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Evidence of the synergistic effect of honey bee pathogens *Nosema ceranae* and *Deformed wing virus*

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Running Head: Synergistic effect of *Nosema ceranae* and DWV

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Abstract

Nosema ceranae and *Deformed wing virus* (DWV) are two of the most prevalent pathogens currently attacking Western honey bees, *Apis mellifera,* and often simultaneously infect the same hosts. Here we investigated the effect of *N. ceranae* and *Deformed wing virus* (DWV) interactions in infected honey bees under lab conditions and at different nutrition statuses. Our results showed that *Nosema* could accelerate DWV replication in infected bees in a dose-dependent manner at the early stages of DWV infection. When bees were restricted from pollen nutrition, inoculation with $1*10^4$ and $1*10^5$ spores/bee could cause a significant increase in DWV titer, while inoculation with $1*10^3$ spores / bee did not show any significant effect on the DWV titer. When bees were provided with pollen, only inoculation with $1*10^5$ spores / bee showed significant effect on DWV titer. However, our results also showed that the two pathogens did not act synergistically when the titer of DWV reached a plateau. This study suggests that the synergistic effect of *N. ceranae* and DWV is dosage- and nutrition- dependent and that the synergistic interactions between the two pathogens could have implications on honey bee colony losses.

Keywords: *Apis mellifera*, *Nosema ceranae*, *Deformed wing virus*, synergistic effect, pollen

1. Introduction

Due to the importance of honey bees, *Apis mellifera*, as pollinators of many crops, large-scale losses of honey bee colonies in some countries in recent years have attracted the attention of both the scientific community and the public (Neumann and Carreck, 2010). There is currently insufficient evidence to pinpoint the exact factor as the cause of the complex problems. Nevertheless, several possible contributing factors have been suggested to be responsible for colony losses, either acting solely or in combination (Neumann and Carreck, 2010). Of the factors proposed to be responsible for colony losses, *Deformed wing virus* (DWV) and *Nosema ceranae* emerged as two of the key pathogens negatively impacting bee health and are often reported to be implicated in colony declines (Dainat et al., 2012; Higes et al., 2009; Martin et al., 2012).

Among viruses attacking honey bees, DWV is the most common and prevalent infection in honey bee colonies (Allen and Ball, 1996). The association of DWV with the parasitic mite *Varroa destructor,* was reported to be responsible for the death of millions of honey bee colonies, and has become the most significant threat to apiculture worldwide (Martin et al., 2012). DWV causes overt symptoms of wing deformity resulting in emerging bees that are unable to fly. In the asymptomatic bees, DWV can affect learning behavior, aggressiveness and lifespan. The ~~clinical signs of~~ DWV infection has been suggested as a predictive marker for honey bee colony losses (Dainat and Neumann, 2013). DWV is now proposed to be the most likely candidate responsible for the majority of colony losses (Schroeder and Martin, 2012).

N. ceranae, an emerging microsporidian parasite that causes the serious disease in honey bees known as nosemosis that is characterized by digestive tract problems and consequently metabolic disorders. For decades, nosemosis of European honey bees was exclusively attributed to a single

55 species of Nosema, *N. apis*. In 2005, a natural infection of *N. ceranae*, a species of Nosema
56 which was first found in the Asian honey bee *A. cerana*, was identified in *A. mellifera* colonies in
57 Taiwan (Huang et al., 2007). Shortly thereafter, the infection of *N. ceranae* in *A. mellifera* was
58 reported worldwide (Chen et al., 2008; Higes et al., 2006; Klee et al., 2007) and the disease
59 caused by *N. ceranae* in honey bees was found to be far more prevalent than that caused by *N.*
60 *apis* (Higes et al., 2009). The infection of *N. ceranae* has impacts at both the individual honey
61 bee and colony levels and has been associated with honey bee colony losses (Currie et al., 2010;
62 Higes et al., 2008a) although the impact of this parasite on colony health in some other countries
63 still remains controversial (Genersch et al., 2010; Gisder et al., 2010).

