immune system (Antúnez et
42 al., 2009; Li et al., 2017), behaviour and cognition (Gómez-Moracho et al., 2017),
43 altogether compromising the fitness of individuals and colonies (Goulson et al. 2015;
44 Klein et al., 2017).

45 The microsporidia *Nosema ceranae* is one of the most prevalent parasites of
46 honey bees worldwide and is thought to be a major cause of colony declines (Martín-
47 Hernández et al., 2018). *N. ceranae* invades the epithelial cells of the honey bee
48 midgut where it replicates (García-Palencia et al., 2010; Higes et al., 2007). At the
49 physiological level, this parasite disrupts the carbohydrate (Aliferis et al., 2012;
50 Mayack and Naug, 2009) and lipid (Li et al., 2018) metabolism of the host, which
51 ensures the availability of nutrients for its own replication. This causes an energetic
52 stress on infected honey bees that consume more sucrose solution than non-infected
53 conspecifics (Alaux et al., 2010; Martín-Hernández et al., 2011; but see Aufauvre et
54 al., 2012). *N. ceranae* also alters gene expression in the brain (Holt et al., 2013),
55 inhibits the apoptosis of epithelial cells (Martín-Hernández et al., 2017) and
56 deregulates immune responses (Martín-Hernandez et al., 2018). At the behavioural
57 level, infected honey bees start foraging earlier in life (Goblirsch et al., 2013; Higes et
58 al., 2009; Perry et al., 2015), exhibit more frequent but shorter foraging flights
59 (Dosselli et al., 2016; Dussaubat et al., 2013; Kralj and Fuchs, 2010), show reduced
60 homing abilities (Wolf et al., 2014) and lower olfactory learning performances
61 (Piiroinen and Goulson, 2016; Gage et al., 2018; but see Charbonneau et al., 2016).

62 Over the past ten years, *N. ceranae* has been identified in an increasing
63 number of wild bee species (bumblebees: (Graystock et al., 2013; Li et al., 2012;
64 Plischuk et al., 2009), stingless bees: (Porrini et al. 2017)) as well as in some wasps
65 (Porrini et al., 2017) and butterflies (Malysh et al., 2018). In the field, horizontal
66 transmission to wild pollinators may occur through contamination of flower pollen by
67 infected honey bees. This possibility is particularly concerning since many of these
68 wild pollinators are solitary or present simple social organisations (Michener, 2000),
69 and therefore lack complex social immunity behaviours that allow honey bees to
70 collectively limit infection risks and combat parasites (Cremer et al., 2007).

71 The effects of *N. ceranae* on wild pollinators have been best investigated in
72 bumblebees. Recent studies suggest that *N. ceranae* impairs the cognitive abilities of
73 bumblebees, potentially reducing the foraging performances of entire colonies

(Piiroinen et al., 2016; Piiroinen and Goulson, 2016). However, the diversity of experimental infections protocols used in these studies, and their relatively low success rates (i.e. from 0% in (Piiroinen et al., 2016) to 62% in (Graystock et al., 2013)), make it difficult to draw definitive conclusions and call for more robust standardised approaches. By contrast, 100% of infections are routinely reached in honey bee studies (Doublet et al., 2015; Roberts and Hughes, 2015).

The age of bumblebees, their level of starvation prior to exposure, and the amount of parasite spores to which they are exposed to, are all potentially important parameters that vary across current infection protocols. Highest infections levels were obtained by starving bumblebees of uncontrolled age for 8h before feeding them with 6500 spores (an empirically measured infection intensity in wild colonies) in 40% sucrose solution individually administered with a pipette (Graystock et al., 2013). Bumblebees were then maintained in smalls groups of 10 individuals with *ad libitum* access to 40% sucrose solution. Under these conditions, 62% of the bumblebees showed spores in their gut, which considerably reduced their lifespan. Exposure to higher spore dosages caused different results. Fürst et al., (2014) starved 2-days old bumblebees for 1h before feeding them with 100K spores administered in a 10 µL drop of sucrose and maintained them individually with 50% sucrose and artificial pollen *ad libitum*. Here, 34% of the bumblebees developed an infection but no effect was observed on survival (Fürst et al., 2014). Piiroinen and Goulson (2016) and Piiroinen et al., (2016) administered bumblebees of uncontrolled age 180K and 130K *N. ceranae* spores respectively with a pipette and maintained them in small groups of 10 with *ad libitum* access to 50% sucrose and pollen. In these experiments, where the bumblebees were provided high numbers of spores but starved for only 2h, 3% (Piiroinen and Goulson, 2016) or none (Piiroinen et al., 2016) of the individuals developed an infection.

Another potentially important parameter, so far unexplored, is the nutritional composition of diets provided to bumblebees after parasite exposure. Nutrition is as a key mediator of host-parasite interactions (Ponton et al., 2011) and should therefore be carefully controlled when developing standard infection procedures. Many insects increase their consumption of dietary protein in order to develop stronger immunological responses and combat parasites (Cotter et al., 2011; Lee et al., 2006; Povey et al., 2014). Increasing evidence indicate that bees given a choice of foods can carefully adjust their intake of nutrients to reach target levels maximizing fitness traits (e.g. Vaudo et al., 2016; Kraus et al., 2019; Ruedenauer et al., 2020). Although there is no direct demonstration that diet modifies the ability of bees to fight

110 infections, bumblebees fed a low-protein diet have a reduced immune response
111 (Brunner et al., 2014). Honey bees infected by *Nosema apis* (Rinderer and Elliott,
112 1977) and *N. ceranae* (Jack et al., 2016; Porrini et al., 2011; Tritschler et al., 2017)
113 survive longer when provided protein rich pollen. Since protein consumption is
114 needed for synthesising peptides in immune pathways (Lee et al., 2006; Mason et
115 al., 2014; Povey et al., 2014), these results suggest that bees can adjust their
116 nutrient intake for self-medication (Poissonnier et al., 2018).

