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

Tamara Gómez-Moracho[1,*,a], Tristan Durand[1,*], Mathieu Lihoreau[1]

[1] Research Center on Animal Cognition (CRCA), Center for Integrative Biology (CBI); CNRS, University Paul Sabatier, Toulouse, France

* These authors contributed equally to this work

[a] Author for correspondence: Tamara Gómez-Moracho (tamara.gomez@univ-tlse3.fr)

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 parasite 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 performance in absolute and differential learning, and were slower to solve a reversal learning task than controls. Long-term memory was also slightly reduced. The consistent effect of *N. ceranae* exposure across different types of olfactory learning indicates that its action was not specific to 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

1. Introduction

Pollinators, such as bees, rely on a rich cognitive repertoire to collect pollen and nectar on flowers and feed their brood. These include associative learning and memories of floral traits like odours, shapes, colours, and textures, to identify 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 microsporidia *Nosema ceranae* is one of the most prevalent parasites of bees worldwide with a large range of hosts including honey bees (Fries et al., 1996; Higes et al., 2006), bumblebees (Figueroa et al., 2021; Plischuk et al., 2009), solitary bees (Figueroa et al., 2021; 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). Once in the gut, the spores invade the epithelial cells of the bee where they develop by exploiting its metabolism (Holt et al., 2013). *Nosema* degenerates the gut epithelium of the host (Dussaubat et al., 2012; Higes et al., 2007), alters its metabolism (Martín-Hernández et al., 2011; Mayack and Naug, 2009) and disrupts the immune response (Antúnez et al., 2009), thereby extending its infection time inside the host. In honey bees, this causes a disease (nosemosis) believed to contribute to colony collapse disorder (Cox-Foster et al., 2007; Higes et al., 2009).

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

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

Given the expanding geographical distribution of *N. ceranae* worldwide (Khezri et al., 2018; Klee et al., 2007; Ostroverkhova et al., 2020) and its prevalence in domesticated and wild bees (Plischuk et al., 2009; Porrini et al., 2017; Ravoet et al., 2014), clarifying its influence on host cognition has become an urgent matter. In particular, other critical forms of learning required for efficient foraging, 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 to discriminate flowers. They involve different brain centers and may thus be more or less impacted by the parasite if its effect is specific (Devaud et al., 2015; Giurfa, 2007; Mertes et al., 2021).

Here we build on a recently established method yielding high the 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.

2. Material and methods

2.1. Bumblebee colonies

We used bumblebees (*B. terrestris*) from 14 commercial colonies acquired from Biobest (Westerlo, Belgium). Before the experiments, we randomly sampled 15 bumblebees from each colony and verified the absence of *N. ceranae* (primer: 218MITOC; Martín-Hernández *et al.*, 2007), as well as other common parasites of bumblebees (*N. bombi* (primer : Nbombi-SSU-J; Klee *et al.*, 2006) and *Crithidia bombi* (primer: CB-ITS1; Schmid-Hempel and Tognazzo, 2010)) in a polymerase chain reaction (PCR). 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; Zheng *et al.*, 2014), in a room at 25±1°C under a 12 h light:12 h dark photocycle.

2.2. *N. ceranae* spores

We obtained fresh *N. ceranae* spores from naturally infected honey bee colonies (*Apis mellifera*) maintained at our experimental apiary (University Paul Sabatier - 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. Once 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), we purified the homogenates 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 step was repeated up to three times to obtain a spore solution of 85% purity (Fries et al., 2013). We then 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.

2.3. 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 placed inside each Petri dish a 20 µL drop of the previous inoculum containing 300,000 *N.*

ceranae spores (e.g. exposed bumblebees). Control bumblebees received 20 µL of sucrose solution (20% w/w). To control that all bumblebees received the same parasite dose, 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, in a plastic box (24 x 18 x 9.6 cm) with a dark compartment, simulating the nest of the colony, and a clear compartment containing a gravity feeder with *ad libitum* access to food (Kraus et al., 2019). Since diet can affect host-parasites relationships (Frost et al., 2008) we provided bumblebees an artificial diet with a protein to carbohydrates ratio of 1:207, that was previously showed 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) and 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.

2.4. Behavioural experiments

We tested the cognitive performances of bumblebees using PER at day 7 after parasite exposure, following the protocol by Toda et al., (2009). The day before the behavioural tests we kept diet to low levels (~200 µL/bumblebee) to keep bumblebees motivated for PER experiments. Three hours before the behavioural tests, we collected 20 bumblebees (10 control, 10 exposed) from each microcolony, 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 (i.e. capsule length was adjusted for each bumblebee; Figure 1A). Capsules had two drilled holes, one at the front side and another at the bottom side (adapted from; Toda *et al.*, 2009). We maintained the bumblebees in a horizontal position during experiments so that the front hole allowed the experimenter to easily reach bumblebees' antennae during conditioning, while the bottom hole permitted the air flow avoiding odours to accumulate inside the tube. Bumblebees could slightly move inside the tube. 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). Once in the capsule, we kept the bumblebees in the dark, in an incubator at 28ºC, with no access to food. All bumblebees that finished the conditioning protocols were frozen and kept at -20°C for later analyses of their infection status through PCR.

2.5. Sucrose responsiveness

We tested the sucrose responsiveness of bumblebees to control for potential influences of *N. ceranae* on their reward perception or feeding motivation. During the sucrose sensitivity test, 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 of sucrose, 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 scored a positive response if bumblebees extended their proboscis after a solution presentation. We discarded bumblebees responding to water (i.e. 0% sucrose solution) to avoid the effect of thirst on sucrose responsiveness (Baracchi et al., 2018). The sum of the number of PER responses divided by the number of concentration tested formed the individual gustatory score (Scheiner et al., 2013).

2.6. Conditioning experiments

All conditioning experiments shared the same general protocol (Supplementary Figure S1). An encapsulated bumblebee (Figure 1A) was placed 1.5 cm ahead of the odour delivery setup (described in (Aguiar et al., 2018)) that delivered a continuous stream of odourless air at 1.2 ml/s. An automated odour-releasing machine controlled by a microcomputer (Arduino® Uno) allowed to selectively add vapour from the vials containing the odorants used as conditioned stimulus (CS) (Raiser et al., 2017). We used two odorants as CS: nonanal and phenylacetaldehyde (Palottini et al., 2018), in a 1:100 dilution in mineral oil (Sommerlandt et al., 2014). Before conditioning, we tested the responsiveness of bees 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 (Supplementary Figure S1c) consisted in 15 seconds of odourless airflow (i.e. familiarization), 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) for 2 seconds. 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 (Supplementary Figure S1c). Bumblebees that responded to the odour in the first conditioning trial were discarded from the analyses. We used conditioned responses to calculate three individual scores for each bumblebee, describing its performance during conditioning (i.e. acquisition score), at the end of conditioning (i.e. learning score) or during memory retrieval (i.e. memory score) (Monchanin et al., 2020; see details below). In all experiments, exposed and control bumblebees were conditioned in parallel (i.e. the same day).

