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Experimental infection of red dwarf honeybee, *Apis florea*, with *Nosema ceranae*

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ABSTRACT

This research is the first record of the infection of *Apis florea* by *Nosema ceranae*, a newly identified pathogen of honeybee in Thailand which was initially isolated from *A. florea* workers. Each *Nosema* free-bee was fed 2 μl of 50% (w/v) sucrose solution containing 0, 10,000 20,000 or 40,000 *Nosema* spores/bee. The survival rates of treated bees were significantly lower compared to control bees. Infectivity was not statistically different among the three spore concentrations, whereas no infection was found in control bees. Protein content of control bee hypopharyngeal glands 14 days post inoculation (p.i) was significantly higher (21.47 ± 0.17 mg/bee) compared to all treatments. The infection ratio of bees treated with 40,000 spores/bee increased with time after inoculation. These results suggest that *N. ceranae* has a significant negative effect on honeybee hypopharyngeal gland protein production and contributes to their shortened life span.

Introduction

Nosema is one of the most economically damaging honeybee diseases. It infects ventricular cells of adults after spores are ingested, especially through trophallaxis (Bailey, 1955; Bailey and Ball, 1991). *N. apis* infecting *Apis mellifera* has been reported worldwide and was described more than one hundred years ago by Zander in 1909 (Matheson, 1996; Higes et al., 2007). The microsporidian parasite may also infect *A. cerana* (Singh, 1975). Asiatic honeybee, *A. cerana*, populations have suffered from *N. ceranae* infections (Fries, 1997). Recently, this species also was found in cultivated *A. mellifera* colonies (Higes et al., 2006; Huang et al., 2007). It has become more widely distributed in the past decade by cross infection from *A. cerana* to European honeybees (Klee et al., 2007). *N. ceranae* can cause many negative effects on honeybee production capacity. Although infected bees do not exhibit obvious external disease symptoms, infection of *Nosema* causes digestive disorders, shortened life spans, lower colony population sizes (Malone et al., 1995), and reduction of honey production and crop products that rely on bees for pollination (Anderson and Giacon, 1992; Fries et al., 1992).

There have been no previous reports of *Nosema* infection in honeybees of Thailand. In this report, we examined for the first time experimental infection of *A. florea* by *N. ceranae* isolated from heavily infected *A. florea* workers. Infection rate, ratio of infected to non-infected cells, and bee survival rate were investigated. In addition, there is some evidence that *N. apis* infected bees may have lower levels of protein resulting in a reduced hypopharyngeal glands (Hassanein, 1951; Wang and Moeller, 1971. Therefore, hypopharyngeal gland protein concentrations were also measured.

Materials and methods

Spore preparation

Nosema spores were isolated from heavily infected *A. florea* workers of an infected colony near Samut Songkram Province, Thailand. Honeybee mid guts were removed and transferred to 1.5 ml microcentrifuge tubes containing 200 μl distilled water, and then homogenized and centrifuged at 6000 *g* for 10 min. Supernatant was discarded. To determine each infection dosage, the white sediment was collected and counted using a hemocytometer.

Experimental infection: infection rate

Frames of sealed brood were obtained from three *Nosema* free colonies of *A. florea* located at Burapha University, Chon Buri Province, Thailand. They were kept in an incubator at 34 ± 2 °C with relative humidity between 50 and 55% to provide newly emerged *Nosema* free honeybee workers for experiments. The emergent bees were carefully removed, confined to cages in groups of 50, and kept in the incubator for two days. Two days after eclosion, the bees were each fed with 2 μl of 50% sucrose solution (w/w) in water containing *Nosema* spores isolated from *A. florea* at dosages of 0 (control), 10,000, 20,000, 40,000

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spores/bee. Three replicated cages of 50 bees each were used. Each cage was fitted with 2 gravity feeders, one containing water, and the other sugar syrup (50% w/v sucrose solution) that was replenished during the experiment. Food was prepared using 60 g pollen mixed with 17 ml of 50% sucrose solution (w/v). Each cage was checked daily to remove and count any dead bees.

Infection ratio or parasitic ratio

Light microscopy

Ventriculi of dead bees were individually checked to verify the presence of *Nosema* spores. Three bees from each cage were collected at six, ten and fourteen days post inoculation (p.i.), and their ventriculi were processed for microscopic examination. The midgut was removed and fixed with Bouin's fluid solution for 24 h, washed three times in 70% ethanol or until the solution became colorless, dehydrated with an alcohol series and embedded in melted paraplast. Six μm sections were cut with a rotary microtome (Leica, Germany), stained with PAS and counterstained with light green, and examined under light microscope. The parasitized ratio was calculated as a proportion of infected cells to non-infected cells of the representative tissue areas over one hundred counted cells. Bees from the control group were sacrificed and analyzed to confirm the absence of spores in ventriculi using the same methods.

