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

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Abstract

Pollinators are exposed to numerous parasites and pathogens when foraging on flowers. These biological stressors may affect critical cognitive abilities required for foraging. Here, we tested whether exposure to *Nosema ceranae*, one of the most widespread parasites of honey bees also found in wild pollinators, impacts cognition in bumblebees. We investigated different forms of olfactory learning and memory using conditioning of the proboscis extension reflex. Seven days after feeding parasite spores, bumblebees showed lower performances in absolute, differential, and reversal learning than controls. The consistent observations across different types of olfactory learning indicates a general negative effect of *N. ceranae* exposure that did not specifically target particular brain areas or neural processes. We discuss the potential mechanisms by which *N. ceranae* impairs bumblebee cognition and the broader consequences for populations of pollinators.

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

Introduction

Pollinators, such as bees, rely on a rich cognitive repertoire to collect pollen and nectar on flowers. These include associative learning and memories of floral traits like odours, shapes, colours, and textures, to identify best profitable resources (Giurfa, 2015; Menzel, 2012), and spatial cues to navigate (Collett et al., 2013). Any disruption of these cognitive abilities by

environmental stressors can considerably reduce the foraging performances of bees, ultimately compromising brood development and survival (Klein et al., 2017).

In particular, foraging bees are exposed to a number of parasites that can affect their physiology and behaviour (Gómez-Moracho et al., 2017). The microsporidium *Nosema ceranae* is one of the most prevalent parasites of bees worldwide with a large range of hosts including honey bees (Higes et al., 2006), bumblebees (Plischuk et al., 2009), solitary bees (Ravoet et al., 2014), but also other flower visitors like wasps (Porrini et al., 2017). Insects get infected by ingesting parasite spores from contaminated water or pollen (Higes et al., 2008), or during physical contacts with contaminated individuals (Smith, 2012). The spores invade the gut epithelial cells of the hosts where they develop (Holt et al., 2013). In honey bees, *Nosema* degenerates the gut epithelium (Higes et al., 2007), alters the metabolism (Mayack and Naug, 2009) and disrupts the immune response (Antúnez et al., 2009). This causes a disease (nosemosis) believed to contribute to colony collapse (Cox-Foster et al., 2007).

Nosema infected honey bees also show impaired navigation (Wolf et al., 2014) and increased flight activity (Dussaubat et al., 2013), suggesting that their cognitive abilities are affected by the parasite. Recent studies have explored this possibility from a mechanistic point of view using Pavlovian olfactory conditioning of the proboscis extension reflex (PER) in which harnessed bees are trained to associate an odour, or a combination of odours, with a sucrose reward (Takeda, (1961); for recent reviews see Lavond and Steinmetz (2003); Matsumoto et al., (2012)). However, their results are mixed, presumably because of important variations in parasite exposure protocols, age of bees, and parasite post-exposure duration use in the different studies (Bell et al., 2020; Charbonneau et al., 2016; Gage et al., 2018; Piiroinen and Goulson, 2016; Piiroinen et al., 2016). Only two studies explored these effects in bumblebees. One study suggests a slight impairment of absolute learning (Piiroinen and Goulson, 2016), and both report no effect on memory (Piiroinen et al., 2016, Piiroinen and Goulson 2016). Note however that in these two studies less than 3% of the bumblebees exposed to *N. ceranae* were indeed found contaminated by the parasite (i.e., PCR positive) after the behavioural tests.

Given the expanding geographical distribution of *N. ceranae* worldwide (Klee et al., 2007), its increasing prevalence in wild bees (Plischuk et al., 2009; Porrini et al., 2017; Ravoet et al., 2014), and the potential high fitness costs incurred by bees with impaired cognition (Henry et

al., 2012; Klein et al., 2017; Perry et al., 2015), clarifying its influence on host learning and memory is important for risk assessment. In particular, other critical forms of learning, such as the ability to associate one of two odours with a reward (differential learning) and reverse this association (reversal learning) have so far been unexplored. These types of learning are essential in the everyday life of bees, to discriminate flowers, olfactory landmarks and social partners, and require different brain centers (e.g. functional mushroom bodies are necessary for the acquisition of non-elemental associations but not for elemental associations (Boitard et al., 2015; Devaud et al., 2007; Devaud et al., 2015; Giurfa and Sandoz, 2012). If the effects of the parasite are specific, these types of learning may be more or less impacted. Conversely, if the effects of the parasite are general, all learning types may be impacted.

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

Material and methods

Bumblebees

We used bumblebee workers (*B. terrestris*) from 14 commercial colonies acquired from Biobest (Westerlo, Belgium). Before the experiments, we verified the absence of *N. ceranae* (Martín-Hernández *et al.*, 2007), and other common parasites (*N. bombi* (Klee *et al.*, 2006); *Crithidia bombi* (Schmid-Hempel and Tognazzo, 2010)) in a PCR using 15 bumblebees from each colony. We maintained bumblebees in their original colonies with *ad libitum* access to the syrup provided by the manufacturer and germ-free pollen (honey bee collected pollen exposed to UV light for 12 hours), in a room at 25±1°C under a 12 h light:12 h dark photocycle, until parasite exposure.

N. ceranae spores

We obtained fresh spores from naturally infected honey bee colonies (*Apis mellifera*) maintained at our experimental apiary (University Toulouse III, France). To prepare spore solutions, we dissected the gut of 15 honey bees and crushed them in 15 mL of distilled H_2O.

We confirmed by PCR the presence of *N. ceranae* and the absence of *N. apis* (another common parasite of honey bees) in each homogenate (Martín-Hernández et al., 2007), and purified them following standard protocols (Fries et al., 2013). We centrifuged homogenates in aliquots of 1 mL at 5,000 rpm for 5 minutes and re-suspended the pellet in 500 µL of dH_2O by vortexing. This was repeated three times to obtain a spore solution of 85% purity (Fries et al., 2013). We counted *N. ceranae* spores in an improved Neubauer haemocytometer (Cantwell, 1970) in a light microscope (x400) and adjusted the spore inoculum to 15,000 spores/µL in 20% (w/w) of sucrose solution. Spore solutions were used within the same week they were purified.

Parasite exposure and experimental conditions

We exposed bumblebees to *N. ceranae* as described in Gómez-Moracho *et al.*, (2021). Briefly, we confined individual bumblebees in a Petri dish during 5 h without food. We then exposed some bumblebees to a 20 µL drop of 20% sucrose solution containing 300,000 *N. ceranae* spores. Control bumblebees received 20 µL of sucrose solution (20% w/w). We only used bumblebees that consumed the entire drop of sucrose within the next 2 h. We then allocated bumblebees into microcolonies of 20-25 individuals, containing a gravity feeder with *ad libitum* access to food (Kraus et al., 2019). Since diet can affect host-parasite relationships (Frost et al., 2008) we provided bumblebees an artificial diet with a protein to carbohydrates ratio of 1:207 previously shown to elicit highest *N. ceranae* prevalence in bumblebees (Gómez-Moracho et al., 2021). The diet was made with a fixed total amount of nutrients of 170 g/L (protein + carbohydrates) and 0.5% of vitamin mixture for insects (Sigma, Germany). Carbohydrates were supplied as sucrose (Euromedex, France). Proteins consisted in a mixture of casein and whey (4:1) (Nutrimuscle, Belgium) (Gómez-Moracho et al., 2021). We kept bumblebee microcolonies in a room at 25±1 ºC with a 12 h light: 12 h dark photoperiod until the behavioural tests. Every day, we renewed the diet and removed dead bumblebees.