2.6.1. 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 only requires peripheral brain centers, i.e. the antennal lobes (Giurfa, 2007; Mertes et al., 2021). We trained bumblebees in a spaced 3-trial absolute conditioning learning (Supplementary Figure S1a), with an intertrial interval of 10 minutes, 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. Only bumblebees that responded to the US in the three trials (i.e. efficient bumblebees) were kept for the analyses (Supplementary table S2). Each of these bees received an acquisition score, calculated as the

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

We tested memory retention in efficient bumblebees that made at least one conditioned response in either one of the last two trials. 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. For tests performed after 24 h, bumblebees were fed until satiation with 50% (w/w) sucrose solution right after conditioning. In bees, STM is independent of protein synthesis whereas LTM requires protein synthesis (Menzel, 1999). Studying performances of bumblebees for these two types of memories was thus a mean to explore whether exposure to *N. ceranae* interfered with protein synthesis. During the test, 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 (Villar et al., 2020). For example, when nonanal was used as CS+, phenylacetaldehyde was used as a NOd in the memory retention test, and *vice versa*. Memory formation was considered when bumblebees extended their proboscis only in response to the CS+ odour but not to the NOd, and thus bumblebees received a memory score of 1 (score was 0, otherwise). 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 US after the memory test were discarded for the analyses (i.e. 14.06% in LTM; Supplementary Table S2).

2.6.2. 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 a first phase of differential conditioning that requires the antennal lobes but is not dependant on central brain centers, i.e. mushroom bodies (Devaud et al., 2015), and a second phase of reversal learning that requires both the antennal lobes and the mushroom bodies (Boitard et al., 2015). In the differential learning phase (phase 1), we trained bumblebees to discriminate between two odours. This phase consisted in 10-trial blocks (Supplementary Figure S1b), 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 the reversal learning phase (phase 2) we trained bumblebees to invert the first learnt contingency. The reversal phase started 1h after the end of the differential phase. Here we trained bumblebees in 12 trial-blocks, six 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 with the same level of learning, we analysed reversal learning in bumblebees that responded to A- either in the first and/or second presentation of this odour during the reversal phase (i.e. selection criteria; Supplementary table S3).

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

2.7. Infection status

At the end of the behavioural experiments, we assessed the infection status of every bumblebee in a PCR (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. We checked *N. ceranae* in a monoplex PCR using the primers 218MITOC ((Martín-Hernández et al., 2007). 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 48-well microtitre plates in a S1000™ Thermal Cycler (Biorad, CA). Thermal conditions were a first step of 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 elongation 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, i.e. control, exposed negative or exposed positive. Bumblebees that were not exposed to the parasite but nevertheless showed a positive result in PCR were excluded from the analyses (i.e. 8.21%; 23 out of 280 control bumblebees; Supplementary Tables S1, S2 and S3).

2.8. Statistical analyses

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

3. Results

3.1. Parasite exposure did not influence sucrose responsiveness

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

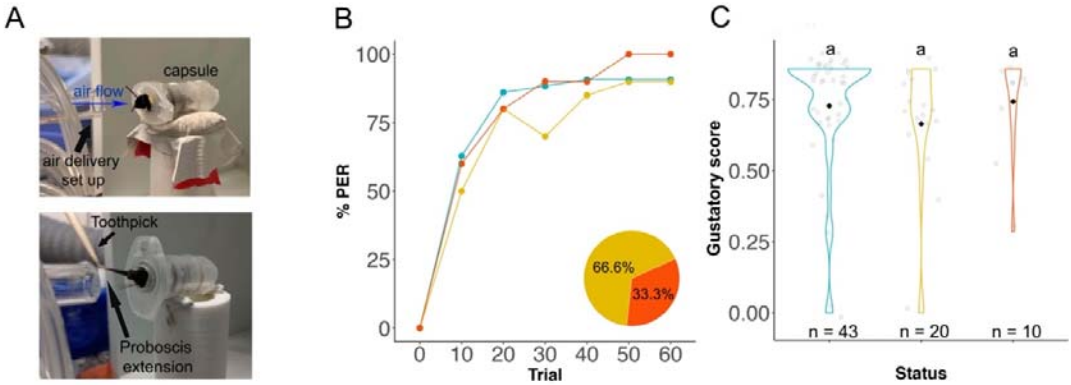


Figure 1. Odour delivery set up and sucrose responsiveness. A) Photo of a bumblebee during odour conditioning. The bumblebee is placed inside the capsule in front of the air delivery set up. After the odour is delivered, sucrose is presented with a toothpick to the bumblebees which extends its proboscis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

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

3.1. Parasite exposure reduced absolute learning but not memory

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

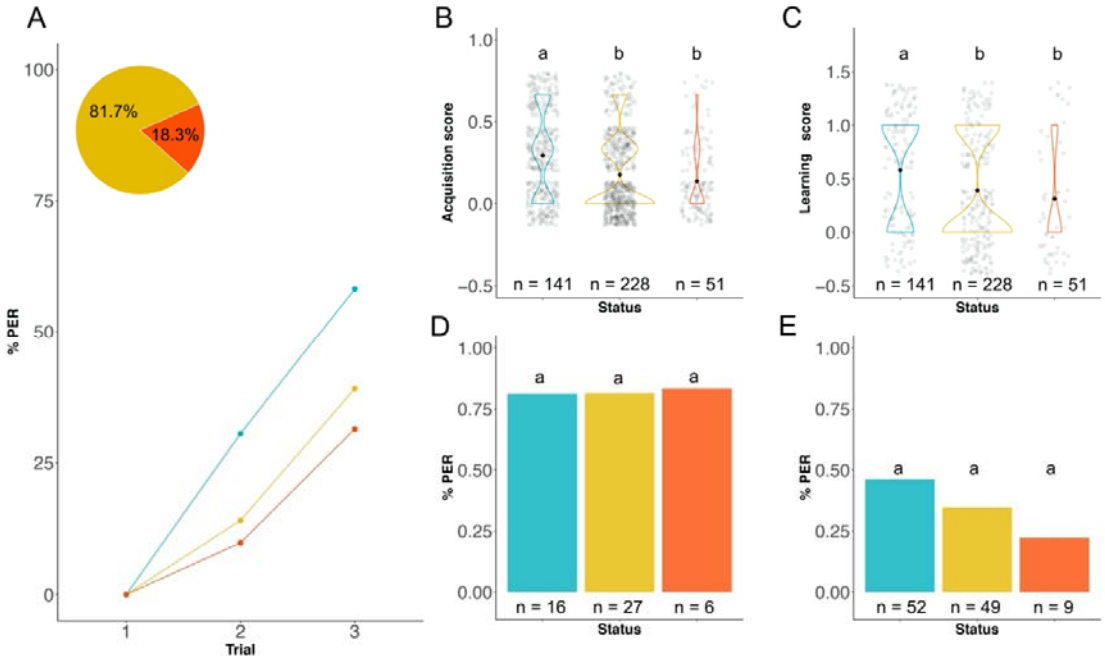


Figure 2. Absolute learning and memory. A) Learning curves show the proportion of control (blue), exposed negative (yellow) and exposed positive (red) bumblebees extending their proboscis to the odour during conditioning. Pie chart shows the percentage of negative and positive exposed bumblebees that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