117 Here we developed an experimental protocol to efficiently infect bumblebees with
118 *N. ceranae* by briefly exposing individuals to controlled amounts of parasite spores
119 and feeding them with artificial diets varying in their protein to carbohydrate ratios.
120 We calibrated our approach by testing two spore dosages and two diets. We
121 analysed parasite prevalence (proportion of infected bumblebees based on
122 polymerase chain reactions, PCR), parasite intensity (spore counts in the gut) and
123 host survival to identify best conditions for infecting bumblebees at sub-lethal doses.

124

125 2. Materials and Methods

126

127 2.1. Bees

128 Experiments were conducted in December 2017. Bumblebee workers (*Bombus*
129 *terrestris*) of unknown age were obtained from two commercial colonies (Biobest,
130 Belgium). Prior to the experiments, 15 workers from each colony were sampled for
131 parasite screening (Graystock et al., 2013). The absence of *N. ceranae* and other
132 common parasites of bumblebees (*N. bombi* and *Crithidia bombi*) in these samples
133 was confirmed in a monoplex PCR with the primers 218MITOC (*N. ceranae:* Martín-
134 Hernández et al., 2007), Nbombi-SSU-J (*N. bombi*: Klee et al., 2006) and CB-ITS1
135 (*C. bombi:* Schmid-Hempel and Tognazzo, 2010).

136 To validate our approach on bumblebees, Honey bees (*Apis mellifera*) were used
137 as positive controls for parasite infection. Honey bee workers of unknown age were
138 obtained from a *Nosema*-free (*N. ceranae* and *N. apis*) colony maintained at our
139 experimental apiary (University Paul Sabatier, Toulouse). The absence of parasite in
140 the colony was confirmed in a duplex PCR with the primers 218MITOC and 321APIS
141 on a sample of 15 workers (Martín-Hernández et al., 2007).

142

143 2.2. Parasites

144 *N. ceranae* spores were obtained from a naturally infected honey bee colony. The
145 abdomens of 15 honey bee foragers were crushed into dH_2O, and the homogenate
146 was examined using light microscopy (x400). Samples showing spores were checked
147 in a duplex PCR (Martín-Hernández et al., 2007) to verify for the presence of *N.*
148 *ceranae* and the absence of *N. apis* (spores of both parasites have similar
149 morphologies; Fries et al., (2006)). Positive honey bee gut homogenates were used
150 to prepare the spore solutions for the experimental infections, following a standard
151 purification protocol (Fries et al., 2013). Spore solutions were prepared no more than
152 one week before the infections. Briefly, 1 mL of the honey bee gut homogenate was
153 centrifuged at 5000 rpm for 5 min. The supernatant containing tissue debris was
154 discarded and the pellet containing the spores was re-suspended in 0.5 mL of dH_2O
155 by vortexing. The sample was centrifuged and washed into dH_2O two times more to
156 obtain a spore solution of 85% purity (Fries et al., 2013). *N. ceranae* spores were
157 counted using an improved Neubauer haemocytometer (Cantwell, 1970) in a light
158 microscope (x400). Counts were made in five squares of 0.2 x 0.2 mm^2 area in both
159 chambers of the haemocytometer and the total number of spores was averaged. The
160 final inoculum concentration was adjusted to either 7500 spores/µL or 15000
161 spores/µL in 20% (v/v) of sucrose solution. All the inocula were prepared the day of
162 the experimental infections.

163

164 2.3. Infections

165 Bumblebees and honey bees were exposed to solutions of *N. ceranae* spores on the
166 same day. Based on preliminary observations showing that different starvation
167 durations are needed to elicit feeding in bumblebees and honey bees, bumblebees
168 and honey bees were manipulated separately. At 9.00 am (GMT+1), 300
169 bumblebees were isolated in empty Petri Dishes (20 mm Ø) and starved for 5h. At
170 12.00 pm, 300 honey bees were isolated in the same conditions but for 2h only. At
171 2.00 pm, bumblebees were individually exposed to a drop of 20 µL of sucrose
172 solution (20% v/v) containing either 0 spore (control), 150K spores or 300K spores.
173 Honey bees were exposed to a drop of sucrose solution containing either 0 spore
174 (control) or 150K spores. The sucrose was delivered with a micropipette in the Petri
175 dish of each bee. Only the bees that consumed the entire drop within 2 two hours
176 were kept for the experiments (272 out of the 300 bumblebees, 258 out of 300 honey
177 bees, see details in supplementary Table 1).

