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1 **The gut parasite *Nosema ceranae* impairs** 2 **olfactory learning in bumblebees**

3

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5

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10

11 **Abstract**

12 Pollinators are exposed to numerous parasites and pathogens when foraging on flowers. These
13 biological stressors may affect critical cognitive abilities required for foraging. Here, we
14 tested whether exposure to *Nosema ceranae*, one of the most widespread parasite of honey
15 bees also found in wild pollinators, impacts cognition in bumblebees. We investigated
16 different forms of olfactory learning and memory using conditioning of the proboscis
17 extension reflex. Seven days after feeding parasite spores, bumblebees showed lower
18 performance in absolute and differential learning, and were slower to solve a reversal learning
19 task than controls. Long-term memory was also slightly reduced. The consistent effect of *N.*
20 *ceranae* exposure across different types of olfactory learning indicates that its action was not
21 specific to particular brain areas or neural processes. We discuss the potential mechanisms by
22 which *N. ceranae* impairs bumblebee cognition and the broader consequences for populations
23 of pollinators.

24 **Keywords** : Bumble bees ; *Bombus terrestris*; learning and memory ; PER

25

26 1. Introduction

27

28 Pollinators, such as bees, rely on a rich cognitive repertoire to collect pollen and nectar on
29 flowers and feed their brood. These include associative learning and memories of floral traits
30 like odours, shapes, colours, and textures, to identify profitable resources (Giurfa, 2015;
31 Menzel, 2012), and spatial cues to navigate (Collett et al., 2013). Any disruption of these
32 cognitive abilities by environmental stressors can considerably reduce the foraging
33 performances of bees, ultimately compromising brood development and survival (Klein et al.,
34 2017).

35 In particular, foraging bees are exposed to a number of parasites that can affect their
36 physiology and behaviour (Gómez-Moracho et al., 2017). The microsporidia *Nosema ceranae*
37 is one of the most prevalent parasites of bees worldwide with a large range of hosts including
38 honey bees (Fries et al., 1996; Higes et al., 2006), bumblebees (Figueroa et al., 2021; Plischuk
39 et al., 2009), solitary bees (Figueroa et al., 2021; Ravoet et al., 2014), but also other flower
40 visitors like wasps (Porrini et al., 2017). Insects get infected by ingesting parasite spores from
41 contaminated water or pollen (Higes et al., 2008), or during physical contacts with
42 contaminated individuals (Smith, 2012). Once in the gut, the spores invade the epithelial cells
43 of the bee where they develop by exploiting its metabolism (Holt et al., 2013). *Nosema*
44 degenerates the gut epithelium of the host (Dussaubat et al., 2012; Higes et al., 2007), alters
45 its metabolism (Martín-Hernández et al., 2011; Mayack and Naug, 2009) and disrupts the
46 immune response (Antúnez et al., 2009), thereby extending its infection time inside the host.
47 In honey bees, this causes a disease (nosemosis) believed to contribute to colony collapse
48 disorder (Cox-Foster et al., 2007; Higes et al., 2009).

49 Infected honey bees also show impaired navigation (Wolf et al., 2014) and increased flight
50 activity (Dussaubat et al., 2013) suggesting their cognitive abilities are affected. Few studies
51 have explored this possibility and their results so far are mixed (Bell et al., 2020;
52 Charbonneau et al., 2016; Gage et al., 2018; Piironen et al., 2016; Piironen and Goulson,
53 2016). Most of these studies were conducted on honey bees using Pavlovian olfactory
54 conditionings of the proboscis extension reflex (PER) in which harnessed bees are trained to
55 associate an odour or a combination of odours (conditioned stimulus: CS) with a sucrose

56 reward (unconditioned stimulus: US) (Takeda, 1961; Bitterman *et al.*, 1983). In absolute
57 conditioning protocols (when an odour is paired to a reward), honey bees showed reduced
58 learning between days 7 and 23 after parasite exposure ((Bell *et al.*, 2020; Gage *et al.*, 2018;
59 Piironen and Goulson, 2016); but see (Charbonneau *et al.*, 2016)). Results on memory are
60 less clear. Short-term memory was impaired after 7 and 15 days (Gage *et al.*, 2018), but not
61 after 9 (Piironen and Goulson, 2016) or 23 days (Bell *et al.*, 2020). Long-term memory was
62 affected after 7 days but not after 15 days (Gage *et al.*, 2018). Only two studies explored these
63 effects on other pollinators, namely bumblebees. One suggests a slight impairment of absolute
64 learning (Piironen and Goulson, 2016), and both report an absence of effect on memory (i.e.
65 2 h-2.5 h memory; Piironen *et al.*, 2016, Piironen and Goulson 2016). Note however that in
66 these two studies less than 3% of the bumblebees exposed to the parasite showed signs of
67 infection after the behavioural tests (i.e. PCR positive).

68 Given the expanding geographical distribution of *N. ceranae* worldwide (Khezri *et al.*, 2018;
69 Klee *et al.*, 2007; Ostroverkhova *et al.*, 2020) and its prevalence in domesticated and wild
70 bees (Plischuk *et al.*, 2009; Porrini *et al.*, 2017; Ravoet *et al.*, 2014), clarifying its influence on
71 host cognition has become an urgent matter. In particular, other critical forms of learning
72 required for efficient foraging, such as the ability to associate one of two odours with a reward
73 (differential learning) and reverse this association (reversal learning) have so far been
74 unexplored. These types of learning are essential to discriminate flowers. They involve
75 different brain centers and may thus be more or less impacted by the parasite if its effect is
76 specific (Devaud *et al.*, 2015; Giurfa, 2007; Mertes *et al.*, 2021).

77 Here we build on a recently established method yielding high the rates of experimental
78 infection by *N. ceranae* (Gómez-Moracho *et al.*, 2021) to study the impact of the parasite on
79 different cognitive tasks in bumblebees. We used PER conditioning to compare the olfactory
80 learning and memory performances of control bumblebees, bumblebees exposed to the
81 parasite, and bumblebees contaminated by the parasites (PCR positive) at seven days post
82 exposure.

83

84 2. Material and methods

85

86 2.1. Bumblebee colonies

87 We used bumblebees (*B. terrestris*) from 14 commercial colonies acquired from Biobest
88 (Westerlo, Belgium). Before the experiments, we randomly sampled 15 bumblebees from
89 each colony and verified the absence of *N. ceranae* (primer: 218MITOC; Martín-Hernández
90 *et al.*, 2007), as well as other common parasites of bumblebees (*N. bombi* (primer : Nbombi-
91 SSU-J; Klee *et al.*, 2006) and *Crithidia bombi* (primer: CB-ITS1; Schmid-Hempel and
92 Tognazzo, 2010)) in a polymerase chain reaction (PCR). We maintained bumblebees in their
93 original colonies with *ad libitum* access to the syrup provided by the manufacturer and germ-
94 free pollen (honey bee collected pollen exposed to UV light for 12 hours; Zheng *et al.*, 2014),
95 in a room at 25±1°C under a 12 h light:12 h dark photocycle.

96

97 2.2. *N. ceranae* spores

98 We obtained fresh *N. ceranae* spores from naturally infected honey bee colonies (*Apis*
99 *mellifera*) maintained at our experimental apiary (University Paul Sabatier - Toulouse III,
100 France). To prepare spore solutions, we dissected the gut of 15 honey bees and crushed them
101 in 15 mL of distilled H₂O. Once we confirmed by PCR the presence of *N. ceranae* and the
102 absence of *N. apis* (another common parasite of honey bees) in each homogenate (Martín-
103 Hernández *et al.*, 2007), we purified the homogenates following standard protocols (Fries *et*
104 *al.*, 2013). We centrifuged homogenates in aliquots of 1 mL at 5,000 rpm for 5 minutes and
105 re-suspended the pellet in 500 µL of dH₂O by vortexing. This step was repeated up to three
106 times to obtain a spore solution of 85% purity (Fries *et al.*, 2013). We then counted *N.*
107 *ceranae* spores in an improved Neubauer haemocytometer (Cantwell, 1970) in a light
108 microscope (x400) and adjusted the spore inoculum to 15,000 spores/µL in 20% (w/w) of
109 sucrose solution. Spore solutions were used within the same week they were purified.

110

111 2.3. Parasite exposure and experimental conditions

112 We exposed bumblebees to *N. ceranae* as described in Gómez-Moracho *et al.*, (2021).
113 Briefly, we confined individual bumblebees in a Petri dish during 5 h without food. We then
114 placed inside each Petri dish a 20 µL drop of the previous inoculum containing 300,000 *N.*

115 *ceranae* spores (e.g. exposed bumblebees). Control bumblebees received 20 μ L of sucrose
116 solution (20% w/w). To control that all bumblebees received the same parasite dose, we only
117 used bumblebees that consumed the entire drop of sucrose within the next 2 h. We then
118 allocated bumblebees into microcolonies of 20-25 individuals, in a plastic box (24 x 18 x 9.6
119 cm) with a dark compartment, simulating the nest of the colony, and a clear compartment
120 containing a gravity feeder with *ad libitum* access to food (Kraus et al., 2019). Since diet can
121 affect host-parasites relationships (Frost et al., 2008) we provided bumblebees an artificial
122 diet with a protein to carbohydrates ratio of 1:207, that was previously showed to elicit
123 highest *N. ceranae* prevalence in bumblebees (Gómez-Moracho et al., 2021). The diet was
124 made with a fixed total amount of nutrients of 170 g/L (protein + carbohydrates) and 0.5% of
125 vitamin mixture for insects (Sigma, Germany). Carbohydrates were supplied as sucrose
126 (Euromedex, France) and proteins consisted in a mixture of casein and whey (4:1)
127 (Nutrimuscle, Belgium) (Gómez-Moracho et al., 2021). We kept bumblebee microcolonies in
128 a room at 25 ± 1 °C with a 12 h light: 12 h dark photoperiod until the behavioural tests. Every
129 day, we renewed the diet and removed dead bumblebees.

130

131 2.4. Behavioural experiments

132 We tested the cognitive performances of bumblebees using PER at day 7 after parasite
133 exposure, following the protocol by Toda et al., (2009). The day before the behavioural tests
134 we kept diet to low levels (~ 200 μ L/bumblebee) to keep bumblebees motivated for PER
135 experiments. Three hours before the behavioural tests, we collected 20 bumblebees (10
136 control, 10 exposed) from each microcolony, chilled them in ice for 5 min and restrained them
137 in a modified 2 mL Eppendorf tube (hereafter, capsule) that we cut in length to fit each
138 bumblebee (i.e. capsule length was adjusted for each bumblebee; Figure 1A). Capsules had
139 two drilled holes, one at the front side and another at the bottom side (adapted from; Toda *et*
140 *al.*, 2009). We maintained the bumblebees in a horizontal position during experiments so that
141 the front hole allowed the experimenter to easily reach bumblebees' antennae during
142 conditioning, while the bottom hole permitted the air flow avoiding odours to accumulate
143 inside the tube. Bumblebees could slightly move inside the tube. We found these conditions
144 better suited to perform PER experiments with bumblebees than the classical vertical

145 harnessing used for honey bees (Giurfa and Sandoz, 2012), in which bumblebees appeared
146 paralysed (unpublished data). Once in the capsule, we kept the bumblebees in the dark, in an
147 incubator at 28°C, with no access to food. All bumblebees that finished the conditioning
148 protocols were frozen and kept at -20°C for later analyses of their infection status through
149 PCR.