Table 1. Tukey Pairwise comparisons for absolute and reversal learning. Significant 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

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

3.2. Parasite exposure reduced differential and reversal learning

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

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

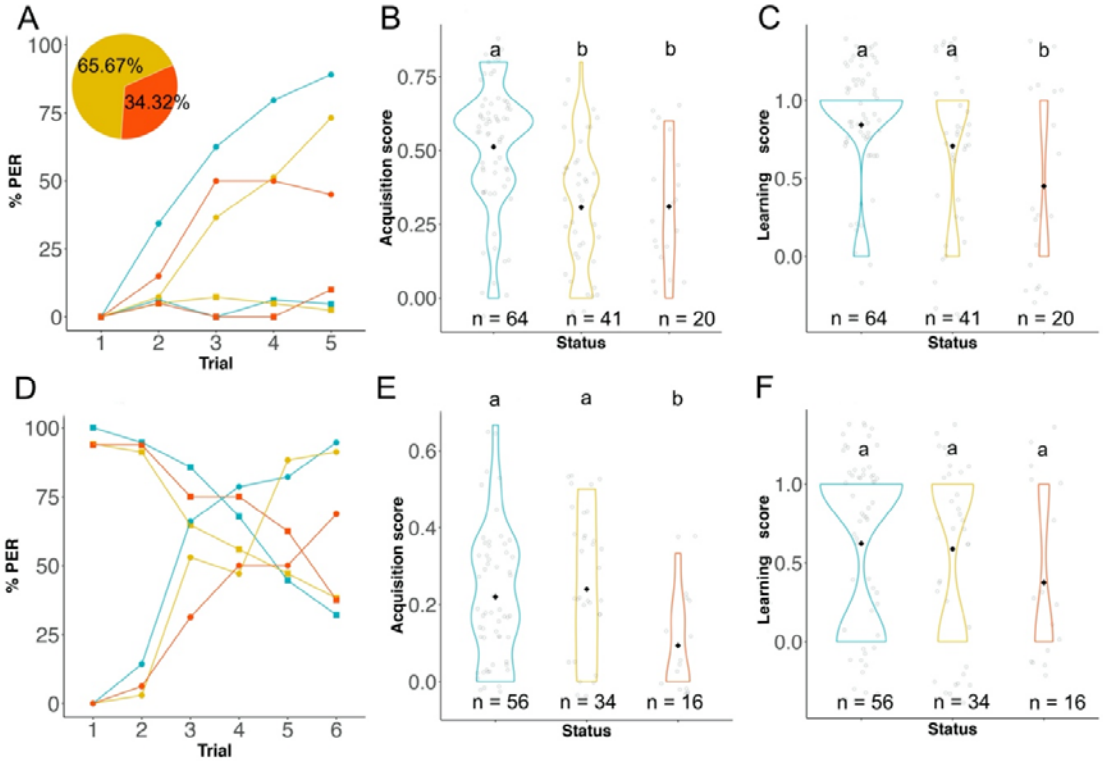


Figure 3. Reversal learning. **A-C)** Differential learning phase. **A)** Percentage of PER responses to rewarded (A+, circle) and unrewarded (B-, square) odours by control (blue), exposed negative (yellow) and exposed positive (red) bumblebees. Pie chart shows the percentage of exposed negative and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

The second phase of reversal learning is the proper reversal task in which the bumblebee must learn to respond to the previously unrewarded odour (now B+) and stop responding to the previously rewarded odour (now A-) (Figure 3D-F). This form of learning involves the antennal lobes but is also dependent on the mushroom bodies (Boitard et al., 2015). We analysed reversal learning of 85.82% of bumblebees that finished the differential phase, as they responded to A- in any of the two first presentations of this odour during reversal phase (59 controls, 37 exposed negative, 19 exposed positive; see details in Supplementary Table S3). All bumblebees reduced their response to A- in favour of B+ over trials (Figure 3D). However, bumblebees with different infection statuses reached significantly different acquisition scores (Figure 3E; GLMM, status, X^2 = 6.042, df = 2, p = 0.048). Exposed positive bumblebees showed a slightly lower acquisition score than controls (Tukey test: p = 0.049; Table 1) and exposed negative bumblebees (Tukey test: p = 0.056, Table 1), suggesting they had a lower ability to reverse the task. By the end of training, bumblebees of all infection statuses reversed the task, as shown by the lack of difference between their learning scores (Binomial GLMM, status: X^2 = 0.641, df = 2, p = 0.725). Note however that just one trial before the end of training (trial 5), bumblebees with different infection statuses differed in their learning scores (Binomial GLMM, status; X^2 = 8.940, df = 2, p = 0.011). At trial 5, exposed positive bumblebees were unable to accomplish the task correctly compared to controls (Tukey test; z = -2.505, p = 0.032) and exposed negative bumblebees (Tukey test; z = -2.759, p = 0.015). Thus overall, exposure to *N. ceranae* also impaired the reversal phase of reversal learning. Interestingly, 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 longer time to be established (see Supplementary Text S1).

4. Discussion

Bees are exposed to a number of parasites that can affect cognitive abilities involved in crucial behaviour (Koch et al., 2017; Schmid-Hempel, 2013). Previous studies exploring the effect of the widespread parasite *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 et al., 2016; Piiroinen and Goulson, 2016). Here, we ran a suite of standard olfactory cognitive assays showing that feeding bumblebees spores of this parasite consistently impairs different types of olfactory learning seven days after exposure. This suggests that the influence of *N. ceranae* exposure on bumblebee cognition is not specific to a particular brain area.

