

RESEARCH ARTICLE

Nosema ceranae parasitism impacts olfactory learning and memory and neurochemistry in honey bees (*Apis mellifera*)

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ABSTRACT

Nosema sp. is an internal parasite of the honey bee, *Apis mellifera*, and one of the leading contributors to colony losses worldwide. This parasite is found in the honey bee midgut and has profound consequences for the host's physiology. *Nosema* sp. impairs foraging performance in honey bees, yet, it is unclear whether this parasite affects the bee's neurobiology. In this study, we examined whether *Nosema* sp. affects odor learning and memory and whether the brains of parasitized bees show differences in amino acids and biogenic amines. We took newly emerged bees and fed them with *Nosema ceranae*. At approximate nurse and forager ages, we employed an odor-associative conditioning assay using the proboscis extension reflex and two bioanalytical techniques to measure changes in brain chemistry. We found that nurse-aged bees infected with *N. ceranae* significantly outperformed controls in odor learning and memory, suggestive of precocious foraging, but by forager age, infected bees showed deficits in learning and memory. We also detected significant differences in amino acid concentrations, some of which were age specific, as well as altered serotonin, octopamine, dopamine and L-dopa concentrations in the brains of parasitized bees. These findings suggest that *N. ceranae* infection affects honey bee neurobiology and may compromise behavioral tasks. These results yield new insight into the host–parasite dynamic of honey bees and *N. ceranae*, as well as the neurochemistry of odor learning and memory under normal and parasitic conditions.

KEY WORDS: Proboscis extension reflex, Pathogen, Insect brain, Associative learning, Amino acid, Biogenic amine

INTRODUCTION

Nosema sp. is an internal parasite of the honey bee, *Apis mellifera*, and one of the most significant factors contributing to colony losses (Goulson et al., 2015). Given the global importance of honey bee pollination to the reproduction of floral species and to agricultural productivity, it is important to understand how *Nosema* sp. parasitism affects honey bee health. *Nosema* sp. is an example of a microsporidian: a group of spore-forming, unicellular parasites classified as fungi (Fries, 2010). Bees typically become infected with *Nosema* sp. through the ingestion of spores found in contaminated food and water, by cleaning contaminated combs

(Higes et al., 2010) or during trophallaxis (Smith, 2012). Once infected, spores begin to thrive in the epithelial cells of the midgut. Over time, cell walls rupture and spores are excreted through the fecal matter (Chen et al., 2009). At very high levels of infection, the symptoms of *Nosema* sp. infection resemble those of dysentery. When infected, bees, which are naturally hygienic and excrete outside of the hive, defecate in and around the hive, spreading the infection to other workers, drones and the queen (Higes et al., 2009; Alaux et al., 2011).

Nosema ceranae, the *Nosema* species used in this study, affects several areas of honey bee physiology. It induces gene expression changes in nutritional, metabolic and hormonal pathways in the midgut and fat body, and alters gene expression in the brain (Holt et al., 2013; McDonnell et al., 2013; Mayack et al., 2015). *Nosema ceranae* obtains energy for replication from the honey bee midgut, harming the bee's epithelial cells and development as the infection grows (Higes et al., 2007; Holt et al., 2013). This effect increases oxidative stress in the bee (Mayack and Naug, 2009) and causes nutrient-sensitive structures, like the hypopharyngeal glands, to become smaller and lose function (Wang and Moeller, 1969, 1971; Alaux et al., 2010; Corby-Harris et al., 2016; Jack et al., 2016). Portions of the midgut proteome responsible for energy production, protein regulation and antioxidant defense are also altered (Vidau et al., 2014; Kurze et al., 2016). This evidence is part of a growing body of knowledge that *N. ceranae* disrupts nutrient digestion and metabolism in its host. As a result, infected honey bees show increased hunger, increased sucrose sensitivity (Mayack and Naug, 2009) and are less likely to share food with nestmates via trophallaxis (Naug and Gibbs, 2009).

At the behavioral systems level, less is known about *Nosema* sp. infection but there are intriguing observations related to foraging behavior. Bees infected with *Nosema* sp. are more likely to forage at a younger age than uninfected bees (Wang and Moeller, 1970; Dussaubat et al., 2013; Goblirsch, 2013; Lecocq et al., 2016; Natsopoulou et al., 2016). Precocious foraging alters the age structure of the population in the colony and can lead to colony losses (Wang and Moeller, 1970; Goblirsch et al., 2013; Higes et al., 2008, 2009; Barron, 2015; Perry et al., 2015). There are reports of infected bees being unable to return to the hive and generally exhibiting poor foraging performance (Kralj and Fuchs, 2010; Dussaubat et al., 2010, 2013; Wolf et al., 2014). Infected bees are also more likely to engage in riskier behavior, such as increased foraging trips during adverse weather conditions (Woyciechowski and Kozłowski, 1998) and robbing other hives for resources (Kuszevska and Woyciechowski, 2014). *Nosema* sp. also affects the number of flights taken and the average flight duration (Dussaubat et al., 2013; Alaux et al., 2014; Dosselli et al., 2016).

These observations gave rise to our hypothesis that *Nosema* sp. may affect foraging behavior through a decline in cognitive ability. Several neurological tasks are involved in successful foraging: processes that include spatial navigation, visual processing, and odor

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learning and memory (Hammer and Menzel, 1995). Given that energy metabolism is altered with *Nosema* sp. infection, it is possible that these foraging tasks, which are metabolically expensive, are impaired with infection. It becomes necessary, then, to determine whether *Nosema* sp., a gut-dwelling pathogen, has specific effects on the brain.

We sought to address this question by asking the following. (1) Does *Nosema* sp. interfere with the ability to associate an odor with a reward? (2) Does *Nosema* sp. impair memory of the odor association? And lastly, (3) if there are changes in odor learning and memory, how does *Nosema* sp. alter neurochemistry such that amino acids and key behavior-regulating biogenic amines are affected?

In this study, we used a forward-paired odor-associative conditioning assay using the proboscis extension reflex (PER). Caged bees were inoculated with *N. ceranae* on the day of emergence and tested at approximate nurse and forager ages. We employed two bioanalytical techniques to measure amino acids and biogenic amines in brain tissue at these ages. We found that nurse-aged bees with *N. ceranae* significantly outperformed controls in memory performance. At forager age, however, infected bees were slower to learn and had reduced memory performance. We found significant differences in amino acid concentrations, as well as altered serotonin, octopamine, dopamine and L-dopa concentrations in the brain.

MATERIALS AND METHODS

Animals

Brood frames were collected from colonies at the Carl Hayden Bee Research Center locations in Southern Arizona between March and July, 2016. Frames were taken from European colonies, *Apis mellifera ligustica* Spinola 1806, headed by queens from Pendell Apiaries (Stonyford, CA, USA). Brood frames were taken from three or more hives at a time to ensure a sufficient number of emerging brood and to control for colony effects. Bees less than 12 h old were inoculated with 100,000 spores of *Nosema* sp. After inoculation, bees were separated into cages according to treatment, with 50 bees per cage. Bees were kept in a Binder BD (E2) incubator (Binder GmbH, Tuttlingen, Germany) at 31.7°C with 50% relative humidity under constant dark conditions.

Feeding

Bee-collected pollen was removed from pollen traps in the surrounding Tucson, AZ, USA area on 26 February 2016 and kept at -20°C until use. Each cage was given an insert with 3 g of pollen, and 50% sucrose solution and water *ad libitum*. Pollen, sucrose and water were changed every 7 days. Cages were checked daily and dead bees were removed.

Nosema inoculum

Spores were collected from bees found at the entrance of an infected hive the day before, or the day of, the inoculation. Over the course of the experiments, multiple hives were used as the source of *Nosema* spores.