150

151 2.5. Sucrose responsiveness

152 We tested the sucrose responsiveness of bumblebees to control for potential influences of *N.*
153 *ceranae* on their reward perception or feeding motivation. During the sucrose sensitivity test,
154 we presented seven sucrose solutions to each bumblebee, from concentrations of 0% (pure
155 water) to 60% (w/w), with increments of 10% (Graystock et al., 2013). For each concentration
156 of sucrose, we touched the antennae of the bumblebee with a toothpick soaked in the
157 corresponding sucrose solution to elicit PER. We presented solutions in an increasing
158 concentration gradient with an inter-trial interval of 5 minutes between concentrations. We
159 scored a positive response if bumblebees extended their proboscis after a solution
160 presentation. We discarded bumblebees responding to water (i.e. 0% sucrose solution) to
161 avoid the effect of thirst on sucrose responsiveness (Baracchi et al., 2018). The sum of the
162 number of PER responses divided by the number of concentration tested formed the
163 individual gustatory score (Scheiner et al., 2013).

164

165 2.6. Conditioning experiments

166 All conditioning experiments shared the same general protocol (Supplementary Figure S1).
167 An encapsulated bumblebee (Figure 1A) was placed 1.5 cm ahead of the odour delivery setup
168 (described in (Aguiar et al., 2018)) that delivered a continuous stream of odourless air at 1.2
169 ml/s. An automated odour-releasing machine controlled by a microcomputer (Arduino® Uno)
170 allowed to selectively add vapour from the vials containing the odorants used as conditioned
171 stimulus (CS) (Raiser et al., 2017). We used two odorants as CS: nonanal and
172 phenylacetaldehyde (Palottini et al., 2018), in a 1:100 dilution in mineral oil (Sommerlandt et
173 al., 2014). Before conditioning, we tested the responsiveness of bees to sucrose by touching

174 both antennae with a toothpick soaked in 50% (w/w) sucrose solution without allowing them
175 to lick. Bumblebees extending their proboscis were considered motivated and kept for the
176 experiments. Conditioning trials (Supplementary Figure S1c) consisted in 15 seconds of
177 odourless airflow (i.e. familiarization), followed by 6 seconds of CS, and 3 seconds of
178 unconditioned stimulus (US) (i.e. 50% sucrose solution applied with a toothpick on the
179 bumblebee's antennae), with 2 seconds of overlap between CS+US, and 20 seconds of
180 odourless airflow (Aguiar et al., 2018). The inter-trial interval was 10 minutes. An air
181 extractor was placed behind the bumblebee to prevent odorant accumulation during CS
182 delivery. Bumblebees extending their proboscis within 3 seconds of US presentation (i.e. 2s
183 CS+US and 1s US) were allowed to lick the toothpick soaked in sucrose (50%, w/w) for 2
184 seconds. In unrewarded trials (see reversal learning and memory tests) no US was applied.
185 We scored a conditioned response if the bumblebee extended its proboscis to the odour
186 delivery before sucrose presentation (Supplementary Figure S1c). Bumblebees that responded
187 to the odour in the first conditioning trial were discarded from the analyses. We used
188 conditioned responses to calculate three individual scores for each bumblebee, describing its
189 performance during conditioning (i.e. acquisition score), at the end of conditioning (i.e.
190 learning score) or during memory retrieval (i.e. memory score) (Monchanin et al., 2020; see
191 details below). In all experiments, exposed and control bumblebees were conditioned in
192 parallel (i.e. the same day).

193

194 2.6.1. Absolute learning, short-term memory and long-term memory

195 We tested the effects of *N. ceranae* on the ability of bumblebees to associate an odour with a
196 reward. This form of learning only requires peripheral brain centers, i.e. the antennal lobes
197 (Giurfa, 2007; Mertes et al., 2021). We trained bumblebees in a spaced 3-trial absolute
198 conditioning learning (Supplementary Figure S1a), with an intertrial interval of 10 minutes,
199 that was shown to generate robust long-term memory in bees (Menzel et al., 2001). We used
200 the same rewarded odour (CS+) during training of a given bumblebee, but both nonanal and
201 phenylacetaldehyde were used as a CS+ for different bumblebees. Only bumblebees that
202 responded to the US in the three trials (i.e. efficient bumblebees) were kept for the analyses
203 (Supplementary table S2). Each of these bees received an acquisition score, calculated as the

204 sum of PER responses (i.e. 0-2) divided by the number of trials (i.e. 3), and a learning score of
205 1 if they responded to CS+ in the last trial or 0 if they did not respond.

206 We tested memory retention in efficient bumblebees that made at least one conditioned
207 response in either one of the last two trials. We performed tests either 1 h (i.e. short-term
208 memory; STM) or 24 h (i.e. long-term memory; LTM) after the last acquisition trial. For tests
209 performed after 24 h, bumblebees were fed until satiation with 50% (w/w) sucrose solution
210 right after conditioning. In bees, STM is independent of protein synthesis whereas LTM
211 requires protein synthesis (Menzel, 1999). Studying performances of bumblebees for these
212 two types of memories was thus a mean to explore whether exposure to *N. ceranae* interfered
213 with protein synthesis. During the test, we presented bumblebees the two odorants without
214 any reward: the odour used as a CS+ to test for memory formation, and the second odour as a
215 novel odorant (NOd), to control for potential generalization (Villar et al., 2020). For example,
216 when nonanal was used as CS+, phenylacetaldehyde was used as a NOd in the memory
217 retention test, and *vice versa*. Memory formation was considered when bumblebees extended
218 their proboscis only in response to the CS+ odour but not to the NOd, and thus bumblebees
219 received a memory score of 1 (score was 0, otherwise). Just after the memory test, we tested
220 the motivation of bumblebees by touching their antennae with a toothpick soaked with 50%
221 (w/w) sucrose solution. Bumblebees that did not respond to US after the memory test were
222 discarded for the analyses (i.e. 14.06% in LTM; Supplementary Table S2).

223

224 2.6.2. Reversal learning

225 We tested the effects of *N. ceranae* on the ability of bumblebees to learn to discriminate two
226 odours and reverse the task. This form of learning involves a first phase of differential
227 conditioning that requires the antennal lobes but is not dependant on central brain centers, i.e.
228 mushroom bodies (Devaud et al., 2015), and a second phase of reversal learning that requires
229 both the antennal lobes and the mushroom bodies (Boitard et al., 2015). In the differential
230 learning phase (phase 1), we trained bumblebees to discriminate between two odours. This
231 phase consisted in 10-trial blocks (Supplementary Figure S1b), five with each odour that was
232 either paired with the US (A+) or unpaired (B-), presented in a pseudo-random order. The

233 rewarded and unrewarded odours were randomized on different training days. Bumblebees
234 that did not respond to US in two or more trials were discarded for the analyses and for
235 reversal phase. In the reversal learning phase (phase 2) we trained bumblebees to invert the
236 first learnt contingency. The reversal phase started 1h after the end of the differential phase.
237 Here we trained bumblebees in 12 trial-blocks, six with each odour presented in a pseudo-
238 random order. The previously rewarded odour was not associated with a reward anymore (A-)
239 while the previously unrewarded odour became rewarded (B+). To start with the same level
240 of learning, we analysed reversal learning in bumblebees that responded to A- either in the
241 first and/or second presentation of this odour during the reversal phase (i.e. selection criteria;
242 Supplementary table S3).

243 We analysed the performance of bumblebees in each phase separately by attributing them an
244 acquisition score and a learning score for each phase. For each trial, bees received 1 point if
245 they made a correct response, i.e. if they responded to CS+, but not to CS-, and 0 points
246 otherwise. The acquisition score consisted in the sum of the number of correct responses by
247 the bumblebee during conditioning divided by the number of trials. The learning score
248 consisted in the performance of a correct or an incorrect (1/0) response by the bumblebee in
249 the last trial of conditioning.

250

251 2.7. Infection status

252 At the end of the behavioural experiments, we assessed the infection status of every
253 bumblebee in a PCR (Martín-Hernández et al., 2007). For each bumblebee, the entire gut was
254 extracted, homogenised in sterile dH₂O and vortexed with 2 mm glass beads (Labbox
255 Labware, Spain). Genomic DNA was extracted using Proteinase K (20 mg/mL; Euromedex,
256 France) and 1 mM of Tris-EDTA Buffer (pH = 8). A sample with *N. ceranae* spores was
257 included in each round of extraction as positive control. We checked *N. ceranae* in a
258 monoplex PCR using the primers 218MITOC ((Martín-Hernández et al., 2007). PCRs were
259 performed with the Taq Polymerase Direct Loading Buffer (5 U/μL; MP Biomedicals, CA)
260 following manufacturer's instructions. We used a final volume of 25 μL with 0.4 μM of each
261 pair of primers (Martín-Hernández et al., 2007), 200 μM of dNTPs (Jena Biosciences,

262 Germany), 0.48 µg/µL of BSA (Sigma, Germany) and 2.5 µL of DNA sample. PCR reactions
263 were carried out in 48-well microtitre plates in a S1000™ Thermal Cycler (Biorad, CA).
264 Thermal conditions were a first step of 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 61.8°C
265 for 45 s and 72 °C for 2 min, and a final elongation step of 72 °C for 7 min. The length of
266 PCR products (i.e. 218 pb) was checked in a 1.2% agarose gel electrophoresis stained with
267 SYBR Safe DNA Stain (Edvotek, Washington DC). Positive and negative controls of PCR
268 were run in parallel. Based on the PCR results we classified bumblebees in three different
269 infection statuses, i.e. control, exposed negative or exposed positive. Bumblebees that were
270 not exposed to the parasite but nevertheless showed a positive result in PCR were excluded
271 from the analyses (i.e. 8.21%; 23 out of 280 control bumblebees; Supplementary Tables S1,
272 S2 and S3).

273

274 2.8. Statistical analyses

275 All statistical analyses were conducted in RStudio (version 1.4.1106). We evaluated the effect
276 of parasite exposure and infection on the scores of sucrose responsiveness, acquisition,
277 learning and memory. The individual gustatory scores, and the acquisition scores obtained
278 during the absolute and reversal conditioning experiments were standardized and analysed in
279 a Generalized Linear Mixed Model (GLMM) using Template Model Builder (package
280 [glmmTMB]; (Brooks et al., 2017)). The response to the first and last sucrose concentrations,
281 as well as the learning and memory scores were analysed in a binomial GLMM (package
282 [lme4]; (Bates et al., 2015)). In all models, bee infection status was used as a fixed factor,
283 while bee identity and odorant were used as random factors. We performed Tukey post-hoc
284 pairwise comparisons (package [multcomp]; (Hothorn et al., 2008)) to assess the relationship
285 between the three bee statuses.