64 A synergistic effect between DWV and *N. ceranae* is highly plausible, since honey bees have
65 often been reported to harbor two pathogens simultaneously (van Engelsdorp et al., 2010). *N.*
66 *ceranae* is a pathogen that causes extensive damage to the midgut epithelial ventricular cells
67 (Fries, 2010). This infection could then create access for other pathogens such as DWV that
68 could be spread by fecal–oral transmission (Chen and Siede, 2007), to pass across the midgut
69 protective barrier and get into the haemolymph. Moreover, it has also been shown that *N.*
70 *ceranae* can actively suppress the immune response in honey bees (Antúnez et al., 2009),
71 making *N. ceranae*-infected colonies more susceptible to viral infections.

72 The present study examined the effects of *N. ceranae* and DWV interactions in the co-infected
73 bees under different infective doses of pathogens and nutritional conditions. We provided
74 evidence of the synergistic effect of *N. ceranae* and DWV in infected bees, however, the
75 synergistic interactions between the two pathogens is dosage- and nutrition- dependent. Our
76 results also showed that the two pathogens did not act synergistically when the titer of DWV
77 reached a plateau.

2. Materials and Methods

2.1 Colony selection

Colonies maintained in the experimental apiary at the USDA-ARS Bee Research Lab, Beltsville, Maryland, USA, were monitored and regularly treated for *Varroa* mite infection , which is positively associated with virus titersin honey bee colonies (Yang and Cox-Foster, 2005). Colonies without any symptoms of *Varroa* infestation were surveyed for *Nosema* infection using a routine spore counting method (Shimanuki and Knox, 2000) and PCR analysis to confirm species status (Chen et al., 2009a). The result of PCR analysis showed that only *N. ceranae* was present in the examined bee colonies in our study. The colonies with infections higher than $5*10^6$ spores per bee on average were selected as colony sources for spore purification. Colonies with undetectable *Nosema* infection were selected for honey bee virus detection. For each colony, thirty adult workers were randomly sampled and pooled for RNA extraction. The presence of seven honey bee viruses, namely Acute bee paralysis virus (ABPV), Black queen cell virus (BQCV), Chronic bee paralysis virus (CBPV), DWV, Isreali acute paralysis virus (IAPV), Kashmir bee virus (KBV), Sacbrood bee virus (SBV), were checked by RT-PCR using previously reported primers (Ai et al., 2012). Colonies infected with DWV, but not any other viruses, were selected for further analysis. Ten newly emerged bees from each colony were subjected to the quantification of DWV individually by qRT-PCR method To ensure bees used in each experiment had approximately an equal amount of DWV infection from the beginning, only those colonies with variation of relative titer of DWV less than 2.0 among the 10 sampled bees were selected as sources of experimental bees.

2.2 Spore purification and bee preparation

N. ceranae spores were isolated by Percoll centrifugation method (Chen et al., 2009b). After dissecting the intestinal tracts, the midguts were macerated in distilled water using a manual tissue grinder followed by the suspension being filtered through a No. 4 Whatman filter paper. The resulting suspension was cleaned by centrifugation with a Percoll gradient at 3,000g three times and finally resuspended in distilled water. The spore concentration was determined by counting with a hemocytometer chamber and the suspension was prepared for use by mixing with 50% (v/v) sucrose syrup.

Frames of sealed brood obtained from two selected colonies with detectable DWV infection, but undetectable *Nosema* infection, were kept in an incubator at 34 ± 2^{o}C to provide newly emerged workers. During a 12-hour period, the frames were checked every hour to collect newly emerged workers to decrease the possibility that the bees would consume pollen or honey from the frames, which could affect our experiment by altering the bees' nutrition status and / or, with low possibility, infect the bees with *Nosema* spores (Higes et al., 2008b). Bees observed being parasitized by *Varroa* mites, which were encountered very occasionally, were excluded. Worker bees were individually fed using a syringe with 2 μl of 50% sucrose syrup containing a specific amount of spores for inoculation. The bees that did not consume the entire droplet were discarded.

2.3 Experimental setup

In the first experiment, four groups were set up (Table S1). Two of them were inoculated with $1*10^4$ spores per bee. The other two served as controls. In the second experiment, eight groups were set up with 2 groups serving as controls (Table S1). The rest were divided into groups of two inoculated with $1*10^3$, $1*10^4$ or $1*10^5$ spores per bee. Each group was composed of three replicates of 30 honey bees in each cage. They were kept in an incubator at 30 ± 2^{o}C, 70 ± 5%

RH and fed *ad libitum* with 50% sucrose syrup solution. To compare the effects of different nutrition status, one of the two groups, in either test or control groups, was additionally provided with pollen in a 1.5ml Eppendorf tube. To avoid viable *Nosema* spores (Higes et al., 2008b) and / or other pathogens contaminating the pollen, pollen freshly collected at the hive entrances with pollen traps were treated with UV-light. For the treatment, pollen was ground into fine powder, spread into petri-dishes in thin layers and exposed to UV-light for 12 hours. During this period, pollen was stirred every 2 hours to allow complete exposure.