A single infected bee abdomen was crushed with a mortar and pestle in 1 ml of water; 10 µl was transferred to a hemocytometer for spore counts. Five squares were counted and the following equation was applied to yield 50,000 spores per 1 µl:

$$\begin{aligned} \text{No. of spores counted / no. of squares counted} \\ = \text{no. of spores in 4 ml,} \end{aligned} \quad (1)$$

$$\begin{aligned} \text{No. of spores in 4 ml} \times 250,000 \\ = \text{total no. of spores in 1 ml of water,} \end{aligned} \quad (2)$$

$$\begin{aligned} \text{Total no. of spores in 1 ml of water / 1000 } \mu\text{l} \\ = \text{no. of spores per } \mu\text{l of sample.} \end{aligned} \quad (3)$$

Once the *Nosema* suspension was determined, the sample was spun down, the supernatant removed, and the *Nosema* pellet reconstituted with a 50% sucrose solution for the desired volume. Each bee was placed into an Eppendorf tube with a hole large enough for a proboscis to extend through. Bees were hand fed 2 µl of *Nosema* inoculum (for a delivery of 100,000 spores per bee) to the proboscis. Controls were fed 2 µl of 50% sucrose solution. Each bee was observed feeding, either by observing proboscis extension through the hole in the tube or by manually stimulating the antennae with sucrose, eliciting extension and feeding. Pollen, sucrose and water were withheld from cages for 1 h after feeding to ensure infection. A sample of the inoculated bees was sent to the Bee Research Center in Beltsville, MD, USA (ARS-USDA) for identification of *Nosema* species. *Nosema* DNA was extracted from infected and control bee abdomens as described in Fries et al. (2013). *Nosema ceranae* was confirmed to be the source of the infection according to band size (for details, see Fries et al., 2013). *Nosema apis* was not detected.

Spore counts

Spore counts of day 7 and day 15 bees were determined from the abdomen of a bee whose brain was analyzed for biogenic amines. Spores were counted using a hemocytometer and calculated as reported in Fries et al. (2013). On day 7, *Nosema*-infected bees had, on average, 14,575,000 spores ($\pm 13,203,254$ s.d., $N=20$). Two out of 20 control bees revealed *Nosema* spores (800,000 and 30,900,000) on day 7. Chemical analysis for these two bees placed them in the *Nosema*-infected category, a predetermined measure. On day 15, spore counts of *Nosema*-infected bees increased to an average of 109,390,104 spores ($\pm 39,526,537$ s.d., $N=16$). No spores were found in day 15 control bees ($N=16$).

Learning and memory

Learning and memory experiments took place between March and May 2016 in four replicates. The night before associative-learning tests, sucrose was removed from the cage between 17:00 h and 18:00 h. The next morning, bees were held in a 1 ml pipette tip cut so that the body was restrained and the neck free to rotate. Wax was used around the opening for further restraint. Bees were tested for the PER by applying a wooden applicator soaked in 50% sucrose to the tip of the antennae. Bees were not allowed to lick. If the bee did not exhibit a strong PER (rapid full extension) it was not used in the study.

Bees that were 7 and 15 days old were assessed for associative odor learning in a forward-paired conditioning paradigm. Clove oil (diluted 1:1000 in mineral oil; Sigma) was placed on filter paper in a 10 µl volume and inserted into a 0.5 ml glass syringe. The syringe was placed at a distance of 1 in from the bee and connected to a solenoid-controlled air stream. The solenoid was powered by an Interval Generator 1830 (W.P. Instruments, Sarasota, FL, USA) to deliver a 5 s odor pulse (7 km h^{-1}). Three seconds into the pulse, a wooden applicator soaked with 50% sucrose was presented to the antennae. The bee was allowed to lick for 1 s. This sequence was repeated for three trials spaced 10 min apart. Three odor conditioning trials was found to be the least number of trials needed for long-term memory (Menzel et al., 2001). Three trials were chosen to assess a potentially subtle difference in learning and memory with *Nosema* infection, which may be masked with a stronger conditioning paradigm of more spaced trials. All

experiments were performed between 10:00 h and 12:00 h under red light. Vacuum suction was applied continuously throughout the experiment.

Animals were tested for odor learning and memory at three time points after conditioning: 1 h, 4 h and 24 h. These are approximate periods of time when memory traces occur and are indicative of late short-term, mid-term and early long-term memory (Menzel, 2001). At each time point, the bee was presented with the 5 s odor pulse and scored on proboscis extension immediately following the odor. At each time point, the bee was tested twice.

At the end of the experimental day, all animals were fed until satiation, typically between 12 and 16 μl of 50% sucrose between 17:00 h and 18:00 h. Feeding was performed away from the odor delivery area to ensure that place conditioning did not occur. Bees were restrained overnight at room temperature in a covered box with 1–2 in of water to maintain humidity. Memory tests at 24 h were performed the following day.

Amino acid analysis of brain tissue and pollen

Whole brains (including antennal and optic lobes) were dissected from 7 and 15 day old bees between July and August 2016 ($N=79$). Bees were flash frozen in liquid nitrogen between 14:00 h and 17:00 h and stored at -80°C until dissection. Each brain was rapidly dissected (in an average of 3 min), weighed using a Sartorius CP2P microscale (Sartorius, Goettingen, Germany), and frozen in liquid nitrogen before being transferred to -80°C until analysis.

Each brain was homogenized using a bead beater for 30 s (100 mg of 1.0 mm beads, 500 μl of deionized water); 200 μl aliquots of brain homogenate were subjected to one of three analyses to control for losses with digestion:

(1) Conventional acid hydrolysis: 500 μl of 6 mol l^{-1} HCl with 4% thioglycolic acid was added to the sample, sealed in an inert atmosphere and digested at 70°C for 24 h; 50 μl aliquots were filtered and dried down for derivatization. (2) Base hydrolysis: 600 μl of 4 mol l^{-1} NaOH was added to the sample, sealed in an inert atmosphere and digested at 90°C for 4 h; 200 μl aliquots were filtered, neutralized with 6 mol l^{-1} HCl and dried down before derivatization. (3) Sodium azide acid hydrolysis: 780 μl of 6 mol l^{-1} HCl, 20 μl of 1% phenol (in 6 mol l^{-1} HCl), 100 μl of 12 mol l^{-1} HCl and 100 μl of 8% sodium azide were added to the sample, sealed and then digested at 70°C for 24 h (Manneburg et al., 1995); 25 μl aliquots were transferred to 2 ml amber glass vials for derivatization.

Conventional acid hydrolysis with chloroformate derivatization was used to quantify all amino acids, with the exception of tryptophan, cysteine and arginine. Tryptophan and cysteine are destroyed under acidic conditions and arginine cannot be derivatized using chloroform. Tryptophan was recovered using base hydrolysis with chloroformate derivatization. Cysteine and arginine were quantified with sodium azide acid hydrolysis followed by phenylisothiocyanate derivatization. The latter method enables cysteine to be quantified in its oxidative form, cysteic acid, and arginine in a phenylthiocarbamyl derivative. Asparagine and glutamine are hydrolyzed to their acidic forms and are reported here as asparagine/aspartic acid and glutamine/glutamic acid, respectively (Fountoulakis and Lahm, 1998).

Conventional acid-hydrolyzed and base-hydrolyzed samples were analyzed using the EZ:faast Amino Acid Analysis Kit for Protein Hydrolysates by Gas Chromatography–Mass Spectrometry (Phenomenex, Torrance, CA, USA). The re-dissolved chloroformate derivatives were analyzed by EI GC-MS on an Agilent 7890A gas chromatography system coupled with a 5975C EI mass spectrometer detector; 1 μl extracts were injected into a Zebra

ZB-50 capillary column (Phenomenex; 30 m \times 0.25 mm ID \times 0.25 μm film) in a 1:15 split mode with an injector temperature of 250°C and helium as the carrier gas (1.1 mL min^{-1}). Separation was achieved using an oven program with an initial temperature of 110°C that was increased to 320°C at a rate of $30^{\circ}\text{C min}^{-1}$, as recommended by the EZ:faast protocol for amino acid analysis. Chloroformate derivatives were identified by comparison of retention times and mass fragmentation patterns with derivatized standards, and quantified using major ions (secondary ion mass spectrometry).