286

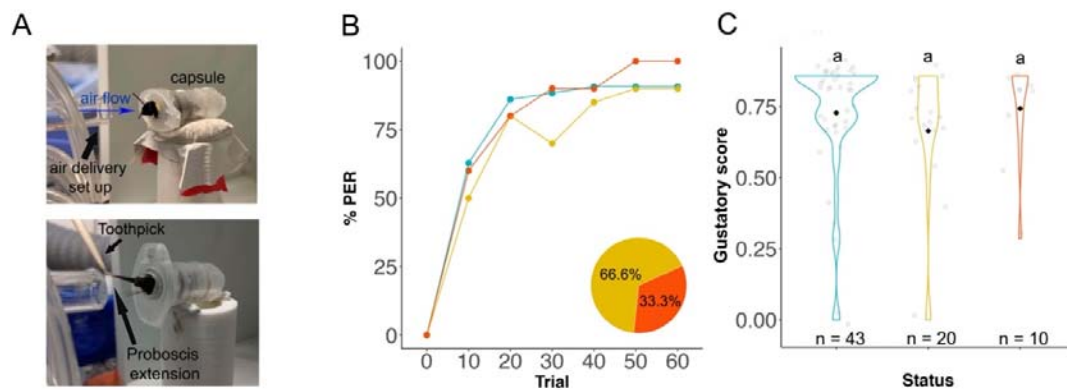
287 3. Results

288

289 3.1. Parasite exposure did not influence sucrose responsiveness

290 We first tested the influence of *N. ceranae* on sucrose responsiveness by studying the
291 response to different sucrose concentrations in 73 bumblebees (43 controls, 20 exposed
292 negative, 10 exposed positive; see details in Supplementary Table S1). Overall, 71.23% of
293 these bumblebees responded to sucrose (Figure 1B and C). This percentage was similar across
294 the three infection statuses ($X^2 = 2.236$, $df = 2$, $p = 0.326$). Bumblebees of the three infection
295 statuses responded similarly to the lowest sucrose concentration (Figure 1B; Binomial
296 GLMM, $X^2 = 0.920$, $df = 2$, $p = 0.631$), reached similar gustatory scores (Figure 1C; GLMM,
297 $X^2 = 1.686$, $df = 2$, $p = 0.430$) and similar final level of response to the highest sucrose
298 concentration (Figure 1B; Binomial GLMM, $X^2 = 0.217$, $df = 2$, $p = 0.897$). Therefore,
299 exposure to *N. ceranae* affected neither the sucrose sensitivity nor the feeding motivation of
300 bumblebees.

301



302

303 **Figure 1. Odour delivery set up and sucrose responsiveness.** A) Photo of a bumblebee
304 during odour conditioning. The bumblebee is placed inside the capsule in front of the air
305 delivery set up. After the odour is delivered, sucrose is presented with a toothpick to the
306 bumblebees which extends its proboscis to drink the reward. B-C) Sucrose responsiveness
307 test. B) Proportion of control (blue), exposed negative (yellow) and exposed positive (red)
308 bumblebees responding to an increase gradient of sucrose concentrations. Pie chart represents
309 the percentage of exposed negative and exposed positive bees (n total=30). C) Violin plots
310 show gustatory score of bumblebees as the sum of all responses for each bumblebee. Black
311 dot represents the mean score for each infection status. Hollow dots represent the score of

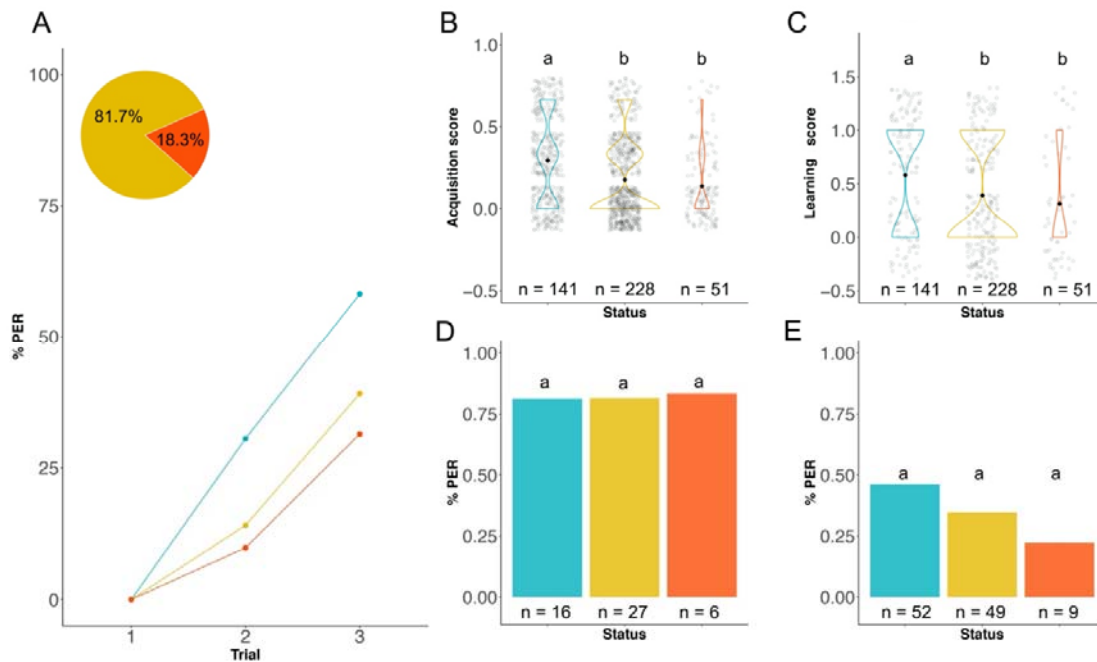
312 each individual. n is the sample size. Letters above violin plots represent significant
313 differences between status (GLMM; $p < 0.05$). n is the sample size.

314

315 3.1. *Parasite exposure reduced absolute learning but not memory*

316 We then tested the influence of *N. ceranae* exposure on learning and memory by submitting
317 bumblebees to an absolute learning task followed by a memory test either 1 h or 24 h later.
318 This type of learning involves the antennal lobes (Giurfa, 2007; Mertes et al., 2021). We
319 analysed absolute conditioning in 420 bumblebees (141 controls, 228 exposed negative, 51
320 exposed positive; see details in Supplementary table S2). The proportion of bumblebees
321 showing a conditioned response increased over trials in the three infection statuses (Figure
322 2A). Exposed bumblebees (either negative or positive) had significantly lower acquisition
323 scores (Figure 2B; GLMM, status: $X^2 = 31.558$, $df = 2$, $p < 0.001$) and learning scores (Figure
324 2C; Binomial GLMM, status, $X^2 = 16.037$, $df = 2$, $p < 0.001$) than controls. Both acquisition
325 and learning scores were similar for negative and positive exposed bumblebees (Table 1).

326



327

328 **Figure 2. Absolute learning and memory.** A) Learning curves show the proportion of
 329 control (blue), exposed negative (yellow) and exposed positive (red) bumblebees extending
 330 their proboscis to the odour during conditioning. Pie chart shows the percentage of negative
 331 and positive exposed bumblebees that finished the conditioning (N total = 279). B-C) Violin
 332 plots for acquisition score (B; sum of correct responses divided by the number of trials for
 333 each bumblebee) and learning score (C; correct response at the last trial). Black dot represents
 334 the mean score for each infection status. Hollow dots represent the score of each individual.
 335 D) Short-term memory. Bar plots show the proportion of bumblebees responding to odour 1 h
 336 after training. E) Long-term memory. Bar plots show the proportion of bumblebees
 337 responding to odour 24 h after training. Letters above violin plots and bar plots represent
 338 significant differences between status (GLMM; $p < 0.05$). n is the sample size.

339

340 **Table 1. Tukey Pairwise comparisons for absolute and reversal learning.** Significant
 341 results ($P < 0.001$) are highlighted in bold.

Test		Contrast	z	P
Absolute learning	Acquisition score	control – exposed negative	-5.140	<0.001
		control – exposed positive	-4.378	<0.001

		exposed negative – exposed positive	-1.017	0.56
	Learning score	control – exposed negative	-3.513	0.001
		control – exposed positive	-3.174	0.004
		exposed negative – exposed positive	-0.996	0.572
Reversal learning – differential phase	Acquisition score	control – exposed negative	-4.251	<0.001
		control – exposed positive	-3.211	0.003
		exposed negative – exposed positive	0.106	0.993
	Learning score	control – exposed negative	-1.653	0.222
		control – exposed positive	-3.323	0.002
		exposed negative – exposed positive	-1.981	0.133
Reversal learning – reversal phase	Acquisition score	control – exposed negative	0.152	0.987
		control – exposed positive	-2.337	0.049
		exposed negative – exposed positive	-2.279	0.056

342

343 We analysed short-term and long-term memory formation of efficient bumblebees that
 344 responded to odour at least once in any of the last two last trials (41.52% in short-term
 345 memory and 42.51% in long-term memory; Supplementary Table S2). A high proportion of
 346 the bumblebees ($82.02\% \pm 0.011$, mean \pm SE) remembered the rewarded odour 1h after training.
 347 This proportion was similar in the three infection statuses (Binomial GLMM, status: $X^2 =$
 348 0.013 , $df = 2$, $p = 0.993$), indicating that *N. ceranae* did not affect short-term memory (Figure
 349 2D). A much lower proportion of the bumblebees ($34.35\% \pm 0.06$, mean \pm SE) remembered the
 350 rewarded odour after 24 h. Exposed bumblebees (either negative or positive) showed a lower
 351 proportion of correct responses than controls, although this was not significantly different
 352 (Figure 2E; Binomial GLMM, status: $X^2 = 2.265$, $df = 2$, $p = 0.322$). Here presumably, the
 353 non-significant result is due to the low amount of exposed positive individuals (9
 354 bumblebees).

