Spore Loads May Not be Used Alone as a Direct Indicator of the Severity of *Nosema ceranae* Infection in Honey Bees *Apis mellifera* (Hymenoptera:Apidae)

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ABSTRACT *Nosema ceranae* Fries et al., 1996, a microsporidian parasite recently transferred from Asian honey bees *Apis cerana* F., 1793, to European honey bees *Apis mellifera* L., 1758, has been suspected as one of the major culprits of the worldwide honey bee colony losses. Spore load is a commonly used criterion to describe the intensity of *Nosema* infection. In this study, by providing *Nosema*-infected bees with sterilized pollen, we confirmed that pollen feeding increased the spore loads of honey bees by several times either in the presence or absence of a queen. By changing the amount of pollen consumed by bees in cages, we showed that spore loads increased with an increase in pollen consumption. *Nosema* infections decrease honey bee longevity and transcription of vitellogenin, either with or without pollen feeding. However, the reduction of pollen consumption had a greater impact on honey bee longevity and vitellogenin level than the increase of spore counts caused by pollen feeding. These results indicate that spore loads may not be used alone as a direct indicator of the severity of *N. ceranae* infection in honey bees.

KEY WORDS *Nosema ceranae*, spore load, pollen, longevity, vitellogenin

Honey bees play important ecological and economic roles as pollinators of many crops and wild plants (Calderone 2012). Since 2006, disastrous colony losses of managed honey bees, *Apis mellifera* L., 1758, have been reported in Europe and North America. Among factors that are suspected to contribute to bee losses, the infection of *Nosema ceranae* Fries et al., 1996 that recently made a host jump from Asian honey bees *Apis cerana* F., 1793, to *A. mellifera* impacts honey bees at both colony and individual levels (Higes et al. 2007, Paxton et al. 2007, Villa et al. 2013). *N. ceranae* infection has been reported to be associated with physiological and behavior changes in honey bees (Goblirsch et al. 2013) and with honey bee colony losses in several countries worldwide (Higes et al. 2009, Bacandritsos et al. 2010, Currie et al. 2010, Neumann and Carreck 2010). However, there is uncertainty whether colony losses in several countries from colder climates could be attributed to *N. ceranae* infection (Gisder et al. 2010, Hedtke et al. 2011, Stevanovic et al. 2013).

Nosema infection levels have traditionally been estimated based on the mean spore count per bee in a representative sample (Furgala and Hyser 1969, Shimanuki and Knox 2000). A close relationship between spore count and the degree of infection has been established for this disease and has been used as a parameter to evaluate a colony's need for treatment. However, the relationship between spore count and level of *Nosema* infection may not be correlated as previously thought (Doull 1965). In a field study, the spore count was not correlated with the health status of whole colonies that were naturally infected by *N. ceranae* (Higes et al. 2008b). Recently, Meana et al. (2010) proposed that spore count is not a reliable parameter of *N. ceranae* infection because the data may be affected by the hour and site of sampling (Meana et al. 2010).

As the most nutritious food for bees (reviewed in [Huang 2012]), pollen is essential to individual bee and colony development. Pollen limitation decreases brood rearing (Kleinschmidt and Kondos 1976) and worker longevity (Knox et al. 1971) which, ultimately, affects colony productivity. Better pollen nutrition leads to an enhanced immune response (Alaux et al. 2010), thus, increasing the resistance to other stresses, e.g., *Varroa* parasitism (Janmaat and Winston 2000) and pesticides (Wahl and Kurt 1983). In an early study, Rinderer and Elliott (1977) found that caged bees, provided with pollen, lived longer than those without pollen when they were infected with *Nosema*

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apis Zander, 1909 (Rinderer and Elliott 1977). Recently, this was also found to be true when bees were inoculated with *N. ceranae* spores (Porrini et al. 2011). In both studies, when the bees were provided with pollen, they exhibited significantly higher (two to six times) spore loads than those bees that did not feed on pollen. These results provided insight into the relationship between pollen feeding and *Nosema* spore counts. However, bee-collected pollen may be contaminated with *Nosema* spores during collection, providing a source of infection (Higes et al. 2008a). This possibility was not considered in prior studies, leading to possible artifacts.