Sodium azide hydrolyzed samples were analyzed using a modified method from Elkin and Wasynczuk (1987). The method consists of a neutralization step using a 2:2:1 mixture of methanol:water:triethylamine (TEA) (v/v), followed by a 20 min derivatization using a 7:1:1:1 mixture of methanol:TEA:water:PITC (v/v). Methanol washes were applied to remove interfering compounds before being dried down. Because of their time and light sensitivity, the phenylthiocarbamyl amino acids were only re-dissolved with a 5 mmol l^{-1} solution of disodium hydrogen phosphate containing 5% acetonitrile (pH 7.4) directly before being analyzed.

The re-dissolved phenylthiocarbamyl derivatives were analyzed by reverse-phase high-performance liquid chromatography–photodiode array (HPLC–PDA) on a ThermoFisher Scientific Spectra System coupled with a Finnigan Surveyor PDA Plus Detector; 20 μl of the extract was injected and separated using a Pico-Tag column (3.9 \times 150 mm) with a linear gradient pattern adopted from Kwanyuen and Burton (2010). Consisting of two eluents, the gradient started with 100% of eluent A, a mixture of 150 mmol l^{-1} $\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$, 0.05% TEA and 6% acetonitrile, pH 6.1, and finished with 100% eluent B, a 6:4 acetonitrile:water (v/v) mixture. A column temperature of 38°C was maintained, the flow rate was 1 mL min^{-1} throughout and the detection wavelength was set to 254 nm. Phenylisothiocyanate derivatives were quantified and identified by comparison of retention times with derivatized standards.

Amino acid composition of pollen was analyzed using the same procedure. Pollen was homogenized using a mortar and pestle with liquid nitrogen. Six 10 mg samples were used for hydrolysis and derivatization steps (Fig. S1).

Biogenic amine analysis of brain tissue

Whole brains were dissected from 7 and 15 day old bees taken from the same cage as those used for behavioral experiments. A bee was placed into a scintillation vial and chilled on ice until immobile (between 2 and 5 min). The brain was rapidly dissected, weighed and placed into 50 μl of chilled 0.1 mol l^{-1} perchloric acid. The brain was ground manually, frozen in liquid nitrogen and transferred to -80°C until analysis. Because levels of neurotransmitters are known to fluctuate throughout the day (Kloppenborg et al., 1999; Gage et al., 2013, 2014), all brains ($N=42$) were dissected between 14:00 h and 17:00 h and each dissection was alternated between treatments to control for any time of day variances between 14:00 h and 17:00 h. The abdomen of the same bee was saved at -20°C for *Nosema* spore counts.

Sample volume was brought to 100 μl in 0.1 mol l^{-1} perchloric acid. Benzoylation was performed by adding 200 μl of 200 mmol l^{-1} carbonate buffer followed by 100 μl 2% v/v benzoyl chloride in acetonitrile. The mixture was manually agitated. Two liquid–liquid extraction steps were performed using 200 μl each of dichloromethane, followed by two washes of the dichloromethane layer with 200 μl of basified water each (adjusted to pH 8 with ammonium hydroxide) to minimize the carryover of unwanted species, such as benzoic acid and salt products. The solution was evaporated to dryness using a Savant Speedvac

Concentrator (ThermoFisher Scientific). The resulting product was reconstituted in 50:50 H₂O:acetonitrile with 0.1% formic acid for electrospray compatibility. Solutions were diluted in 50:50 H₂O:acetonitrile with 0.1% formic acid to bring signals into the linear dynamic range of the instrument.

Detection and quantification were performed with an AB Sciex QStar Elite (Applied Biosystems, Foster City, CA, USA). Samples were injected using a 20 µl injection loop, with 50:50 H₂O:acetonitrile with 0.1% formic acid spray solvent flowing at 5 µl min⁻¹. The most abundant fragment peak for benzoylated compounds is the 105 m/z benzoyl fragment. The area of the 105 m/z peak was quantified over the course of the 5 min collection. Samples were run in duplicate, values were averaged for each sample and concentrations were calculated from a standard curve.

Statistics

JMP 12.0.1 was used for all statistics. The behavioral results were analyzed for the effect of *N. ceranae* infection using a Wilcoxon (rank sums) test with a chi-square approximation. This test was applied separately for learning trials 2 and 3, and memory testing at 1, 4 and 24 h. The amino acid concentrations were normalized with a log₁₀ transformation. Amino acids and biogenic amines were individually analyzed using a two-way, full factorial ANOVA with a *post hoc* Tukey HSD test. All error bars reported are s.e.m. All tests employed $\alpha=0.05$ and a 95% confidence interval.

RESULTS

Odor-associative learning and memory in nurse- and forager-aged bees

At 7 days old, bees infected with *N. ceranae* learned to associate an odor with a reward, similar to control bees (Fig. 1A). Memory

performance, however, differed with infection. *Nosema*-infected bees showed increased PER when tested for memory of the conditioned odor (Fig. 1C). Significant increases in PER occurred with *Nosema* at 1 h [$\chi^2(1, N=122)=25.98, P<0.0001$] and 4 h [$\chi^2(1, N=106)=5.14, P=0.02$], and continued at 24 h [$\chi^2(1, N=94)=3.28, P=0.07$] (Fig. 1A,C).

When bees were 15 days old, differences in trial learning emerged (Fig. 1B). Control bees had higher PER than *Nosema*-infected bees at trial 2 [$\chi^2(1, N=67)=3.058, P=0.08$] and significantly higher PER at trial 3 [$\chi^2(1, N=67)=3.99, P=0.04$]. When PER performance was compared between days 7 and 15, *Nosema*-infected bees did not improve in trial learning. *Nosema*-infected bees showed an average of 61.2% PER (trial 3) to the conditioned odor on day 7 and 56.7% PER (trial 3) on day 15 [$\chi^2(1, N=67)=0.14, P=0.71$]. Control bees, in contrast, showed a significant increase in trial learning, from 50% on day 7 to 80% on day 15 [$\chi^2(1, N=60)=5.83, P=0.02$] (Fig. 1A,B).

Day 15 also revealed differences in memory performance between the treatment groups. *Nosema*-infected bees had a significant deficit in memory at the 1 h time point [$\chi^2(1, N=148)=6.41, P=0.01$] (Fig. 1D). Odor memory tested at 4 and 24 h was lower in comparison with that of control bees, but this difference was not significant at either time.

Amino acid concentrations in the whole brain of nurse- and forager-aged bees

Altogether, the ANOVA results showed that 15 out of 18 amino acids in the brain were significantly affected by *N. ceranae* (Figs 2 and 3, Tables 1 and 2). Additionally, 13 out of 18 amino acids were significantly affected by age (7–15 days old) (Figs 2 and 3, Tables 1