Dead bees were removed daily and sucrose syrup was changed every two days. Five bees were collected from each cage on days 2, 4, 6, 8, 12 in the first experiment and days 2, 4, 8 in the second experiment for DWV quantification.

2.4 RNA extraction and qRT-PCR analysis

Total RNA was isolated from individual bees using TRIzol® Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Briefly, each bee was homogenized in 1ml TRIzol Reagent, shaken vigorously for 30 seconds and then incubated at room temperature for 3 minutes. Following precipitation and centrifugation, the resultant RNA pellets were resuspended in 250µl nuclease-free water. The RNA concentration was measured using a NanoDrop™ spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The titer of DWV was quantified by one-step SYBR Green real-time qRT-PCR. The expression of a housekeeping gene, β-actin, in each sample was also measured for normalization of virus quantification results. The primer pairs for DWV and β-actin were previously reported (Prisco et al., 2011). RT-PCR reactions were carried out in a 20-µl reaction volume, containing 10 µl of 2× Brilliant® SYBR® Green QRT-PCR Master Mix (Stratagene, La Jolla, CA), 0.4 µM each of forward and reverse primers, and 250 ng of template RNA. The thermal profile for the

one-step RT-PCR was as follows: one cycle at 50°C for 30 min, one cycle at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 sec, 55 °C for 45s, and 72 °C for 30 sec. After amplification, a dissociation curve was constructed using 81 complete cycles of incubation where the temperature was increased by 0.5°C/cycle, beginning at 55°C and ending at 95°C to verify presence of a single product. Negative controls (no template) were included in each run of the reaction and yielded no products. *Nosema* infection of each bee was verified by qRT-PCR using *N. ceranae* primers (Chen et al., 2009a). qRT-PCR was replicated three times for each sample to address the variability of the analysis process. The relative quantity of DWV and *N. ceranae* was calculated by subtracting the cycle threshold (Ct) of DWV from Ct of the reference gene (β-actin) (Chaimanee et al., 2012), averaged between runs and compared between groups.

2.5 Statistical analysis

Data sets were subjected to descriptive analysis and outliers exceeding 1.5 times the length of the box away from either the lower or upper quartiles were excluded. Normality of data sets was tested using the Shapiro-Wilk test. Data sets meeting normal distribution were compared with the parametric student's t-test. Non-normal distribution data sets were analyzed using the lower powered nonparametric Mann-Whitney U test. Data is shown in average±SE. p-values below 0.05 were considered significant.3. Results

3.1 DWV proliferation in cage bees

All of the individual bees tested at day 0 had detectable DWV in our study, which enabled us to conduct the study without additionally inoculating virus. Our data showed ready proliferation of DWV in the cage bees. The relative quantity of DWV increased from -16.33±1.03 at day 0 to 7.00±0.21 (calculation of four groups) at day 6 in the first experiment (Figure 1), which demonstrated an approximate 10 million fold change in the 6 days. Minor increase was observed

from day 6 to day 12, showing a plateau period after day 6. Despite DWV titers at day 0, 2, 4 in the second experiment being relatively higher than those at corresponding days in the first experiment, the values at day 8 were similar in both experiments (1^{st} vs 2^{nd} experiment: 7.51±0.17 VS 7.33±0.15), suggesting that a plateau period existed in both trials.

3.2 Effect of *N. ceranae* infection on DWV

Quantification of *N. ceranae* revealed that the level of Nosema infection increased after inoculation in our experiments (Figure S1), which ensured the effect, if any, that *Nosema* infection in honey bees was established. During the cage experiments, no server mortality occurred as only one to five bees died in each cage. The mortality rate was not significantly different amongst the groups (Kruskal-Wallis H, $p>0.05$).