In the current study, by using bee pollen sterilized by treatment with ultraviolet (UV) light, we investigated the effect of pollen on the development of *Nosema* spores to see whether the increase of spores was due to the contamination of *Nosema* spores in pollen and to see whether the effect of pollen consumption, if there any, on spore counts was dose-dependent. Further, we investigated the effect of pollen consumption on the expression of vitellogenin, a biomarker of general robustness, and life span of *Nosema*-infected bees.

Materials and Methods

Colony Selection. Colonies maintained in the experimental apiary of U.S. Department of Agriculture–Agricultural Research Service (USDA-ARS) Bee Research Lab, Beltsville, MD, were surveyed for *N. ceranae* infection with a routine spore count method (Shimanuki and Knox 2000). The colonies with infection levels higher than 5×10^6 spores per bee on average were selected as sources for spore purification and those with undetectable infections were selected to provide experimental bees.

***Nosema* Spore Purification and Inoculation.** *N. ceranae* spores were isolated by Percoll centrifugation (Chen et al. 2009). Whole midguts were macerated in distilled water using a manual tissue grinder. The suspension was filtered through No. 4 Whatman filter paper. The resulting suspension was further cleaned by centrifugation with a Percoll gradient at 3,000 g and resuspended in distilled water. The spore concentration was determined by counting with a hemocytometer chamber. The suspension for inoculation was freshly prepared before use with 50% (vol:vol) sucrose syrup.

Frames of sealed brood, obtained from four selected healthy colonies, were kept in an incubator at 34 ± 2°C to provide newly emerged *Nosema*-free honey bees. Young *Nosema*-free honey bees were starved for 2 h and individually fed using a syringe with 2 μl of 50% sucrose syrup containing 20,000 spores. Bees that did not consume the entire droplet were discarded.

Evaluation of the Effect of UV Radiation on *N. ceranae* Spore Viability. Freshly purified spores were diluted to the concentration of 1×10^7 spore per milliliter in water. A spore solution of 1 ml was spread into a petri dish (3 cm in diameter) and exposed to UV light (254 nm, 8 watt) for 0, 5, 15, 30, 45, or 60 min in a crosslinker chamber (UVP HL-2000 Hybrilinker, CA). The spore solution was recovered into a 1.5-ml tube and centrifuged at 5,000 rpm for 3 min, and then the pellet was suspended in 200 μl H_2O. Treatment under room conditions served as the controls. Spore viability was evaluated with LIVE/DEAD Sperm Viability Kit (L7011, Molecular Probes, Eugene, OR) by mixing the 200 μl spore solution with 8 μl propidium iodide and 1.5 μl SYBR 14 dye. The mixture was incubated in the dark at room temperature for 20 min. Ten microliter of the mixture was used to check the viability of spores. Live (green) or dead (red) spores were counted with a hemocytometer (Hausser Bright-Line) under a fluorescence microscope. The procedure was repeated five times and each sample was counted three times.

Effect of Pollen Feeding on *N. ceranae* Spore Development. Two kinds of multifloral bee-collected pollen were used in the experiment. One was freshly collected at hive entrances using pollen traps in the summer of 2012 and directly used without any storage (fresh pollen, FP). The other one was collected using pollen traps in the spring of 2009 and stored at 4°C until used (aged pollen, AP). Both pollen sources were confirmed to carry *N. ceranae* spores by PCR detection (Higes et al. 2008a). To kill the spores, the pollen was ground into fine powder, spread in petri dishes in thin (≈2 mm) layers, and exposed to UV light for 12 h. During this period, the pollen was stirred every 2 h to allow complete penetration of UV light.

In total, 10 groups were set up: five with bees fed with *Nosema* spores and five with no *Nosema* exposure. Each group was composed of three replicates with 30 honey bees in each cage. They were kept in an incubator at 30 ± 2°C and fed ad libitum with 50% sugar syrup solution or 50% sucrose syrup solution with one of the following additives: fresh pollen without UV treatment (FP), fresh pollen with UV treatment (UVFP), aged pollen without UV treatment (AP), or aged pollen with UV treatment (UVAP). At day 12 and 18, three bees from each cage were sampled for spore counting. Dead bees were removed daily. The number of bees that died between day 12 and day 18, post infection, were counted ($N = 11 \approx 18$ for each group). To obtain the *Nosema* spore counts, the whole abdomen from each bee was placed into a sterile Eppendorf microtube and thoroughly ground. One milliliter of water was added, and the spore count for each bee was calculated with a hemocytometer (Hausser Bright-Line).

