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Short Communication

# Within-host competition among the honey bees pathogens *Nosema ceranae* and Deformed wing virus is asymmetric and to the disadvantage of the virus

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ABSTRACT

Two pathogens co-infecting a common host can either interact positively (facilitation), negatively (competition) or act independently. A correlative study has suggested that two pathogens of the honey bee, *Nosema ceranae* and Deformed wing virus (DWV), interact negatively within a host (Costa et al., 2011). To test this hypothesis, we sequentially co-infected honey bees with these pathogens in a reciprocally crossed experimental design. Prior establishment in the host ventriculus by *N. ceranae* inhibited DWV while prior infection by DWV did not impact *N. ceranae*, highlighting an asymmetry in the competitive interaction between these emerging pathogens.



## 1. Introduction

Co-infection of a host by multiple pathogens is widespread, particularly in social insects where transmission of microbes may be facilitated by the high density of individuals in a colony, high genetic relatedness between nest mates and frequent social interactions (Schmid-Hempel, 1998). Indeed, multiple pathogen infections are widely observed in honey bees (Cox-Foster et al., 2007; Ravoet et al., 2013; Runckel et al., 2011) and interactions between emerging pathogens have been considered a major cause of global colony mortality (Cornman et al., 2012; Doublet et al., 2014; Evans and Schwarz, 2011). Co-infecting pathogens may act independently of each other in the host, or interact positively, when one proliferates due to the presence of the other, or negatively, when pathogens suppress each other (Cox, 2001). In a recent study, Costa et al. (2011) observed in host honey bee ventriculi a negative correlation in pathogen loads between the microsporidian *Nosema ceranae* and Deformed wing virus (DWV), two emerging pathogens associated with bee mortality (Fürst et al., 2014; Higes et al., 2008; Nazzi et al., 2012). To explore putative competition between these pathogens in adult honey bee midguts, we performed sequential experimental oral infections (i.e. one pathogen after the other), giving a potential advantage of prior establishment to the first inoculated pathogen over the second, and compared their performance in terms of pathogen load per bee.

## 2. Material and methods

Five colonies of honey bees were used as a source of pupae, and bees were mixed in cages across treatments. Worker honey bees that emerged in the laboratory were kept two days in an incubator with 50% sucrose solution before inoculation. Pathogens were fed to individual bees in 10 µl of 50% sucrose solution. For each treatment, bees were fed twice, at day 2 and day 6 post emergence, for sequential feeding of pathogens. Two competition treatments were tested: Nosema/DWV treatment (N/D), where *N. ceranae* spores were fed first (day 2) and DWV second (day 6), and DWV/Nosema (D/N), where DWV was fed first and *N. ceranae* spores second (day 2 and day 6 respectively). Following the same nomenclature, four treatments with only one pathogen were used as controls: Control/Nosema (C/N), Nosema/Control (N/C), Control/DWV (C/D), and DWV/Control (D/C). Additionally, a double control treatment (C/C) was included, where bees were fed twice with a control solution. During the experiment, bees were maintained in metal cages, placed in an incubator at 30 °C ± 1 °C and 50% relative humidity, and fed with 50% sucrose solution *ad libitum*, as recommended by Williams et al. (2013). All treatments were run in triplicate, with 16 bees per cage.

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Spores of *N. ceranae* used for inoculations were obtained from artificial propagations in adult honey bees kept in the laboratory. Spores were isolated following a triangulation method (Fries et al., 2013) and counted using a Fuchs-Rosenthal hemocytometer. Each worker bee was inoculated with $10^5$ spores. DWV was obtained from symptomatic bees crushed in cold 0.5 M PBS (pH 8), filtered through cotton wool, and subsequently centrifuged at 4 °C for 15 min at 15,000*g*, before carefully extracting the supernatant and diluting in PBS (Bailey and Ball, 1991). This extract was then injected into uninfected pupae for propagation. After 6 days, injected pupae were crushed and viral particles extracted as above. Extracts were placed into clean aliquots and checked for the presence of DWV and co-propagation of other viruses using qRT-PCR (see Supplementary Table S1). Each inoculum contained $10^7$ genome equivalents of DWV, with a non-significant amount of Chronic bee paralysis virus (CBPV < 0.001%) contamination. Control inoculum was prepared from uninfected pupae and was devoid of viral contamination.

Nine days after the second pathogen feeding and before the onset of significant mortality, five bees were randomly sampled per treatment per replicate (except for the third N/C replicate with only 2 surviving hosts). Sampled bees were flash-killed in liquid nitrogen and conserved in RNAlater ICE (Ambion, USA) at −20 °C. Total RNA from individual midguts was extracted using an RNeasy Mini Kit in a Qiacube robot (Qiagen). Pathogens and reference gene RP49 were quantified by qRT-PCR, using standard 10-fold dilutions of cloned fragments for absolute quantification (see Supplementary Table S1). Relative quantification of DWV was calculated by the ratio of DWV to RP49 copy numbers. The potential presence of other co-occurring pathogens in experimental samples was examined using RT-qPCR and reverse-transcriptase multiplex-ligation probe dependent amplification (RT-MLPA; De Smet et al. (2012)). Amplified fragments from RT-MLPA were visualized on a QIAxcel (Qiagen) with an acceptance threshold of 0.1 relative fluorescence units. Six bees with unsuccessful *N. ceranae* infections (three from N/C and three from N/D treatments) and two bees with *N. ceranae* contamination (C/D treatment) were discarded from the analysis, as well as one bee from the D/N treatment with unsuccessful viral infection. To estimate *N. ceranae* spore number in bees, a linear regression between qPCR Cq values and actual spore numbers in a bee midgut was calculated using five randomly selected bees ($y = -11.773x + 109.35$; $R^2 = 0.95$).