Exposure to *N. ceranae* in food clearly 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 solve to efficiently forage on flowers (Giurfa, 2013). This result agrees with a previous study reporting a slightly reduced absolute learning in bumblebees exposed to *N. ceranae* (Piiroinen and Goulson, 2016). Here we provide evidence that these reduced cognitive performances were not due to an effect of the parasite on odour perception or motivation to feed, and give insight on where the parasite might act in the nervous system. In bees, sucrose perception through the antennae, olfactory absolute learning, differential learning and reversal learning all require processing of olfactory information through the antennal lobes (de Brito Sanchez, 2011; Giurfa, 2007; Mertes et al., 2021) while reversal learning also requires the mushroom bodies (Devaud et al., 2015). The fact that all types of learning were impaired and that sucrose sensitivity was not, indicates 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 olfactory memory. 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* impaired neither short-term nor long-term memory. Our protocol imposed that only efficient bumblebees (i.e. that responded to sucrose in the three conditioning trials) with at least one conditioned response would be selected for the memory test, as bumblebees only had three trials to learn the stimulus-reward association. Nearly all bees exposed to *Nosema* (either positive or negative in PCR) remembered the

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

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 in a prevalence that usually ranges from 4.76% to 21% (Figueroa et al., 2021; Graystock et al., 2013; Li et al., 2012; Sinpoo, 2018), or even higher (i.e. 72%; Arbulo et al., 2015). Whether or not bumblebees are suitable hosts for *N. ceranae* replication is confirmed in future experiments, our results imply that bumblebees are strongly 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 (Klee et al., 2007; 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 21.38% in comparison to previous studies (Piiroinen et al., 2016; Piiroinen and Goulson, 2016), which allowed the comparison 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 barely discriminated odours. These bees were also slower at inverting odour associations than negative exposed individuals. Even if bumblebees in all statuses developed a novel odour association by the end of the experiment, positive exposed bumblebees acquired this ability only in the last trial, contrary to the other two groups, suggesting that infection may further interfere with cognition, however further experiments are needed to tackle this specific question.

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 by increasing oxidative responses (Kurze et al., 2016) or modulating the expression of antimicrobial peptides (Antúnez et al., 2009; Botías et al., 2020; Sinpoo, 2018). Stimulation of

the immune system of bees 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 (Alghamdi et al., 2008; Mobley and Gegear, 2018). It is thus possible that the observed effects on cognition in bumblebees exposed to *N. ceranae* were caused by an activation of the immune response. Infection with *N. ceranae* was shown to downregulate the expression of genes in the honey bee brain (Doublet et al., 2016; McDonnell et al., 2013), some of them linked to sensory, as odorant binding proteins (Badaoui et al., 2017) and chemosensory proteins (CSP1; (Doublet et al., 2016)). *N. ceranae* also interferes with the metabolism of serotonin by, for example, inhibiting the expression of the dopa descarboxylase encoding gene (Aufauvre et al., 2014), a biogenic amine that plays a crucial role in memory formation in Drosophila (Sitaraman et al., 2008). Modulation of gene expression in the brain may lead to changes in behaviour and cognition. Interestingly, we found no effect of parasite exposure on cognition at two days post exposure (Supplementary Text S1). Whether *N. ceranae* exerts an effect on the immune response of honey bees at this time is unknown, as most studies tested this response later than three days after parasite exposure (Antúnez et al., 2009; Chaimanee et al., 2012; Sinpoo, 2018). In bumblebees the immune response is activated just 1 h after feeding *Crithidia bombi* (Riddell et al., 2011). However, overexpression of aminopeptides is observed 12 h after infection (Riddell et al., 2011) and, although it varies among aminopeptides, it can last even for 10 days when it is triggered by LPS injection (Mobley and Gegear, 2018). Then, it is possible that at two days post exposure the stress generated by the parasite may have not affected yet the nervous system.

Since olfactory learning is essential for bumblebees to forage, this sublethal effect of *N. ceranae* exposure on individual cognition can impact the collective performance and compromise colony development. Commercial and wild bumblebee colonies exhibit physiological and behavioural differences as a result of different selective pressures (Velthuis and Doorn, 2006). They may show different responses to parasites. Parasite loads in the field can range from a few spores to several thousands, as some honey bees can harbour even 10 thousands of *Nosema* 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 nature, estimated in 6800 spores per individual (Graystock et al., 2013). Feeding this quantity of spores infected 62% of bumblebees which neither suffer a reduction of their gustatory response (Graystock et al., 2013), but no further effects in cognition were analysed. Further studies are thus needed to analyse the effects of *N.* ceranae, and their possible interactions with other stressors, in the field in order to understand their real impact on colony dynamics.

5. Acknowledgements

We thank Philipp Heeb, Cristian Pasquaretta and Maria Eugenia Villar for constructive discussions. We thank Charline Schartier who helped running some experiments during an internship. We are also grateful to Martin Giurfa for lending the automated PER conditioning setup.

6. Funding

TGM was funded by a postdoctoral grant of the Fyssen foundation. While writing, TGM, TD and ML were funded by the CNRS and grants of the Agence Nationale de la Recherche (project POLLINET ANR-16-CE02-0002-01, project 3DNaviBee ANR-19-CE37-0024, project BEE-MOVE ANR-20-ERC8-0004-01), the European Regional Development Fund (project ECONECT), and the Agence de la Transition Ecologique (project LOTAPIS) to ML.

7. References

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

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

Antúnez, K., Martín-Hernández, R., Prieto, L., Meana, A., Zunino, P., Higes, M., 2009. Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (Microsporidia). Environ. Microbiol. 11, 2284–2290. https://doi.org/10.1111/j.1462-2920.2009.01953.x

Arbulo, N., Antúnez, K., Salvarrey, S., Santos, E., Branchiccela, B., Martín-Hernández, R., Higes, M., Invernizzi, C., 2015. High prevalence and infection levels of *Nosema ceranae* in bumblebees *Bombus atratus* and *Bombus bellicosus* from Uruguay. J. Invertebr. Pathol. 130, 165–168. https://doi.org/10.1016/j.jip.2015.07.018

Aufauvre, J., Misme-Aucouturier, B., Viguès, B., Texier, C., Delbac, F., Blot, N., 2014. Transcriptome analyses of the honeybee response to *Nosema ceranae* and insecticides. PLoS ONE 9, e91686. https://doi.org/10.1371/journal.pone.0091686

Badaoui, B., Fougeroux, A., Petit, F., Anselmo, A., Gorni, C., Cucurachi, M., Cersini, A., Granato, A., Cardeti, G., Formato, G., Mutinelli, F., Giuffra, E., Williams, J.L., Botti, S., 2017. RNA-sequence analysis of gene expression from honeybees (*Apis mellifera*) infected with *Nosema ceranae*. PLOS ONE 12, e0173438. https://doi.org/10.1371/journal.pone.0173438

Baracchi, D., Rigosi, E., de Brito Sanchez, G., Giurfa, M., 2018. Lateralization of sucrose responsiveness and non-associative learning in honeybees. Front. Psychol. 9, 425. https://doi.org/10.3389/fpsyg.2018.00425

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects models using **lme4**. J. Stat. Softw. 67. https://doi.org/10.18637/jss.v067.i01

Bell, H.C., Montgomery, C.N., Benavides, J.E., Nieh, J.C., 2020. Effects of *Nosema ceranae* (Dissociodihaplophasida: Nosematidae) and flupyradifurone on olfactory learning in honey bees, *Apis mellifera* (Hymenoptera: Apidae). J. Insect Sci. 20. https://doi.org/10.1093/jisesa/ieaa130

Bitterman, M.E., Menzel, R., Fietz, A., Schäfer, S., 1983. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). J. Comp. Psychol. 97, 107–119. https://doi.org/10.1037/0735-7036.97.2.107