178

179 2.4. Artificial diets

180 Immediately after parasite exposure, bees were allocated to one of two artificial diets,
181 which generated 10 experimental groups. The number of individuals in each group
182 varied between 44 and 50 for bumblebees, and 47 and 77 for honey bees
183 (supplementary Table 1). Diets were liquid solutions containing either a low protein to
184 carbohydrate ratio (P:C 1:150, hereafter "low-protein diet") or a high protein to
185 carbohydrate ratio (P:C 1:5, hereafter "high-protein diet"). The two diets were
186 prepared with a fixed total amount of nutrients (P + C content of 170 g/L).
187 Carbohydrates were supplied as sucrose (Euromedex, France). Proteins consisted in
188 a mixture of casein and whey (4:1) (Nutrimuscle, Belgium). Both diets contained
189 0.5% of vitamin mixture for insects (Sigma, Germany).

190

191 2.5. Survival

192 Bumblebees and honey bees were individually kept in a Petri dish with a hole (1 cm
193 Ø) on the top lid, in which was placed a gravity feeder containing one of the two
194 diets. The feeder consisted in a 1.5 mL Eppendorf tube with a hole at its basis
195 through which the bees could insert their proboscis and ingest liquid food. All bees
196 were kept in two identical incubators (Pol-Eko, Poland) at 26ºC, with a photoperiod of
197 12h light: 12h dark, for 21 days. Each incubator contained the same proportion of
198 bees from the 10 experimental groups (supplementary Table 1). Every day, diets
199 were renewed and dead bees were stored at -20 ºC for molecular analyses. Bees
200 that survived the entire experiment were freeze-killed on day 21.

201

202 2.6. Prevalence and intensity of *N. ceranae*

203 Parasite prevalence (presence in the gut of bees) was assessed in bumblebees and
204 honey using PCR (see example Figure 1A). The gut of each bee was extracted and
205 homogenised in dH_2O and vortexed with 2 mm glass beads (Labbox Labware,
206 Spain). Genomic DNA was extracted using Proteinase K (20 mg/mL; Euromedex,
207 France) and 1 mM of Tris-EDTA Buffer (pH = 8). PCR conditions were adapted from
208 Martín-Hernández et al., (2007) for a monoplex PCR with the 218MITOC primers
209 specific for *N. ceranae*. PCR reactions were carried out in 48-well microtitre plates in
210 a S1000™ Thermal Cycler (Biorad, CA). Each reaction contained 1.5 U of Taq
211 Polymerase (5 U/µL; MP Biomedicals, CA), 1x PCR Direct Loading Buffer (MP

212 Biomedicals, CA), 0.4 µM of each pair of primers (Martín-Hernández et al., 2007),
213 200 µM of dNTPs (Jena Biosciences, Germany), 0.48 µg/µL of BSA (Sigma,
214 Germany) and 2.5 µL of DNA sample in a final volume of 25 µL. Thermal conditions
215 consisted in an initial denaturing step of 94 °C for 2 min, followed by 35 cycles of 94
216 °C for 30 s, 61.8ºC for 45 s and 72 °C for 2 min, with a final elongation step of 72 °C
217 for 7 min. The length of PCR products (i.e. 218 pb) was checked in a 1.2% agarose
218 gel electrophoresis stained with SYBR Safe DNA Stain (Edvotek, Washington DC).

219 Parasite intensity (spore load in the gut) was estimated in positive PCR
220 bumblebee samples under a light microscope (x400). The number of spores was
221 counted as described above (see Section 1.2 and example Figure 2A). Random
222 samples of *Nosema*-negative PCR bumblebees were also screened to confirm the
223 absence of parasite spores.

224

225 2.7. Statistical analyses

226 Bumblebees were analysed for parasite prevalence, parasite intensity and survival.
227 Honey bees were used as positive control for parasite infection, and were therefore
228 only analysed for parasite prevalence. All analyses were performed in R v. 1.0.143
229 (R Development Core Team). All means are shown with standard errors (mean ±
230 S.E).

231 The effects of diet, spore dosage and their interaction on parasite prevalence
232 (proportion of individuals infected after parasite exposure) were tested using
233 Generalized Linear Models with a binomial error structure (binomial GLMs). The
234 effect of diet, spore dosage and their interaction on the proportion of bees showing *N.*
235 *ceranae* spores in their gut were tested using a binomial GLM. The effect of diet,
236 spore dosage and their interaction on parasite intensity (number of spores per bee)
237 were tested using a negative binomial GLM (because of the large over-dispersion of
238 the data). The parasite loads of individuals bees that survived until the end of the
239 experiment (day 21) and those that died before were compared using a negative
240 binomial GLM with a binary predictor (e.g. dead or alive at day 21) as fixed effect.
241 GLMs with binomial distribution errors were fitted using the *glm* function in the R
242 package "stats". GLMs with a negative binomial error distribution were fitted using
243 the *glm.nb* function in the R package “MASS” (Venables and Ripley, 2002). All the
244 models were tested for interactions among predictors (e.g. diet, dosage and PCR
245 results). Interactions that did not improve the fitting were removed from the models
246 using the Akaike information criterion (AIC) for model comparison (Akaike, 1985).

247 The survival of bumblebees was analysed using a Kaplan-Meier test curve
248 with the function *survfit* in the R package "Survival" (Therneau and Grambsch, 2000).
249 The effects of spore dosage, diet, infectious status and their interactions were
250 analysed using Cox proportional-hazards regression models (function *coxph* in the R
251 package "Survival"), followed by a Tukey post-hoc test to account for pairwise
252 comparisons. In all models, colony origin and incubator identity were included as
253 random factors.