## 3. Results and discussion

Inoculated bees developed infections (Figs. S1–S3). Sequential inoculation of worker honey bees by *N. ceranae* and DWV revealed an asymmetric competitive interaction between the two pathogens. Inoculation by DWV had no impact on the load of *N. ceranae* spores in a honey bee's midgut, both when the virus was inoculated before (Figs. 1 and S1; Mann–Whitney with 2-tailed Monte-Carlo correction $U = 94$; $p = 0.653$) or after the microsporidian ($U = 46.5$; $p = 0.621$). Conversely, prior establishment of *N. ceranae* had a significant negative impact on the load of DWV (Fig. 2; $U = 41$; $p = 0.047$; see Supplementary Figs. S2 and S3). Though *N. ceranae* also seemed to inhibit DWV titres when the microsporidian was inoculated after the virus, the effect was not significant ($U = 64$; $p = 0.079$). No other viral pathogen was associated with experimental treatments (see Supplementary Table S2).

Competitive suppression of DWV by *N. ceranae* may be due to direct competition of pathogens for host resources or space in the midgut. Indeed, *N. ceranae* induces a degeneration of the epithelial gut cells and reduces their capacity to self-repair (Dussaubat et al., 2012). This suggests that biological cell functions are compromised by *N. ceranae*, and microsporidian-infected cells might not be

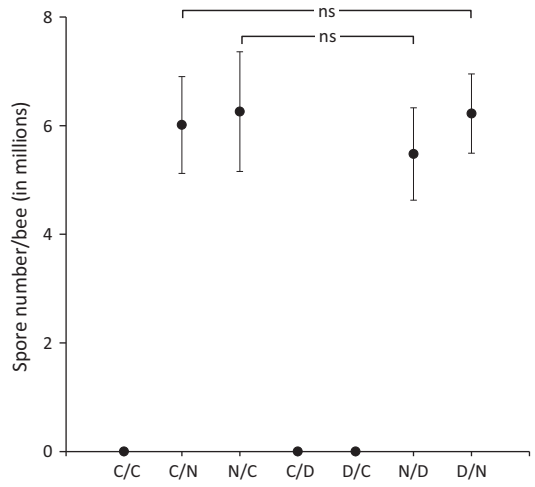


**Fig. 1.** Average estimated number of *N. ceranae* spores (±sem) per honey bee midgut from the seven treatments. Abbreviations of the treatments are on the *x*-axis in chronological order of infection at day 2 and day 6 post-eclosion: C = control, N = *N. ceranae*, D = DWV. As an example, treatment C/N means that bees first received a sugar solution then, four days later, a sugar solution with *N. ceranae* spores.

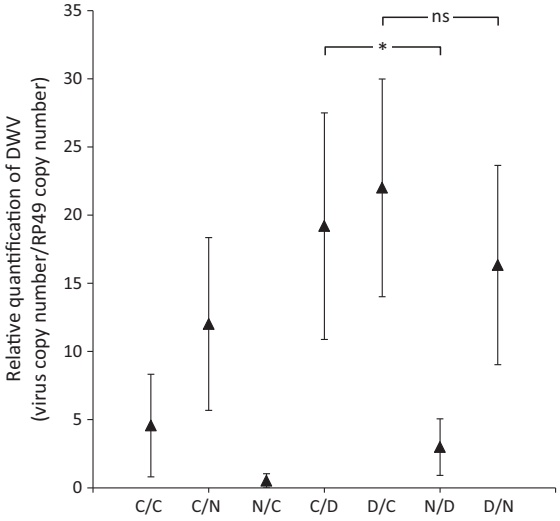


**Fig. 2.** Average relative quantification of DWV (±sem) per honey bee midgut from the seven treatments (reference gene: RP49). Abbreviations of the treatments are on the *x*-axis in chronological order. C = control, N = *N. ceranae*, D = DWV (see also legend to Fig. 1).

suitable for RNA virus replication, or that *N. ceranae* infection limits the number of host cells available for viral infection, thereby limiting viral load. Conversely, viral infection does not seem to reduce the susceptibility or suitability of host cells for microsporidian infection.

Alternatively, suppression of DWV by *N. ceranae* might be mediated by immune priming of the host. Although, *N. ceranae* has been shown to induce immune suppression in honey bees (Antúnez et al., 2009; Aufauvre et al., 2014; Chaimanee et al., 2012), recent transcriptomic and proteomic studies demonstrate that *N. ceranae* infection is associated with oxidative stress in the ventriculus, which may constitute the main cellular immune response of the honey bee midgut to microsporidia, and potentially responsible of the cellular damage of the gut epithelium (Dussaubat et al., 2012; Vidau et al., 2014). In the mosquito *Aedes*