Behavioural experiments

We tested the cognitive performances of bumblebees of unknown age using PER at day 7 after parasite exposure. The day before the behavioural tests we kept diets to low levels (~200 µL/bumblebee) to keep bumblebees motivated for the PER experiments. Three hours before the behavioural tests we collected bumblebees from the microcolonies, chilled them in ice for 5 min and restrained them in a modified 2 mL Eppendorf tube (hereafter, capsule) that we cut

in length to fit each bumblebee (adapted from Toda *et al.*, (2009); Figure 1A). Bumblebees were tested in the horizontal position and could move forward and backward inside the tube, and therefore retract their head (Toda et al., 2009). We found these conditions better suited to perform PER experiments with bumblebees than the classical vertical harnessing used for honey bees (Giurfa and Sandoz, 2012), in which bumblebees appeared paralysed (unpublished data). In this approach we obtained comparable to better learning performances than previous studies (e.g. 58.1% in our study vs. 44% in Laloi et al., (1999) or 57% in Piiroinen et al., (2016)). Once in the capsule, we kept the bumblebees in the dark, in an incubator at 28ºC, with no access to food. Bumblebees were left in the capsules for 3h before the experiments, and during the whole duration of each conditioning protocol (i.e., a total of 4h for sucrose responsiveness test, 5h for absolute learning with short term memory test, 6h for reversal learning, and 28h for absolute learning with 24h memory test). All bumblebees that finished the conditioning protocols were kept at -20°C for later analyses of their infection status through PCR.

Sucrose responsiveness

We tested the sucrose responsiveness of bumblebees to control for potential influences of *N. ceranae* on their reward perception or feeding motivation. We presented seven sucrose solutions to each bumblebee, from concentrations of 0% (pure water) to 60% (w/w), with increments of 10% (Graystock et al., 2013). For each concentration, we touched the antennae of the bumblebee with a toothpick soaked in the corresponding sucrose solution to elicit PER. We presented solutions in an increasing concentration gradient with an inter-trial interval of 5 minutes between concentrations. We discarded all the bumblebees responding to water (i.e., 0% sucrose solution) to avoid the effect of thirst on sucrose responsiveness (Baracchi et al., 2018), and those showing an inconsistent response (i.e., bumblebees responding to lower but not higher sucrose concentrations; Table 1) (Scheiner et al., 2003). Since sucrose concentrations were systematically presented in the same increasing order to the bumblebees, we calculated a gustatory score for each bumblebee based on the lowest concentration at which they responded. Bumblebees whose first response was observed following exposure to 10% sucrose had a score of 1, whereas bumblebees that responded for first time to 60% sucrose had a score of 6. Therefore, the lower the score, the lower the sucrose sensitivity threshold of the bumblebee.

Conditioning experiments

All experiments shared the same general protocol (Figure 1A). An encapsulated bumblebee (Figure 1B) was placed 1.5 cm ahead of an automated conditioning setup (described in (Aguiar et al., 2018)) delivering a continuous stream of odourless air at 1.2 ml/s to which specific odours were selectively added (Raiser et al., 2017). We used two odorants as conditioned stimulus (CS): nonanal and phenylacetaldehyde (Palottini et al., 2018; Sommerlandt et al., 2014), in a 1:100 dilution in mineral oil. Before conditioning, we tested the responsiveness of bumblebees to sucrose by touching both antennae with a toothpick soaked in 50% (w/w) sucrose solution without allowing them to lick. Bumblebees extending their proboscis were considered motivated and kept for the experiments. Conditioning trials (Figure 1Ac) consisted in 15 seconds of odourless airflow, followed by 6 seconds of CS, and 3 seconds of unconditioned stimulus (US) (i.e. 50% sucrose solution applied with a toothpick on the bumblebee's antennae), with 2 seconds of overlap between CS+US, and 20 seconds of odourless airflow (Aguiar et al., 2018). The inter-trial interval was 10 minutes. An air extractor was placed behind the bumblebee to prevent odorant accumulation during CS delivery. Bumblebees extending their proboscis within 3 seconds of US presentation (i.e., 2s CS+US and 1s US) were allowed to lick the toothpick soaked in sucrose (50%, w/w). In unrewarded trials (see reversal learning and memory tests) no US was applied. We scored a conditioned response if the bumblebee extended its proboscis to the odour delivery before sucrose presentation. Bumblebees that responded to the odour in the first conditioning trial were discarded from the analyses. We used conditioned responses to calculate individual scores for each bumblebee describing its performance during conditioning (i.e., acquisition score) (Monchanin et al., 2020; see details below). Exposed and control bumblebees were always conditioned in parallel.

Absolute learning, short-term memory and long-term memory

We tested the effects of *N. ceranae* on the ability of bumblebees to associate an odour with a reward. This form of learning primarily requires peripheral brain centers, (i.e. the antennal lobes) as it can be observed in bees with non-functional central brain (Giurfa and Sandoz, 2012). We trained bumblebees in a spaced 3-trial absolute conditioning learning (Figure 1Aa) that was shown to generate robust long-term memory in bees (Menzel et al., 2001). We used the same rewarded odour (CS+) during training of a given bumblebee, but both nonanal and phenylacetaldehyde were used as a CS+ for different bumblebees. For each of these bees we calculated an acquisition score (sum of PER responses (i.e., 0-2)), and compared the learning

curve to assess the increase of PER responses over trials. Bumblebees that did not respond to the US in at least one trial were considered not motivated and were removed from the learning analyses (i.e. 30.3% control and 29.6% exposed bumblebees; Table 1).

We tested memory retention of bumblebee responders that showed at least one conditioned response in either one of the last two trials (Simcock et al., 2018; Wright et al., 2015) (i.e. 41.3% of bumblebees conditioned for STM and 30-68% of bumblebees conditioned for LTM; see details in Table 1). We performed tests either 1 h (i.e., short-term memory; STM) or 24 h (i.e., long-term memory; LTM) after the last acquisition trial. Bumblebees were tested either for 1 h or 24 h, but never for both because of the risk of reconsolidation or extinction of memory when the CS is presented several times without a reward to bees (Bouton and Moody, 2004). For tests performed after 24 h, bumblebees were fed until satiation with 50% (w/w) sucrose solution right after conditioning, unfortunately 9-20% of responder bumblebees died before the test (Table 1). In bees, LTM is dependent on protein synthesis whereas STM is not (Menzel, 2001). Studying these two types of memories was thus a mean to explore whether exposure to *N. ceranae* interfered with protein synthesis. We presented bumblebees the two odorants without any reward: the odour used as a CS+ to test for memory formation, and the second odour as a novel odorant (NOd), to control for potential generalization (Matsumoto et al., 2012). For example, when nonanal was used as CS+, phenylacetaldehyde was used as a NOd, and *vice versa*. Bumblebees responding only to CS+, and not to NOd were considered to have generated a specific memory to the rewarded odour. Other responses registered were response to NoD alone (inverted response), both odours (general response) or none of the odours (no memory). Just after the memory test, we tested the motivation of bumblebees by touching their antennae with a toothpick soaked with 50% (w/w) sucrose solution. Bumblebees that did not respond to the US were discarded for the analyses (i.e., 14.06% of alive responder bumblebees in LTM; Table 1). Sample sizes dropped between learning and memory tests because of the selection protocol of responders, mortality in tubes, and loss of motivation by the bumblebees (see details in Table 1).