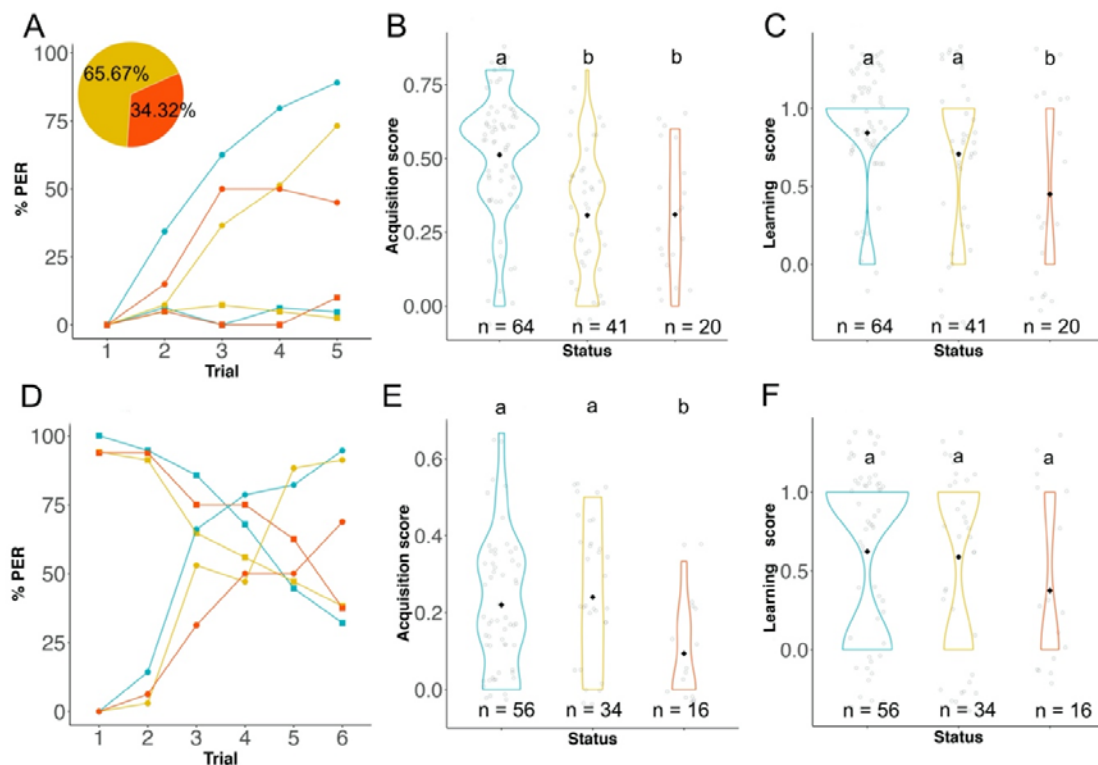
355

356 3.2. Parasite exposure reduced differential and reversal learning

357 Having showed an effect of *N. ceranae* exposure on absolute learning, we questioned whether
 358 this effect was observed in learning tasks involving different brain areas using a reversal

359 learning protocol. The first phase of reversal learning is a differential learning task in which
360 the bumblebee must learn to respond to rewarded odour (A+) and not respond to the
361 unrewarded odour (B-). This form of learning involves the antennal lobes and is not
362 dependant on the central brain centers, i.e. mushroom bodies (Devaud et al., 2015). We
363 analysed 134 bumblebees during the first phase of reversal learning (67 controls, 44 exposed
364 negative, 23 exposed positive; see details in Supplementary Table S3). Bumblebees in the
365 three infection statuses increased their response to A+ over trials, but not to B- (Figure 3A),
366 indicating that they were equally able to discriminate the odours. However, parasite exposure
367 affected acquisition scores (GLMM, status, $X^2 = 22.039$, $df = 2$, $p < 0.001$; Figure 3B).
368 Exposed bumblebees (positive or negative) showed similar acquisition scores (Tukey test: $p >$
369 0.05 ; Table 1) and these scores were significantly lower than the scores of controls (Tukey
370 test: $p < 0.05$; Table 1). Parasite exposure also affected the final level of learning (Binomial
371 GLMM, status, $X^2 = 11.168$, $df = 2$, $p = 0.003$; Figure 2C). Exposed positive bumblebees had
372 the lowest learning score. Their score was significantly lower than that of controls (Tukey
373 test: $p = 0.002$; Table 1) but similar to that exposed negative bumblebees (Tukey test: $p =$
374 0.133 ; Table 1). Thus overall, exposure to *N. ceranae* reduced differential learning
375 performances.

376



377

378 **Figure 3. Reversal learning.** **A-C)** Differential learning phase. **A)** Percentage of PER
 379 responses to rewarded (A+, circle) and unrewarded (B-, square) odours by control (blue),
 380 exposed negative (yellow) and exposed positive (red) bumblebees. Pie chart shows the
 381 percentage of exposed negative and positive bumblebees that finished the differential phase (n
 382 total = 67). Violin plots of **B)** acquisition scores (i.e. sum of the correct responses divided by
 383 the number of trials for each bee) and **C)** learning scores (i.e. performance of bumblebees at
 384 last trial) of bumblebees with different infection statuses. Black dot represents the mean score
 385 of each status and hollow dots the score of each individual. **D-F)** Reversal learning phase. **D)**
 386 Curves show the increase in the percentage of PER response to B+ over A- over trials. **E)**
 387 Acquisition score. **F)** learning scores. Letters above violin plots show significant differences
 388 between infection statuses in the acquisition (GLMM, $p < 0.05$) and learning (Binomial
 389 GLMM) scores. n is the sample size.

390

391 The second phase of reversal learning is the proper reversal task in which the bumblebee must
392 learn to respond to the previously unrewarded odour (now B+) and stop responding to the
393 previously rewarded odour (now A-) (Figure 3D-F). This form of learning involves the
394 antennal lobes but is also dependent on the mushroom bodies (Boitard et al., 2015). We
395 analysed reversal learning of 85.82% of bumblebees that finished the differential phase, as
396 they responded to A- in any of the two first presentations of this odour during reversal phase
397 (59 controls, 37 exposed negative, 19 exposed positive; see details in Supplementary Table
398 S3). All bumblebees reduced their response to A- in favour of B+ over trials (Figure 3D).
399 However, bumblebees with different infection statuses reached significantly different
400 acquisition scores (Figure 3E; GLMM, status, $X^2 = 6.042$, $df = 2$, $p = 0.048$). Exposed
401 positive bumblebees showed a slightly lower acquisition score than controls (Tukey test: $p =$
402 0.049 ; Table 1) and exposed negative bumblebees (Tukey test: $p = 0.056$, Table 1), suggesting
403 they had a lower ability to reverse the task. By the end of training, bumblebees of all infection
404 statuses reversed the task, as shown by the lack of difference between their learning scores
405 (Binomial GLMM, status: $X^2 = 0.641$, $df = 2$, $p = 0.725$). Note however that just one trial
406 before the end of training (trial 5), bumblebees with different infection statuses differed in
407 their learning scores (Binomial GLMM, status; $X^2 = 8.940$, $df = 2$, $p = 0.011$). At trial 5,
408 exposed positive bumblebees were unable to accomplish the task correctly compared to
409 controls (Tukey test; $z = -2.505$, $p = 0.032$) and exposed negative bumblebees (Tukey test; $z =$
410 -2.759 , $p = 0.015$). Thus overall, exposure to *N. ceranae* also impaired the reversal phase of
411 reversal learning. Interestingly, we found no effect of *N. ceranae* exposure on either phase of
412 reversal learning when bumblebees were tested at 2 days post exposure, suggesting that stress
413 due to parasite exposure or parasite infection requires longer time to be established (see
414 Supplementary Text S1).

415

416 4. Discussion

417

418 Bees are exposed to a number of parasites that can affect cognitive abilities involved in
419 crucial behaviour (Koch et al., 2017; Schmid-Hempel, 2013). Previous studies exploring the
420 effect of the widespread parasite *N. ceranae* on absolute olfactory learning and memory in

421 bees reported contrasting results, presumably because of differences in conditioning protocols
422 and infection rates across studies (Bell et al., 2020; Charbonneau et al., 2016; Gage et al.,
423 2018; Piironen et al., 2016; Piironen and Goulson, 2016). Here, we ran a suite of standard
424 olfactory cognitive assays showing that feeding bumblebees spores of this parasite
425 consistently impairs different types of olfactory learning seven days after exposure. This
426 suggests that the influence of *N. ceranae* exposure on bumblebee cognition is not specific to a
427 particular brain area.

428 Exposure to *N. ceranae* in food clearly impaired the ability of bumblebees to associate an
429 odour with a reward (absolute learning), discriminate two odours (differential learning), and
430 learn an opposite association (reversal learning). These are fundamental cognitive operations
431 a bee must solve to efficiently forage on flowers (Giurfa, 2013). This result agrees with a
432 previous study reporting a slightly reduced absolute learning in bumblebees exposed to *N.*
433 *ceranae* (Piironen and Goulson, 2016). Here we provide evidence that these reduced
434 cognitive performances were not due to an effect of the parasite on odour perception or
435 motivation to feed, and give insight on where the parasite might act in the nervous system. In
436 bees, sucrose perception through the antennae, olfactory absolute learning, differential
437 learning and reversal learning all require processing of olfactory information through the
438 antennal lobes (de Brito Sanchez, 2011; Giurfa, 2007; Mertes et al., 2021) while reversal
439 learning also requires the mushroom bodies (Devaud et al., 2015). The fact that all types of
440 learning were impaired and that sucrose sensitivity was not, indicates that *N. ceranae* did not
441 specifically target the antennal lobes or the mushroom bodies. Rather, it likely impacted the
442 learning processes in general.

443 By contrast, we found no evidence that *N. ceranae* influenced olfactory memory. During
444 training animals learn and form short-term memories that are later consolidated and
445 transformed into stable long-term memories (Menzel and Muller, 1996) after protein synthesis
446 (Menzel, 2001). In our experiments, *N. ceranae* impaired neither short-term nor long-term
447 memory. Our protocol imposed that only efficient bumblebees (i.e. that responded to sucrose
448 in the three conditioning trials) with at least one conditioned response would be selected for
449 the memory test, as bumblebees only had three trials to learn the stimulus-reward association.
450 Nearly all bees exposed to *Nosema* (either positive or negative in PCR) remembered the

451 association of the odour with the reward 1 h after training. While exposed bumblebees
452 showed reduced responses to the odour alone 24 h after training than controls, this tendency
453 was not significant and may result from low sample size as only 29.44% of the efficient
454 exposed bumblebees were eligible to analyse their memory (i.e. efficient bumblebees, with at
455 least one conditioned response during learning, that responded to sucrose after memory test).

456 It has recently been questioned whether bumblebees are natural hosts of *N. ceranae* based on
457 the lack of evidence of parasitic forms inside host cells (Gisder et al., 2020). Several studies
458 have nevertheless reported *N. ceranae* in wild bumblebees in a prevalence that usually ranges
459 from 4.76% to 21% (Figuroa et al., 2021; Graystock et al., 2013; Li et al., 2012; Sinpoo,
460 2018), or even higher (i.e. 72%; Arbulo et al., 2015). Whether or not bumblebees are suitable
461 hosts for *N. ceranae* replication is confirmed in future experiments, our results imply that
462 bumblebees are strongly impacted by an acute exposure to the parasite. Such exposure may be
463 extremely frequent in nature due to the high prevalence of *N. ceranae* in honey bees (Klee et
464 al., 2007; Runckel et al., 2011) that contaminate flowers with spores through physical contact
465 or in their faeces (Graystock et al., 2015). Our protocol of parasite exposure significantly
466 increased the infection rate of bumblebees to 21.38% in comparison to previous studies
467 (Piiroinen et al., 2016; Piiroinen and Goulson, 2016), which allowed the comparison of
468 cognitive traits in bumblebees in the three infection statuses. Bumblebees that tested positive
469 to *N. ceranae* showed a tendency for lower cognitive performances than negative exposed
470 bumblebees. They reached the lowest learning during the absolute conditioning and barely
471 discriminated odours. These bees were also slower at inverting odour associations than
472 negative exposed individuals. Even if bumblebees in all statuses developed a novel odour
473 association by the end of the experiment, positive exposed bumblebees acquired this ability
474 only in the last trial, contrary to the other two groups, suggesting that infection may further
475 interfere with cognition, however further experiments are needed to tackle this specific
476 question.

