Effects of *Nosema apis*, *N. ceranae*, and coinfections on honey bee (*Apis mellifera*) learning and memory

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Western honey bees (*Apis mellifera*) face an increasing number of challenges that in recent years have led to significant economic effects on apiculture, with attendant consequences for agriculture. Nosemosis is a fungal infection of honey bees caused by either *Nosema apis* or *N. ceranae*. The putative greater virulence of *N. ceranae* has spurred interest in understanding how it differs from *N. apis*. Little is known of effects of *N. apis* or *N. ceranae* on honey bee learning and memory. Following a Pavlovian model that relies on the proboscis extension reflex, we compared acquisition learning and long-term memory recall of uninfected (control) honey bees versus those inoculated with *N. apis*, *N. ceranae*, or both. We also tested whether spore intensity was associated with variation in learning and memory. Neither learning nor memory differed among treatments. There was no evidence of a relationship between spore intensity and learning, and only limited evidence of a negative effect on memory; this occurred only in the co-inoculation treatment. Our results suggest that if *Nosema* spp. are contributing to unusually high colony losses in recent years, the mechanism by which they may affect honey bees is probably not related to effects on learning or memory, at least as assessed by the proboscis extension reflex.

Western honey bees (*Apis mellifera*) are ecologically and economically important pollinators world-wide, with pollination services contributing billions of dollars annually¹,². For reasons that appear multi-causal³–⁶, honey bee colonies have in recent years been suffering significant mortality in regions around the world, likely at an unprecedented rate⁷. Causes of mortality include pesticides, shortages of forage, improper management by beekeepers, and parasites⁸–¹⁵. Among the latter are two species of microsporidian fungi, *Nosema apis* and *N. ceranae*. Although many signs of pathology have been identified for infections with *N. apis* and *N. ceranae*, effects of these parasites on honey bee acquisition learning (hereafter, learning) and long-term memory recall (hereafter, memory) are not well studied. Wright (ref. 16) found that fungal infection by *Metarhizium anisopliae* was associated with both enhanced and impaired learning in honey bees, depending on a variety of other variables, including infection with *Nosema apis*. Here, we test directly whether *Nosema* spp. parasitism affects learning and memory in honey bees.

*Nosema apis* was the historic species infecting *A. mellifera* honey bees¹⁷, but probably early in this century, *N. ceranae* became an invasive parasite of *A. mellifera*, transferring from Asian honey bees *A. cerana*¹⁸–²³. Currently, *N. ceranae* essentially matches *N. apis*’s nearly global distribution²², and the two species can co-infect honey bees²⁴,²²,²⁵. Some theory predicts that co-infections select for increased virulence because of within-host competition for resources²⁶–²⁸. Although co-infections occur, *N. ceranae* has become the predominant species in many regions¹²,¹¹,²⁹,³⁰, which suggests that *N. ceranae* may be a better competitor than *N. apis*²⁴,³¹. This raises questions about the nature of differences between the two species; there is ongoing debate about which species is more virulent²²–³⁴, which could relate to competitive ability and explain higher mortality caused by *N. ceranae* than *N. apis*²²,³⁰.

Mortality from parasites may be a direct consequence of pathology to a host, or indirect wherein behaviour is modified. For example, parasitic infections can impair cognition, both in vertebrate¹⁵–³⁶ and invertebrate hosts³⁷–⁴².
were there differences within treatments (all were affected to a greater extent later on in infections as a consequence of cumulative pathology. Tests were performed 7 and 14 days post-inoculation (d.p.i.) to evaluate whether learning and memory were significantly affected in bees infected with both Nosema spp. treatments (all Kruskal-Wallis \( \chi^2 \) values \( > 8.8, P = 0.003 \)). At 14 d.p.i, there were no significant differences in spore intensities among treatments (Fig. 1).

There were no significant differences in learning or memory (both indexed by the number of positive PER responses; see methods) among treatments at either 7 or 14 d.p.i. (Table 1, Fig. 2). When all honey bees were pooled, learning 14 d.p.i. was significantly better than at 7 d.p.i. (Kruskal Wallis \( \chi^2 \) = 8.8, \( P = 0.003 \), but this pattern was not significant within treatment groups (all \( \chi^2 < 2.7 \), all \( P > 0.10 \)). When all honey bees were pooled, there was no significant difference in memory between bees tested at 7 versus 14 d.p.i (\( \chi^2 < 0.1 \), \( P = 0.95 \), nor were there differences within treatments (all \( \chi^2 < 2.3 \), all \( P > 0.13 \)).