The effect of *Nosema* infection on DWV titer could be observed at day 4 in the first experiment. When bees were restricted from pollen, *Nosema* infection significantly increased DWV titer ($p<0.001$). While, when they were supplemented with pollen feeding, there was no significant difference between *Nosema*-infected bees and non-infected bees on DWV titers ($p=0.58$). However, a significant difference existed among *Nosema*-infected bees when they were fed with and without pollen ($p=0.002$), indicating pollen feeding compensated the effect of *Nosema* infection, which was also supported by the significant difference observed at day 8 on *Nosema*-infected bees fed with and without pollen ($p<0.001$). No significant difference was found between non-infected bees fed with or without pollen at any other time point, including comparisons at days 2, 6, and 12.

3.3 Dosage effect of *N. ceranae* infection on DWV

With a higher titer of DWV at day 0, bees in the second experiment had relatively higher DWV titers at day 2 and 4 compared with those in the first experiment. The significant difference

193 between *Nosema*-infected and non-infected bees occurred as early as two days after inoculation
194 (Figure 2). When bees were not fed with pollen, significant increases on DWV titer were found
195 when they were inoculated with $1*10^4$ and $1*10^5$ spores/bee (p=0.001; p=0.001). The increase
196 of DWV titer was not significant when bees were inoculated with $1*10^3$ spores/bee (p=0.411).
197 When they were supplemented with pollen, significant increase in DWV titer could only be
198 found when bees were inoculated with $1*10^5$ spores/bee (p=0.036). Bees inoculated with $1*10^3$
199 and $1*10^4$ spores/bee had relatively higher DWV titer, while the differences were not significant
200 compared with the control groups (p=0.265; p=0.072). No significant difference could be
201 observed in any comparison at day 4. At day 8, bees inoculated with $1*10^5$ spores/bee were
202 found to have significantly higher DWV compared with those inoculated with $1*10^3$ spores/bee
203 when they were fed with pollen (p=0.045), although the increase was not significant when they
204 were compared with control groups. Due to the variation among different runs of qRT-PCR and
205 the absence of using standard samples in different runs, groups between pollen and non-pollen
206 groups were not compared in the second experiment since they were not tested in the same runs.
207

207 Discussion

208 Synergistic effects between various pesticides and the microsporidian parasite *N. ceranae*,
209 were found to increase honey bee mortality in laboratory assays (Alaux et al., 2010a; Pettis et al.,
210 2012). Although the potentially synergistic effect between DWV and *N. ceranae* had been tested
211 before, the idea had not been supported by previously published experimental results. Costa and
212 colleagues (2011) found no significant correlation between *N. ceranae* and DWV at the whole
213 bee level (Costa et al., 2011). In the midgut they also found no evidence of synergistic effects,
214 but possibly an antagonistic effect, since a negative correlation between *N. ceranae* and DWV
215 was found. In a field scale study, any synergistic effect between DWV and *N. ceranae* was
216 lacking (Hedtke et al., 2011). Dussaubat and colleagues (2012) also mentioned they found no
217 synergistic effect between DWV and *N. ceranae* Martin and colleagues (2013) reported that
218 there was no significant difference in spore counts between colonies infected with DWV and
219 colonies in which DWV was not detected and that there was no significant correlation between
220 DWV loads and *N. ceranae* spore counts found (Martin et al., 2013). However, synergistic
221 effects between *N. apis* and several honey bee viruses such as filamentous virus (FV), bee virus
222 Y (BVY) and BQCV was reported (Bailey et al., 1983). More recently, it was reported that *N.*
223 *ceranae* and CBPV act synergistically on CBPV replication in winter bees (Toplak et al., 2013).
224 Given the high prevalence of *N. ceranae* and DWV in the array of pathogens infecting honey
225 bees, we present important insight into their pathological effect. As previously done by Costa
226 and colleagues (2011), we tested the potential effect of *N. ceranae* infection on DWV
227 proliferation in laboratory conditions. However, instead of comparing at one time point, we
228 compared at five different time points post inoculation, which was proved to be vital to
229 demonstrate any significant differences. DWV titer increased readily with millions folds change

in cage bees and reached a plateau within a few days. Bees with different backgrounds may take different times to complete the changes and significant differences may only be detectable at different time points in different trials, as indicated by the comparison between the two experiments in our study. Sampling at the time points when DWV titer has reached the plateau, any effect of *Nosema* infection could be masked. This highlights the importance of establishing controlled experimental designs across empirically determined informative time points in attempts to clearly characterize the relationship between the two pathogens, which was also suggested by Schwarz and Evans (2013) for honey bee immunity studies (Schwarz and Evans, 2013).