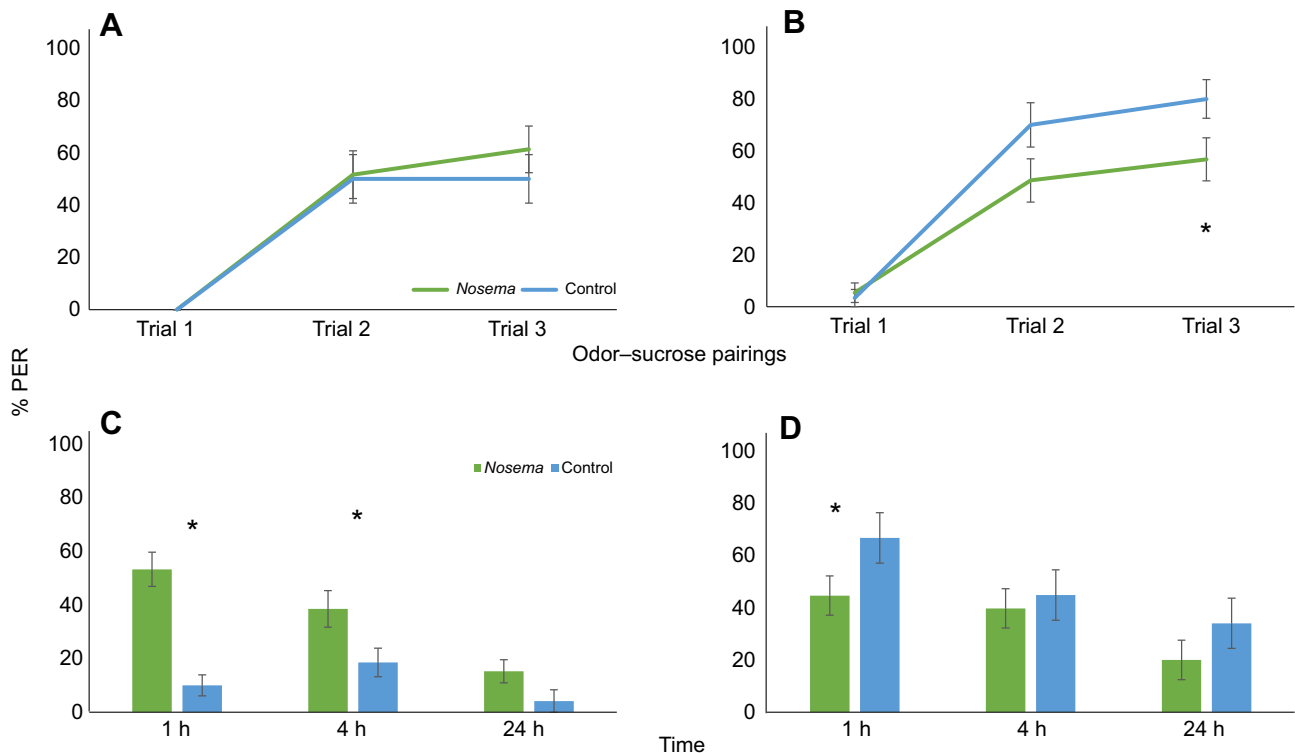


Fig. 1. Honey bee olfactory learning and memory in 7 and 15 day old bees treated with *Nosema ceranae*. (A,B) Proboscis extension response (PER) was measured during trial learning for *Nosema*-infected and control bees on day 7 (A; $N=31, 30$) and day 15 (B; $N=37, 30$). (C,D) Learning and memory of the conditioned odor in day 7 (C) and day 15 (D) bees was tested 1, 4 and 24 h after trial learning. Data were collected between March and May using caged bees. Significance was determined using separate Wilcoxon tests. *1B: $P=0.04$, *1C: $P<0.0001$ and $P=0.02$, *1D: $P=0.01$.

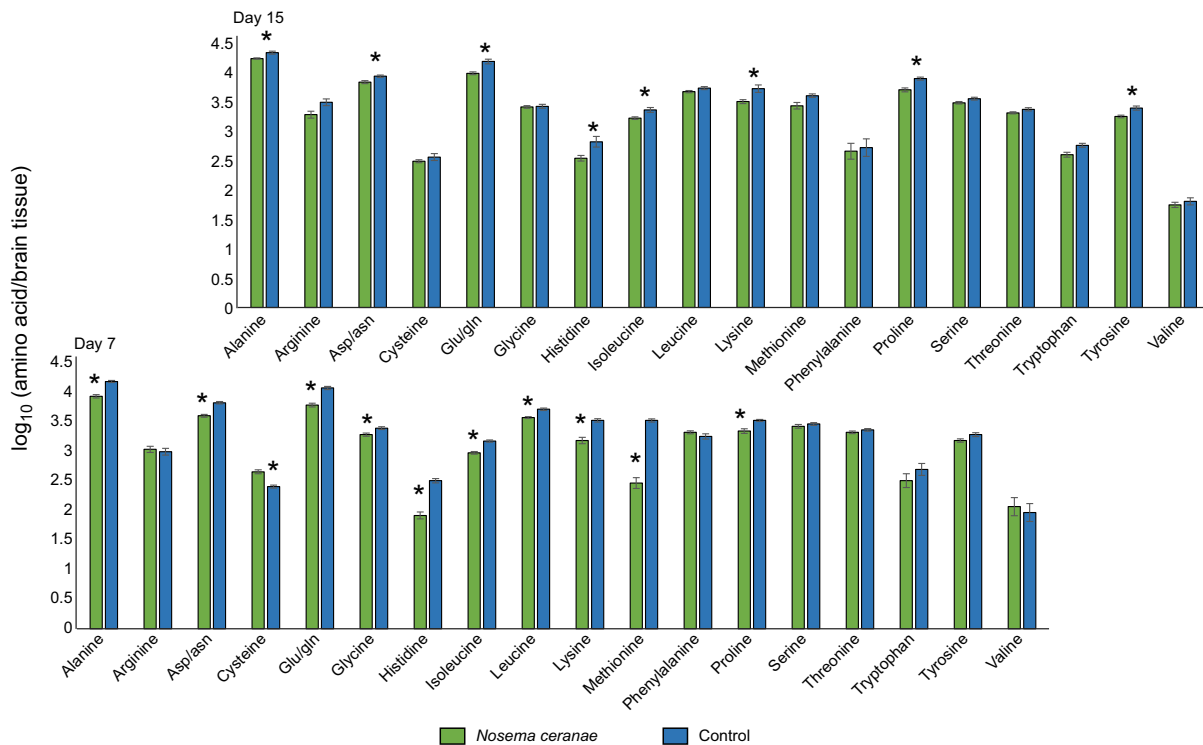


Fig. 2. Amino acid levels in honey bees at 7 and 15 days of age with *N. ceranae*. Amino acid concentrations (ng mg⁻¹ brain tissue) were measured in whole brain homogenates in *N. ceranae*-infected bees ($N=19, 20$) and controls ($N=20, 20$). Data were analyzed using a two-way ANOVA with a *post hoc* Tukey HSD to test for age and infection. Asterisks denote significance and error bars denote s.e.m.

and 2). Six amino acids – alanine, arginine, asparagine/aspartic acid, cysteine, histidine and methionine – showed a significant interaction effect with infection and age.

At 7 days old, bees infected with *N. ceranae* showed significant concentration differences in 11 amino acids: alanine, asparagine/aspartic acid, cysteine, glutamine/glutamic acid, glycine, histidine, isoleucine, lysine, methionine, proline and tyrosine (Fig. 2, Tables 1 and 2). Of these, four amino acids – cysteine, glycine, leucine and methionine – showed a significant difference in concentration with infection at day 7 only. *Nosema*-infected bees had significantly lower levels of each of these amino acids, with the exception of cysteine. A closer look at the interaction effect between infection and age ($P<0.0001$) revealed that cysteine levels were significantly higher in *Nosema*-infected bees on day 7 ($P<0.0001$, Tukey HSD) but not on day 15 ($P=0.58$, Tukey HSD). Cysteine levels in *Nosema*-infected bees also significantly decreased with age from day 7 to day 15 ($P=0.004$, Tukey HSD). Control bees, in contrast, showed increased levels of cysteine with age ($P=0.05$, Tukey HSD).

At 15 days old, bees infected with *N. ceranae* showed significant concentration differences in eight amino acids: alanine, asparagine/aspartic acid, glutamine/glutamic acid, histidine, isoleucine, lysine, proline and tyrosine (Fig. 2, Tables 1 and 2). The concentrations of the first seven amino acids were also significantly lower with infection on day 7. The concentration of tyrosine, however, was significantly lower only on day 15 in *Nosema*-infected bees (day 7, $P=0.10$; day 15, $P=0.01$, Tukey HSD). Arginine, too, may be affected by *N. ceranae* at day 15: arginine levels were lower on day 15 with a borderline P -value ($P=0.06$, Tukey HSD) but were similar on day 7 ($P=0.95$, Tukey HSD). The interaction term between infection and age, however, was significant with arginine levels ($P=0.03$) (Tables 1 and 2, Figs 2 and 3).