Boitard, C., Devaud, J.-M., Isabel, G., Giurfa, M., 2015. GABAergic feedback signaling into the calyces of the mushroom bodies enables olfactory reversal learning in honey bees. Front. Behav. Neurosci. 9, 1–13. https://doi.org/10.3389/fnbeh.2015.00198

Botías, C., Jones, J.C., Pamminger, T., Bartomeus, I., Hughes, W.O.H., Goulson, D., 2020. Multiple stressors interact to impair the performance of bumblebee (*Bombus terrestris*) colonies. J. Anim. Ecol. 1365-2656.13375. https://doi.org/10.1111/1365-2656.13375

Brooks, M., E., Kristensen, K., Benthem, K., J. ,van, Magnusson, A., Berg, C., W., Nielsen, A., Skaug, H., J., Mächler, M., Bolker, B., M., 2017. glmmtmb balances speed and flexibility among packages for zero-inflated Generalized Linear Mixed Modeling. R J. 9, 378. https://doi.org/10.32614/RJ-2017-066

Cantwell, G., 1970. Standard methods for counting *Nosema* spores. Am. Bee J. 110, 222–223.

Chaimanee, V., Chantawannakul, P., Chen, Y., Evans, J.D., Pettis, J.S., 2012. Differential expression of immune genes of adult honey bee (*Apis mellifera*) after inoculated by *Nosema ceranae*. J. Insect Physiol. 58, 1090–1095. https://doi.org/10.1016/j.jinsphys.2012.04.016

Charbonneau, L.R., Hillier, N.K., Rogers, R.E.L., Williams, G.R., Shutler, D., 2016. Effects of *Nosema apis*, *N. ceranae*, and coinfections on honey bee (*Apis mellifera*) learning and memory. Sci. Rep. 6, 22626. https://doi.org/10.1038/srep22626

Collett, M., Chittka, L., Collett, T.S., 2013. Spatial memory in insect navigation. Curr. Biol. 23, R789–R800. https://doi.org/10.1016/j.cub.2013.07.020

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., Quan, P.-L., Briese, T., Hornig, M., Geiser, D.M., Martinson, V., vanEngelsdorp, D., Kalkstein, A.L., Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchison, S.K., Simons, J.F., Egholm, M., Pettis, J.S., Lipkin, W.I., 2007. A metagenomic survey of microbes in honey bee Colony Collapse Disorder. Science 318, 283–287. https://doi.org/10.1126/science.1146498

de Brito Sanchez, M.G., 2011. Taste perception in honey bees. Chem. Senses 36, 675–692. https://doi.org/10.1093/chemse/bjr040

Devaud, J.-M., Papouin, T., Carcaud, J., Sandoz, J.-C., Grünewald, B., Giurfa, M., 2015. Neural substrate for higher-order learning in an insect: Mushroom bodies are necessary for configural discriminations. Proc. Natl. Acad. Sci. 112, E5854–E5862. https://doi.org/10.1073/pnas.1508422112

Doublet, V., Paxton, R.J., McDonnell, C.M., Dubois, E., Nidelet, S., Moritz, R.F.A., Alaux, C., Le Conte, Y., 2016. Brain transcriptomes of honey bees (*Apis mellifera*) experimentally infected by two pathogens: Black queen cell virus and *Nosema ceranae*. Genomics Data 10, 79–82. https://doi.org/10.1016/j.gdata.2016.09.010

Dussaubat, C., Brunet, J.-L., Higes, M., Colbourne, J.K., Lopez, J., Choi, J.-H., Martín-Hernández, R., Botías, C., Cousin, M., McDonnell, C., Bonnet, M., Belzunces, L.P., Moritz, R.F.A., Le Conte, Y., Alaux, C., 2012. Gut pathology and responses to the Microsporidium *Nosema ceranae* in the honey bee *Apis mellifera*. PLoS ONE 7, e37017. https://doi.org/10.1371/journal.pone.0037017

Dussaubat, C., Maisonnasse, A., Crauser, D., Beslay, D., Costagliola, G., Soubeyrand, S., Kretzchmar, A., Le Conte, Y., 2013. Flight behavior and pheromone changes associated to *Nosema ceranae* infection of honey bee workers (*Apis mellifera*) in field conditions. J. Invertebr. Pathol. 113, 42–51. https://doi.org/10.1016/j.jip.2013.01.002

Figueroa, L.L., Compton, S., Grab, H., McArt, S.H., 2021. Functional traits linked to pathogen prevalence in wild bee communities. Sci. Rep. 11, 7529. https://doi.org/10.1038/s41598-021-87103-3

Fries, I., Chauzat, M.-P., Chen, Y.-P., Doublet, V., Genersch, E., Gisder, S., Higes, M., McMahon, D.P., Martín-Hernández, R., Natsopoulou, M., Paxton, R.J., Tanner, G., Webster, T.C., Williams, G.R., 2013. Standard methods for *Nosema* research. J. Apic. Res. 52, 1–28. https://doi.org/10.3896/IBRA.1.52.1.14

Fries, I., Feng, F., da Silva, A., Slemenda, S.B., Pieniazek, N.J., 1996. *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). Eur. J. Protistol. 32, 356–365. https://doi.org/10.1016/S0932-4739(96)80059-9

Frost, P.C., Ebert, D., Smith, V.H., 2008. Bacterial infection changes the elemental composition of *Daphnia magna*. J. Anim. Ecol. 77, 1265–1272. https://doi.org/10.1111/j.1365-2656.2008.01438.x

Gage, S.L., Kramer, C., Calle, S., Carroll, M., Heien, M., DeGrandi-Hoffman, G., 2018. *Nosema ceranae* parasitism impacts olfactory learning and memory and neurochemistry in honey bees (*Apis mellifera*). J. Exp. Biol. 221, jeb161489. https://doi.org/10.1242/jeb.161489

Gisder, S., Horchler, L., Pieper, F., Schüler, V., Šima, P., Genersch, E., 2020. Rapid gastrointestinal passage may protect *Bombus terrestris* from becoming a true host for *Nosema ceranae*. Appl. Environ. Microbiol. 86. https://doi.org/10.1128/AEM.00629-20

Giurfa, M., 2015. Learning and cognition in insects: learning and insect cognition. Wiley Interdiscip. Rev. Cogn. Sci. 6, 383–395. https://doi.org/10.1002/wcs.1348

Giurfa, M., 2013. Cognition with few neurons: higher-order learning in insects. Trends Neurosci. 36, 285–294. https://doi.org/10.1016/j.tins.2012.12.011

Giurfa, M., 2007. Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. J. Comp. Physiol. A 193, 801–824. https://doi.org/10.1007/s00359-007-0235-9

Giurfa, M., Sandoz, J.-C., 2012. Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. Learn. Mem. 19, 54–66. https://doi.org/10.1101/lm.024711.111