Honey bee queens produce pheromones that modulate several aspects of the feeding behavior and physiology of worker bees and are critical for colony social organization (Fisher and Grozinger 2008, Trhlin and Rajchard 2011). To investigate whether or not there is any difference between queenright and queenless conditions on the effect of pollen feeding on *N. ceranae*-infected bees, another four groups were set up. Each group was composed of three replicates with 30 inoculated worker bees in each cage. Bees in each group were fed ad libitum with 50% sucrose syrup solution or 50% sucrose syrup solution with sterilized

multifloral pollen. For queenright conditions, each cage was provided a 1-yr old mated queen. Five bees were sampled from each cage at day 8 and day 12 and spores were counted as formerly described.

Effect of the Amount of Pollen Consumed by Bees on *N. ceranae* Spore Development. Fresh multifloral bee pollen was collected in the spring of 2013. Pollen was treated with UV light as in 2.4.1. Seven groups were set up. Each group was composed of three replicates of 40 honey bees, with 30 of them destined to determine longevity and the other 10 randomly sampled on day 12 postinfection for spore counting and RNA extraction. Groups 1 and 2, which served as controls without any spore inoculation, were fed with only 50% sucrose syrup or 50% sucrose syrup and pollen for the whole experimental period, respectively. Each cage in groups 3, 4, 5, 6, and 7 was provided with 50% sucrose syrup and 0, 0.24, 0.48, 0.71, and 0.95 g pollen, respectively, where 0.95 g is the amount of pollen consumed by 40 *Nosema*-infected bees for their whole lives as determined in preliminary studies.

Dead bees were counted and removed daily until all the bees had died. The mean longevity of the bees in each group was calculated. *Nosema* spores were counted as previously described.

RNA Extraction and qRT-PCR Analysis. Total RNA was isolated from individual bees sampled at day 12 using TRIzol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Briefly, each bee was homogenized in 1 ml TRIzol Reagent, shaken vigorously for 30 s, and then incubated at room temperature for 2–3 min. Following precipitation and centrifugation, the resultant RNA pellets were resuspended in 100 μl nuclease-free water. The RNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The expression of vitellogenin (Vg) was quantified by one-step SYBR Green real-time qRT-PCR. The expression of a housekeeping gene, β-actin, was measured in each sample for normalization of real time qRT-PCR results. The primer pairs for Vg and β-actin were previously reported (Prisco et al. 2011). RT-PCR reactions were carried out in a 25-μl reaction volume, containing 12.5 μl of 2× Brilliant SYBR Green QRT-PCR Master Mix (Stratagene, LA Jolla, CA), 0.4 μM of forward and reverse primers each, and 0.5 μg 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 s, 55°C for 1 min, and 72°C for 30 s. After amplification, a dissociation curve was constructed using 81 complete cycles of incubation where the temperature was increased by 0.5°C per cycle, beginning at 55°C and ending at 95°C to verify presence of a single product.

qRT-PCR was replicated three times for each sample. The relative expression of Vg was calculated by subtracting the cycle threshold (Ct) of Vg from Ct of the reference gene and compared between groups.

Statistical Analysis. Mann–Whitney *U* test was used to compare the spore counts between groups, and *P* values <0.05 were considered significant. SPSS 16.0 was used for data analysis. The relationship between pollen consumption and *Nosema* spore load was analyzed using a spearman rang correlation, with $P < 0.05$ being the level of significance. The same tests were done for the relationship between pollen consumption and honey bee longevity and between pollen consumption and the level of Vg expression.