Within Nosema treatments, two of 12 correlations between spore intensity and learning and memory were significant: Nosema spp.-infected bees with higher spore intensities learned better 14 d.p.i. whereas co-inoculated honey bees had reduced memory 14 d.p.i. if they had higher spore intensities (Table 2).
Initially N. ceranae inoculations produced lower spore intensities compared to the other Nosema treatments, but spore intensities were equivalent among Nosema treatments by 14 d.p.i. Regardless, we found no differences in

### Table 1.

Learning and memory (mean number of positive PERs in 8 trials for all bees within a treatment) did not differ among treatments 7 or 14 d post-inoculation. \( N \) is total number of bees tested in each treatment (for 8 conditioning and 8 extinction trials).

| Variable | Days post-inoculation | Treatment | Kruskal-Wallis statistics |
|----------|-----------------------|-----------|---------------------------|
|          |                       | Control   | N. apis | N. ceranae | Co-inoculation | \( \chi^2 \) | \( P \) |
| Learning | 7                     | 89        | 2.7     | 85         | 2.2           | 80       | 2.2 | 83       | 2.6 | 2.9 | 0.40 |
| Learning | 14                    | 68        | 3.4     | 64         | 2.7           | 38       | 2.6 | 46       | 3.2 | 4.7 | 0.20 |
| Memory   | 7                     | 82        | 0.6     | 62         | 0.5           | 65       | 0.6 | 69       | 0.8 | 2.9 | 0.41 |
| Memory   | 14                    | 58        | 0.6     | 48         | 0.5           | 28       | 0.3 | 37       | 0.4 | 5.1 | 0.17 |

### Table 2.

Within treatment Spearman correlations between spore intensities and learning and memory. Significant results in bold.

| Variable | Days post-inoculation | Nosema apis | Nosema ceranae | Co-inoculation |
|----------|-----------------------|-------------|----------------|----------------|
|          |                       | \( r_s \) | \( p \) | \( r_s \) | \( p \) | \( r_s \) | \( p \) |
| Learning | 7                     | 85        | 0.08 | 0.47 | 80 | 0.10 | 0.07 | 83 | 0.09 | 0.43 |
| Memory   | 7                     | 82        | 0.02 | 0.86 | 65 | 0.39 | 0.59 | 69 | <0.01 | 0.99 |
| Learning | 14                    | 64        | 0.21 | 0.02 | 38 | 0.14 | 0.39 | 46 | 0.09 | 0.57 |
| Memory   | 14                    | 48        | 0.10 | 0.90 | 28 | 0.11 | 0.56 | 37 | −0.32 | 0.05 |

### Discussion

Initially N. ceranae inoculations produced lower spore intensities compared to the other Nosema treatments, but spore intensities were equivalent among Nosema treatments by 14 d.p.i. Regardless, we found no differences in
learning or memory among treatments either at 7 or 14 d.p.i., at least within the PER paradigm we used. We did observe better learning with higher spore intensities within *N. apis*-infected bees at 7 d.p.i., and poorer memory within co-infected bees with increased spore intensity at 14 d.p.i. The former result may indicate greater hunger and therefore more responsiveness whereas the latter result supports the hypothesis that co-infections result in increased virulence. In 10 other tests, we observed no significant effects of spore intensity, so that on the whole we obtained limited evidence of effects of *Nosema* on learning and memory in honey bees, at least as assessed by PER.

Effects on learning and memory may vary with age, caste (e.g., nurse bee versus forager), satiation level, nutrition, experience, and genotype. Moreover, differences in responsiveness between two genotypic strains can occur within 0 to 2 d of emergence. However, bees in this study were all of the Buckfast genetic strain, were sampled from the same colonies, emerged within a day or two of each other, received the same food, and experienced similar conditions in the cage prior to PER trials and in the trials themselves. Thus, we reduced potential influences on responses to rewards and increased our ability to detect possible effects on learning and memory.

Learning and memory were not significantly related to spore intensity among or within treatments in bees 7 d.p.i. One explanation for this is that *Nosema* spores have not reached pathological levels by 7 d.p.i. This may explain why we observed no effects of spore intensity on honey bee learning and memory at 7 d.p.i., but did find some effects at 14 d.p.i.

Honey bees were tested at 7 and 14 d.p.i. to provide a range in spore intensity on which to test learning and memory and to evaluate effects of cumulative pathology. Temporal patterns we observed in spore intensities were consistent with other studies. At 12 d.p.i., Forsgren and Fries (ref. 24) found *N. ceranae* and *N. apis* spores had roughly equal intensities, possibly due to lack of space for more spores in the ventriculus. There is mixed evidence for whether one species of *Nosema* has a competitive advantage in co-infections.