Gómez-Moracho, T., Durand, T., Pasquaretta, C., Heeb, P., Lihoreau, M., 2021. Artificial diets modulate infection rates by *Nosema ceranae* in bumblebees. Microorganisms 9, 158. https://doi.org/10.3390/microorganisms9010158

Gómez-Moracho, T., Heeb, P., Lihoreau, M., 2017. Effects of parasites and pathogens on bee cognition: Bee parasites, pathogens and cognition. Ecol. Entomol. 42, 51–64. https://doi.org/10.1111/een.12434

Graystock, P., Goulson, D., Hughes, W., 2015. Parasites in bloom: flowers aid dispersal and

transmission of pollinator parasites within and between bee species. Proc. R. Soc. B Biol. Sci. 282. https://doi.org/10.1098/rspb.2015.1371

Graystock, P., Yates, K., Darvill, B., Goulson, D., Hughes, W.O.H., 2013. Emerging dangers: Deadly effects of an emergent parasite in a new pollinator host. J. Invertebr. Pathol. 114, 114–119. https://doi.org/10.1016/j.jip.2013.06.005

Higes, M., García-Palencia, P., Martín-Hernández, R., Meana, A., 2007. Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). J. Invertebr. Pathol. 94, 211–217. https://doi.org/10.1016/j.jip.2006.11.001

Higes, M., Martín, R., Meana, A., 2006. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. J. Invertebr. Pathol. 92, 93–95. https://doi.org/10.1016/j.jip.2006.02.005

Higes, M., Martín-Hernández, R., Botías, C., Bailón, E.G., González-Porto, A.V., Barrios, L., del Nozal, M.J., Bernal, J.L., Jiménez, J.J., Palencia, P.G., Meana, A., 2008. How natural infection by *Nosema ceranae* causes honeybee colony collapse. Environ. Microbiol. 10, 2659–2669. https://doi.org/10.1111/j.1462-2920.2008.01687.x

Higes, M., Martín-Hernández, R., Garrido-Bailón, E., González-Porto, A.V., García-Palencia, P., Meana, A., del Nozal, M.J., Mayo, R., Bernal, J.L., 2009. Honeybee colony collapse due to *Nosema ceranae* in professional apiaries. Environ. Microbiol. Rep. 1, 110–113. https://doi.org/10.1111/j.1758-2229.2009.00014.x

Holt, H.L., Aronstein, K.A., Grozinger, C.M., 2013. Chronic parasitization by *Nosema* microsporidia causes global expression changes in core nutritional, metabolic and behavioral pathways in honey bee workers (*Apis mellifera*). BMC Genomics 14, 799. https://doi.org/10.1186/1471-2164-14-799

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. Biom. Z. 50, 346–363. https://doi.org/10.1002/bimj.200810425

Khezri, M., Moharrami, M., Modirrousta, H., Torkaman, M., Salehi, S., Rokhzad, B., Khanbabai, H., 2018. Molecular detection of *Nosema ceranae* in the apiaries of Kurdistan province, Iran. Vet. Res. Forum Int. Q. J. 9, 273–278.

https://doi.org/10.30466/vrf.2018.32086

Klee, J., Besana, A.M., Genersch, E., Gisder, S., Nanetti, A., Tam, D.Q., Chinh, T.X., Puerta, F., Ruz, J.M., Kryger, P., Message, D., Hatjina, F., Korpela, S., Fries, I., Paxton, R.J., 2007. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. J. Invertebr. Pathol. 96, 1–10. https://doi.org/10.1016/j.jip.2007.02.014

Klee, J., Tek Tay, W., Paxton, R.J., 2006. Specific and sensitive detection of *Nosema bombi* (Microsporidia: Nosematidae) in bumble bees (*Bombus spp.*; Hymenoptera: Apidae) by PCR of partial rRNA gene sequences. J. Invertebr. Pathol. 91, 98–104. https://doi.org/10.1016/j.jip.2005.10.012

Klein, S., Cabirol, A., Devaud, J.-M., Barron, A.B., Lihoreau, M., 2017. Why bees are so vulnerable to environmental stressors. Trends Ecol. Evol. 32, 268–278. https://doi.org/10.1016/j.tree.2016.12.009

Koch, H., Brown, M.J., Stevenson, P.C., 2017. The role of disease in bee foraging ecology. Curr. Opin. Insect Sci. 21, 60–67. https://doi.org/10.1016/j.cois.2017.05.008

Kraus, S., Gómez-Moracho, T., Pasquaretta, C., Latil, G., Dussutour, A., Lihoreau, M., 2019. Bumblebees adjust protein and lipid collection rules to the presence of brood. Curr. Zool. 65, 437–446. https://doi.org/10.1093/cz/zoz026

Kurze, C., Dosselli, R., Grassl, J., Le Conte, Y., Kryger, P., Baer, B., Moritz, R.F.A., 2016. Differential proteomics reveals novel insights into *Nosema*–honey bee interactions. Insect Biochem. Mol. Biol. 79, 42–49. https://doi.org/10.1016/j.ibmb.2016.10.005

Li, J., Chen, W., Wu, J., Peng, W., An, J., Schmid-Hempel, P., Schmid-Hempel, R., 2012. Diversity of *Nosema* associated with bumblebees (*Bombus spp.*) from China. Int. J. Parasitol. 42, 49–61. https://doi.org/10.1016/j.ijpara.2011.10.005

Mallon, E.B., Brockmann, A., Schmid-Hempel, P., 2003. Immune response inhibits associative learning in insects. Proc. R. Soc. Lond. B Biol. Sci. 270, 2471–2473. https://doi.org/10.1098/rspb.2003.2456

Martín-Hernández, R., Botías, C., Barrios, L., Martínez-Salvador, A., Meana, A., Mayack, C., Higes, M., 2011. Comparison of the energetic stress associated with experimental *Nosema ceranae* and *Nosema apis* infection of honeybees (*Apis mellifera*). Parasitol. Res. 109, 605–612. https://doi.org/10.1007/s00436-011-2292-9

Martín-Hernández, R., Meana, A., Prieto, L., Salvador, A.M., Garrido-Bailon, E., Higes, M., 2007. Outcome of Colonization of *Apis mellifera* by *Nosema ceranae*. Appl. Environ. Microbiol. 73, 6331–6338. https://doi.org/10.1128/AEM.00270-07

Mayack, C., Naug, D., 2009. Energetic stress in the honeybee *Apis mellifera* from *Nosema ceranae* infection. J. Invertebr. Pathol. 100, 185–188. https://doi.org/10.1016/j.jip.2008.12.001

McDonnell, C.M., Alaux, C., Parrinello, H., Desvignes, J.-P., Crauser, D., Durbesson, E., Beslay, D., Le Conte, Y., 2013. Ecto- and endoparasite induce similar chemical and brain neurogenomic responses in the honey bee (*Apis mellifera*). BMC Ecol. 13, 25. https://doi.org/10.1186/1472-6785-13-25