Reversal learning

We tested the effects of *N. ceranae* on the ability of bumblebees to learn to discriminate two odours and reverse the task. This form of learning involves two phases. The differential conditioning phase (phase 1) requires the antennal lobes but is not dependant on central brain centers, (i.e., mushroom bodies) (Boitard et al., 2015; Devaud et al., 2007). The reversal

learning phase (phase 2) requires both functional antennal lobes and mushroom bodies to be observed (Boitard et al., 2015; Devaud et al., 2007). In phase 1, we trained bumblebees to discriminate between two odours. This consisted in 10 trials (Figure 1Ab), five with each odour that was either paired with the US (A+) or unpaired (B-), presented in a pseudo-random order. The rewarded and unrewarded odours were randomized on different training days. Bumblebees that did not respond to US in two or more trials were discarded for the analyses and for reversal phase. In phase 2, we trained bumblebees to invert the first learnt contingency. This reversal phase started 1h after the end of the differential phase. Here we trained bumblebees in 12 trials, 6 with each odour presented in a pseudo-random order. The previously rewarded odour was not associated with a reward anymore (A-) while the previously unrewarded odour became rewarded (B+). To start from the same level of learning, in the first trial of reversal phase we presented the odour A-, and kept for the analyses only the bumblebees that extended their proboscis (Table 1). We analysed the performance of bumblebees in each phase separately by attributing them an acquisition score (sum of all trials where the bee responded to CS+ but not to CS-) for each phase.

Infection status

We assessed the infection status of bumblebees that finished the tests in a PCR using the primers 218MITOC (Martín-Hernández et al., 2007). These primers are 100% specific for *N. ceranae* and have a 0% error rate (false negatives) when reactions containing DNA equivalent for 2000 spores (Martín-Hernández et al., 2007). For each bumblebee, the entire gut was extracted, homogenised in sterile dH2O and vortexed with 2 mm glass beads (Labbox Labware, Spain). Genomic DNA was extracted using Proteinase K (20 mg/mL; Euromedex, France) and 1 mM of Tris-EDTA Buffer (pH = 8). A sample with *N. ceranae* spores was included in each round of extraction as positive control. PCRs were performed with the Taq Polymerase Direct Loading Buffer (5 U/µL; MP Biomedicals, CA) following manufacturer's instructions. We used a final volume of 25 µL with 0.4 µM of each pair of primers (Martín-Hernández et al., 2007), 200 µM of dNTPs (Jena Biosciences, Germany), 0.48 µg/µL of BSA (Sigma, Germany) and 2.5 µL of DNA sample. PCR reactions were carried out in a S1000™ Thermal Cycler (Biorad, CA). Thermal conditions were 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 61.8ºC for 45 s and 72 °C for 2 min, and a final step of 72 °C for 7 min. The length of PCR products (i.e., 218 pb) was checked in a 1.2% agarose gel electrophoresis stained with SYBR Safe DNA Stain (Edvotek, Washington DC). Positive and negative controls of PCR were run in parallel. Based on the PCR results we classified bumblebees in three different

infection statuses: control, *Nosema* exposed negative (NE-) or *Nosema* exposed positive (NE+). Bumblebees that were not exposed to the parasite but nevertheless showed a positive result in PCR were excluded from the analyses (i.e. 6.26%; 23 out of 367 control bumblebees). These positives may be due to the fact that despite our precautions before starting the experiments (PCR screening of about 10% of the workers in the colonies, use of UV-treated pollens), it is possible that the commercial colonies we used were not fully free of parasites. Additionally, we cannot completely exclude low levels of cross-contaminations between treatments during manipulations.

Statistical analyses

All analyses were conducted in RStudio (version 4.1.0). We evaluated the effects of parasite exposure and infection on learning curves, gustatory, acquisition, learning and memory scores. The proportion of responses to the different sucrose concentration was analysed in a Generalized Linear Mixed Model (GLMM) (package [lme4]; (Bates et al., 2015)), with infection status and concentration as fixed factors. Learning curves of absolute and reversal learning experiments were analysed in a binomial GLMM with infection status, trial, and the interaction between them as fixed factor. Whenever the interaction was not significant it was removed from the model. The response to rewarded and unrewarded odours during reversal learning were analysed separately. To determine the ability of bumblebees to learn to differentiate a rewarded from an unrewarded odour (i.e., differential phase) and the opposite (i.e., reversal phase) in the reversal learning experiment, we studied the interaction of infection status, trial and reward in a binomial GLMM, followed by a Tukey pairwise comparison applying the function *lsmeans* (package [emmeans]). All these models included colony and bee identity as random factors. Gustatory scores for sucrose responsiveness, and acquisition scores during learning experiments were analyzed with a linear model. These models included bee infection status as fixed factor and colony of origin as random factor. We performed Tukey post-hoc pairwise comparisons (package [multcomp]; (Hothorn et al., 2008)) to assess the relationship between the three bee infection statuses. The effect of infection status on the ability of bumblebees to generate specific memory (i.e., response to CS+ only) or no memory (i.e., response to any odor) was compared with a Chi-square. Raw data are available online (doi:10.5281/zenodo.4376362).

Results

Parasite exposure did not influence sucrose responsiveness

We tested responsiveness to different sucrose concentrations in 63 consistent bumblebees (37 controls, 16 NE-, 10 NE+; Table 1). Bumblebees increased their response to sucrose solution with concentration (Figure 2A; GLMM, Estimate = 0.098, SE = 0.009, $p < 0.001$) in the three infection statuses (GLMM, infection status: $x^2 = 4.754$, df = 2, p = 0.09). Gustatory scores ranged from 1.32 ± 1.22 (mean ± SE) in controls, 1.43 ± 0.24 in NE- and 1.80 ± 0.41 in NE+ bumblebees, but did not differ between infection status (Figure 2B; LM, F = 1.077, p = 0.347). Therefore, exposure to *N. ceranae* neither affected the sucrose sensitivity nor the feeding motivation of bumblebees.

Parasite exposure reduced absolute learning but not memory

We analysed absolute conditioning in 420 bumblebees (141 controls, 228 NE-, 51 NE+; Table 1). The proportion of bumblebees showing a conditioned response to CS+ increased with trials (GLMM; trial: $x^2 = 58.432$, df = 2, $p < 0.001$), but this trend was lower for NE- (Estimate = -1.056, SE = 0.2950, p = 0.00034) and NE+ (Estimate = -1.388, SE = 0.474, p = 0.003) bumblebees than for controls (Figure 3). Likewise, exposed bumblebees (either NE- or NE+) had significantly lower acquisition scores (Figure 3B; LM, infection status: $x^2 = 15.897$, df = 2, $p < 0.001$) than controls. Acquisition scores were similar for NE- and NE+ (Tukey $p > 0.05$; Table S1).