Pollen is the most nutritive food for bees, providing sufficient proteins, minerals and other nutrients. It is essential to the health of individual bees and colonies. Pollen is not only the principal protein source to bees but increases the honey bee immune system (Alaux et al., 2010b), thereby increasing honey bee resistance to diseases and other stresses,. Our results showed pollen feeding could reduce the effects of *Nosema* infection on DWV titer when bees were inoculated with $1*10^5$ spores per bee, despite pollen feeding increasing *N. ceranae* spore production of honey bees by several times (Zheng et al., 2014). Field bees normally have free access to pollen. Moreover, the dosage of $1*10^5$ spores per bee is relatively high since the spore counts in infected bees reached 37.6 million on average, 8 days post inoculation which is rarely seen in field conditions (Martin et al., 2013). These give explanations as to why no synergistic effect had been found in field bees so far.

A bimodal distribution of normalized levels of DWV was observed in honey bee pupae in a previously reportedstudy (Moore et al., 2011). In our study, normalized DWV levels were not

252 distributed in a bimodal pattern (Figure S2). The difference between our results and the previous
253 finding may be due to the fact that adult bees instead of pupae were used in our study.

254 Both DWV and *N. ceranae* are known to cause negative impact on honey bee health. Our
255 results suggested that DWV and *Nosema* could act synergistically in infected colonies but that
256 the synergistic effect is dosage- and nutrition- dependent.

257

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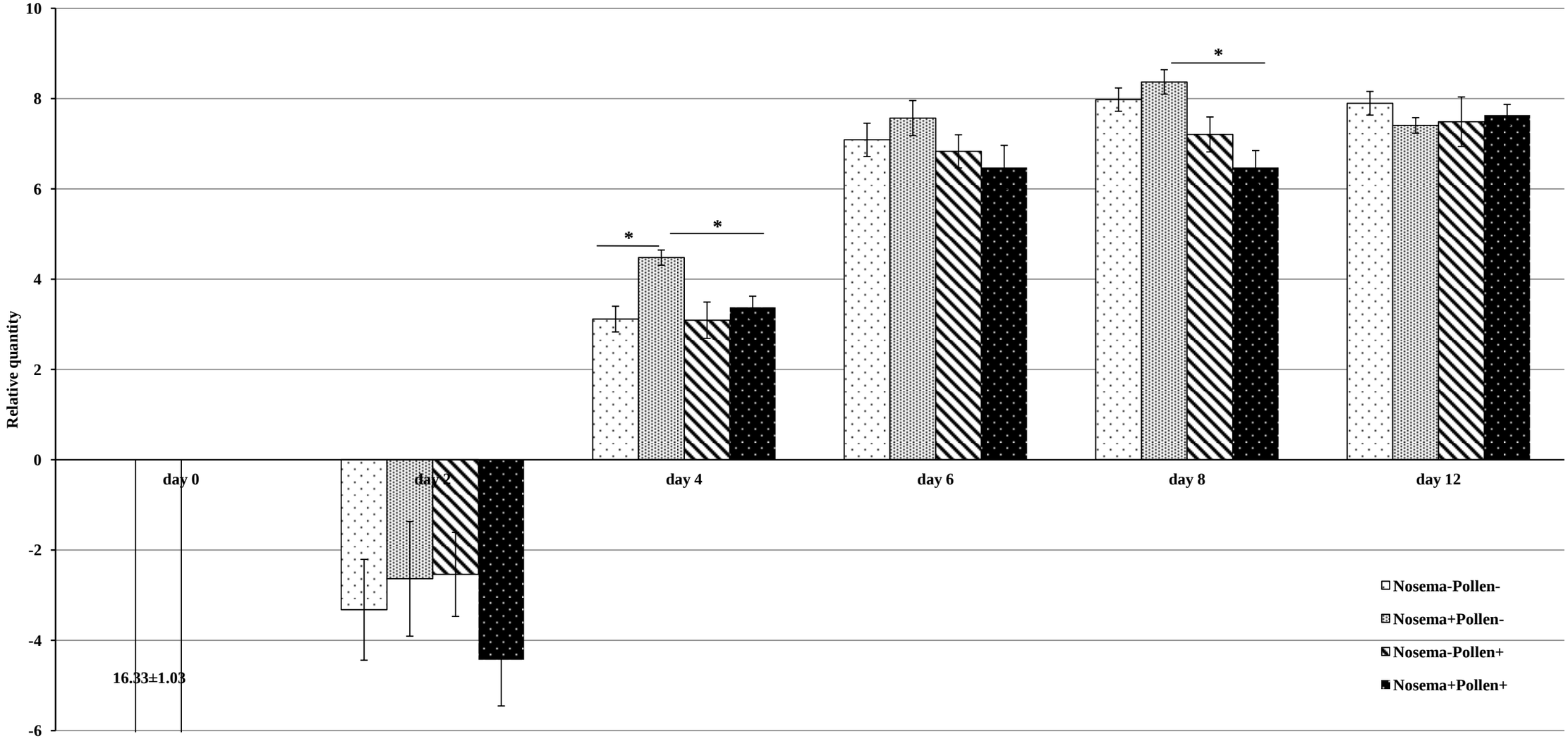
Figure captions