Meana, A., Martín-Hernández, R., Higes, M., 2010. The reliability of spore counts to diagnose *Nosema ceranae* infections in honey bees. J. Apic. Res. 49, 212–214. https://doi.org/10.3896/IBRA.1.49.2.12

Menzel, R., 2012. The honeybee as a model for understanding the basis of cognition. Nat. Rev. Neurosci. 13, 758–768. https://doi.org/10.1038/nrn3357

Menzel, R., 2001. Searching for the memory trace in a mini-brain, the honeybee. Learn. Mem. 8, 53–62. https://doi.org/10.1101/lm.38801

Menzel, R., 1999. Memory dynamics in the honeybee. J. Comp. Physiol. [A] 185, 323–340. https://doi.org/10.1007/s003590050392

Menzel, R., Muller, U., 1996. Learning and memory in honeybees: from behavior to neural substrates. Annu. Rev. Neurosci. 19, 379–404. https://doi.org/10.1146/annurev.ne.19.030196.002115

Menzel, Randolf, Manz, G., Menzel, Rebecca, Greggers, U., 2001. Massed and spaced learning in honeybees: the role of cs, us, the intertrial interval, and the test interval. Learn. Mem. 8, 198–208. https://doi.org/10.1101/lm.40001

Mertes, M., Carcaud, J., Sandoz, J.-C., 2021. Olfactory coding in the bumble bee antennal lobe. bioRxiv 2020.12.31.424929. https://doi.org/10.1101/2020.12.31.424929

Mobley, M.W., Gegear, R.J., 2018. Immune-cognitive system connectivity reduces bumblebee foraging success in complex multisensory floral environments. Sci. Rep. 8, 5953. https://doi.org/10.1038/s41598-018-24372-5

Monchanin, C., Drujont, E., Devaud, J.-M., Lihoreau, M., Barron, A.B., 2020. Heavy metal pollutants have additive negative effects on honey bee cognition. bioRxiv 2020.12.11.421305. https://doi.org/10.1101/2020.12.11.421305

Ostroverkhova, N.V., Konusova, O.L., Kucher, A.N., Kireeva, T.N., Rosseykina, S.A., 2020. Prevalence of the Microsporidian *Nosema spp*. in honey bee populations (*Apis mellifera*) in some ecological regions of North Asia. Vet. Sci. 7. https://doi.org/10.3390/vetsci7030111

Palottini, F., Estravis Barcala, M.C., Farina, W.M., 2018. Odor learning and its experience-dependent modulation in the South American native bumblebee *Bombus atratus* (hymenoptera: apidae). Front. Psychol. 9, 603. https://doi.org/10.3389/fpsyg.2018.00603

Piiroinen, S., Botías, C., Nicholls, E., Goulson, D., 2016. No effect of low-level chronic neonicotinoid exposure on bumblebee learning and fecundity. PeerJ 4, e1808. https://doi.org/10.7717/peerj.1808

Piiroinen, S., Goulson, D., 2016. Chronic neonicotinoid pesticide exposure and parasite stress differentially affects learning in honeybees and bumblebees. Proc. R. Soc. B Biol. Sci. 283, 20160246. https://doi.org/10.1098/rspb.2016.0246

Plischuk, S., Martín-Hernández, R., Prieto, L., Lucía, M., Botías, C., Meana, A., Abrahamovich, A.H., Lange, C., Higes, M., 2009. South American native bumblebees (Hymenoptera: Apidae) infected by *Nosema ceranae* (Microsporidia), an emerging pathogen of honeybees (*Apis mellifera*). Environ. Microbiol. Rep. 1, 131–135.

https://doi.org/10.1111/j.1758-2229.2009.00018.x

Porrini, M.P., Porrini, L.P., Garrido, P.M., de Melo e Silva Neto, C., Porrini, D.P., Muller, F., Nuñez, L.A., Alvarez, L., Iriarte, P.F., Eguaras, M.J., 2017. *Nosema ceranae* in South American native stingless bees and social wasp. Microb. Ecol. 74, 761–764. https://doi.org/10.1007/s00248-017-0975-1

Raiser, G., Galizia, C.G., Szyszka, P., 2017. A high-bandwidth dual-channel olfactory stimulator for studying temporal sensitivity of olfactory processing. Chem. Senses 42, 141–151. https://doi.org/10.1093/chemse/bjw114

Ravoet, J., De Smet, L., Meeus, I., Smagghe, G., Wenseleers, T., de Graaf, D.C., 2014. Widespread occurrence of honey bee pathogens in solitary bees. J. Invertebr. Pathol. 122, 55–58. https://doi.org/10.1016/j.jip.2014.08.007

Riddell, C.E., Sumner, S., Adams, S., Mallon, E.B., 2011. Pathways to immunity: temporal dynamics of the bumblebee (*Bombus terrestris*) immune response against a trypanosomal gut parasite: Bumblebee immune genes. Insect Mol. Biol. 20, 529–540. https://doi.org/10.1111/j.1365-2583.2011.01084.x

Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D., Andino, R., DeRisi, J.L., 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, *Nosema*, and *Crithidia*. PLoS ONE 6, e20656. https://doi.org/10.1371/journal.pone.0020656

Scheiner, R., Abramson, C.I., Brodschneider, R., Crailsheim, K., Farina, W.M., Fuchs, S., Grünewald, B., Hahshold, S., Karrer, M., Koeniger, G., Koeniger, N., Menzel, R., Mujagic, S., Radspieler, G., Schmickl, T., Schneider, C., Siegel, A.J., Szopek, M., Thenius, R., 2013. Standard methods for behavioural studies of *Apis mellifera*. J. Apic. Res. 52, 1–58. https://doi.org/10.3896/IBRA.1.52.4.04

Schmid-Hempel, P., 2013. Evolutionary parasitology: the integrated study of infections, immunology, ecology, and genetics. Oxford University Press.

Schmid-Hempel, R., Tognazzo, M., 2010. Molecular divergence defines two distinct lineages

of *Crithidia bombi* (Trypanosomatidae), parasites of bumblebees. J. Eukaryot. Microbiol. 57, 337–345. https://doi.org/10.1111/j.1550-7408.2010.00480.x

Sinpoo, C., 2018. Impact of *Nosema ceranae* and *Nosema apis* on individual worker bees of the two host species (*Apis cerana* and *Apis mellifera*) and regulation of host immune response. J. Insect Physiol. 8.