We analysed short-term and long-term memory formation of bumblebee responders (i.e. those that showed at least one conditioned response at either trials 2 and/or 3: 41.3% in short-term memory and 50% in long-term memory; Table 1). One hour after training bumblebees either responded to the CS+ only (i.e. specific memory; 82.02%±0.011, mean±SE) or did not respond to any odour (i.e. no memory), with no bumblebees responding to NOd (i.e. neither generalizers nor inverters; Figure 3C). The proportion of bumblebees with specific memory was similar in the three infection statuses ($x^2 = 0.013$, df = 2, p = 0.993), indicating that *N. ceranae* did not affect short-term memory (Figure 3C). A much lower proportion of the bumblebees (33.03%±6.35, mean ±SE) showed specific memory for the conditioned odour after 24 h (Figure 3D). Two bumblebees generalized their responses (one Control and one NE-), and only one NE- bumblebee inverted its response (i.e. response to NOd only). The proportion of bumblebees showing specific memory to CS+ did not differ between infection

statuses (Figure 2E; $x^2 = 2.349$, df = 2, p = 0.310), presumably because of the low amount of NE+ individuals (9 bumblebees).

Parasite exposure reduced differential and reversal learning

We analysed differential learning in 125 bumblebees (64 controls, 41 NE-, 20 NE+; Table 1). The proportion of bumblebees responding to A+ was affected by the interaction between the infection status and trial (GLMM; infection status × trial: $x^2 = 7.987$, df = 2, p = 0.018). Responses increased with trials in Control and NE- bumblebees (Estimate = 1.220, SE = 0.1879, $p < 0.001$), but not in NE+ (Estimate = -0.732, SE = 0.299, p = 0.014). Control bumblebees discriminated the two odours (i.e. higher proportion of responses to the A+ than B-) at trial 2 (Tukey; z = 3.674, p = 0.044), NE- at trial 4 (Tukey: z = 4.083, p = 0.009), but NE+ bumblebees did not show this ability nor at trial 5 (Tukey: z = 2.514, p = 0.665), even if the proportion of NE- that responded to B- was zero in previous trials (Figure 4A). Parasite exposure affected acquisition scores (GLMM, infection status, $x^2 = 29.978$, df = 2, $p < 0.001$; Figure 4B). NE bumblebees (positive or negative) showed similar acquisition scores (Tukey test: $p > 0.05$; Table S1) and these scores were significantly lower than the scores of controls (Tukey test: $p < 0.05$; Table S1). Thus overall, exposure to *N. ceranae* reduced differential learning performances. Exposed bumblebees were slower (i.e. NE-) or unable (i.e. NE+) to solve the task.

We analysed reversal learning in 103 bumblebees that finished the differential phase, and that responded to A- in the first trial of the reversal phase (56 controls, 32 NE-, 15 NE+; Table 1). All bumblebees reduced their response to A- over trials (GLMM, trial: Estimate = -1.187, SE = 0.134; $p<0.001$) and increased their response to B+ (GLMM, trial; Estimate = 1.215, SE = 0.124; $p<0.001$) (Figure 4D). Infection status did not affect the response to A- (GLMM; infection status: $x^2 = 9$, df = 2, p = 0.389). The proportion of bumblebees responding to B+ was not different between controls and NE- (Estimate = -0.579, SE = 0.348, p = 0.096). However, it was significantly reduced in NE+ (Estimate = -1.597, SE = 0.450, $p < 0.001$). Control bumblebees reversed their response to odours earlier, at trial 5 (i.e. higher proportion of bees responding to B+ than A-; Tukey: z = 4.478, p = 0.002), while NE- did it at trial 6 (Tukey: z=4.281, p = 0.006), and NE+ never did it (Tukey; trial 6: z= 2.103, p= 0.960). This was also reflected in the acquisition scores, which were significantly different between bumblebees of different infection statuses (Figure 4E; GLMM, infection status, $x^2 = 6.783$, p = 0.033). NE+ bumblebees had lower acquisition scores than controls (Tukey test: p = 0.053;

Table S1) and NE- bumblebees (Tukey test: p = 0.044, Table S1), suggesting they had a lower ability to reverse the task. Thus overall, exposure to *N. ceranae* also impaired the reversal phase of reversal learning. We found no effect of *N. ceranae* exposure on either phase of reversal learning when bumblebees were tested at 2 days post exposure, suggesting that stress due to parasite exposure or parasite infection requires a longer time to be established (Text S1).

Discussion

Bees are exposed to a number of parasites that can affect cognitive abilities supporting crucial behaviour (Koch et al., 2017; Schmid-Hempel, 2013). Previous studies exploring the effect of *N. ceranae* on absolute olfactory learning and memory in bees reported contrasting results, presumably because of differences in conditioning protocols and infection rates across studies (Bell et al., 2020; Charbonneau et al., 2016; Gage et al., 2018; Piiroinen and Goulson, 2016; Piiroinen et al., 2016). Here, we ran a suite of standard olfactory cognitive assays showing that feeding bumblebees spores of this parasite consistently impaired different types of olfactory learning but not memory seven days after exposure.

Exposure to *N. ceranae* in food impaired the ability of bumblebees to associate an odour with a reward (absolute learning), discriminate two odours (differential learning), and learn an opposite association (reversal learning). These are fundamental cognitive operations a bee must display to efficiently forage on flowers (Giurfa and Sandoz, 2012). Our results agree with a previous study reporting a reduced absolute learning in *N. ceranae* exposed bumblebees (Piiroinen and Goulson, 2016). We also found that *N. ceranae* did not affect sucrose responsiveness, contrary to observations in honey bees in which it was found to increase their hunger (Mayack and Naug, 2009). The parasite may thus not produce the same energetic stress observed previously in honey bees (Mayack and Naug, 2009), where *N. ceranae* seems to be a more specific parasite (van der Steen et al., 2022). However, further experiments are needed to confirm this since protocols in these previous studies differed slightly from ours. For instance, under our conditions we cannot discard the possibility that response to high sucrose concentrations was due to sensitization as no water was used between sucrose concentrations. In bees, sucrose perception through the antennae and olfactory learning require processing of olfactory information through the antennal lobes

(Giurfa and Sandoz, 2012). While simple forms of associations can be acquired only with functional antennal lobes, others types of learning (i.e. reversal learning, configural learning) also require information processing in the mushroom bodies (Devaud et al. 2007; Devaud et al. 2015; Boitard et al. 2015). In our experiments, the fact that all types of learning were impaired and that sucrose sensitivity was not, suggests that *N. ceranae* did not specifically target the antennal lobes or the mushroom bodies. Rather, it likely impacted the learning processes in general.

By contrast, we found no evidence that *N. ceranae* influenced memory or sucrose sensitivity. During training animals learn and form short-term memories that are later consolidated and transformed into stable long-term memories (Menzel and Muller, 1996) after protein synthesis (Menzel, 2001). In our experiments, *N. ceranae* neither impaired short-term nor long-term memory.