Three amino acids – serine, threonine and tryptophan – showed an overall effect of *N. ceranae*, but did not show a significant difference with age-matched controls at either day 7 or day 15 (Table 2). At each of these ages, serine, threonine and tryptophan levels were reduced in *Nosema*-infected bees.

Thirteen amino acids were affected by age (Fig. 3, Table 2). Twelve increased with age: alanine, arginine, asparagine/aspartic acid, glutamine/glutamic acid, glycine, histidine, isoleucine, lysine, methionine, proline, serine, and tyrosine; and one decreased with age: phenylalanine. Only one of the amino acids we tested, valine, showed no significant effect due to infection or age ($F_{3,61}=1.41$, $P=0.25$; infection: $F=0.029$, $P=0.87$; age: $F=3.63$, $P=0.06$; infection \times age: $F=0.36$, $P=0.55$). Valine, however, was not normally distributed in our dataset and may require further testing. Like phenylalanine, valine had a decreasing trend with age from day 7 to day 15.

Six compounds had a significant interaction effect with *N. ceranae* infection and age (Fig. 3, Table 2). Five of these amino acids – alanine, asparagine/aspartic acid, cysteine, histidine and methionine – showed the greatest differences with infection with age-matched controls on day 7, and less disparity on day 15. Methionine was perhaps the strongest example. On day 7, methionine levels of *Nosema*-infected bees were significantly lower than those of controls ($P<0.0001$, Tukey HSD), though, by day 15, methionine levels were similar ($P=0.15$, Tukey HSD). Thus, methionine significantly increased with age in infected bees ($P<0.0001$, Tukey HSD) while remaining similar in controls ($P=0.43$, Tukey HSD). Arginine levels, conversely, were similar on day 7 with infection ($P=0.95$, Tukey HSD) but, by day 15, *Nosema*-infected bees had lower levels ($P=0.06$, Tukey HSD). Both infected ($P=0.03$, Tukey HSD) and control groups ($P<0.0001$, Tukey HSD) showed significantly higher levels of arginine with age.

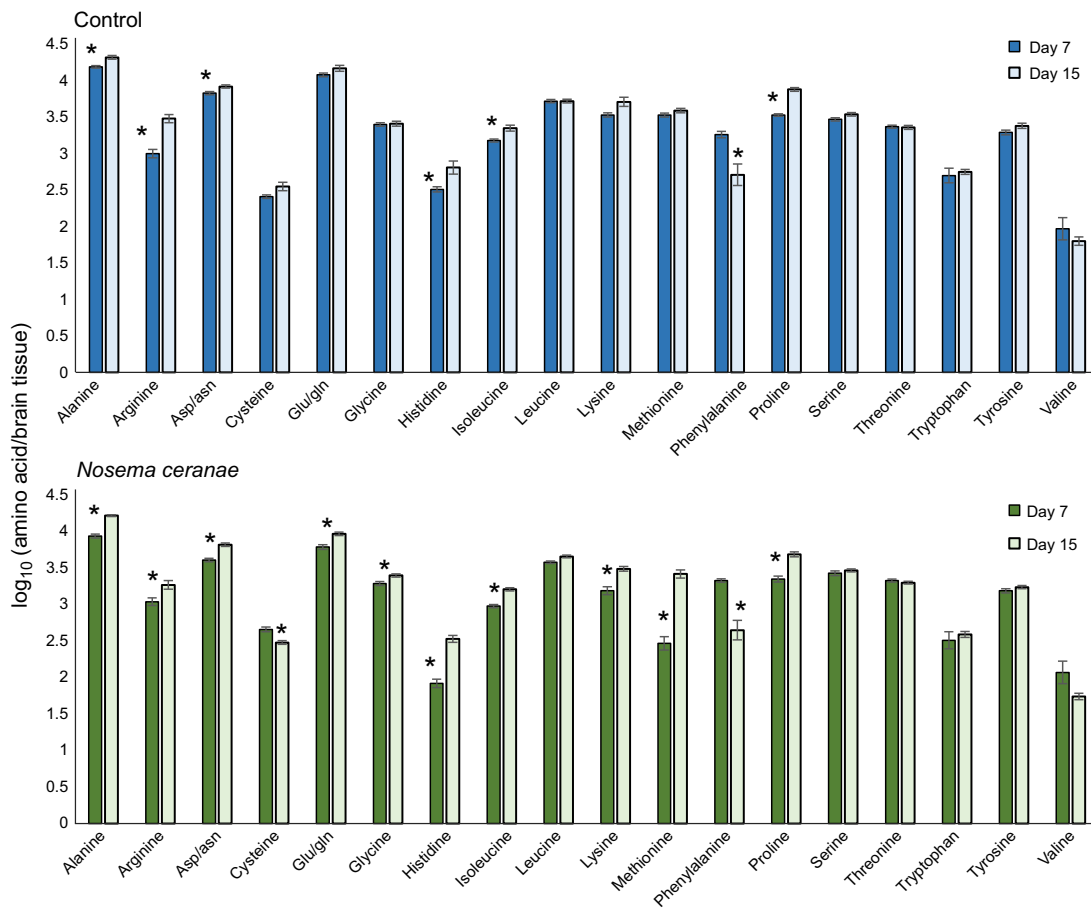


Fig. 3. Variation in amino acid levels in *Nosema*-infected and control bees with age. Amino acid concentrations (ng mg^{-1} brain tissue) presented in Fig. 2 were rearranged to present the effect of age. Data were analyzed using a two-way ANOVA with a *post hoc* Tukey HSD to test for age and infection. Asterisks denote significance and error bars denote s.e.m.

Neurotransmitter levels in nurse- and forager-aged bees

To further examine the effect of *N. ceranae* on brain chemistry, biogenic amines were measured to understand how these key behavior-regulating chemical messengers might be affected (Table 2, Fig. 4). Overall, two amino acids – serotonin and dopamine – were affected by *Nosema* infection, and octopamine was affected by age. L-Dopa, the precursor to dopamine, was the only compound that showed a significant interaction effect with infection and age.

At 7 days old, bees infected with *N. ceranae* showed significant concentration differences in serotonin ($F_{3,39}=4.83$, $P=0.0059$; infection: $F=12.43$, $P=0.0011$; age: $F=1.09$, $P=0.30$; infection \times age: $F=2.45$, $P=0.12$) and L-dopa ($F_{3,38}=3.59$, $P=0.02$; infection: $F=3.50$, $P=0.069$; age: $F=1.51$, $P=0.22$; infection \times age: $F=4.47$, $P=0.041$). *Post hoc* analyses showed significantly higher levels of serotonin in bees infected with *N. ceranae* on day 7 than in age-matched controls ($P=0.001$, Tukey HSD), but no difference was found on day 15 ($P=0.16$, Tukey HSD). L-Dopa, however, did not show a significant effect for infection ($P=0.069$), but did show a significant interaction effect between infection and age ($P=0.041$). *Post hoc* analyses showed a significant effect of infection on L-dopa on day 7 only ($P=0.01$, Tukey HSD) and not on day 15 ($P=0.85$, Tukey HSD). Dopamine, too, showed a significant infection effect overall, being elevated on both day 7 and day 15, but, this significance was not specific to either age ($F_{3,37}=3.37$, $P=0.11$; infection: $F=4.21$, $P=0.047$; age: $F=3.44$, $P=0.07$; infection \times age: $F=0.02$, $P=0.87$).

Octopamine was the only biogenic amine that varied significantly with age ($F_{3,38}=2.84$, $P=0.05$; infection: $F=3.24$, $P=0.079$; age: $F=6.31$, $P=0.016$; infection \times age: $F=1.195$, $P=0.28$). Levels of octopamine were higher in the whole brain on day 15 than on day 7 in both control bees and *Nosema*-infected bees. In *Nosema*-infected bees, however, there was very little difference in octopamine levels between nurses and foragers ($P=0.71$, Tukey HSD). Octopamine levels averaged 14.2 ng mg^{-1} brain tissue on day 7 and 18.3 ng mg^{-1} on day 15. In control bees, however, there was a more pronounced effect with age. At nurse age, octopamine levels averaged 5.77 ng mg^{-1} brain tissue on day 7 and 16.3 ng on day 15 ($P=0.09$, Tukey HSD). Though there appears to be a large difference in nurse bees with *Nosema* infection (*Nosema*: 14.2 ± 3.17 , control: 5.77 ± 3.46), this effect was not significant ($P=0.22$, Tukey HSD).