Sitaraman, D., Zars, M., LaFerriere, H., Chen, Y.-C., Sable-Smith, A., Kitamoto, T., Rottinghaus, G.E., Zars, T., 2008. Serotonin is necessary for place memory in *Drosophila*. Proc. Natl. Acad. Sci. 105, 5579–5584. https://doi.org/10.1073/pnas.0710168105

Smith, M.L., 2012. The honey bee parasite *Nosema ceranae*: transmissible via food exchange? PLOS ONE 7, e43319. https://doi.org/10.1371/journal.pone.0043319

Sommerlandt, F.M.J., Rössler, W., Spaethe, J., 2014. Elemental and non-elemental olfactory learning using PER conditioning in the bumblebee, *Bombus terrestris*. Apidologie 45, 106–115. https://doi.org/10.1007/s13592-013-0227-4

Takeda, K., 1961. Classical conditioned response in the honey bee. J. Insect Physiol. 6, 168–179. https://doi.org/10.1016/0022-1910(61)90060-9

Toda, N.R.T., Song, J., Nieh, J.C., 2009. Bumblebees exhibit the memory spacing effect. Naturwissenschaften 96, 1185–1191. https://doi.org/10.1007/s00114-009-0582-1

Velthuis, H.H.W., Doorn, A. van, 2006. A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie 37, 421–451. https://doi.org/10.1051/apido:2006019

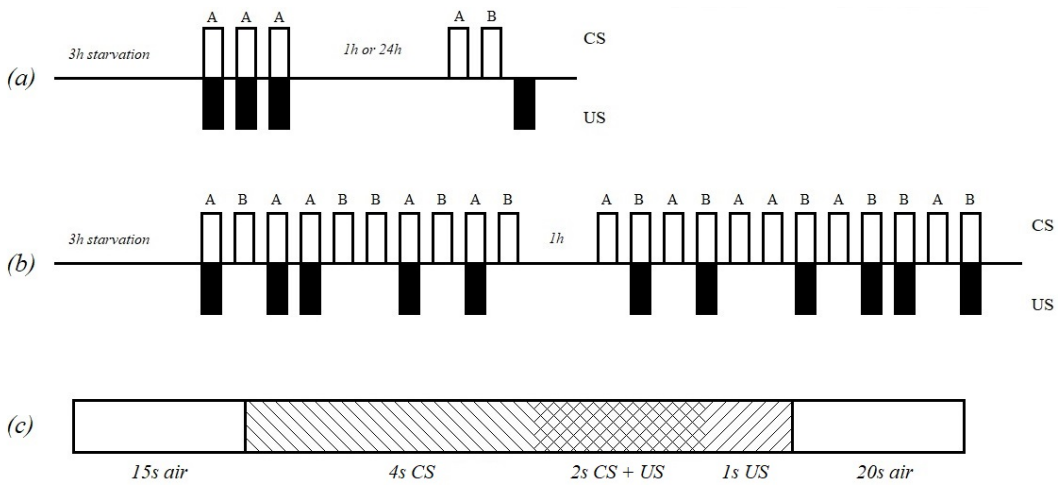
Villar, M.E., Marchal, P., Viola, H., Giurfa, M., 2020. Redefining single-trial memories in the honeybee. Cell Rep. 30, 2603-2613.e3. https://doi.org/10.1016/j.celrep.2020.01.086

Wolf, S., McMahon, D.P., Lim, K.S., Pull, C.D., Clark, S.J., Paxton, R.J., Osborne, J.L., 2014. So near and yet so far: harmonic radar reveals reduced homing ability of *Nosema* infected honeybees. PLoS ONE 9, e103989. https://doi.org/10.1371/journal.pone.0103989

Zheng, H.-Q., Lin, Z.-G., Huang, S.-K., Sohr, A., Wu, L., Chen, Y.P., 2014. Spore loads may

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

Supplementary Materials



Supplementary Figure S1. 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.

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

Treatment	**Control**	**Exposed**
Fed	48	30
Positive in PCR	5	10
Did not finish the test	0	0
Total	**43**	**30**

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

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

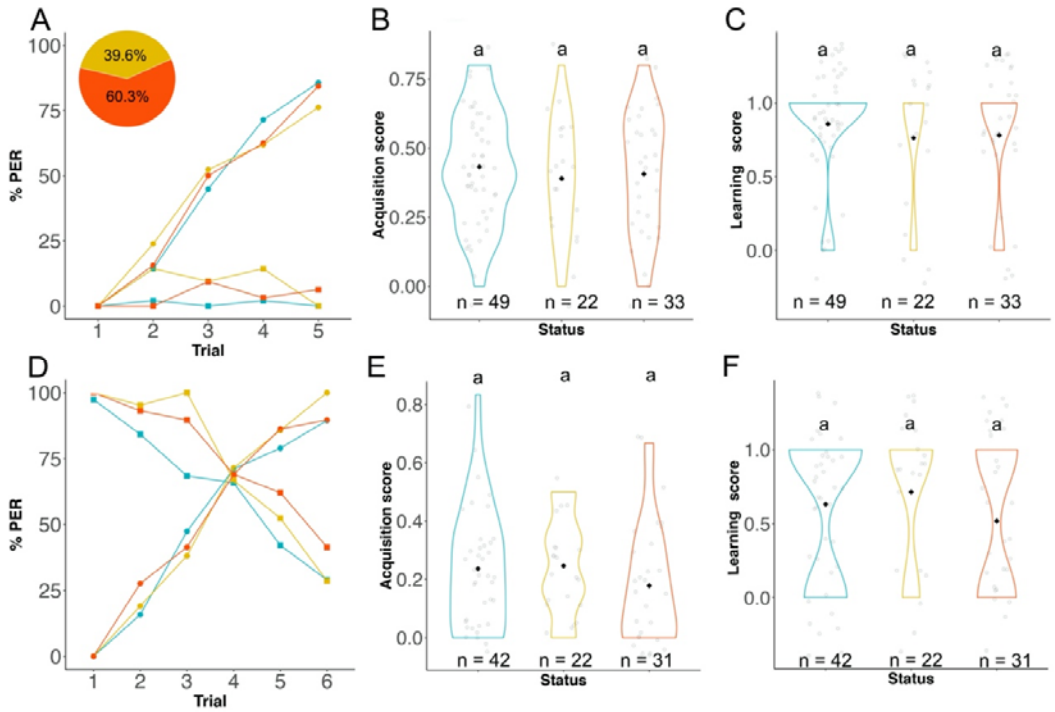
Supplementary Table S3. Number of bumblebees analysed in reversal learning. Exposed positive bumblebees are shown into brackets. 17.48% of the bumblebees died during the differential phase and 11.25% were not motivated (i.e. did not respond to US in two or more trials). 11.94% of the bumblebees that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

	Control	***Exposed***
Selected for training	87	96
Died during differential	14	18

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

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

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



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

positive (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. Violin plots of **B)** acquisition scores (i.e. sum of the correct responses divided by the number of trials for each bee) and **C)** learning scores (i.e. performance of bumblebees at last trial) of bumblebees with different status. Black dots represent the mean score of each status. Hollow dots represent the score of each individual. **D-F)** Reversal learning phase. **D)** Curves show the increase in the proportion of PER responses to B+ over A- over trials. **E)** Acquisition and **F)** learning scores during reversal phase. Letters above violin plots show significant differences between status in the acquisition (GLMM, $p < 0.05$) and learning (Binomial GLMM) scores. n is the sample size.

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

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