



# A novel bee host cannot detect a microbial parasite, in contrast to its original host

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## Abstract

Organisms that can detect parasites may have a greater likelihood of avoiding exposure to them. We would expect hosts that share an evolutionary history with a parasite to be more likely to detect and avoid it compared to novel hosts. *Nosema ceranae* is a gut parasite of the Asian honey bee, *Apis cerana*, that has relatively recently been detected in the western honey bee, *Apis mellifera*. Using a Proboscis Extension Response assay, we found that *A. cerana* was significantly more likely than *A. mellifera* to avoid sucrose solutions with concentrations above  $1 \times 10^6$  *N. ceranae* spores per mL. However, neither species avoided the sucrose solutions with lower *N. ceranae* concentrations, similar to those detected on flowers.

**Keywords** *Apis cerana* · *Apis mellifera* · Behavior · *Nosema ceranae* · Parasite detection · Proboscis extension response

Parasitic infection can drive the evolution of host defense mechanisms to decrease the effects of infection (Hall et al. 2011). While hosts usually display a wide array of these defense mechanisms, they can also prevent infection by detecting the presence of a parasite, recognizing it as a threat, and avoiding contact. This suite of behaviors might be more likely to be displayed by a host that has a shared evolutionary history with a parasite than by a novel host.

*Nosema ceranae* is a gut microsporidian of the Asian honey bee, *Apis cerana*, that has relatively recently been detected in the western or European honey bee, *Apis mellifera* (Botias et al. 2012; Higes et al. 2006). *Nosema ceranae* reduces the lifespan of experimentally infected *A. mellifera* and *A. cerana* workers (Sinpoo et al. 2018) via changes in physiology and immunity (Paris et al. 2018), which can in turn affect colony health (Higes et al. 2008). *Nosema ceranae* can be effectively transmitted on flowers (Purkiss and Lach 2019), and therefore the ability of foraging workers to detect the parasite might enable them to reduce the risk of becoming infected.

We hypothesized that *A. cerana* would be more likely than *A. mellifera* to avoid *N. ceranae* due to its shared evolutionary history with the parasite. To test our hypothesis, we used a Proboscis Extension Response assay (Takeda 1960) and measured the responsiveness of bees to sucrose solutions with increasing concentrations of *N. ceranae* spores. Proboscis Extension Response assays are commonly used to test whether bees can discriminate between different olfactory stimuli (Hladun et al. 2012; Mustard et al. 2020; Raza et al. 2019; Takeda 1960). Social insects have a highly developed sense of olfaction that they use in association with hygienic behaviors to prevent the spread of infection in the colony (Gramacho and Spivak 2003; McAfee et al. 2017), and there is evidence that fungi and associated spores have distinctive smells (Yanagawa et al. 2010, 2012). We prepared fresh spore solutions daily as per Ferguson et al. (2018). Though we cannot rule out that other microorganisms, such as viruses, were present in the solutions, we used standard methods to manipulate only the spore concentration. We would expect that any other microorganisms that may be present would be randomly distributed in various concentrations in the different spore solutions and thus not affect the bees' response to *N. ceranae* in a confounding manner.

We used one hive per bee species and collected foraging bees as they exited. We tested a cohort of 20 bees each day for each bee species for 6 days. We restrained the bees in individual holders and starved them (1 h for *A. cerana* and 3 h for the larger *A. mellifera*), after which we contacted

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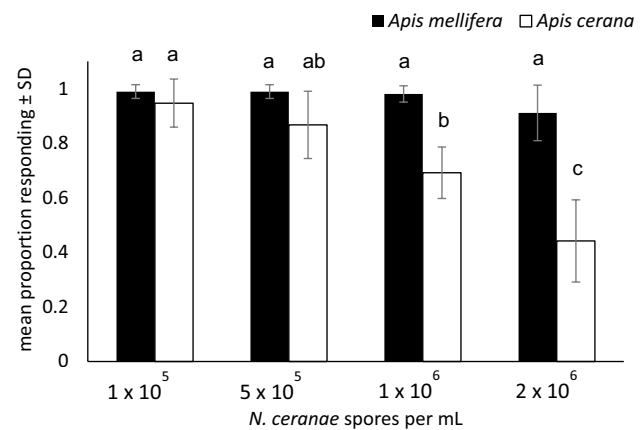
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the bees' antennae with a piece of filter paper soaked in 50% w:v sucrose solution. We interpreted extension of the proboscis as interest in consuming the solution (Reinhard 2019). The 14 *A. mellifera* and 23 *A. cerana* that did not respond to this initial stimulus appeared to be dying, most likely due to handling during capture and placement in the holders, and were removed from the trial, leaving 106 *A. mellifera* and 97 *A. cerana*. We then offered sucrose solutions with increasing concentrations of *N. ceranae* spores similar to the range of *N. ceranae* concentrations found on flowers (Purkiss and Lach 2019):  $1 \times 10^5$ ,  $5 \times 10^5$ ,  $1 \times 10^6$ , and  $2 \times 10^6$  spores per mL. Each spore solution was alternated with a spore-free 50% w:w sucrose solution to test the bees' continued motivation for sugar. Assayed bees were frozen until dissection to determine spore count with a hemocytometer (Fries et al. 2013).