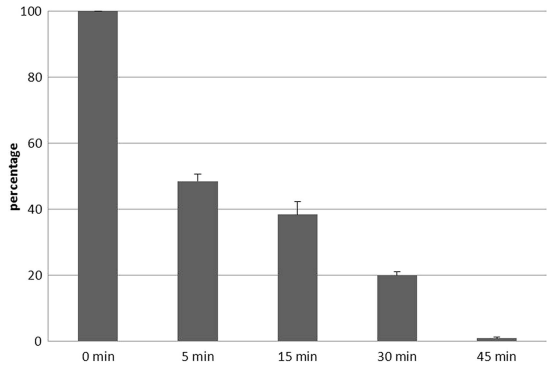

Fig. 1. Effect of UV radiation on *N. ceranae* spore viability. The percentage of viable spores after UV treatment is shown (average ± SE).

Results

UV Radiation Killed *N. ceranae* Spores. No viability loss was observed when spores were exposed to white light under room conditions. When they were exposed to UV light, the viability was reduced by 51.6% within 5 min and 99% after 45-min exposure (Fig. 1). The results indicated that UV radiation was able to efficiently kill *N. ceranae* spores.

Pollen Feeding Increased *N. ceranae* Spore Loads. In the first cage experiment, no *Nosema* infection was found throughout the experiment in the control group where bees were fed with sucrose only. However, in the control group additionally fed with fresh pollen, six out of the 18 bees collected at day 12 and 18 postinfection were found to be positive with *Nosema* infection, with an average of 3.37 ± 1.28 (SD) million spores at day 12 and 11.15 ± 3.62 (SD) million at day 18. This indicated that the spores contaminating pollen were viable and infective. No bees were infected in the control group fed with UV-treated fresh pollen, indicating that the UV treatment efficiently killed the spores in pollen. Bees fed with pollen that was several years old, either with or without UV treatment, did not cause any infection, indicating the spores had lost viability during storage.

At both day 12 and 18, out of all the groups inoculated with *Nosema*, the group fed with pollen had significantly more spores than the group fed with sucrose only (Mann–Whitney test, $P_{(12d,\ AP\ versus\ sucrose)} = 0.002$, *P* for other comparisons <0.001, Fig. 2). The average spore counts in the bees fed with fresh pollen were as high as 121 million per bee at day 18. There was no difference between the spore counts of inoculated bees fed with fresh pollen or UV-treated fresh pollen

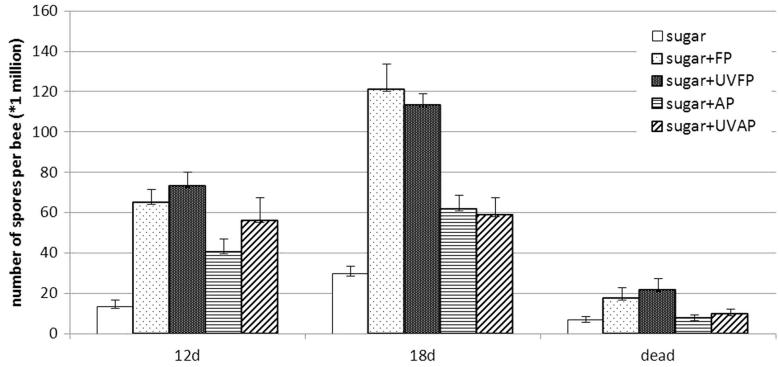

Fig. 2. Effect of pollen feeding on *Nosema* spore loads of 12-d-old, 18-d-old, and dead bees. Only data of inoculated bees are shown (average ± SE). FP, UVFP, AP and UVAP indicate fresh pollen, UV-treated fresh pollen, aged pollen, and UV-treated aged pollen, respectively.

either at day 12 or 18 (Fig. 2), indicating that the major cause of the elevated spore counts was due to the pollen itself and not from spores contaminating the pollen. At day 12, bees fed with aged pollen without UV treatment had considerably lower spore counts than those fed with UV treated aged pollen. This difference, however, was not significant ($P = 0.2$).

Spore counts of the bees that died between day 12 and 18 were much lower than those of the bees collected at day 12 and 18 alive ($P < 0.001$ for all comparisons). No significant difference was found between dead bees that had been fed with pollen with or without UV treatment, either for fresh pollen or aged pollen. Dead bees from fresh pollen groups had significantly higher spores counts than those of the sucrose group. There was no significant difference in spore counts between the aged pollen groups and the sucrose group.