It has recently been questioned whether bumblebees are natural hosts of *N. ceranae* based on the lack of evidence of parasitic forms inside host cells (Gisder et al., 2020). Several studies have nevertheless reported *N. ceranae* in wild bumblebees at low (e.g. 4.76%; (Sinpoo, 2018)) and high prevalence (e.g. 72%; (Arbulo et al., 2015)). Whether or not bumblebees are suitable hosts for *N. ceranae* replication, our results imply they are impacted by an acute exposure to the parasite. Such exposure may be extremely frequent in nature due to the high prevalence of *N. ceranae* in honey bees (Runckel et al., 2011) that contaminate flowers with spores through physical contact or in their faeces (Graystock et al., 2015). Our protocol of parasite exposure significantly increased the infection rate of bumblebees to 28% in comparison to previous studies (Piiroinen and Goulson, 2016; Piiroinen et al., 2016), which allowed the evaluation of cognitive traits in bumblebees in the three infection statuses. Bumblebees that tested positive to *N. ceranae* showed a tendency for lower cognitive performances than negative exposed bumblebees. They reached the lowest learning during the absolute conditioning and did not discriminate odours, suggesting that infection may interfere with some aspects of cognition. However further experiments are needed to tackle this question, as the lower performance of exposed bumblebees positive to *N. ceranae* could also be related to a worse health status and therefore higher susceptibility to become infected.

Through which mechanism may the parasite impair learning? Ingestion of *N. ceranae* spores exerts a stress that can reduce cognition. *N. ceranae* is known to alter the immune system of

bees, for example by modulating the expression of antimicrobial peptides (Antúnez et al., 2009; Botías et al., 2020; Sinpoo, 2018). Stimulation of the immune system with non-pathogenic elicitors, as lipopolysaccharides (LPS), was shown to reduce learning abilities in honey bees, that were less able to associate an odour with a reward (Mallon et al., 2003), and bumblebees, that showed lower performances in odour (Mobley and Gegear, 2018) and colour differential learning tasks (Mobley and Gegear, 2018). It is thus possible that the observed effects of *N. ceranae* exposure on bumblebee cognition were caused by an activation of the immune response. Exposed positive bumblebees showed lower learning performance than exposed negative bumblebees during differential and reversal learning tasks, suggesting a further effect of parasite infection, rather than just exposure. In honey bees, infection with *N. ceranae* was shown to downregulate the expression of genes in the brain (Doublet et al., 2016), some of which are linked to olfaction (Badaoui et al., 2017; Doublet et al., 2016), potentially leading to changes in behaviour and cognition. Whether *N. ceranae* downregulates gene expression in the brain of bumblebees needs to be addressed. Interestingly, none of these effects were observed at two days post exposure (Text S1). So far it is unknown if *N. ceranae* triggers the immune response at this time. In honey bees, the earliest effects have been observed after three days (Chaimanee et al., 2012).

Since olfactory learning is essential for foraging, this sublethal effect of *N. ceranae* exposure on bumblebee cognition can compromise colony foraging success, as well as chemical communication between bees, ultimately leading to colony collapse. Parasite loads in the field can range from a few to thousands spores (Meana et al., 2010). Here we used a substantially higher spore loads of *N. ceranae* to infect commercially reared bumblebees than the actual infection rates found in wild bumblebees (e.g. 6800 spores per individual (Graystock et al., 2013)). Commercial and wild bumblebee colonies exhibit physiological and behavioural differences as a result of different selective pressures (Velthuis and Doorn, 2006), and may, therefore, show different susceptibility to parasites. Therefore, further studies are needed to analyse the effects of different concentrations of *N.* ceranae spores, and their possible interactions with other stressors in the field in order to assess their real impact on wild pollinators. Beyond bees, these effects may also have broader fundamental consequences for plants and parasites. From the plant perspective, an impaired flower constancy by pollinators may increase pollen transfer between incompatible flowers of different species, and therefore reduce pollination efficiency. From the parasite perspective, foraging errors due to impaired learning by bees may decrease they tendency for flower constancy. This would be beneficial

for the parasite as it may favour its spread across flower species and thus possibly increase the range of hosts.

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Figures and Table

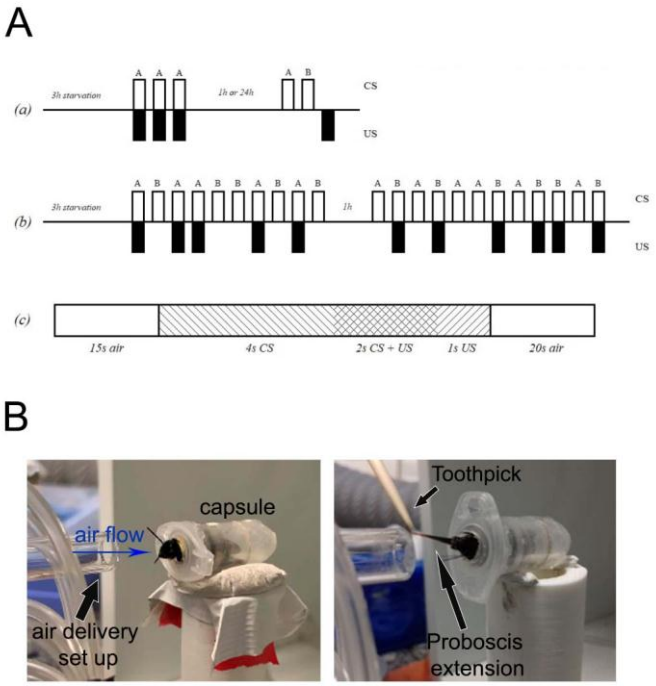

Figure 1. **Conditioning protocols and odor delivery setup.** A) Schematic representation of the PER protocols used in cognitive assays using two odorants (A and B). (a) Sequences used in the absolute learning and memory tasks. (b) Sequences used for rewarded and unrewarded trials in the differential and reversal tasks. (c) Sequence of events used in every trial. White bars represent odourless air flow before and after conditioning. Odour (CS) alone is represented by right diagonal lines. Sucrose (US) alone is represented by left diagonal lines. The crossing pattern shows the overlap of CS and US presentation. B) Odour delivery setup. Photo of a bumblebee during odour conditioning. The bumblebee is placed inside the capsule in front of the air delivery set up. After the odour is delivered, sucrose is presented with a toothpick to the bumblebees which extends its proboscis to drink the reward.