254

255 # 3. Results

256

257 ## 3.1. Parasite prevalence

258 To assess infection rates, bumblebees and honey bees were analysed with PCR for
259 the presence of parasites after the 21 days of the experiment. In total, 46.15% of the
260 bumblebees (84 out of 182, Figure 1B and Table 1) and 83.8% of the honey bees
261 (119 out of 142, Figure 1C) exposed to *N. ceranae* were PCR positive, which
262 validates our protocol. Note however that a proportion of control (non-exposed)
263 individuals (5.5% of bumblebees, 19.8% honey bees) were found PCR positive
264 (Figures 1B and 1C). This suggests that our colonies were not entirely free of
265 parasites prior to the experiments or that horizontal cross-contamination occurred
266 during the experiments. All these infected control bees were excluded from further
267 analyses.

268 Diets significantly influenced the proportion of bees that became infected after
269 exposure to the parasite (Figures 1B and 1C; Table 1). A significantly larger number
270 of bumblebees became infected when fed the low-protein diet than when fed the
271 high-protein diet ($GLM_{binomial}$: Estimate = 1.186 (± 0.313), z = 3.79, $p < 0.001$),
272 irrespective of spore dosage ($GLM_{binomial}$: Estimate = 0.4870 (± 0.313), z = 1.503, p =
273 0.133). Likewise, a larger number of honey bees were infected when fed the low-
274 protein than when fed the high protein-diet ($GLM_{binomial}$: Estimate = 1.298 (± 0.538), z
275 = 2.41, $p = 0.016$). Therefore, feeding a low-protein diet induced highest infection
276 rates in both species. Interestingly all the bumblebees that were fed the low-protein
277 diet and that survived until day 21 were PCR positive to *N. ceranae* (6 bumblebees
278 for 150K, 9 bumblebees for 300K). By contrast, the only bumblebee that was fed the
279 high-protein diet and that survived until day 21 (300K) was not infected.

280

281 3.2. Parasite loads

282 To assess the intensity of the infection, the gut homogenates of dead PCR positive
283 bumblebees were screened under microscope for the presence of *N. ceranae*
284 spores.

285 Spores were observed in bumblebee guts for both diets (Figures 2B-E; see
286 supplementary Table 1 for details). Comparing the presence of spores in
287 bumblebees that did not die on the same day yield some information about the
288 dynamics of spore production. For the low-protein diet, spores were seen from day 3
289 to day 20 post exposure with 150K spores (Figure 2B) and from day 4 to day 20 post
290 exposure with 300K spores (Figure 2C). Spores were never observed between day 8
291 and day 15. This may be due to the low mortality rate of bumblebees on these days.
292 For the high-protein diet, spores were seen from day 2 to 8 post exposure with 150K
293 spores (Figure 2D), and from day 3 to day 9 post exposure with 300K spores (Figure
294 2E).

295 Overall 30.9% (26 out of 84) of the infected bumblebees showed *N. ceranae*
296 spores in their gut (Table 1). This estimation of prevalence based on spore loads is
297 lower than that based on PCR screening (Figure 1B and Table 1) because, here, the
298 intracellular stages of the parasite (e.g. meronts, sporonts) cannot be detected. The
299 proportion of bumblebees with spores in their gut was similar irrespective of the diet
300 ($GLM_{binomial_diet}$: Estimate = 0.176 (± 0.489), z = 0.36, p = 0.718) and the spore
301 dosage ($GLM_{binomial_spore\ dosage}$: Estimate = 0.109 (± 0.477), z = 0.23, p = 0.819). Spore
302 loads were highly variable across individuals, with an average of 367.31 (± 72.03)
303 spores per bumblebee. The spore dosage the bees were exposed to had no effect
304 on the spore loads found in their gut ($GLM_{negative\text{-}binomial}$: Estimate = -0.002 (± 0.002),
305 z = -0.73, p = 0.464). On the contrary, the diet had a significant effect on spore load
306 ($GLM_{negative\text{-}binomial}$: Estimate = 1.294 (± 0.347), z = 3.73, p < 0.001), leading to higher
307 amounts of spores in bumblebees fed the high-protein diet (662.50 ± 222.71 spores
308 per bee) than in bumblebees fed the low-protein diet (182.81 ± 40.99 spores per
309 bee).

310 Considering bumblebees that were fed the low-protein diet and that survived
311 until day 20 post exposure, only 4 out of 6 individuals exposed to 150K spores (175 ±
312 87.5 spores per bee; Figure 2B) and 4 out 9 individuals exposed to 300K spores (125
313 ± 62.5 spores per bee; Figure 2C) showed spores in their gut. These bumblebees
314 showed similar spore loads than those fed the same diet but that died before day 20
315 post exposure ($GLM_{negative\text{-}binomial:}$ Estimate = -0.363 (± 0.389), z = -0.93, p = 0.350).

316

317 3.3. Survival

318 To test the effect of spore dosage and diet on the physiology of bumblebees, survival
319 was analysed during the 20 days after parasite exposure. Here the infection status of
320 each bumblebee was based on the PCR results.