No significant difference was found between queenright and queenless conditions regardless of whether bees were fed with pollen or not. However, in both queenright or queenless conditions, bees fed with pollen had significantly higher number of spores both at day 8 and day 12 ($P < 0.001$; Fig. 3).

Spore Loads Associated With the Amount of Pollen Consumed. In the experiment investigating the effect of the amount of pollen consumed by bees, no infection was found in control groups fed with sucrose or sucrose and UV-treated pollen. Spore loads of *Nosema*-infected bees increased from 21.8 ± 6.0 (SE) million per bee of bees fed only with sucrose to 91.0 ± 11.0 million per bee of bees fed with sucrose and pollen in the whole period, with a significant positive correlation between spore loads and pollen consumption (Fig. 4, $r = 0.97$, $P = 0.005$).

Honey bee longevity increased from 17.2 to 24.2 d when bees were provided with pollen. *Nosema* infection decreased longevity from 17.2 to 13.8 d when bees were fed only with sucrose syrup. When bees were fed with pollen, longevity decreased from 24.2 to 22.6 d with *Nosema* infection. As bees infected with *Nosema* spores increased their pollen consumption, their longevity increased gradually from 13.8 to 22.6 d, with a significant correlation between longevity and pollen consumption (Fig. 5, $r = 0.97$, $P = 0.008$).

As reported previously, pollen feeding significantly increased the relative expression level of Vg when bees were not infected with *N. ceranae* ($P = 0.000$). *Nosema* infection decreased the relative expression level of Vg for bees fed with or without pollen, while the difference was only significant for bees fed with pollen ($P_{\text{pollen}+} = 0.013$; $P_{\text{pollen}-} = 0.073$). Meanwhile, the relative expression level of Vg of *Nosema*-infected bees was positively correlated with the amount of pollen consumed significantly (Fig. 6, $r = 0.95$, $P = 0.014$).

For both longevity and Vg levels, the groups of *Nosema*-infected bees fed with pollen had significantly higher values than those of uninfected bees fed without pollen ($P_{\text{longevity}} = 0.049$; $P_{\text{Vg}} = 0.006$), indicating the absence of pollen alone seemed to have caused a larger reduction than the *Nosema* infection itself.

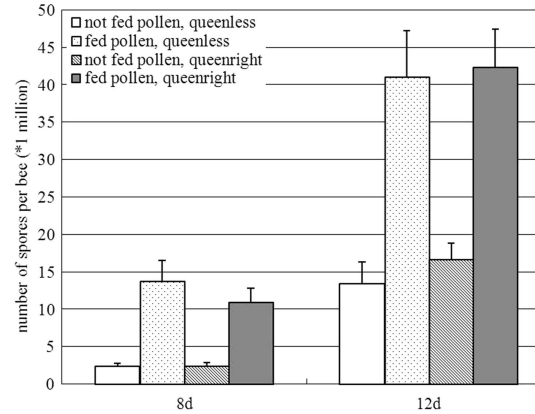

Fig. 3. *N. ceranae* spore production of bees fed with or without pollen under queenright and queenless conditions (average ± SE).

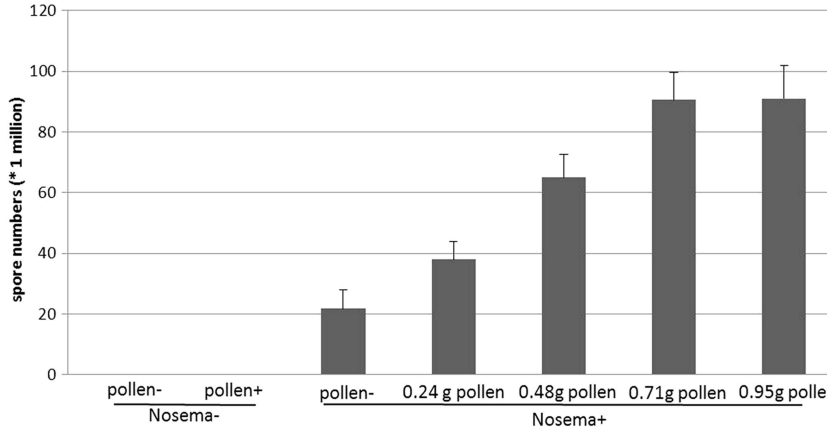

Fig. 4. Effect of the amount of pollen consumed on spore loads of bees (average ± SE).