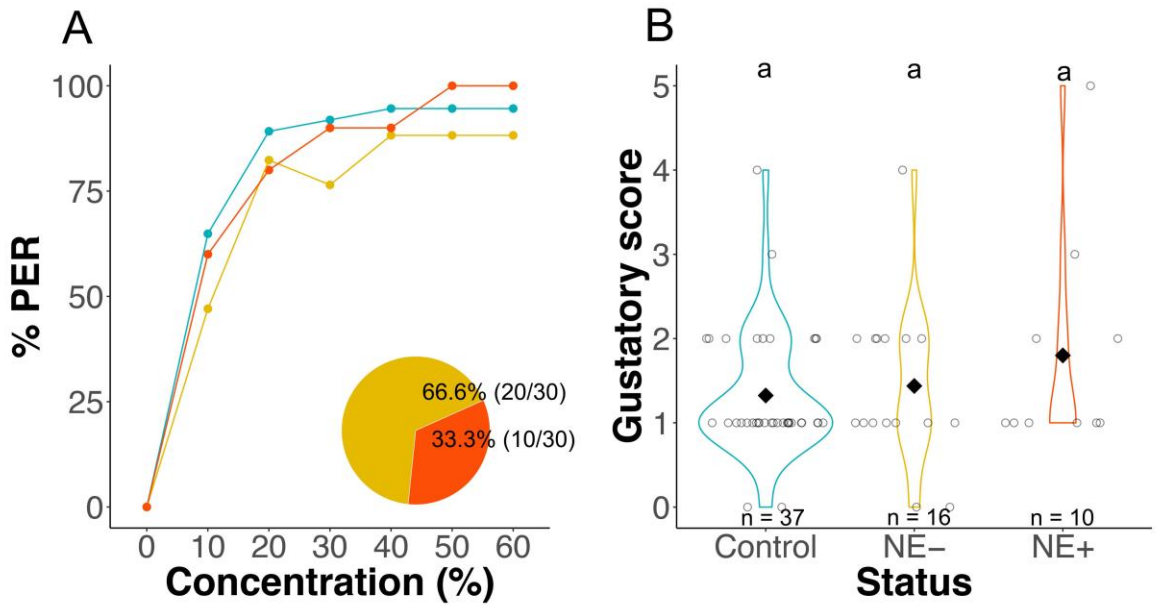

Figure 2. Sucrose responsiveness. A) Proportion of control (blue, n = 37), *Nosema* exposed negative (NE-; yellow, n = 16) and *Nosema* exposed positive (NE+; red, n = 10) bumblebees responding to an increase gradient of sucrose concentrations. Pie chart represents the percentage of NE- and NE+ bees (n total=30). **B)** Violin plots show gustatory score of bumblebees as the sum of all responses for each bumblebee. Black diamonds represent the mean score for each infection status. White dots represent the score of each individual. n is the sample size. Differing letters above violin plots represent significant differences between infection status (GLMM; $p < 0.05$). n is the sample size.

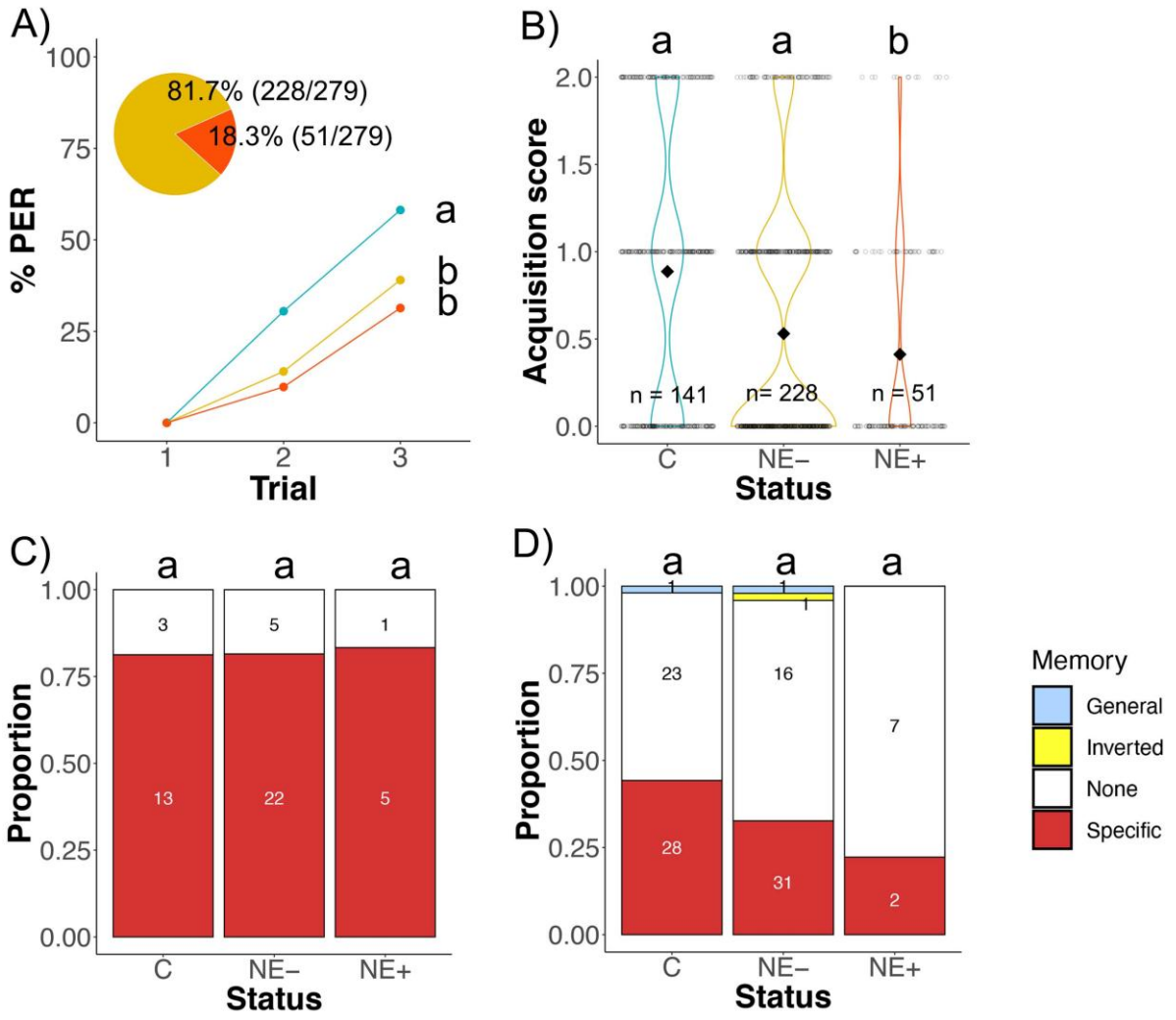

Figure 3. Absolute learning and memory. A) Learning curves show the percentage of control (C; blue, n =141), exposed negative (NE-; yellow, n = 228) and exposed positive (NE+; red, n=51) bumblebees extending their proboscis to the odour during conditioning. Pie chart shows the percentage of **NE-** and **NE+** bumblebees that finished the conditioning (N total = 279). **B)** Violin plots for acquisition score (B; sum of correct responses). Black diamonds represent the mean score for each infection status. White dots represent the score of each individual. n shows the sample size. **C-D)** Short-term and long-term memory. Bar plots show the proportion of bumblebees responding to both CS+ and NoD (i.e general memory, blue), CS+ only (specific memory, red), NoD only (inverted memory, yellow), or none odours (no memory, white) 1 h (C; STM) and 24 h (D; LTM) after training. Numbers inside the bars represent the sample size. Letters above violin plots and bar plots represent significant differences between infection status (GLMM; $p < 0.05$).