321 Overall, bumblebees fed the high-protein diet had lower survival than
322 bumblebees fed the low-protein diet (Figure 3; Cox: estimate = - 0.75 (± 0.14), z = -
323 5.17, $p < 0.001$). Bumblebees fed the high-protein diet (Figure 3A) showed similar
324 mortality rate irrespective of spore dosage (Table 2) and infectious status
325 (supplementary Table 2). By contrast, bumblebees fed the low-protein diet (Figure
326 3B) had a significantly reduced survival when exposed to spores than when non-
327 exposed (Table 2). For this diet, 50% of exposed bumblebees died between days 6
328 and 7 post exposure. This lethal time 50 (LT_{50}) was not reached for the non-exposed
329 (control) bumblebees. Interestingly, in the low-protein diet, infected bumblebees
330 tended to die faster than control bumblebees, but survived significantly longer than
331 bumblebees exposed and non-infected (Figure 3B; supplementary Table 2).
332 Therefore, both parasite exposure and infection had negative effects on the survival
333 of bumblebees maintained on the low-protein diet.

334

335 # 4. Discussion

336

337 Several recent studies suggest that *N. ceranae* negatively affect bee behaviour and
338 cognition, which may have dramatic consequences for colony growth and survival
339 (Gage et al., 2018; Piiroinen and Goulson, 2016). In bumblebees, these results
340 remain difficult to interpret due to the low efficiency of experimental infection
341 protocols. Here we exploited recent insight into the nutritional ecology of bees (e.g.
342 Vaudo et al., 2016; Kraus et al., 2019; Ruedenauer et al., 2020) to develop a reliable
343 method to infect bumblebees with *N. ceranae*. We identified key effects of diet
344 composition on bumblebee infection rates and survival, indicating a complex
345 interaction between diet, parasite development and host health.

346 Overall 46% of the bumblebees exposed to *N. ceranae* spores were infected,
347 a percentage that increased up to 70% when only considering bumblebees that were
348 exposed to 300K spores and maintained on a low-protein high-carbohydrate diet.
349 These levels of prevalence are similar to those obtained by Graystock et al., (2013)

350 (62% infection rate) who used a 20 to 45 times lower infection dose (measured in
351 naturally infected colonies: 6500 spores/bee) administered with a micropipette
352 directly into the bee mouth parts. These rates of successful infections are higher than
353 all previous studies where bumblebees were exposed to an order of 10^5 spores/bee
354 either delivered as a drop of solution in a Petri Dish (39%; Fürst et al., 2014) or with a
355 micropipette directly into the bee mouth (0-3%; Piiroinen and Goulson, 2016;
356 Piiroinen et al., 2016).

357 We did not find any clear effect of parasite dosage (150K or 300K spores per
358 bee) on prevalence and survival. Comparing our results to that of previous studies
359 does not indicate such effect neither, which suggests that above a certain
360 concentration of parasite spores (e.g. 6500 spores/bee in Graystock et al. 2013),
361 parasite dosage is not an important factor determining the success of an infection. By
362 contrast, we found a strong effect of diet on parasite prevalence and spore loads.
363 Previous studies indicate that pollen intake affects honey bee physiology and
364 tolerance to *N. ceranae* (Basualdo et al., 2014; Di Pasquale et al., 2013). Using
365 artificial diets with controlled amounts of protein and carbohydrates, we demonstrate
366 that food macronutrient balance substantially influences both the infection rate and
367 the survival of bumblebees. A higher number of bumblebees were infected when fed
368 the low-protein high-carbohydrate diet, suggesting that different blends of nutrients
369 differently affect the germination of the parasite (but see Jack et al., 2016). The
370 digestion of the different macronutrients in the bumblebee gut, whose microbiota is
371 mostly composed by sugar-fermentative bacteria (Mohr and Tebbe, 2006), may lead
372 to changes in the gut environment that could favour the parasite germination, such as
373 shifts in pH (Wittner and Weiss, 1999). Furthermore, because *N. ceranae* exploits the
374 metabolism of carbohydrates to obtain energy from its host (Mayack and Naug,
375 2009) a higher carbohydrate consumption by infected bumblebees may benefit the
376 establishment of *N. ceranae*. Alike previous studies on honey bees that showed that
377 the supplement of pollen in diet increase the parasite intensity (Basualdo et al., 2014;
378 Fleming et al., 2015; Jack et al., 2016), we found that bumblebees with access to
379 higher amounts of protein show higher spore loads in their gut, suggesting that
380 proteins benefit the replication of the parasite.

381 The effect of diet was also evident on bumblebee survival. *N. ceranae*
382 reduced the survival of bumblebees, specifically in the low-protein high-carbohydrate
383 diet, where mortality rate increased by day 3 post exposure. This result is consistent
384 with the time described for *N. ceranae* to produce new spores in the honey bee gut
385 (Higes et al., 2007). Similarly to Graystock et al., (2013), we found that most of the