Figure 1. Effect of *Nosema* infection on honey bee DWV proliferation with / without pollen feeding. The relative quantity of DWV (y-axis) was calculated by subtracting the cycle threshold (Ct) of DWV from Ct of the reference gene. The results are based on the data of experiment 1. "*" indicates $p<0.05$.

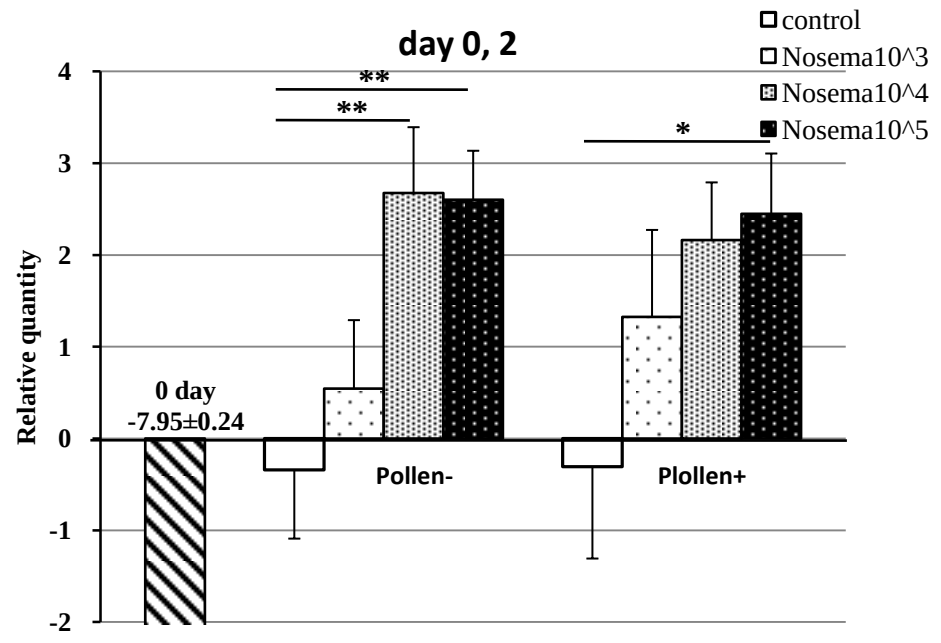
Figure 2. Dosage effect of *Nosema* infection on DWV titer at different nutrition statuses. The relative quantity of DWV (y-axis) was calculated by subtracting the cycle threshold (Ct) of DWV from Ct of the reference gene. The results are based on the data of experiment 2. Please note the different scale between figures. "*" indicates $p<0.05$; "**" indicates $p<0.01$.