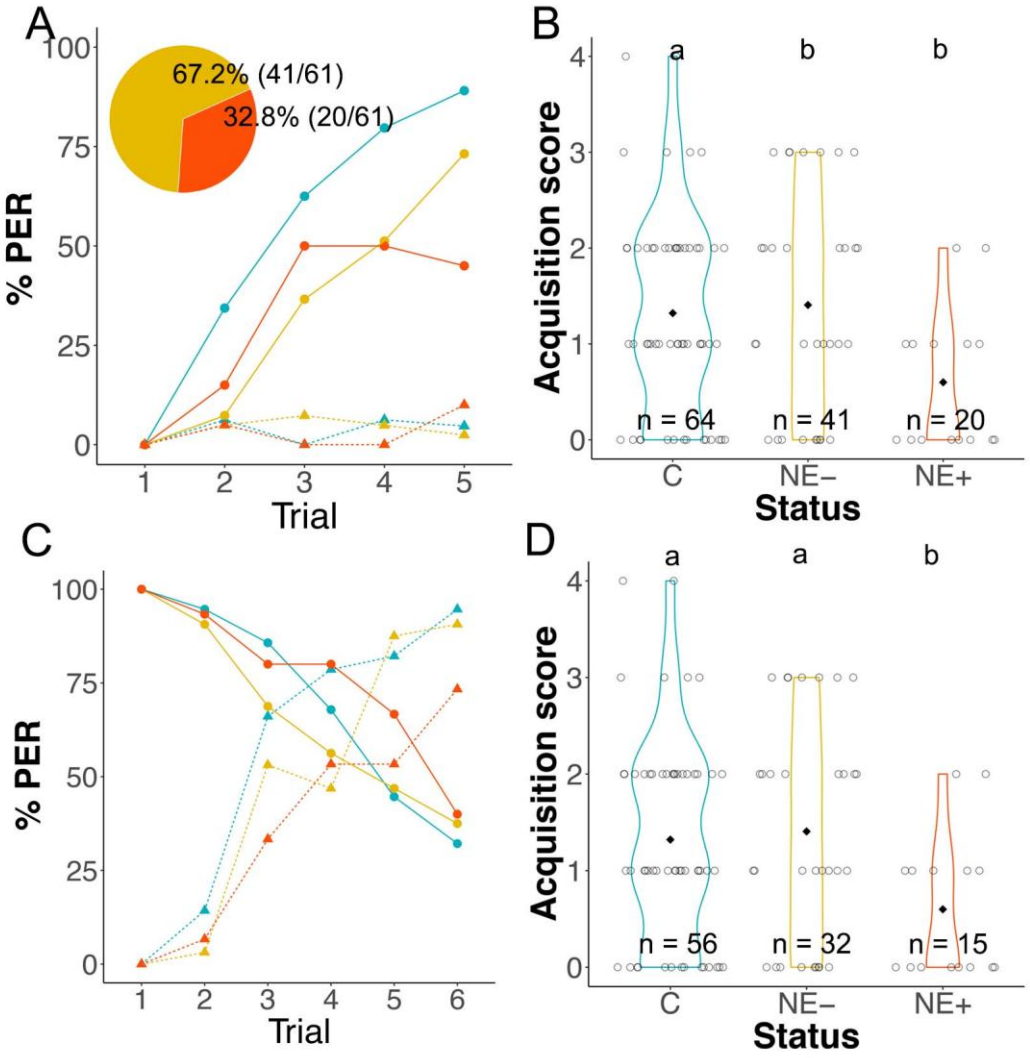

Figure 4. Reversal learning. **A-B)** Differential learning phase. **A)** Percentage of PER responses to rewarded (A+, circle) and unrewarded (B-, triangle) odours by control (blue, n = 64), exposed negative (NE-, yellow, n = 41) and exposed positive (NE+, red, n = 20) bumblebees. Pie chart shows the percentage of NE- and NE+ bumblebees that finished conditioning (n total = 61). **B)** Violin plots of acquisition scores (i.e., sum of the correct responses divided by the number of trials for each bee). Black diamonds represent the mean score of each infection status. White dots are the scores for each individual. **C-D)** Reversal learning phase. **C)** Curves show the increase in the percentage of PER response to B+ over A- over trials in control (n = 56), NE- (n = 32) and NE+ (n = 15) bumblebees. **D)** Acquisition scores. Letters above violin plots show significant differences between infection statuses (GLMM, $p < 0.05$). n is the sample size.

Table 1. Details of samples sizes for each experiment after 7 days post exposure.

Test	Selected and discarded individuals	Control	NE-	NE+
Sucrose Sensitivity	Final sample size	37	16	10
	Total discarded	11	4[a]	-
	Inconsistent	6	4	0
	Positive PCR	5	10[b]	-
Absolute conditioning	Final sample size	141	228	51
	Total discarded	93	132[a]	-
	Died during training	4	9	
	Escaped	0	1	
	Not motivated	71	122	
	PCR positive	18	51[b]	
Short-term memory (STM)	Final sample size	16	27	6
	Total discarded	20	40	9
	Not Responders	20	40	9
Long-term memory (LTM)	Final sample size	52	49	9
	Total discarded	53	112	27
	Not responders	33	93	25
	Died before test	15	7	1
	Not motivated	5	12	1
Differential learning	Final sample size	64	41	20
	Total discarded	21	35[a]	-
	Died during test	12	18	
	Unmotivated	6	11	
	Response to US at first presentation	3	6	
	Positive PCR	0	20[b]	
Reversal learning	Final sample size	56	32	15
	Total discarded	8	9	5
	Died during test	6	6	3
	Failed response to A-	2	3	2

[a] All exposed individuals (NE- and NE+) as the final status of bumblebees was confirmed in those that finished the tests; [b] Exposed positive individuals showed in column NE+.

Table S1. Tukey Pairwise comparisons for absolute and reversal learning.
Significant results (P<0.001) are highlighted in bold.

Test		Contrast	z	P
Absolute learning	Learning curve	**C: trial2 vs trial3**	**-4.741**	**<0.001**
		NE-: trial2 vs trial3	**-5.970**	**<0.001**
		NE+: trial2 vs trial3	-2.661	0.089
		Trial2: C vs. NE-	**3.710**	**0.002**
		Trial2: C vs. NE+	**2.827**	**0.053**
		Trial2: NE- vs. NE+	0.831	0.961
		Trial3: C vs. NE-	**3.514**	**0.005**
		Trial3: C vs. NE+	**3.242**	**0.015**
		Trial3: NE- vs. NE+	1.070	0.893
	Acquisition score	**C – NE-**	**3.679**	**<0.001**
		C – NE+	**2.961**	**0.008**
		NE- – NE+	0.605	0.814
Reversal learning – differential phase	Acquisition score	**C – NE-**	**4.706**	**<0.001**
		C – NE+	**4.102**	**<0.001**
		NE- – NE+	0.336	0.928
Reversal learning – reversal phase	Acquisition score	control – NE-	-0.345	0.936
		control – NE+	**2.348**	**0.053**
		NE- – NE+	2.429	0.044

Reversal learning at two days post exposure.