Histamine was not significantly affected by either infection or age ($F_{3,32}=1.72$, $P=0.18$; infection: $F=2.01$, $P=0.16$; age: $F=1.70$, $P=0.20$; infection \times age: $F=0.42$, $P=0.52$). Generally, histamine was lower in infected bees than in controls (day 7: $4.71\text{--}5.91$, day 15: $5.74\text{--}8.97 \text{ ng mg}^{-1}$ of brain tissue), a trend not observed for the other biogenic amines.

DISCUSSION

Reports of *Nosema*-infected bees foraging differently prompted us to consider whether *N. ceranae*, a pathogen that resides in the midgut, affects the brain of the honey bee. We focused on

Table 1. Compounds found to vary significantly with *Nosema ceranae* infection in the whole brain of the honey bee

Compounds significant between control/ <i>Nosema</i>	<i>P</i> -value (two-way ANOVA, <i>post hoc</i> Tukey HSD)	Effect of <i>Nosema</i>	<i>N</i> (<i>Nosema</i> , control)
Day 7			
Alanine	<0.0001	Decrease	17, 19
Asparagine/aspartic acid	<0.0001	Decrease	17, 20
Glutamine/glutamic acid	<0.0001	Decrease	17, 20
Histidine	<0.0001	Decrease	17, 18
Isoleucine	<0.0001	Decrease	19, 20
Lysine	<0.0001	Decrease	19, 18
Proline	0.0003	Decrease	19, 18
<i>Cysteine</i>	<0.0001	Increase	18, 17
<i>Glycine</i>	0.02	Decrease	18, 20
<i>Leucine</i>	0.0003	Decrease	15, 20
<i>Methionine</i>	<0.0001	Decrease	18, 20
<i>L-Dopa</i>	0.04	Increase	11, 7
<i>Serotonin</i>	0.006	Increase	11, 8
Day 15			
Alanine	0.02	Decrease	16, 19
Asparagine/aspartic acid	0.01	Decrease	19, 19
Glutamine/glutamic acid	0.0002	Decrease	17, 18
Histidine	0.009	Decrease	20, 15
Isoleucine	0.004	Decrease	19, 17
Lysine	0.009	Decrease	19, 20
Proline	0.0002	Decrease	19, 19
<i>Tyrosine</i>	0.01	Decrease	18, 20
<i>Arginine</i>	0.06	Decrease	18, 16

P-values reported are those from Tukey HSD *post hoc* analyses comparing *Nosema*-infected bees with age-matched controls. The italicized compounds varied significantly only on day 7 or day 15.

examining odor learning and memory performance in the laboratory. Odor learning and memory are necessary for successful foraging and involve the coordination of several areas

of the insect brain, including the antennal lobe, mushroom bodies and the subesophageal zone (reviewed in Gauthier and Grünewald, 2012). If odor learning and memory was disrupted by *N. ceranae* infection, it would suggest that the infection had brain-specific effects. We would also expect to find dysregulation in amino acids and biogenic amines in the brain that regulate signaling pathways.

We found that *N. ceranae* infection does affect odor learning and memory and that the effects are age specific. *Nosema*-infected bees showed significantly enhanced odor learning and memory performance at nurse age. This effect may be an indicator of accelerated maturation in response to *N. ceranae* infection and may lead to precocious foraging. Precocious foraging has been reported elsewhere in *Nosema*-infected honey bees (Wang and Moeller, 1970; Dussaubat et al., 2013; Goblirsch, 2013; Lecocq et al., 2016; Natsopoulou et al., 2016) and we can add that infected bees demonstrate heightened odor learning and memory performance at nurse age, suggesting an increased physiological capacity for mechanisms of memory.

The impact of *N. ceranae* in odor learning and memory was found to change with the age of the bee. By forager age, average spore counts rose to over a 100 million per bee from approximately 14 million at nurse age. In trial learning, an indication of learning acquisition, *Nosema*-infected bees showed reduced PER at trials 2 and 3. This suggests that at this age and level of infection, *Nosema*-infected bees may be slower to associate an odor with a reward. When memory of the odor association was tested, *Nosema*-infected bees showed a significant deficit 1 h after training. These results suggest that forager bees with a *Nosema* infection may be compromised as a result of deficits in odor learning and memory. These results, however, contrast with those of the Charbonneau et al. (2016) study which found a limited effect of *Nosema* sp. in odor learning and memory using PER. We speculate that the difference in results may be due to the strength of the conditioning paradigm used. We employed three spaced odor–sucrose pairings as the minimum conditioning required for long-term odor memory

Table 2. Compounds in the honey bee whole brain and the effects of *Nosema ceranae* and age using individual, full-factorial two-way ANOVAs

Compounds	d.f.	<i>F</i> -value ANOVA	<i>P</i> -value ANOVA	Effect tests, <i>F</i> -values			Effect tests, <i>P</i> -values		
				<i>Nosema</i> effect	Age effect	<i>Nosema</i> ×age	<i>Nosema</i> effect	Age effect	<i>Nosema</i> ×age
Alanine	3, 67	54.34	<0.0001*	62.42	89.07	12.62	<0.0001*	<0.0001*	0.0007*
Arginine	3, 68	14.69	<0.0001*	2.24	38.27	4.92	0.14	<0.0001*	0.030*
Asparagine/aspartic acid	3, 71	32.89	<0.0001*	50.38	46.85	7.18	<0.0001*	<0.0001*	0.0091*
Cysteine	3, 65	8.57	<0.0001*	6.22	0.35	18.61	0.015*	0.56	<0.0001*
Glutamine/glutamic acid	3, 69	26.83	<0.0001*	61.67	17.79	2.36	<0.0001*	<0.0001*	0.13
Glycine	3, 73	4.74	0.0044*	5.49	6.07	3.29	0.02*	0.016*	0.074
Histidine	3, 64	38.95	0.0094*	54.24	59.17	7.17	<0.0001*	<0.0001*	0.0094*
Isoleucine	3, 70	31.07	<0.0001*	38.88	57.51	1.25	<0.0001*	<0.0001*	0.27
Leucine	3, 68	8.44	<0.0001*	20.61	3.88	3.033	<0.0001*	0.053	0.08
Lysine	3, 72	20.59	<0.0001*	34.24	24.62	1.72	<0.0001*	<0.0001*	0.19
Methionine	3, 70	90.95	<0.0001*	123.94	83.4	64.98	<0.0001*	<0.0001*	<0.0001*
Phenylalanine	3, 61	12.79	<0.0001*	0.0001	38.05	0.39	0.99	<0.0001*	0.53
Proline	3, 71	56.94	<0.0001*	38.66	13.02	0.0074	<0.0001*	<0.0001*	0.93
Serine	3, 70	3.38	0.023*	5.49	35.49	6.7	0.02*	0.036*	0.56
Threonine	3, 72	2.12	0.1	5.64	0.53	0.081	0.02*	0.47	0.78
Tryptophan	3, 72	1.7	0.17	4.44	0.73	0.019	0.039*	0.4	0.89
Tyrosine	3, 72	7.12	0.0003*	15.21	5.62	0.36	0.0002*	0.02*	0.55
Valine	3, 61	1.41	0.25	0.029	3.63	0.36	0.87	0.06	0.55
Histamine	3, 32	1.72	0.18	2.01	1.71	0.42	0.17	0.201	0.52
Octopamine	3, 38	2.85	0.05	3.24	6.32	1.19	0.079	0.016*	0.28
Serotonin	3, 39	4.84	0.0059*	12.43	1.09	2.46	0.0011*	0.302	0.13
Dopamine	3, 37	2.19	0.11	4.22	3.46	0.024	0.047*	0.071	0.88
<i>L-Dopa</i>	3, 38	3.59	0.022*	3.5	1.51	4.47	0.069	0.23	0.041*

Asterisks denote significance.