477 Through which mechanism may the parasite impair learning? Ingestion of *N. ceranae* spores
478 exerts a stress that can reduce cognition. *N. ceranae* is known to alter the immune system of
479 bees by increasing oxidative responses (Kurze et al., 2016) or modulating the expression of
480 antimicrobial peptides (Antúnez et al., 2009; Botías et al., 2020; Sinpoo, 2018). Stimulation of

481 the immune system of bees with non-pathogenic elicitors, as lipopolysaccharides (LPS), was
482 shown to reduce learning abilities in honey bees, that were less able to associate an odour with
483 a reward (Mallon et al., 2003), and bumblebees, that showed lower performances in odour
484 (Mobley and Gegear, 2018) and colour differential learning tasks (Alghamdi et al., 2008;
485 Mobley and Gegear, 2018). It is thus possible that the observed effects on cognition in
486 bumblebees exposed to *N. ceranae* were caused by an activation of the immune response.
487 Infection with *N. ceranae* was shown to downregulate the expression of genes in the honey
488 bee brain (Doublet et al., 2016; McDonnell et al., 2013), some of them linked to sensory, as
489 odorant binding proteins (Badaoui et al., 2017) and chemosensory proteins (CSP1; (Doublet
490 et al., 2016)). *N. ceranae* also interferes with the metabolism of serotonin by, for example,
491 inhibiting the expression of the dopa decarboxylase encoding gene (Aufauvre et al., 2014), a
492 biogenic amine that plays a crucial role in memory formation in *Drosophila* (Sitaraman et al.,
493 2008). Modulation of gene expression in the brain may lead to changes in behaviour and
494 cognition. Interestingly, we found no effect of parasite exposure on cognition at two days post
495 exposure (Supplementary Text S1). Whether *N. ceranae* exerts an effect on the immune
496 response of honey bees at this time is unknown, as most studies tested this response later than
497 three days after parasite exposure (Antúnez et al., 2009; Chaimanee et al., 2012; Sinpoo,
498 2018). In bumblebees the immune response is activated just 1 h after feeding *Crithidia bombi*
499 (Riddell et al., 2011). However, overexpression of aminopeptides is observed 12 h after
500 infection (Riddell et al., 2011) and, although it varies among aminopeptides, it can last even
501 for 10 days when it is triggered by LPS injection (Mobley and Gegear, 2018). Then, it is
502 possible that at two days post exposure the stress generated by the parasite may have not
503 affected yet the nervous system.

504 Since olfactory learning is essential for bumblebees to forage, this sublethal effect of *N.*
505 *ceranae* exposure on individual cognition can impact the collective performance and
506 compromise colony development. Commercial and wild bumblebee colonies exhibit
507 physiological and behavioural differences as a result of different selective pressures (Velthuis
508 and Doorn, 2006). They may show different responses to parasites. Parasite loads in the field
509 can range from a few spores to several thousands, as some honey bees can harbour even 10
510 thousands of *Nosema* spores (Meana et al., 2010). Here we used a substantially higher spore
511 loads of *N. ceranae* to infect commercially reared bumblebees than the actual infection rates

512 found in nature, estimated in 6800 spores per individual (Graystock et al., 2013). Feeding this
513 quantity of spores infected 62% of bumblebees which neither suffer a reduction of their
514 gustatory response (Graystock et al., 2013), but no further effects in cognition were analysed.
515 Further studies are thus needed to analyse the effects of *N. ceranae*, and their possible
516 interactions with other stressors, in the field in order to understand their real impact on colony
517 dynamics.

518

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520

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525

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532

533 7. References

534

535 Aguiar, J.M.R.B.V., Roselino, A.C., Sazima, M., Giurfa, M., 2018. Can honey bees
536 discriminate between floral-fragrance isomers? *J. Exp. Biol.* 221, jeb180844.
537 <https://doi.org/10.1242/jeb.180844>

538 Alghamdi, A., Dalton, L., Phillis, A., Rosato, E., Mallon, E.B., 2008. Immune response
539 impairs learning in free-flying bumble-bees. *Biol. Lett.* 4, 479–481.
540 <https://doi.org/10.1098/rsbl.2008.0331>

- 541 Antúnez, K., Martín-Hernández, R., Prieto, L., Meana, A., Zunino, P., Higes, M., 2009.
542 Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema*
543 *ceranae* (Microsporidia). Environ. Microbiol. 11, 2284–2290. [https://doi.org/10.1111/j.1462-](https://doi.org/10.1111/j.1462-2920.2009.01953.x)
544 [2920.2009.01953.x](https://doi.org/10.1111/j.1462-2920.2009.01953.x)
- 545 Arbulo, N., Antúnez, K., Salvarrey, S., Santos, E., Branchiccela, B., Martín-Hernández, R.,
546 Higes, M., Invernizzi, C., 2015. High prevalence and infection levels of *Nosema ceranae* in
547 bumblebees *Bombus atratus* and *Bombus bellicosus* from Uruguay. J. Invertebr. Pathol. 130,
548 165–168. <https://doi.org/10.1016/j.jip.2015.07.018>
- 549 Aufauvre, J., Misme-Aucouturier, B., Viguès, B., Texier, C., Delbac, F., Blot, N., 2014.
550 Transcriptome analyses of the honeybee response to *Nosema ceranae* and insecticides. PLoS
551 ONE 9, e91686. <https://doi.org/10.1371/journal.pone.0091686>
- 552 Badaoui, B., Fougeroux, A., Petit, F., Anselmo, A., Gorni, C., Cucurachi, M., Cersini, A.,
553 Granato, A., Cardeti, G., Formato, G., Mutinelli, F., Giuffra, E., Williams, J.L., Botti, S.,
554 2017. RNA-sequence analysis of gene expression from honeybees (*Apis mellifera*) infected
555 with *Nosema ceranae*. PLOS ONE 12, e0173438.
556 <https://doi.org/10.1371/journal.pone.0173438>
- 557 Baracchi, D., Rigosi, E., de Brito Sanchez, G., Giurfa, M., 2018. Lateralization of sucrose
558 responsiveness and non-associative learning in honeybees. Front. Psychol. 9, 425.
559 <https://doi.org/10.3389/fpsyg.2018.00425>
- 560 Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects models
561 using **lme4**. J. Stat. Softw. 67. <https://doi.org/10.18637/jss.v067.i01>
- 562 Bell, H.C., Montgomery, C.N., Benavides, J.E., Nieh, J.C., 2020. Effects of *Nosema ceranae*
563 (Dissociodihaplophasida: Nosematidae) and flupyradifurone on olfactory learning in honey
564 bees, *Apis mellifera* (Hymenoptera: Apidae). J. Insect Sci. 20.
565 <https://doi.org/10.1093/jisesa/ieaa130>
- 566 Bitterman, M.E., Menzel, R., Fietz, A., Schäfer, S., 1983. Classical conditioning of proboscis
567 extension in honeybees (*Apis mellifera*). J. Comp. Psychol. 97, 107–119.
568 <https://doi.org/10.1037/0735-7036.97.2.107>

- 569 Boitard, C., Devaud, J.-M., Isabel, G., Giurfa, M., 2015. GABAergic feedback signaling into
570 the calyces of the mushroom bodies enables olfactory reversal learning in honey bees. *Front.*
571 *Behav. Neurosci.* 9, 1–13. <https://doi.org/10.3389/fnbeh.2015.00198>
- 572 Botías, C., Jones, J.C., Pamminger, T., Bartomeus, I., Hughes, W.O.H., Goulson, D., 2020.
573 Multiple stressors interact to impair the performance of bumblebee (*Bombus terrestris*)
574 colonies. *J. Anim. Ecol.* 1365-2656.13375. <https://doi.org/10.1111/1365-2656.13375>
- 575 Brooks, M., E., Kristensen, K., Benthem, K., J., van, Magnusson, A., Berg, C., W., Nielsen,
576 A., Skaug, H., J., Mächler, M., Bolker, B., M., 2017. glmmTMB balances speed and flexibility
577 among packages for zero-inflated Generalized Linear Mixed Modeling. *R J.* 9, 378.
578 <https://doi.org/10.32614/RJ-2017-066>
- 579 Cantwell, G., 1970. Standard methods for counting *Nosema* spores. *Am. Bee J.* 110, 222–223.
- 580 Chaimanee, V., Chantawannakul, P., Chen, Y., Evans, J.D., Pettis, J.S., 2012. Differential
581 expression of immune genes of adult honey bee (*Apis mellifera*) after inoculated by *Nosema*
582 *ceranae*. *J. Insect Physiol.* 58, 1090–1095. <https://doi.org/10.1016/j.jinsphys.2012.04.016>
- 583 Charbonneau, L.R., Hillier, N.K., Rogers, R.E.L., Williams, G.R., Shutler, D., 2016. Effects
584 of *Nosema apis*, *N. ceranae*, and coinfections on honey bee (*Apis mellifera*) learning and
585 memory. *Sci. Rep.* 6, 22626. <https://doi.org/10.1038/srep22626>
- 586 Collett, M., Chittka, L., Collett, T.S., 2013. Spatial memory in insect navigation. *Curr. Biol.*
587 23, R789–R800. <https://doi.org/10.1016/j.cub.2013.07.020>
- 588 Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., Quan, P.-
589 L., Briese, T., Hornig, M., Geiser, D.M., Martinson, V., vanEngelsdorp, D., Kalkstein, A.L.,
590 Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchison, S.K., Simons, J.F., Egholm, M., Pettis, J.S.,
591 Lipkin, W.I., 2007. A metagenomic survey of microbes in honey bee Colony Collapse
592 Disorder. *Science* 318, 283–287. <https://doi.org/10.1126/science.1146498>
- 593 de Brito Sanchez, M.G., 2011. Taste perception in honey bees. *Chem. Senses* 36, 675–692.
594 <https://doi.org/10.1093/chemse/bjr040>