Reduced learning and memory could arise if parasites interfere with neural signalling processes. Others have found that increased *Nosema* spp. spore intensity in bees was associated with energetic stress, which could affect neural signalling. In any case, increased consumption of food in response to parasitic infection is not uncommon in insects (but see ref. 57 for a review of vertebrates wherein anorexia is the dominant response to parasite infection). In previous studies, co-infected honey bees were significantly more responsive to sucrose and consumed significantly higher amounts, indicating increased appetite and overall hunger.

Parasitism can disrupt ecologically significant components of cognition in animals and impairments to learning and memory could have significant detrimental effects on honey bee colony survival. However, our results provide only limited and contradictory evidence that *Nosema* spp. infections have damaging effects on learning and memory.

**Methods**

**Source of spores.** When spores are frozen, *N. apis* has higher rates of infectivity than *N. ceranae*, therefore, frozen spores were used only to generate fresh spore stock for experimental inoculations (additional details in ref. 61). A spore homogenate was created from naturally infected dead, frozen honey bees collected in eastern Canada. Abdomens of 50 bees were added to 50 mL of distilled water and crushed using a mortar and pestle. Homogenate suspensions were vortexed and viewed under phase-contrast light microscopy using a haemocytometer to count spores (Hausser Bright-Line, 1/400 cm, 0.1 mm depth). Homogenate was diluted with distilled water and assessed repeatedly using a haemocytometer until an equal spore amount of 125,000 spores per μl was assessed by PER.

**Source of honey bees for generation of fresh spore stock.** Honey bees were collected from a colony in Coldbrook, Nova Scotia, Canada. Fifty honey bees were collected from hive entrances to first confirm that a colony was free of *Nosema* spp. To verify this, honey bees were freeze-killed, suspensions of their tissues created, and *Nosema* spp. spores counted, using a haemocytometer as above. In addition to being *Nosema*-free, colonies had not been treated chemically against *Nosema* spp. or Shorten to *V. destructor* mites, limiting potential for chemotherapies to affect learning or memory.

After confirming that honey bees were *Nosema* spp.-free, newly emerged honey bees from the same colony were used to generate fresh, even-aged spore stock for experimental inoculations. A frame with brood that was 2–3 d before eclosion was placed in a mesh bag in a nucleus box and immediately transferred to a temperature-controlled and humidity-controlled growth chamber, maintained at 33°C and 45 ± 2% RH. Approximately 50 newly emerged honey bees were placed into each of two rectangular 17 × 12 × 13 cm plywood cages with removable Plexiglas sides and a wire mesh top. Honey bees were provided sucrose solution (50% w/w in water) administered *ad libitum* through a plastic syringe suspended from the wire mesh top of the cage. Food was removed after 2 d and honey bees were starved overnight in preparation for inoculations.

**Inoculation of honey bees to generate fresh spore stock.** Honey bees were cooled in cages for ease of handling, grasped by the thorax with tweezers, and individually fed 5 μl of 50% w/w sucrose in water solution containing 125,000 spores per μl of either *N. apis* or *N. ceranae*. After force-feeding, honey bees were fed *ad libitum* on sucrose solution and, in the following days, individual honey bees were selected, freeze-killed, and spor species confirmed as above. New spore homogenate was kept at room temperature for no more than an hour and used to inoculate experimental honey bees. Fresh spores were obtained for subsequent inoculations by crushing honey bees that had gone through PER trials (see below).
Preparing honey bees for PER testing. Frames with capped brood were collected according to previously described techniques on four occasions between July and September 2010 to provide newly emerged honey bees for inoculation and PER testing. At each occasion, after emergence, 20 honey bees were transferred to one of eight cages (same construction as above) with two cages [7- and 14-d post-inoculation (d.p.i.) honey bees] for each of four treatment groups: control (uninoculated), N. apis, N. ceranae, or co-inoculation. Honey bees were provided sucrose ad libitum for 2 d and then starved overnight in preparation for inoculations. Treatment groups were force fed 3 μl sucrose solution and 2 μl spore solution containing equal numbers of fresh spores as described above, achieved by dilutions41,53,68,69. The co-inoculated group received 1 μl each of N. apis and N. ceranae combined with 3 μl sucrose solution. Control honey bees were given sucrose solution to feed on ad libitum53. All cages were kept in a growth chamber as described above.