386 infected bumblebees (67.5%) died without showing spores in their gut, suggesting
387 that the parasite was not able to complete its cycle at a detectable level (i.e. produce
388 new spores). The lack of spores in the bumblebee guts could be due to a migration of
389 the parasite to the fat body of their host where it cannot reproduce (Graystock et al.,
390 2013). Alternately, this could be a consequence of a high virulence of the parasite.
391 Emergent parasites can be very virulent for a novel host causing its death before
392 reproducing and releasing their offspring (Schmid-Hempel, 1998). Although it is not
393 clear when *N. ceranae* started to colonise bumblebee hosts (including *B. terrestris*),
394 the parasite was first identified in bumblebees in 2005 (Plischuk et al., 2009),
395 implying a short coevolution time in which the parasite may not have had time to fully
396 adapt its virulence to its diverse host species. Interestingly, the absence of parasite
397 spores in the gut of bumblebees that died at different times could reflect the ability of
398 bumblebees to clear the parasite. The fact that infected bumblebees fed the low-
399 protein high-carbohydrate diet survived longer than exposed non-infected
400 bumblebees fed the same diet suggests that the mechanisms allowing parasite
401 clearance incur a higher metabolic cost, here manifested as a reduction of
402 bumblebee lifespan, than parasite tolerance. Nonetheless, given that only dead
403 bumblebees were checked for spores and that spore loads are generally higher in
404 live bees than in dead ones (Zheng et al., 2014), studying the dynamics of *N.*
405 *ceranae* spore production in live bumblebees (for instance by analysing faeces) will
406 be necessary to determine whether these insects are able to get rid of the parasite.

407 The fact that bumblebees showed low parasite prevalence but died faster
408 when fed a high-protein low-carbohydrate diet suggests that immunity and lifespan
409 are maximized at different nutritional balances. Several studies show how an
410 animals' diet can differently influence the expression of key life-history traits, forcing
411 animals to trade-off between optimizing multiple traits simultaneously (e.g. Bunning
412 et al., 2016, 2015; Rapkin et al., 2018). In insects, lifespan is typically enhanced on
413 high-carbohydrate diets whereas reproduction is maximized on high-protein diets
414 (fruit flies: Lee et al., 2008; Fanson et al., 2009; Reddiex et al., 2013; Jensen et al.,
415 2015; Semaniuk et al., 2018, crickets: Maklakov et al., 2008). Immunity and
416 reproduction also display differences in nutritional requirements (fruit flies: Ponton et
417 al., 2011, 2015; decorated crickets: Rapkin et al., 2018; leafworm: Cotter et al.,
418 2011). Honey bees survive longer on high-carbohydrate diets (Altaye et al., 2010)
419 and pollen (the main source of protein) favours the survival of individuals infected
420 with *N. ceranae* (Basualdo et al., 2014; Fleming et al., 2015; Jack et al., 2016; Zheng
421 et al., 2014). Whether sterile bumblebee workers trade-off between over-ingesting

422 protein and under-ingestion of carbohydrates in order to reduce parasite
423 establishment at the expense of a shorter lifespan is an open question. In these
424 social insects, such individual strategy may considerably reduce the risks of
425 contamination of other workers within the colony (Poissonnier et al., 2018).

426 Developing standard protocols to characterize the sublethal effects of parasites
427 and pathogens on bees has become a major challenge for understanding bee
428 population declines (Gómez-Moracho et al., 2017). Here we demonstrated that diet is
429 key in determining infection rates of bumblebees exposed to *N. ceranae*. Highest
430 infection rates and longest survivals were obtained with a high-carbohydrate low-
431 protein diet, thereby providing ideal conditions for investigating potential effects of *N.*
432 *ceranae* on bee behaviour and cognition. Other parameters, not tested here, may
433 also be of importance and investigated in future studies. For instance, both our study
434 and that of Graystock et al. (2013) starved bumblebees for at least 5 h prior to
435 parasite exposure, which may have increased the probability of parasite
436 establishment. Variations in the age of the bumblebee tested may also explain some
437 of these differences. In the only study that controlled for age, Fürst et al., (2014)
438 infected 2-days old bumblebees (post eclosion from the pupa) and obtained a lower
439 infection rate than that presented here and no effect on bumblebee mortality,
440 suggesting that young bumblebees are less susceptible to the parasite. Finally, we
441 cannot exclude differences in the virulence of the *N. ceranae* strains used for
442 experimental infection across studies (but see Van der Zee et al., 2014). We hope
443 that our infection protocol will facilitate further studies on the interactions between *N.*
444 *ceranae* and bumblebees in order to better assess the risks this emergent parasite
445 represents for wild pollinators.

446

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721 # Tables and Table legends

722 **Table 1. Number of bumblebees exposed to *N. ceranae*, infected with parasite**
723 **(PCR positive), and showing spores in their gut.** The percentage of bumblebees
724 infected relative to those exposed, and the percentage of bumblebees showing
725 spores in their gut relative to the number of PCR positive individuals are given into
726 brackets.