- 595 Devaud, J.-M., Papouin, T., Carcaud, J., Sandoz, J.-C., Grünewald, B., Giurfa, M., 2015.
596 Neural substrate for higher-order learning in an insect: Mushroom bodies are necessary for
597 configural discriminations. *Proc. Natl. Acad. Sci.* 112, E5854–E5862.
598 <https://doi.org/10.1073/pnas.1508422112>
- 599 Doublet, V., Paxton, R.J., McDonnell, C.M., Dubois, E., Nidelet, S., Moritz, R.F.A., Alaux,
600 C., Le Conte, Y., 2016. Brain transcriptomes of honey bees (*Apis mellifera*) experimentally
601 infected by two pathogens: Black queen cell virus and *Nosema ceranae*. *Genomics Data* 10,
602 79–82. <https://doi.org/10.1016/j.gdata.2016.09.010>
- 603 Dussaubat, C., Brunet, J.-L., Higes, M., Colbourne, J.K., Lopez, J., Choi, J.-H., Martín-
604 Hernández, R., Botías, C., Cousin, M., McDonnell, C., Bonnet, M., Belzunces, L.P., Moritz,
605 R.F.A., Le Conte, Y., Alaux, C., 2012. Gut pathology and responses to the Microsporidium
606 *Nosema ceranae* in the honey bee *Apis mellifera*. *PLoS ONE* 7, e37017.
607 <https://doi.org/10.1371/journal.pone.0037017>
- 608 Dussaubat, C., Maisonnasse, A., Crauser, D., Beslay, D., Costagliola, G., Soubeyrand, S.,
609 Kretzchmar, A., Le Conte, Y., 2013. Flight behavior and pheromone changes associated to
610 *Nosema ceranae* infection of honey bee workers (*Apis mellifera*) in field conditions. *J.*
611 *Invertebr. Pathol.* 113, 42–51. <https://doi.org/10.1016/j.jip.2013.01.002>
- 612 Figueroa, L.L., Compton, S., Grab, H., McArt, S.H., 2021. Functional traits linked to
613 pathogen prevalence in wild bee communities. *Sci. Rep.* 11, 7529.
614 <https://doi.org/10.1038/s41598-021-87103-3>
- 615 Fries, I., Chauzat, M.-P., Chen, Y.-P., Doublet, V., Genersch, E., Gisder, S., Higes, M.,
616 McMahon, D.P., Martín-Hernández, R., Natsopoulou, M., Paxton, R.J., Tanner, G., Webster,
617 T.C., Williams, G.R., 2013. Standard methods for *Nosema* research. *J. Apic. Res.* 52, 1–28.
618 <https://doi.org/10.3896/IBRA.1.52.1.14>
- 619 Fries, I., Feng, F., da Silva, A., Slemenda, S.B., Pieniasek, N.J., 1996. *Nosema ceranae* n. sp.
620 (Microspora, Nosematidae), morphological and molecular characterization of a
621 microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). *Eur. J.*
622 *Protistol.* 32, 356–365. [https://doi.org/10.1016/S0932-4739\(96\)80059-9](https://doi.org/10.1016/S0932-4739(96)80059-9)

- 623 Frost, P.C., Ebert, D., Smith, V.H., 2008. Bacterial infection changes the elemental
624 composition of *Daphnia magna*. J. Anim. Ecol. 77, 1265–1272.
625 <https://doi.org/10.1111/j.1365-2656.2008.01438.x>
- 626 Gage, S.L., Kramer, C., Calle, S., Carroll, M., Heien, M., DeGrandi-Hoffman, G., 2018.
627 *Nosema ceranae* parasitism impacts olfactory learning and memory and neurochemistry in
628 honey bees (*Apis mellifera*). J. Exp. Biol. 221, jeb161489. <https://doi.org/10.1242/jeb.161489>
- 629 Gisder, S., Horchler, L., Pieper, F., Schüller, V., Šima, P., Genersch, E., 2020. Rapid
630 gastrointestinal passage may protect *Bombus terrestris* from becoming a true host for *Nosema*
631 *ceranae*. Appl. Environ. Microbiol. 86. <https://doi.org/10.1128/AEM.00629-20>
- 632 Giurfa, M., 2015. Learning and cognition in insects: learning and insect cognition. Wiley
633 Interdiscip. Rev. Cogn. Sci. 6, 383–395. <https://doi.org/10.1002/wcs.1348>
- 634 Giurfa, M., 2013. Cognition with few neurons: higher-order learning in insects. Trends
635 Neurosci. 36, 285–294. <https://doi.org/10.1016/j.tins.2012.12.011>
- 636 Giurfa, M., 2007. Behavioral and neural analysis of associative learning in the honeybee: a
637 taste from the magic well. J. Comp. Physiol. A 193, 801–824. <https://doi.org/10.1007/s00359-007-0235-9>
- 639 Giurfa, M., Sandoz, J.-C., 2012. Invertebrate learning and memory: fifty years of olfactory
640 conditioning of the proboscis extension response in honeybees. Learn. Mem. 19, 54–66.
641 <https://doi.org/10.1101/lm.024711.111>
- 642 Gómez-Moracho, T., Durand, T., Pasquaretta, C., Heeb, P., Lihoreau, M., 2021. Artificial
643 diets modulate infection rates by *Nosema ceranae* in bumblebees. Microorganisms 9, 158.
644 <https://doi.org/10.3390/microorganisms9010158>
- 645 Gómez-Moracho, T., Heeb, P., Lihoreau, M., 2017. Effects of parasites and pathogens on bee
646 cognition: Bee parasites, pathogens and cognition. Ecol. Entomol. 42, 51–64.
647 <https://doi.org/10.1111/een.12434>
- 648 Graystock, P., Goulson, D., Hughes, W., 2015. Parasites in bloom: flowers aid dispersal and

- 649 transmission of pollinator parasites within and between bee species. *Proc. R. Soc. B Biol. Sci.*
650 282. <https://doi.org/10.1098/rspb.2015.1371>
- 651 Graystock, P., Yates, K., Darvill, B., Goulson, D., Hughes, W.O.H., 2013. Emerging dangers:
652 Deadly effects of an emergent parasite in a new pollinator host. *J. Invertebr. Pathol.* 114, 114–
653 119. <https://doi.org/10.1016/j.jip.2013.06.005>
- 654 Higes, M., García-Palencia, P., Martín-Hernández, R., Meana, A., 2007. Experimental
655 infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). *J. Invertebr.*
656 *Pathol.* 94, 211–217. <https://doi.org/10.1016/j.jip.2006.11.001>
- 657 Higes, M., Martín, R., Meana, A., 2006. *Nosema ceranae*, a new microsporidian parasite in
658 honeybees in Europe. *J. Invertebr. Pathol.* 92, 93–95. <https://doi.org/10.1016/j.jip.2006.02.005>
- 659 Higes, M., Martín-Hernández, R., Botías, C., Bailón, E.G., González-Porto, A.V., Barrios, L.,
660 del Nozal, M.J., Bernal, J.L., Jiménez, J.J., Palencia, P.G., Meana, A., 2008. How natural
661 infection by *Nosema ceranae* causes honeybee colony collapse. *Environ. Microbiol.* 10,
662 2659–2669. <https://doi.org/10.1111/j.1462-2920.2008.01687.x>
- 663 Higes, M., Martín-Hernández, R., Garrido-Bailón, E., González-Porto, A.V., García-Palencia,
664 P., Meana, A., del Nozal, M.J., Mayo, R., Bernal, J.L., 2009. Honeybee colony collapse due
665 to *Nosema ceranae* in professional apiaries. *Environ. Microbiol. Rep.* 1, 110–113.
666 <https://doi.org/10.1111/j.1758-2229.2009.00014.x>
- 667 Holt, H.L., Aronstein, K.A., Grozinger, C.M., 2013. Chronic parasitization by *Nosema*
668 microsporidia causes global expression changes in core nutritional, metabolic and behavioral
669 pathways in honey bee workers (*Apis mellifera*). *BMC Genomics* 14, 799.
670 <https://doi.org/10.1186/1471-2164-14-799>
- 671 Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric
672 models. *Biom. J. Biom. Z.* 50, 346–363. <https://doi.org/10.1002/bimj.200810425>
- 673 Khezri, M., Moharrami, M., Modirrousta, H., Torkaman, M., Salehi, S., Rokhzad, B.,
674 Khanbabai, H., 2018. Molecular detection of *Nosema ceranae* in the apiaries of Kurdistan
675 province, Iran. *Vet. Res. Forum Int. Q. J.* 9, 273–278.

- 676 <https://doi.org/10.30466/vrf.2018.32086>
- 677 Klee, J., Besana, A.M., Genersch, E., Gisder, S., Nanetti, A., Tam, D.Q., Chinh, T.X., Puerta,
678 F., Ruz, J.M., Kryger, P., Message, D., Hatjina, F., Korpela, S., Fries, I., Paxton, R.J., 2007.
679 Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the
680 western honey bee, *Apis mellifera*. J. Invertebr. Pathol. 96, 1–10.
681 <https://doi.org/10.1016/j.jip.2007.02.014>
- 682 Klee, J., Tek Tay, W., Paxton, R.J., 2006. Specific and sensitive detection of *Nosema bombi*
683 (Microsporidia: Nosematidae) in bumble bees (*Bombus spp.*; Hymenoptera: Apidae) by PCR
684 of partial rRNA gene sequences. J. Invertebr. Pathol. 91, 98–104.
685 <https://doi.org/10.1016/j.jip.2005.10.012>
- 686 Klein, S., Cabirol, A., Devaud, J.-M., Barron, A.B., Lihoreau, M., 2017. Why bees are so
687 vulnerable to environmental stressors. Trends Ecol. Evol. 32, 268–278.
688 <https://doi.org/10.1016/j.tree.2016.12.009>
- 689 Koch, H., Brown, M.J., Stevenson, P.C., 2017. The role of disease in bee foraging ecology.
690 Curr. Opin. Insect Sci. 21, 60–67. <https://doi.org/10.1016/j.cois.2017.05.008>
- 691 Kraus, S., Gómez-Moracho, T., Pasquarella, C., Latil, G., Dussutour, A., Lihoreau, M., 2019.
692 Bumblebees adjust protein and lipid collection rules to the presence of brood. Curr. Zool. 65,
693 437–446. <https://doi.org/10.1093/cz/zoz026>
- 694 Kurze, C., Dosselli, R., Grassl, J., Le Conte, Y., Kryger, P., Baer, B., Moritz, R.F.A., 2016.
695 Differential proteomics reveals novel insights into *Nosema*–honey bee interactions. Insect
696 Biochem. Mol. Biol. 79, 42–49. <https://doi.org/10.1016/j.ibmb.2016.10.005>
- 697 Li, J., Chen, W., Wu, J., Peng, W., An, J., Schmid-Hempel, P., Schmid-Hempel, R., 2012.
698 Diversity of *Nosema* associated with bumblebees (*Bombus spp.*) from China. Int. J. Parasitol.
699 42, 49–61. <https://doi.org/10.1016/j.ijpara.2011.10.005>
- 700 Mallon, E.B., Brockmann, A., Schmid-Hempel, P., 2003. Immune response inhibits
701 associative learning in insects. Proc. R. Soc. Lond. B Biol. Sci. 270, 2471–2473.
702 <https://doi.org/10.1098/rspb.2003.2456>