At 2 days post exposure we analysed differential learning in 104 bumblebees (see details in Supplementary Table S2). *N. ceranae* did not affect differential learning nor reversal learning (Supplementary Figure S1). During the differential learning phase, the proportion of responses increased in over trials (GLMM, trial: $X^2 = 69.318$, df = 1, $p < 0.001$) and it was similar for bumblebees in all infection status (GLMM, stauts: $X^2 = 0.113$, df = 2, p = 0.945). All bumblebees were able to discriminate between odours. Control and NE+ did it at trial 4 (Tukey; rewarded vs unrewarded: z = 4.492, p = 0.001, and z = 3.661, p = 0.046), while NE- did it at trial 5, with no bees responding to the unrewarded odour. Likewise, the three infection statuses reached similar acquisition scores (Figure S1B, GLMM, infection status: $X^2 = 0.863$, df = 2, p = 0.649). During the reversal learning phase, bumblebees reversed previous contingency as shown by the decrease over trials in the proportion of responses to A- (GLMM, trial: Estimate = -1.134, SE = 0.139, $p < 0.001$) in favour to B+ (Figure S1D; GLMM, trial: Estimate = 1.372, SE

= 0.256, $p < 0.001$). Infection status did not affect these responses (GLMM, infection status, odour A-: $X^2 = 0.828$, df = 2, $p = 0.660$; response to B+: $X^2 = 1.545$, df = 2, $p = 0.461$). All infection status responded more to B+ than A- by trial 6 (Tukey: $p < 0.001$). Acquisition scores (Figure S1E; GLMM, infection status: $X^2 = 1.756$, df = 2, $p = 0.415$) were not affected by parasite exposure, all groups being able to reverse the task.

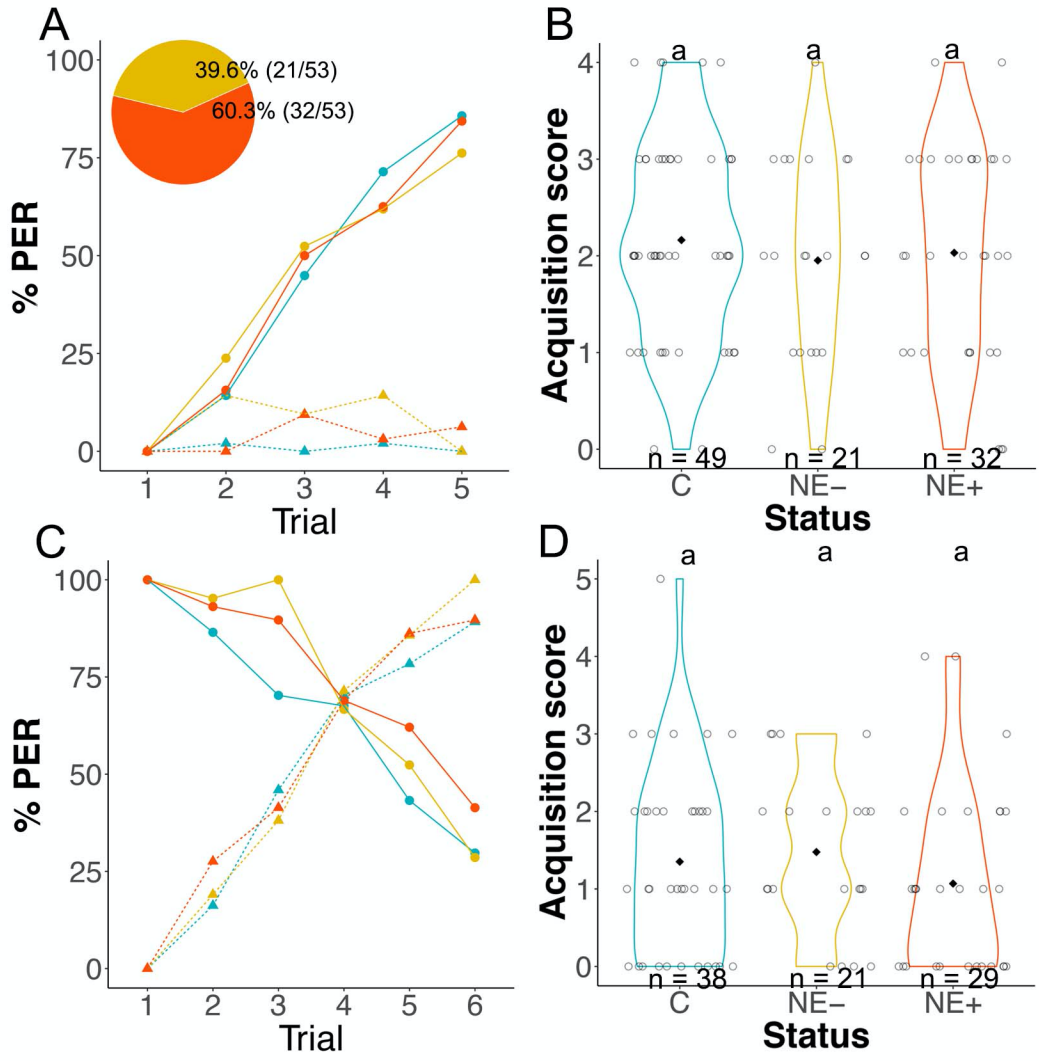

Fig. S1. Reversal learning at 2 days after exposure. A-B) Differential learning phase. **A)** Curve showing the percentage of PER responses to rewarded (A+, circle) and unrewarded (B-, triangle) odours by control (C, blue), exposed negative (NE-, yellow) and exposed positive (NE+, red) bumblebees. Pie chart shows the percentage of exposed bumblebees that finished the phase testing positive (red) and negative (yellow) to *N. ceranae* in a PCR. **B)** Violin plots of acquisition scores. Black dots represent the mean score of each infection status. Hollow dots represent the score of each individual. **C-D)** Reversal learning phase. **C)** Curves show the increase in the proportion of PER responses to B+ over A- over trials. **D)** Acquisition scores during reversal phase. Letters above violin plots show significant differences between infection status in the acquisition scores (GLMM, $p < 0.05$). n is the sample size.

Table S2. Number of bumblebees analysed in reversal learning.

Exposed positive bumblebees are shown into brackets. During the differential phase 10.6% of the bumblebees died, 0.75% did not respond to US in at least two trials, and 6.81% responded to CS at its first presentation. We discarded them from the analyses. We also discarded six control bumblebees that tested positive to *N. ceranae* in the PCR (not shown in the table). In the reversal phase five bumblebees did not fit the selection criteria (i.e. PER to A- in the first trials with this odour during the reversal phase) and were not taken into account for the analyses. 8.82% of bumblebees died during this phase.

Test	Selection criteria	Control	NE+	NE-
Differential learning	Final sample size	49	21	32
	Discarded	16	14[a]	-
	Died	5	9	
	Unmotivated	1	0	
	Response to odour at first presentation	4	5	
Reversal learning	Final sample size	37	21	29
	Discarded	12	0	3
	Died	7	0	2
	Failed selection criteria	5	0	1

[a] All exposed bees (NE- and NE+) as the final status of bumblebees was confirmed in bumblebees that finished the tests.