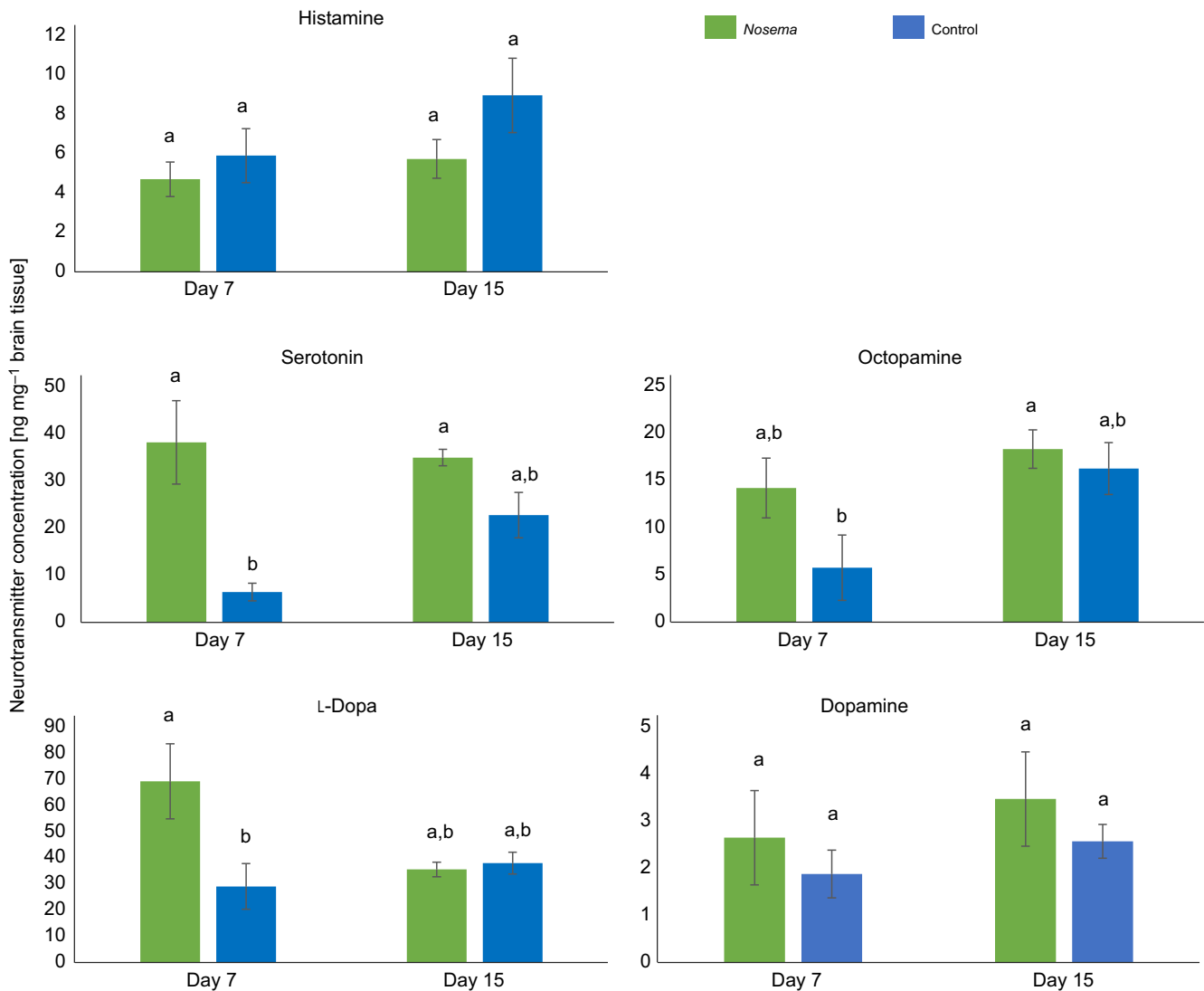


Fig. 4. Biogenic amine concentrations in whole-brain tissue in honey bees with and without *N. ceranae*. Biogenic amine concentrations were measured in whole brain homogenates in *N. ceranae*-infected bees ($N=11$, 11) and controls ($N=8$, 12). Data were analyzed using a two-way ANOVA to test for age and infection. Groups connected by the same letter were not significantly different. Error bars denote s.e.m.

(Menzel, 2001) and tested this association at three time points within 24 h. Charbonneau et al. (2016), in contrast, used eight pairings and tested at 24 h. This latter, robust paradigm is interesting in that, given extensive training, odor memory is similar to that of controls. In the field, this finding may translate to *Nosema*-infected bees needing to make more flower visits than controls to learn and remember odors at a similar rate. *Nosema ceranae* infection and the act of foraging are energetically demanding and it may be unlikely that infected bees are able to compensate in this manner. Alternatively, and perhaps more simply, the difference may be explained by whether caged bees were given pollen. Bees need to consume pollen for growth and development, especially in the first few days after emergence. We noticed that PER performance improved in our preliminary work when pollen was given and it may be why our findings differ from those of the Charbonneau study (in which pollen was not given). Bee-collected pollen also contains fatty acids and deficiencies in fatty acid composition, namely omega-3, do impair odor learning (Arien et al., 2015).

Chemical analyses may shed light on the physiology underlying our behavioral results. We found that a group of seven amino acids –

alanine, asparagine, glutamine, histidine, isoleucine, lysine and proline – were significantly reduced with *N. ceranae* in the brain regardless of worker age. There were also age-specific differences in five amino acids: cysteine, glycine, leucine, methionine and tyrosine. Cysteine was the only amino acid that was significantly increased with *N. ceranae*, but only in nurse-aged bees. This anomaly merits further study into the physiological role of cysteine under parasitic infection. Glycine, too, varied significantly with infection at nurse age only, but was lower with infection. By forager age, tyrosine levels were significantly lower and arginine levels were also lower ($P=0.06$, Tukey HSD). Tyrosine is in the biosynthesis pathways of dopamine and octopamine, and arginine is in the biosynthesis pathway of the gaseous, unconventional neurotransmitter nitric oxide, known to be involved in odor learning and memory (Müller, 1996, Gage et al., 2013, 2014). Serine, threonine and tryptophan were also reduced overall in *Nosema*-infected bees, though their levels were not significantly lower than those in controls at either age. Overall, there were 15 out of 18 amino acids significantly affected by *N. ceranae*, suggesting that the effects of this midgut pathogen on the brain are considerable.

These results raise the possibility of diet supplementation as a means to rescue cognitive impairment and improve overall health. This strategy might include forage with pollens naturally higher in these specific amino acids or a diet supplement beekeepers could use to feed their colonies. It needs to be determined whether an enhanced diet can overcome the nutrient deficiency caused by *N. ceranae*. A goal, moving forward, might be to supplement honey bee nutrition in such a way that *Nosema* sp. infection becomes asymptomatic. We suggest caution with the results of certain amino acids. The measurements of valine, phenylalanine, threonine, methionine, arginine, glycine and glutamine were not normally distributed in all experimental groups.