Diet	Spore dosage	Exposed	Infected	Showing spores
Low-protein	150K	44	23 (52.27%)	7 (30.4%)
	300K	45	31 (68.88%)	9 (29.03%)
High-protein	150K	47	14 (29.78%)	4 (28.5%)
	300K	46	16 (34.78%)	6 (37.5%)
Total		182	84 (46.15%)	26 (30.9%)

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731 **Table 2. Cox Regression Model testing the effect of spore dosage on the**
732 **survival of bumblebees for each diet.**

Diet	Spore dosage	Estimate	S.E.	z	p
High-protein	150K	0.2766	0.2115	1.308	0.191
	300K	0.1440	0.2151	0.669	0.503
Low-protein	150K	1.2595	0.2792	4.511	< 0.001
	300K	1.1021	0.2808	3.925	< 0.001

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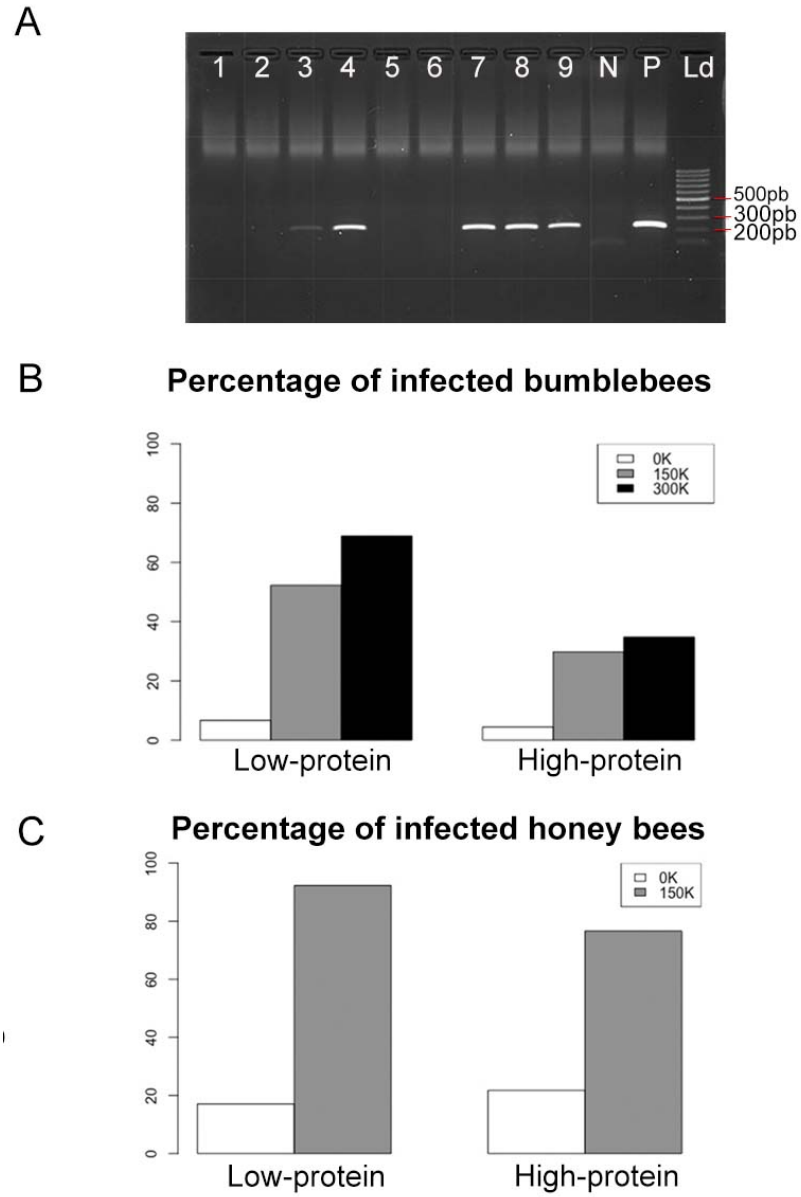
739 Figures and figure legends



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741 **Figure 1. Parasite prevalence**. (A) Agarose electrophoresis gel with negative (lanes
742 1, 2, 5 and 6) and positive (lanes 3, 4, 7-9) bumblebees to *N. ceranae* determined by
743 PCR. N: negative control of PCR; P: positive control of PCR; Ld: molecular size
744 marker (100 pb). Percentage of (B) bumblebees and (C) honey bees infected by *N.*
745 *ceranae* (PCR positive) for all diets and spore dosages. Bumblebee groups
746 contained between 44 and 50 individuals. Honey bee groups contained between 47
747 and 77 individuals (see details in supplementary Table 1).