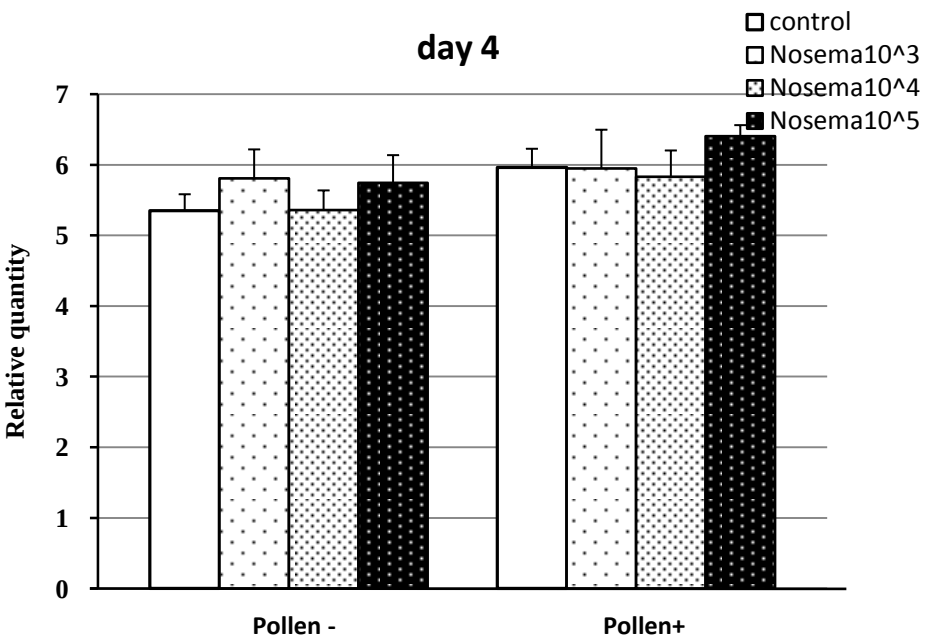
1. DWV titer increased with millions fold change and reached a plateau in only a few days in cage bees.
2. *Nosema* could induce DWV replication in infected bees in a dose-dependent manner.
3. The synergistic effect of *N. ceranae* and DWV may have implication on honey bee colony losses.

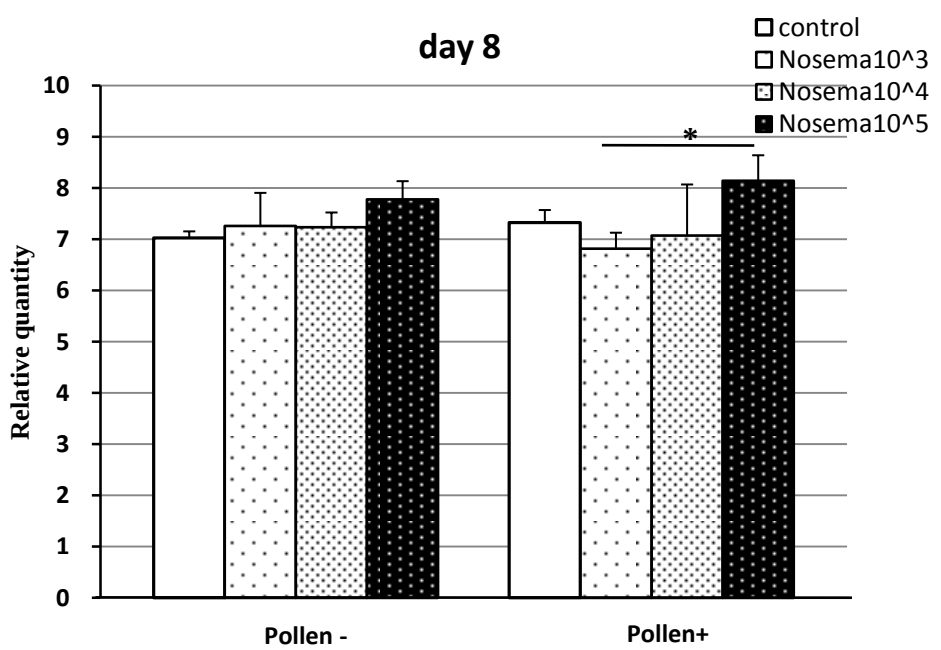
Figure


10
8
6
4
2
0
-2
-4
-6
Relative quantity
day 0
day 2
day 4
day 6
day 8
day 12
*
*
*
16.33±1.03
Nosema-Pollen-
Nosema+Pollen-
Nosema-Pollen+
Nosema+Pollen+

Figure


day 0, 2
control
Nosema10^3
Nosema10^4
Nosema10^5
Relative quantity
4
3
2
1
0
-1
-2
**
**
*
0 day
-7.95±0.24
Pollen-
Plollen+


day 4
control
Nosema10^3
Nosema10^4
Nosema10^5
Relative quantity
7
6
5
4
3
2
1
0
Pollen -
Pollen+


day 8
control
Nosema10^3
Nosema10^4
Nosema10^5
Relative quantity
10
9
8
7
6
5
4
3
2
1
0
*
Pollen -
Pollen+