Biogenic amines, which function as neurotransmitters, neuromodulators and neurohormones, were also examined. (reviewed in Bicker, 1999 and Gauthier and Grünewald, 2012). We hypothesized that if *N. ceranae* infection affected the honey bee brain, we may expect to see differences in biogenic amine levels. We found that serotonin and dopamine levels were significantly higher in *Nosema*-infected bees. Octopamine and L-dopa were also higher but with borderline *P*-values. When we looked more closely at these results, we found that there were significant changes in biogenic amine levels that were specific to age. In nurse-aged bees, serotonin and L-dopa were significantly higher in the brains of *Nosema*-infected bees compared with those of controls. Octopamine levels in infected nurses were also elevated and were similar to those in foragers. From the literature, we know that high levels of serotonin and octopamine are associated with foraging behavior (Wagener-Hulme et al., 1999; Schulz et al., 2002a,b; Barron et al., 2002). In fact, octopamine treatment is sufficient to induce precocious foraging (Schulz and Robinson, 2001) and improves learning and memory in newly emerged bees (Behrends and Scheiner, 2012). Moreover, bees induced to forage precociously show higher, forager-like levels of serotonin and octopamine in their antennal lobes and bees that revert back to nursing have lower levels (Schulz and Robinson, 1999). Our results in the whole brain show similar trends with *N. ceranae* infection. Levels of serotonin and octopamine were elevated in *Nosema*-infected nurses and did not differ from control foragers. We suspect that heightened serotonin and octopamine in infected nurses may underscore our findings of enhanced odor learning and memory, and could be an additional indication that *Nosema*-infected bees at nurse age are precocious foragers. We measured neurotransmitters between March and August 2016 and found a large variation in response to *N. ceranae*; this needs further study. We matched the neurotransmitter data, the bulk of which was collected in the spring, to the time that behavior was performed between March and May. In effect, our studies report the results of caged spring bees fed spring pollen and it is possible that summer and autumn bees respond to *N. ceranae* differently. It is also important to consider that the chemical and behavioral analyses were performed using caged bees of identical age and are, therefore, independent of the social environment experienced in a typical colony setting.

Taken together, it is necessary to consider our results in light of the host–parasite relationship. Precocious foraging could be the bee’s effort to replace nutrients lost to *N. ceranae*, but it could also be advantageous to the parasite. For instance, the behavioral change to foraging for pollen may aid parasite replication within the individual honey bee and increase transmission within the hive. Two studies (Fleming et al., 2015; Jack et al., 2016) and unpublished results in our lab found that *Nosema*-infected bees that were fed pollen had significantly higher spore loads than starved bees, suggesting that spore replication is greater when the host has consumed pollen. A hive rich in pollen would therefore

fuel *N. ceranae* replication within the hive. Moreover, infected bees are known to prefer higher temperatures and are drawn to the population-dense colony center, a possible means to increase in-hive transmission (Campbell et al., 2010). Precocious foraging, too, could provide a means of dispersal for the parasite as infected bees are known to drift or rob other hives, potentially spreading infection (Ushitani et al., 2016). As bees age and spore counts rise, the probability of drifting increases. These observations raise the possibility that behavioral change in *Nosema*-infected bees may not be entirely the host’s response to infection but could also be parasitic manipulation of honey bee behavior.

A number of researchers recognize that true parasitic manipulations are those where the parasite pays a cost to induce a behavioral change, or the specificity of the behavioral change is such that it enhances the fitness of the parasite driven by natural selection (Thomas et al., 2005). It is unclear at this time whether there is such a cost to *N. ceranae*, or whether the changes induced by the parasite are driven by natural selection. *Nosema ceranae* has a relatively short evolutionary history in *A. mellifera* (Klee et al., 2007) and it may be that the proximate changes reported here are host defense mechanisms with possible benefits to *N. ceranae*. Our study was not designed to test these criteria, though these results may add to the discussion of this particular host–parasite dynamic. Below, we put our results into context with other parasite–host relationships.

The biogenic amine changes we observed in infected honey bees resemble those described in other examples of parasitic manipulation. Serotonin, octopamine and dopamine are neuromodulators commonly affected by parasitism (reviewed in Adamo, 2013; Perrot-Minnot and Cezilly, 2013). These neuromodulators can modify neural circuits to accommodate behavioral changes to meet the animal’s immediate survival needs and to adapt to a changing environment. This behavioral plasticity can also come at a price: it can open the animal to manipulation by another organism (Adamo, 2013). Intra- and extra-CNS parasites of gammarids, for example, alter serotonin signaling in the host. Serotonin modulates escape behaviors in crustaceans and it is suggested that parasites of gammarids manipulate serotonin to make the host more susceptible to predation, an effect suggested to enhance parasite transmission (Helluy, 2013). The parasitic wasp *Cotesia congregata*, through an unknown mechanism, elevates octopamine levels in the brain and thoracic and abdominal ganglia in its host larvae, *Manduca sexta* (Adamo, 2005). Normally, hungry caterpillars show reduced feeding, a behavior that increases parasite survival (Adamo, 2005). The parasitic wasp *Ampulex compressa* secretes a variety of substances to ‘zombify’ the cockroach *Periplaneta americana* (Libersat et al., 2009). These substances act upon multiple neurotransmitter systems including the cholinergic, GABAergic, dopaminergic and octopaminergic systems of the host.

In each of these examples, multiple mechanisms occur to affect the behavior of the host, such as changes in the neuromodulatory system, the neuroendocrine system and the immune system (Adamo, 2013). We can only speculate about the honey bee–*N. ceranae* dynamic, but, if it is like other host–parasite examples, *N. ceranae* could be affecting multiple mechanisms to ensure its survival. One possible mechanism by which a gut-dwelling parasite can affect the brain is described by the neuro–immune hypothesis. This theory proposes that parasite-induced behavioral change may be the result of the parasite’s attempt at circumventing or defeating host immune responses (Adamo, 2002). If the parasite can manipulate the immune system, it may not need to reside in the brain to affect behavior. Immune-derived molecules have privileged

routes of transmission to the brain (Dantzer et al., 2008) to affect neural cells; biogenic amines function as neurohormones circulating throughout the insect body and are affected by the immune response through interactions with cytokines (De Simoni and Imeri, 1998). Octopamine, for example, is released during both stress and immune responses in insects and is thought to be one aspect of the immune–neural connection manipulated by the parasitic wasp *C. congregata* in *M. sexta* (Adamo, 2010). The schistosome parasite *Trichobilharzia ocellata* is perhaps the clearest example of a parasite manipulating the immune system. *Trichobilharzia ocellata* secretes schistosomin, a molluscan cytokine-like molecule (de Jong-Brink et al., 2001), into its snail host, *Lymnaea stagnalis*, which suppresses the snail's neuroendocrine cells leading to a reduction in egg laying. The energy from the snail is redirected to support parasitic growth. Something similar may be occurring in honey bees with *Nosema* infection. *Nosema ceranae* has been found to suppress the immune system of the honey bee (Antunez et al., 2009; Holt et al., 2013; Li et al., 2016). It upregulates the naked cuticle gene, *nkd*, a negative regulator of host immune function (Li et al., 2016). This has the effect of suppressing the host's immune response and, when *nkd* is knocked down, several immune genes are upregulated and *N. ceranae* spore loads are reduced (Li et al., 2016). An important distinction to be made, however, is that evasion of the immune system does not indicate parasitic manipulation necessarily: there may be an effect on the immune system that leads to behavioral manipulation over evolutionary time (Adamo, 2002). Given our results that multiple neurotransmitters are affected, there may be potential for parasitic manipulation to develop.

In conclusion, we observed two distinct behaviors to *N. ceranae* infection that occurred with age or length of parasite incubation. We saw evidence of precocious foraging occurring at nurse age, which could be viewed as a novel behavior for the host, and a deleterious behavior with reduced learning and memory performance at forager ages. Considering our chemical data and examples of other host–parasite relationships, we speculate about the relationship between honey bees and *N. ceranae*. These results may have special importance for managed apiaries where colonies live close together. A better understanding of how *N. ceranae* affects the honey bee brain could provide better strategies to curb infection.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.L.G.; Methodology: S.L.G., C.K., S.C., M.C., M.H.; Validation: S.L.G.; Formal analysis: S.L.G., C.K., S.C., G.D.-H.; Investigation: S.L.G., C.K., S.C.; Resources: G.D.-H.; Data curation: S.L.G.; Writing - original draft: S.L.G., C.K., S.C., G.D.-H.; Writing - review & editing: S.L.G., C.K., S.C., M.C., M.H., G.D.-H.; Supervision: M.C., M.H., G.D.-H.; Funding acquisition: M.H., G.D.-H.

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Supplementary information

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