Discussion

UV radiation has been shown to be efficient in inactivating microsporidian spores of several species (Baribeau and Burkhardt 1970, Wilson 1974, Teetor and Kramer 1977, Kelly and Anthony 1979). For example, *Nosema algerae* Vavra and Undeen, 1970 infectivity was reduced by 48% after one minute of UV exposure and 76% after two minutes (Kelly and Anthony 1979). Our results indicated that UV radiation was sufficient to kill *N. ceranae* spores.

By using pollen treated with UV radiation, we confirmed that pollen feeding led to increased *Nosema* spore production in bees. We observed a 3.8≈5.4 times increase of spore loads when feeding sterilized fresh pollen, which is comparable with Rinderer and Elliott 1977 and Porrini et al. 2011. The significant difference in the effect of fresh pollen versus aged pollen on spore loads indicates that the quality of pollen may affect spore production. This could be achieved directly by decreasing the nutrients or indirectly by decreasing the consumption of aged pollen (Pernal and Currie 2000) since the quality, e.g., free radical scavenging activity (Campos et al. 2003) and vitamin content (Hagedorn and Burger 1968), decreases during pollen storage. Further studies are warranted to elucidate the roles of pollen nutrients in the growth and proliferation of the *Nosema* within host cells.

Bees used for counting spores may be another factor causing variation of spore loads, as reported in other published studies. In some studies, live bees were sampled and used for counting spores (Martín-Hernández et al. 2009, Forsgren and Fries 2010, Porrini et al. 2011), while in some others, to avoid sacrificing samples for the primary goals of their studies, dead bees were used to count spores (Rinderer and Elliott 1977, Dussaubat et al. 2012). Spore loads of dead bees, in our study, are comparable with Rinderer and Elliott 1977, but are much lower than the spore loads of living bees. This could be due to *Nosema*-infected bees defecating before death (observed in experiments), while healthy bees do not defecate inside cages. This suggests that lower spore loads of dead bees should be taken into consideration when comparing spore loads among studies, for example, between Rinderer and Elliott 1977 and Porrini et al. 2011, both of which addressed similar scientific questions.

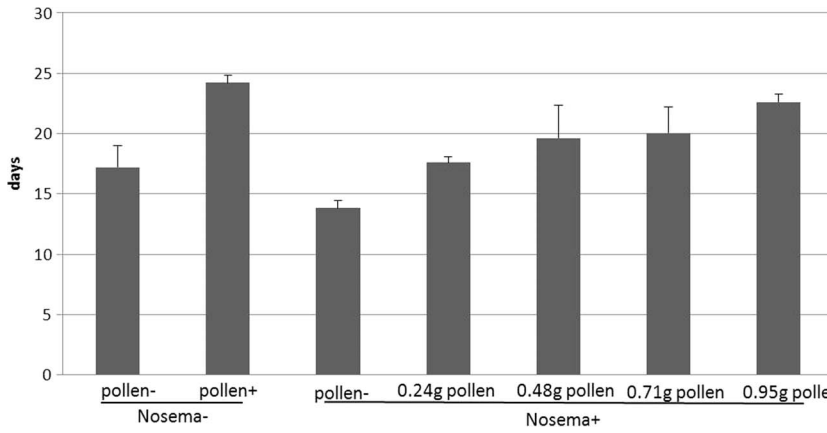

Fig. 5. Effects of pollen feeding and *Nosema* infection on honey bee longevity (average ± SD).