Conditioning and extinction trials. PER trials were run both 7 and 14 d.p.i. to assess learning and memory. Food was removed from cages the night before testing and the following morning, honey bees were cooled in their cages in a 20 °C freezer70, just until no visible signs of movement could be detected. Each honey bee was then loaded into a modified 1000-μl clear pipette tip with the tapered end removed and a small piece of wax securing the honey bee in place, so only the head was exposed and antennae and mouth parts were free to move70. Honey bees were randomly selected relative to parasite treatment, and PER was evaluated blind to treatment to avoid bias71.

Each honey bee’s PER responsiveness to sucrose was checked by applying 1.5 M sucrose solution, delivered on a wooden toothpick, to its left antenna72,73. A honey bee with a positive PER response (extension of proboscis) was fed sucrose for 3 sec and then left in darkness for 3 h. Honey bees that failed to respond were not used further in PER testing72–74.

Each bee received continuous air flow for 15 sec to acclimate it to mechanosensory stimulation. A manual valve controlled continuous air flow and delivery of the stimulus odour; both united at a mixing chamber positioned 10 to 15 mm in front of a honey bee’s head. A vacuum system behind the honey bee continuously removed odour from the testing area and contributed to drawing air and odour over honey bee antennae. Air was dispensed at a rate of approximately 1.0 L per min. Honey bees that spontaneously extended their proboscis in the first trial of the learning phase (air flow or before presentation of sucrose) were taken out of the experiment because this response indicates a previously established odour/reward association70,73. In each conditioning trial, honey bees were presented with the odour geraniol followed by a sucrose reward. Geraniol is common in many plant oils and is produced by the Nasonov gland and used as an attraction signal in worker bees72,76. Additionally, floral odours are learned faster than other odours77 and thus, are often used in conditioning experiments. The conditioned stimulus was prepared by pipetting 3 μl of geraniol onto filter paper that was housed in a syringe44,74–76.

Immediately following the 15-sec acclimation period, the conditioned stimulus was delivered for 6 sec. Three seconds after the onset of odour, sucrose (unconditioned stimulus) was delivered to the left antenna using a wooden toothpick for 1 sec and then to the proboscis for 2 sec of feeding70,72. A positive PER was recorded when the mandibles opened and the proboscis extended in response to the odour but before sucrose delivery; this indicated a learned response. The interval between two successive trials was 9 min during which time we tested the other bees that had been prepared. There were 8 conditioning trials/bee so that a score of 8 indicated maximum conditioned a learned response. The interval between two successive trials was 9 min during which time we tested the other bees that had been prepared. There were 8 conditioning trials/bee so that a score of 8 indicated maximum learning. Following 8 trials, honey bees were fed satiation and kept in darkness at room temperature for 24 h. After these 24 h, 8 extinction trials were done to test memory; these were the same as conditioning trials but without a sucrose reward.

Individuals were scored for the number of times they exhibited PER in response to the odour in conditioning and extinction trials. Following extinction trials, honey bees were freeze-killed and spore counts carried out as above. Conventional PCR was completed on all honey bees in the co-inoculated treatment and a random sample of N. apis and N. ceranae treatments to confirm that no cross-contamination had occurred.

Statistical analyses. Statistical analyses were conducted in SAS version 9.3 (Cary, North Carolina). Data were not normally distributed (Kolmogorov-Smirnov tests for normality) even after transformations; thus, non-parametric tests of raw data were done. Spore intensities, learning, and memory were compared among treatments using Kruskal-Wallis tests, following up with Mann-Whitney U tests where significance was obtained. We tested whether learning and memory were related to spore intensity using Spearman’s rank correlations. Spore intensities, learning, and memory were compared among treatments to confirm that no cross-contamination had occurred.

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**Acknowledgements**

We thank Kevin Spicer for providing colonies from which bees were obtained, and Team Shutler for critical evaluations at all stages. Research was supported by a Natural Sciences and Engineering Research Council of Canada Industrial Postgraduate Scholarship to L.R.C. and to G.R.W. and Acadia University Research Funds to G.R.W. We appreciate the input of a number of reviewers.

**Author Contributions**

L.R.C., D.S. and G.R.W. developed ideas and also methods with N.K.H., all authors were involved in applying for funding. L.R.C. conducted the bulk of the data collection, L.R.C. and D.S. analyzed the data, and L.R.C., N.K.H., G.R.W. and D.S. were involved in writing and editing.

**Additional Information**

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Charbonneau, L. R. et al. Effects of *Nosema apis*, *N. ceranae*, and coinfections on honey bee (*Apis mellifera*) learning and memory. *Sci. Rep.* 6, 22626; doi: 10.1038/srep22626 (2016).

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