We ran a generalized linear mixed model with binomial distribution with the package lme4 in R (Bates et al. 2015; R Core Team 2019) to test for differences in the responsiveness to the spore solutions between the two bee species. We used bee species, spore concentration, and their interaction as fixed effects. We used cohort as a random effect and conducted Tukey post-hoc pairwise comparisons with the package emmeans (Lenth 2019). Models that included spore presence or number as a covariate failed to converge, so we used a z-test comparison of proportions of bees that had *N. ceranae* spores among bees that responded to spore solutions and those that ceased responding. We did not test for differences in response to spore-free solutions over time because all bees responded to each spore-free sucrose solution offered in between spore solutions, and the proportion thus never varied (Choppin and Lach 2022).

The proportion of *A. cerana* responding to the spore solutions significantly decreased as the concentration of spores increased, whereas the proportion of *A. mellifera* responding did not vary significantly as spore concentration increased (Fig. 1, Table 1). All bees continued to extend their proboscis to each spore-free sucrose solution. The continued extension of the proboscis to spore-free solutions enables us to rule out habituation, which is characterized by the diminishing of a response to a repetitive stimulus (Raza et al. 2019). Neither species demonstrated sensitization to the solutions, which is defined by an increase in response to a stimulus following exposure to a strong but different stimulus (Raza et al. 2019). Altogether, we conclude that *A. cerana*, which is the original host of *N. ceranae*, is more likely than *A. mellifera* to detect and avoid high concentrations of spores.

The proportion of bees with spores detected in their guts did not differ between those that responded to all spore solutions (7/46 *A. cerana*, 11/96 *A. mellifera*) and those that stopped responding (5/51 *A. cerana*, 0/10 *A. mellifera*) for



**Fig. 1** Mean proportion  $\pm$ SD of bees that extended their proboscis in response to the different spore solutions for each bee species. Bars with different letters indicate significant differences within species comparisons at  $p < 0.05$

**Table 1** Summary of generalized linear mixed models and their Type 2 Wald chi-squared tests

Response and explanatory variables	$\chi^2$	df	p
Response to <i>Nosema ceranae</i>			
Species	64.5	1	<0.0001
Spore concentration	61.3	3	<0.0001
Species $\times$ Spore concentration	1.33	3	0.72

either bee species (*A. cerana*  $z = -0.81$ ,  $p = 0.42$ ; *A. mellifera*  $z = -1.13$ ,  $p = 0.26$ ).

The non-avoidance displayed by *A. mellifera* could have several explanations. *Apis mellifera* may have continued to extend its proboscis in response to solutions with high *N. ceranae* spore concentrations because it did not detect the spores in the solutions. Indeed, *A. mellifera* have fewer olfactory sensilla than *A. cerana*, which results in lower olfactory responses to floral volatiles (Jung et al. 2014). Moreover, *A. cerana* is more efficient than *A. mellifera* at removing ectoparasitic mites, mainly based on its superior olfaction (Peng et al. 1987). However, *A. mellifera* more often responds to a range of sucrose concentrations than *A. cerana* (Raza et al. 2019), so another possibility is that *A. mellifera* might have detected the spores and perceived them as a threat, but considered the sugar reward worth the risk (Desmedt et al. 2016). Varying the sucrose concentrations to determine if avoidance is more likely when sucrose concentration is low and whether *A. mellifera* is more risk prone as a species compared to *A. cerana* may elucidate whether *A. mellifera* is trading off risk for reward. A third plausible explanation is that *A. mellifera* sensed the spores but did not associate the smell of *N. ceranae* with a threat, and consequently did not avoid the

solutions containing the spores. This lack of threat recognition might arise from the shorter coevolution time of *A. mellifera* with *N. ceranae*.

We might expect that bees that were infected with *N. ceranae* and were experiencing disease may be more inclined to avoid spore solutions if they could detect the spores. The similarity of proportions of bees with spores detected in their gut tissues that continued to respond or stopped responding to spore solutions suggests that infection status is not a predictor of avoidance for either bee species. However, the mere presence of spores may not be indicative of disease, as even spore load is considered a poor indicator of the severity of *N. ceranae* infection (Zheng et al. 2014). A reliable non-destructive indicator of assessing *N. ceranae* disease state in live bees will be needed to test this hypothesis further. In our experiment, neither species would have had the opportunity to learn the risk of infection because spore solutions were not ingested, and the effect of *N. ceranae* on bee health is not immediate.

Neither bee species avoided the solutions with the lowest *N. ceranae* concentrations ( $1 \times 10^5$ ,  $2 \times 10^5$  spores per ml), although a dose of  $1 \times 10^5$  *N. ceranae* spores per mL fed directly to a bee of either species is considered sufficient to ensure 100% infection under laboratory conditions (Fries et al. 2013; Sinpoo et al. 2018). Our tested concentrations are similar to the range of *N. ceranae* spore loads detected on flowers (Purkiss and Lach 2019). It would be useful to determine whether the differences in responses to spore concentrations between species in the laboratory are reflected in bees' foraging behavior in their natural habitat.

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**Author contributions** MC and LL designed the study. MC collected and analyzed the data. Both authors interpreted the data, wrote the manuscript, and approved its final version.

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**Availability of data and material** The datasets generated during and/or analyzed during the current study have been deposited in the Research Data Australia repository and are available at <https://doi.org/10.25903/28xb-cy60>.

## Declarations

**Conflicts of interest/Competing interests** The authors have no relevant financial or non-financial interests to disclose.

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