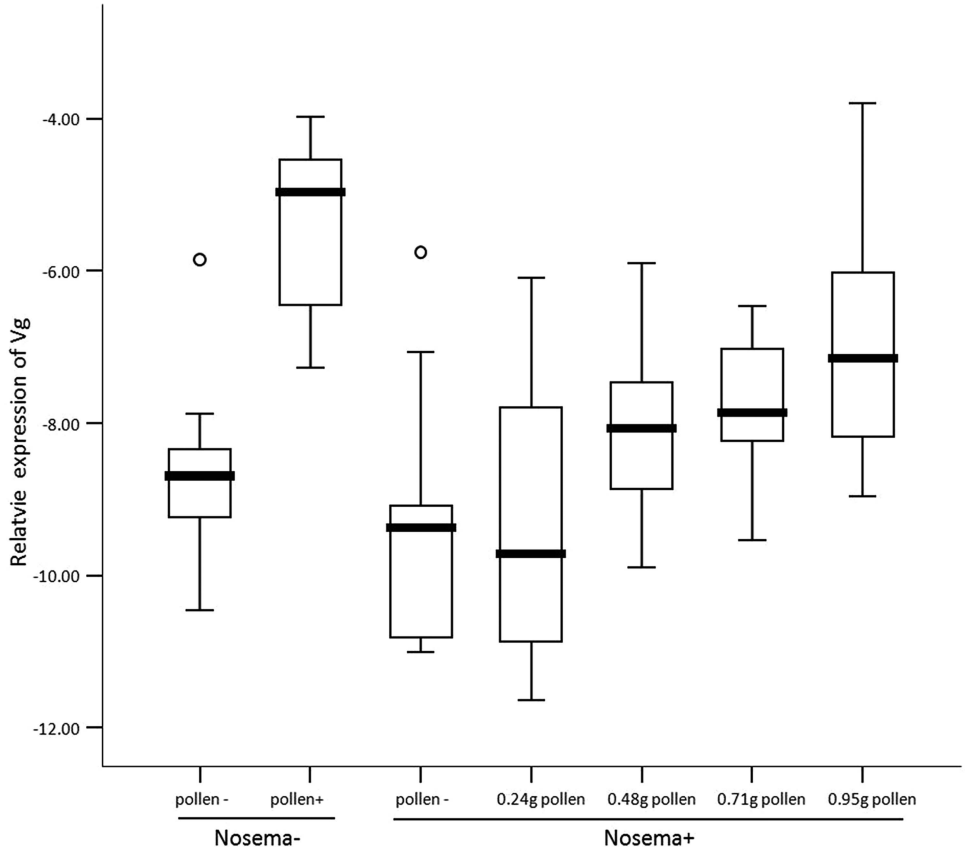

Fig. 6. Effects of pollen feeding and *Nosema* infection on the relative expression level of Vitellogenin. For each box plot, top of box is third quartile, bottom of box is first quartile, top bar is the maximum observation still within 1.5 IQR (interquartile range) of the third quartile, lower bar is the minimum observation still within 1.5 IQR of the first quartile, middle bar is median value, and circles are outliers.

Vg, a precursor protein of egg yolk, has been implicated in honey bee lifespan regulation and is therefore a popular biomarker of general robustness of honey bees (Amdam et al. 2005, Simone et al. 2009). By providing bees with different amounts of pollen, our data clearly showed that there was a significantly positive correlation between level of pollen consumption and the level of Vg expression and that the upregulated Vg expression in turn led to increased longevity in *Nosema*-infected bees.

Though honey bee colonies regulate pollen storage levels around a homeostatic set point (Fewell and Winston 1992), pollen collection of a colony varies as a response to resource variation, e.g., pollen availability (Seeley 1986), and colony conditions, e.g., brood amount (Dreller et al. 1999), queen presence (Free 1967), empty space (Free 1967, Dreller et al. 1999), and genetics (Hellmich et al. 1985, Page et al. 2000). Moreover, pollen consumption of individual bees is related to their age and their tasks in the system of division of labor (Crailsheim et al. 1992), as well as, to the nutritional values of pollen from different plant sources (Schmidt 1984). Based on these facts, pollen consumption of the bees sampled for *Nosema* detection, may vary with sample locations, seasons, colony sources, collection preference on plant sources, individual ages, and tasks. If the results of our study, along with those reported by Rinderer and Elliott 1977 and Porrini et al. 2011, are applicable in the field, this variation will turn into the observed variation of spore loads and lead to bias in the determination of health status of *Nosema*-infected colonies.

In conclusion, the influence of pollen consumption on spore loads in honey bees should be considered when spore load is used as a criterion in studies or diagnosis. Spore load alone is not a reliable health indicator of *Nosema*-infected bees. There is an urgent need to establish standard procedures to accurately, reliably, and meaningfully quantify the health status of *Nosema*-infected honey bees.

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