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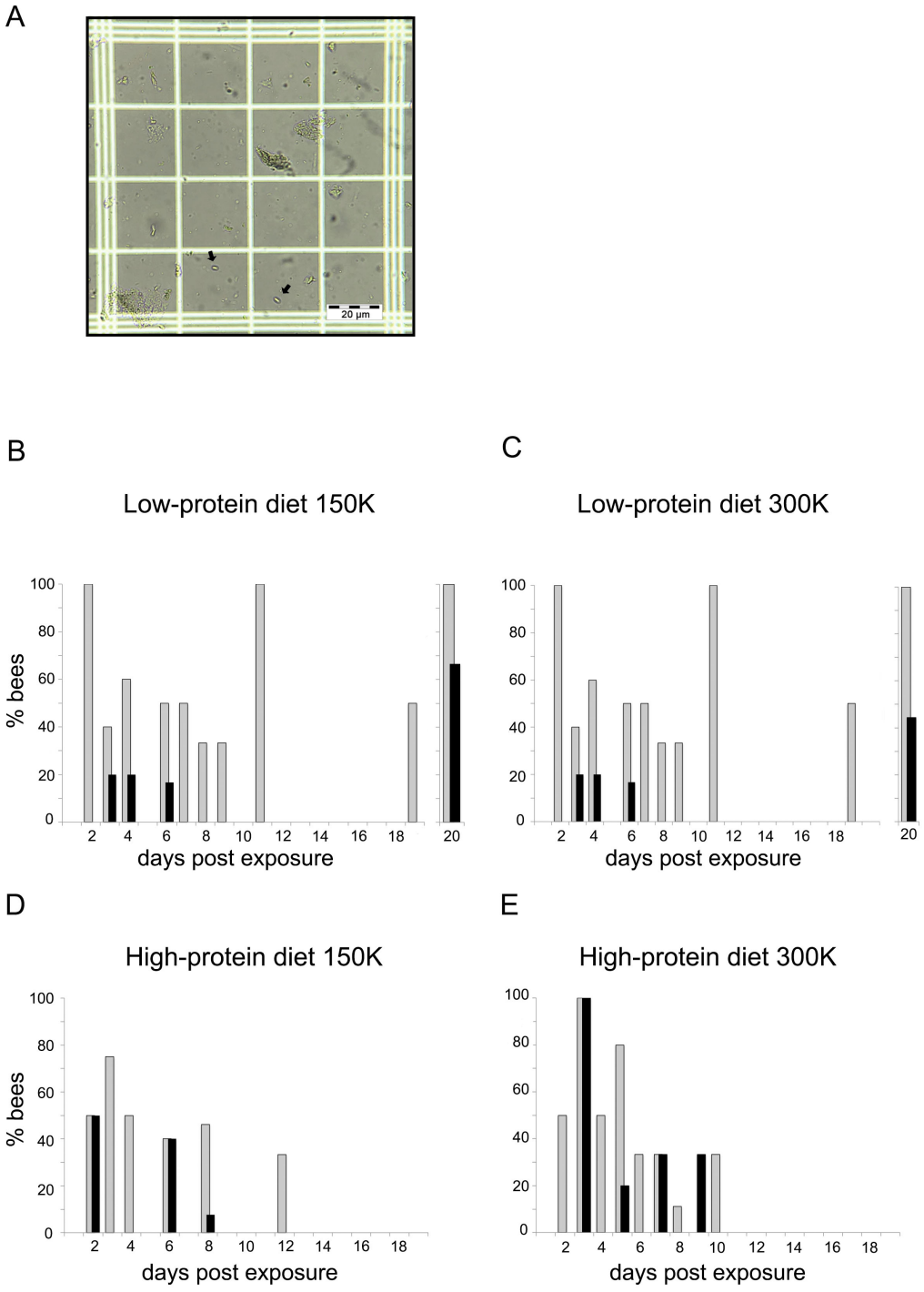

Figure 2. Parasite loads. (A) *N. ceranae* spores observed in an infected bumblebee (optical microscope, x 400). (B-E) Proportion of dead bumblebees at different days post exposure that were positive to *N. ceranae* in a PCR (grey bars) and showed spores in their gut (black bars), for both diets (high- and low-protein diets) and spore dosages (150K and 300K). Each group contained between 44 and 50 individuals (see details in supplementary Table 1).

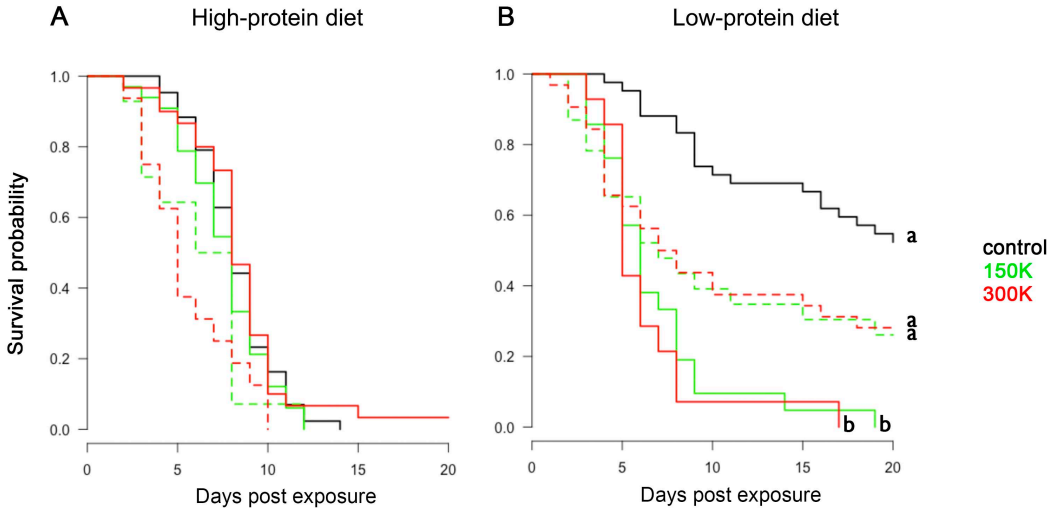

Figure 3. Survival analyses. Survival probability of bumblebees fed the high-protein diet (A) and the low-protein diet (B), for the different spore dosages (control, 150K and 300K) across time. Infected (dashed lines) and non-infected (solid lines) bumblebees are differentiated. Different letters associated to curves indicate statistical differences in survival ($p<0.05$; post-hoc Tukey test after Cox-regression model in a Kaplan Meier test curve, see supplementary Table 3). Each group contained between 44 and 50 individuals (see details in supplementary Table 1).