- 703 Martín-Hernández, R., Botías, C., Barrios, L., Martínez-Salvador, A., Meana, A., Mayack, C.,
704 Higes, M., 2011. Comparison of the energetic stress associated with experimental *Nosema*
705 *ceranae* and *Nosema apis* infection of honeybees (*Apis mellifera*). *Parasitol. Res.* 109, 605–
706 612. <https://doi.org/10.1007/s00436-011-2292-9>
- 707 Martín-Hernández, R., Meana, A., Prieto, L., Salvador, A.M., Garrido-Bailon, E., Higes, M.,
708 2007. Outcome of Colonization of *Apis mellifera* by *Nosema ceranae*. *Appl. Environ.*
709 *Microbiol.* 73, 6331–6338. <https://doi.org/10.1128/AEM.00270-07>
- 710 Mayack, C., Naug, D., 2009. Energetic stress in the honeybee *Apis mellifera* from *Nosema*
711 *ceranae* infection. *J. Invertebr. Pathol.* 100, 185–188.
712 <https://doi.org/10.1016/j.jip.2008.12.001>
- 713 McDonnell, C.M., Alaux, C., Parrinello, H., Desvignes, J.-P., Crauser, D., Durbesson, E.,
714 Beslay, D., Le Conte, Y., 2013. Ecto- and endoparasite induce similar chemical and brain
715 neurogenomic responses in the honey bee (*Apis mellifera*). *BMC Ecol.* 13, 25.
716 <https://doi.org/10.1186/1472-6785-13-25>
- 717 Meana, A., Martín-Hernández, R., Higes, M., 2010. The reliability of spore counts to
718 diagnose *Nosema ceranae* infections in honey bees. *J. Apic. Res.* 49, 212–214.
719 <https://doi.org/10.3896/IBRA.1.49.2.12>
- 720 Menzel, R., 2012. The honeybee as a model for understanding the basis of cognition. *Nat.*
721 *Rev. Neurosci.* 13, 758–768. <https://doi.org/10.1038/nrn3357>
- 722 Menzel, R., 2001. Searching for the memory trace in a mini-brain, the honeybee. *Learn. Mem.*
723 8, 53–62. <https://doi.org/10.1101/lm.38801>
- 724 Menzel, R., 1999. Memory dynamics in the honeybee. *J. Comp. Physiol. [A]* 185, 323–340.
725 <https://doi.org/10.1007/s003590050392>
- 726 Menzel, R., Muller, U., 1996. Learning and memory in honeybees: from behavior to neural
727 substrates. *Annu. Rev. Neurosci.* 19, 379–404.
728 <https://doi.org/10.1146/annurev.ne.19.030196.002115>

- 729 Menzel, Randolph, Manz, G., Menzel, Rebecca, Greggers, U., 2001. Massed and spaced
730 learning in honeybees: the role of cs, us, the intertrial interval, and the test interval. *Learn.*
731 *Mem.* 8, 198–208. <https://doi.org/10.1101/lm.40001>
- 732 Mertes, M., Carcaud, J., Sandoz, J.-C., 2021. Olfactory coding in the bumble bee antennal
733 lobe. *bioRxiv* 2020.12.31.424929. <https://doi.org/10.1101/2020.12.31.424929>
- 734 Mobley, M.W., Gegear, R.J., 2018. Immune-cognitive system connectivity reduces
735 bumblebee foraging success in complex multisensory floral environments. *Sci. Rep.* 8, 5953.
736 <https://doi.org/10.1038/s41598-018-24372-5>
- 737 Monchanin, C., Drujont, E., Devaud, J.-M., Lihoreau, M., Barron, A.B., 2020. Heavy metal
738 pollutants have additive negative effects on honey bee cognition. *bioRxiv* 2020.12.11.421305.
739 <https://doi.org/10.1101/2020.12.11.421305>
- 740 Ostroverkhova, N.V., Konusova, O.L., Kucher, A.N., Kireeva, T.N., Rosseykina, S.A., 2020.
741 Prevalence of the Microsporidian *Nosema spp.* in honey bee populations (*Apis mellifera*) in
742 some ecological regions of North Asia. *Vet. Sci.* 7. <https://doi.org/10.3390/vetsci7030111>
- 743 Palottini, F., Estravis Barcala, M.C., Farina, W.M., 2018. Odor learning and its experience-
744 dependent modulation in the South American native bumblebee *Bombus atratus*
745 (hymenoptera: apidae). *Front. Psychol.* 9, 603. <https://doi.org/10.3389/fpsyg.2018.00603>
- 746 Piironen, S., Botías, C., Nicholls, E., Goulson, D., 2016. No effect of low-level chronic
747 neonicotinoid exposure on bumblebee learning and fecundity. *PeerJ* 4, e1808.
748 <https://doi.org/10.7717/peerj.1808>
- 749 Piironen, S., Goulson, D., 2016. Chronic neonicotinoid pesticide exposure and parasite stress
750 differentially affects learning in honeybees and bumblebees. *Proc. R. Soc. B Biol. Sci.* 283,
751 20160246. <https://doi.org/10.1098/rspb.2016.0246>
- 752 Plischuk, S., Martín-Hernández, R., Prieto, L., Lucía, M., Botías, C., Meana, A.,
753 Abrahamovich, A.H., Lange, C., Higes, M., 2009. South American native bumblebees
754 (Hymenoptera: Apidae) infected by *Nosema ceranae* (Microsporidia), an emerging pathogen
755 of honeybees (*Apis mellifera*). *Environ. Microbiol. Rep.* 1, 131–135.

- 756 <https://doi.org/10.1111/j.1758-2229.2009.00018.x>
- 757 Porrini, M.P., Porrini, L.P., Garrido, P.M., de Melo e Silva Neto, C., Porrini, D.P., Muller, F.,
758 Nuñez, L.A., Alvarez, L., Iriarte, P.F., Eguaras, M.J., 2017. *Nosema ceranae* in South
759 American native stingless bees and social wasp. *Microb. Ecol.* 74, 761–764.
760 <https://doi.org/10.1007/s00248-017-0975-1>
- 761 Raiser, G., Galizia, C.G., Szyszka, P., 2017. A high-bandwidth dual-channel olfactory
762 stimulator for studying temporal sensitivity of olfactory processing. *Chem. Senses* 42, 141–
763 151. <https://doi.org/10.1093/chemse/bjw114>
- 764 Ravoet, J., De Smet, L., Meeus, I., Smaghe, G., Wenseleers, T., de Graaf, D.C., 2014.
765 Widespread occurrence of honey bee pathogens in solitary bees. *J. Invertebr. Pathol.* 122, 55–
766 58. <https://doi.org/10.1016/j.jip.2014.08.007>
- 767 Riddell, C.E., Sumner, S., Adams, S., Mallon, E.B., 2011. Pathways to immunity: temporal
768 dynamics of the bumblebee (*Bombus terrestris*) immune response against a trypanosomal gut
769 parasite: Bumblebee immune genes. *Insect Mol. Biol.* 20, 529–540.
770 <https://doi.org/10.1111/j.1365-2583.2011.01084.x>
- 771 Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D., Andino, R., DeRisi, J.L.,
772 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal
773 prevalence of known viruses, *Nosema*, and *Crithidia*. *PLoS ONE* 6, e20656.
774 <https://doi.org/10.1371/journal.pone.0020656>
- 775 Scheiner, R., Abramson, C.I., Brodschneider, R., Crailsheim, K., Farina, W.M., Fuchs, S.,
776 Grünewald, B., Hahshold, S., Karrer, M., Koeniger, G., Koeniger, N., Menzel, R., Mujagic,
777 S., Radspieler, G., Schmickl, T., Schneider, C., Siegel, A.J., Szopek, M., Thenius, R., 2013.
778 Standard methods for behavioural studies of *Apis mellifera*. *J. Apic. Res.* 52, 1–58.
779 <https://doi.org/10.3896/IBRA.1.52.4.04>
- 780 Schmid-Hempel, P., 2013. Evolutionary parasitology: the integrated study of infections,
781 immunology, ecology, and genetics. Oxford University Press.
- 782 Schmid-Hempel, R., Tognazzo, M., 2010. Molecular divergence defines two distinct lineages

- 783 of *Crithidia bombi* (Trypanosomatidae), parasites of bumblebees. *J. Eukaryot. Microbiol.* 57,
784 337–345. <https://doi.org/10.1111/j.1550-7408.2010.00480.x>
- 785 Sinpoo, C., 2018. Impact of *Nosema ceranae* and *Nosema apis* on individual worker bees of
786 the two host species (*Apis cerana* and *Apis mellifera*) and regulation of host immune
787 response. *J. Insect Physiol.* 8.
- 788 Sitaraman, D., Zars, M., LaFerriere, H., Chen, Y.-C., Sable-Smith, A., Kitamoto, T.,
789 Rottinghaus, G.E., Zars, T., 2008. Serotonin is necessary for place memory in *Drosophila*.
790 *Proc. Natl. Acad. Sci.* 105, 5579–5584. <https://doi.org/10.1073/pnas.0710168105>
- 791 Smith, M.L., 2012. The honey bee parasite *Nosema ceranae*: transmissible via food
792 exchange? *PLOS ONE* 7, e43319. <https://doi.org/10.1371/journal.pone.0043319>
- 793 Sommerlandt, F.M.J., Rössler, W., Spaethe, J., 2014. Elemental and non-elemental olfactory
794 learning using PER conditioning in the bumblebee, *Bombus terrestris*. *Apidologie* 45, 106–
795 115. <https://doi.org/10.1007/s13592-013-0227-4>
- 796 Takeda, K., 1961. Classical conditioned response in the honey bee. *J. Insect Physiol.* 6, 168–
797 179. [https://doi.org/10.1016/0022-1910\(61\)90060-9](https://doi.org/10.1016/0022-1910(61)90060-9)
- 798 Toda, N.R.T., Song, J., Nieh, J.C., 2009. Bumblebees exhibit the memory spacing effect.
799 *Naturwissenschaften* 96, 1185–1191. <https://doi.org/10.1007/s00114-009-0582-1>
- 800 Velthuis, H.H.W., Doorn, A. van, 2006. A century of advances in bumblebee domestication
801 and the economic and environmental aspects of its commercialization for pollination.
802 *Apidologie* 37, 421–451. <https://doi.org/10.1051/apido:2006019>
- 803 Villar, M.E., Marchal, P., Viola, H., Giurfa, M., 2020. Redefining single-trial memories in the
804 honeybee. *Cell Rep.* 30, 2603–2613.e3. <https://doi.org/10.1016/j.celrep.2020.01.086>
- 805 Wolf, S., McMahon, D.P., Lim, K.S., Pull, C.D., Clark, S.J., Paxton, R.J., Osborne, J.L.,
806 2014. So near and yet so far: harmonic radar reveals reduced homing ability of *Nosema*
807 infected honeybees. *PLoS ONE* 9, e103989. <https://doi.org/10.1371/journal.pone.0103989>
- 808 Zheng, H.-Q., Lin, Z.-G., Huang, S.-K., Sohr, A., Wu, L., Chen, Y.P., 2014. Spore loads may

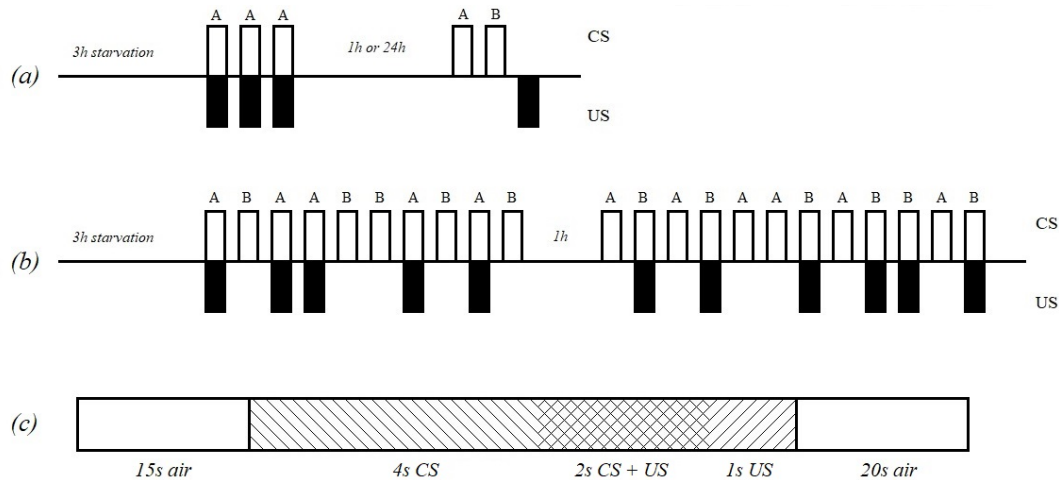
809 not be used alone as a direct indicator of the severity of *Nosema ceranae* infection in honey
810 bees *Apis mellifera* (Hymenoptera:Apidae). J. Econ. Entomol. 107, 2037–2044.
811 <https://doi.org/10.1603/EC13520>

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814 Supplementary Materials

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818 **Supplementary Figure S1. Schematic representation of the PER protocols used in**
 819 **cognitive assays using two odorants (A and B).** (a) Sequences used in the absolute learning
 820 and memory tasks. (b) Sequences used for rewarded and unrewarded trials in the differential
 821 and reversal tasks. (c) Sequence of events used in every trial. White bars represent odourless
 822 air flow before and after conditioning. Odour (CS) alone is represented by right diagonal
 823 lines. Sucrose (US) alone is represented by left diagonal lines. The crossing pattern shows the
 824 overlap of CS and US presentation.

825

826 **Supplementary Table S1. Number of bumblebees tested for sucrose sensitivity at 7 days**
 827 **post exposure.**

<i>Treatment</i>	Control	Exposed
<i>Fed</i>	48	30
<i>Positive in PCR</i>	5	10
<i>Did not finish the test</i>	0	0
Total	43	30

828 **Supplementary Table S2. Number of bumblebees trained in an absolute learning and**
 829 **tested for short-term memory (STM) and long-term memory (LTM).** Exposed positive
 830 bumblebees are shown into brackets. The proportion of bumblebees that failed to respond to
 831 the US in at least 1 trial during conditioning (inefficient bumblebees) was similar in control
 832 (69.13%) and exposed (69.40%) bumblebees. Only bumblebees that showed a positive
 833 conditioning (i.e. conditioned bumblebees) during the learning phase were kept for memory
 834 tests. Mortality within 24 h before LTM was 7.6%. Near 18% of exposed bumblebees and
 835 9.6% of control were not motivated to accomplish the memory test (us.memory = 0;
 836 Chi.square $X^2 = 1.187$, df = 1, p-value = 0.279).

Cognitive test	Bumblebee description	Control	Exposed (+positive)
Absolute learning	<i>Selected for training</i>	234	411
	<i>Died during training</i>	4	9
	<i>Escaped</i>	0	1
	<i>Not efficient</i>	71	122
	<i>PCR positive</i>	18	51
	Efficient	141	228 (+51)
Short-term memory (STM)	<i>Selected for STM</i>	36	67(+15)
	<i>Conditioned</i>	17	27(+6)
	<i>Died before test</i>	1	0
	<i>us.memory = 0</i>	0	0
	Total to analyse	16	27 (+6)
Long-term memory (LTM)	<i>Selected for LTM</i>	105	161(+36)
	<i>Conditioned</i>	72	68(+11)
	<i>Died before test</i>	15	7 (+1)
	<i>us.memory = 0</i>	5	12(+1)
	<i>Response to NoD</i>	1	2 (+0)
	Total to analyse	52	49(+9)

837

838 **Supplementary Table S3. Number of bumblebees analysed in reversal learning.** Exposed
 839 positive bumblebees are shown into brackets. 17.48% of the bumblebees died during the
 840 differential phase and 11.25% were not motivated (i.e. did not respond to US in two or more
 841 trials). 11.94% of the bumblebees that finished the differential phase died during the reversal
 842 phase. Only four bumblebees did not fit the selection criteria (i.e. PER to A- in any of the first
 843 two trials with this odour during the reversal phase). We discarded them from the analyses.

	<i>Control</i>	<i>Exposed</i>
<i>Selected for training</i>	87	96
<i>Died during differential</i>	14	18

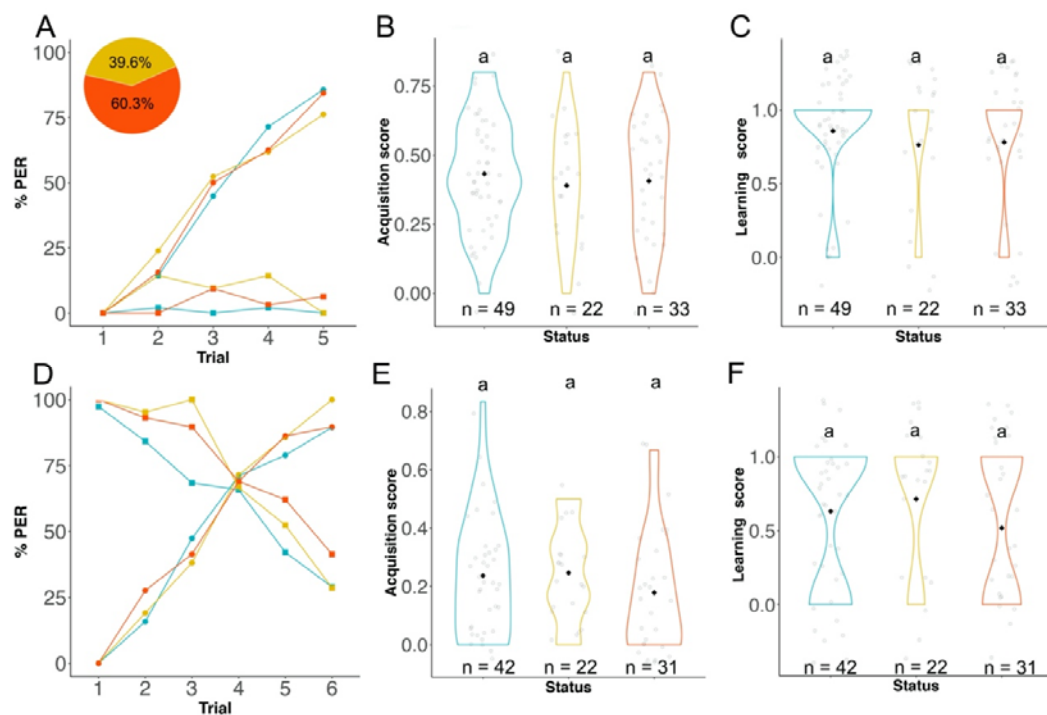
<i>phase</i>		
<i>Unmotivated</i>	6	11
<i>Positive PCR</i>	0	23
Total number of bees used to analyse differential learning	67	44 (+23)
<hr/>		
<i>Died in reversal</i>	6	6 (+3)
<i>Failed selection criteria</i>	2	1 (+1)
Total number of bees used to analyse reversal learning	59	37 (+19)
<hr/>		

844

845 **Supplementary Text S1. Reversal learning at two days post exposure.**

846 At 2 days post exposure we analysed differential learning in 104 bumblebees (see details in
847 Supplementary Table S4). *N. ceranae* did not affect differential learning nor reversal learning
848 (Supplementary Figure S2). During the differential learning phase, bumblebees in all infection
849 statuses increased their response to A+, but not to B-, over trials (Figure S3A), indicating their
850 ability to discriminate between odours. Performance was similar in the three infection statuses
851 that reached similar acquisition score (Figure S3B, GLMM, status: $X^2 = 1.235$, $df = 2$, $p =$
852 0.539) and learning score (Figure S3C; Binomial GLMM, status: $X^2 = 1.185$, $df = 2$, $p =$
853 0.552) by the end of the test. During the reversal learning phase, bumblebees reversed
854 previous contingency as shown by the decrease in the proportion of responses to A- in favour
855 to B+ along trials (Figure S3D). Neither acquisition (Figure S3E; GLMM, status: $X = 2.83$, $df =$
856 2 , $p = 0.242$) nor learning scores (Figure S3F; Binomial GLMM, status: $X = 2.053$, $df = 2$, $p =$
857 0.358) were affected by parasite exposure, all groups being able to reverse the task.

858



859

860 **Supplementary Figure S2. Reversal learning at 2 days after exposure.** A-C) Differential
861 learning phase. A) Curve showing the percentage of PER responses to rewarded (A+, circle)
862 and unrewarded (B-, square) odours by control (blue), exposed negative (yellow) and exposed

863 positive (red) bumblebees. Pie chart shows the percentage of exposed bumblebees that
864 finished the phase testing positive (red) and negative (yellow) to *N. ceranae* in a PCR. Violin
865 plots of **B**) acquisition scores (i.e. sum of the correct responses divided by the number of trials
866 for each bee) and **C**) learning scores (i.e. performance of bumblebees at last trial) of
867 bumblebees with different status. Black dots represent the mean score of each status. Hollow
868 dots represent the score of each individual. **D-F**) Reversal learning phase. **D**) Curves show the
869 increase in the proportion of PER responses to B+ over A- over trials. **E**) Acquisition and **F**)
870 learning scores during reversal phase. Letters above violin plots show significant differences
871 between status in the acquisition (GLMM, $p < 0.05$) and learning (Binomial GLMM) scores.
872 n is the sample size.

873

874 **Supplementary Table S4. Number of bumblebees analysed in reversal learning.** Exposed
875 positive bumblebees are shown into brackets. During the differential phase 10.6% of the
876 bumblebees died and 0.75% did not respond to US in at least two trials. We discarded them
877 from the analyses. We also discarded six control bumblebees that tested positive to *N.*
878 *ceranae* in the PCR. In the reversal phase only one bumblebee did not fit the selection criteria
879 (i.e. PER to A- in any of the first two trials with this odour during the reversal phase) and was
880 not taken into account for the analyses. 7.76% of bumblebees died during this phase.

	<i>Control</i>	<i>Exposed</i>
Fed	61	64
<i>Died in differential</i>	5	9
<i>Unmotivated</i>	1	0
<i>Positive PCR</i>	6	33
Total Differential	49	22 (33)
<i>Died in reversal</i>	6	0 (2)
<i>Failed selection criteria</i>	1	0 (0)
Total Reversal	42	22 (31)

881

882