Transmission electron microscopy

For examination of ultrastructural change, midguts were placed in insect saline (NaCl 75 g/l, Na_2HPO_4 2.38 g/l, KH_2PO_4 2.72 g/l) and prefixed with modified Karnovsky fixative (4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) for 1 h at 4 °C. Tissues were post-fixed in 2% osmium tetroxide in cacodylate buffer for 1 h at room temperature. They were then washed three times in the same buffer for 10 min each. Specimens were contrasted for 12 h in 1% phospotungstic acid dissolved in 50% ethanol, dehydrated in a standard alcohol series (50–100%), cleared in propylene oxide, and embedded in Epon 812-Aradite 502 resins. Tissue sections of 500 μm and 90 nm were cut using an LKB ultramicrotome. The sections were contrasted with 10% uranyl acetate and lead citrate dissolved in 50% ethanol for 15 min and examined under the TEM (JEOL CX 200).

The hypopharyngeal gland protein contents

Three bees were removed from each cage on days six, ten, and fourteen p.i. and stored frozen until analyzed. Bees were then thawed and decapitated and their hypopharyngeal glands removed. The glands from bees of each cage were stored in 50 μl phosphate buffer (pH. 7.8) in 1.5 ml microcentrifuge tubes. Glands were homogenized and then centrifuged at 1000 rpm for 2 min. Supernatant from each tube was used for analysis by Bradford protein assay (Bradford, 1976). Standard curves were prepared using bovine serum albumin (BSA). Protein absorbance was measured at 595 nm against a blank reagent using a Shimadzu UV–visible spectrophotometer (UV-1610). Concentrations of protein (BSA) were plotted against the corresponding absorbance values to generate a linear regression standard curve which was used to predict protein concentration (mg/bee) from absorbance. Data were analyzed using one way ANOVA and appropriate post-tests (e.g., Duncan's Multiple Range Test).

Survival analysis

Each day for 30 days, the number of dead bees per cage was recorded and bodies removed. To evaluate mortality among the treatments, Kaplan–Meier survival curves were generated by plotting the number of surviving bees against days from the initiation of the experiment (Le, 1997).

Results

Infection rates (infectivity)

Control bees were negative and all experimentally infected bees were positive for *Nosema* spores throughout the study. The infection rates of bees treated with 10,000, 20,000 and 40,000 spores/bee were 45.00% ± 1.41, 73.00% ± 4.24 and 68.00% ± 2.82, respectively. The infection rate of bees treated with 10,000 spores/bee was significantly different from bees dosed with 20,000 and 40,000 spores/bee ($F_3 = 89.46$, $P<0.0004$). The highest infection rate was found in bees treated with 20,000 spores/bee, whereas it was the lowest in bees treated 10,000 spores/bee. There was no significantly difference between those treated with 20,000 and 40,000 spores/bee ($P>0.05$).

Infection ratio

The infection ratios of bee treated with 10,000 spores per bee were significantly different ($F_{8,9} = 384.16$; $P<0.0001$) on days 6, 10 and 14 p.i.. There were 4 ± 1.4 cells infected by day 6 p.i. while there were 48 ± 2.8 cells on day 10 p.i. The highest infection ratio was found in bees 14 days p.i. (50 ± 2.8 cells). Similar signs were found in bees treated with 20,000 spores per bee. The infection ratios were 8.5 ± 0.7, 7 ± 2.8 and 79 ± 1.4 cells infected by day 6, 10 and 14 p.i., respectively. In addition, the infection ratios of bees treated with 40,000 spores per bee were significantly different on day 6, 10 and 14 p.i. (10.5 ± 3.5, 78.5 ± 3.5 and 95 ± 1.4 cells, respectively) (Fig. 1).

Light microscopy and transmission electron microscopy

Nosema spores were found in the ventricular cells of infected bees. By day 6 p.i., infection of ventricular epithelium was detected. Each epithelial cell contained spores distributed throughout the cell cytoplasm, but particularly at the apical part of the cell (Figs. 2a, b). The two edges of *Nosema* spores stained positive to PAS, while the middle area stained green with light green (Fig. 2b). Heavy infection was found in bees 14 p.i., where spores were clearly observed at the bottom of the cells near the basement membrane. This appearance was similar to that of days 10 p.i. (Fig. 2b). Degeneration of epithelial cells was also observed at days 10 and 14 p.i. (Figs. 2c, d). Electron micrographs of longitudinal sections of mature spores showed that the spore wall consisted of compact exospores, 25–50 nm thick and a lucent layer endospore (Figs. 2e, f). Infected-ventricular cells were enlarged and cytoplasm contained numerous ribosomes and disorganized organelles. Broken and dispersed microvilli at the free end of ventricular cell could be seen at days 10 and 14 p.i.. Clear zones could be seen surrounding where *Nosema* spore embedded in cell cytoplasm (Fig. 2e).

Protein contents of the hypopharyngeal glands

The mean total protein content of hypopharyngeal glands taken on days 6, 10 and 14 p.i. from bees subjected to all three treatments are shown in Fig. 3. On day 6 p.i., there were no significant differences between bees treated with 10,000 spores/bee and the control ($F_{3,6} = 1.09$, $P<0.3099$), suggesting no effect of *Nosema* on protein production of the glands. However, the mean total protein contents of hypopharyngeal glands of these bees were significantly different from bees treated with 20,000 or 40,000 spores/bee ($F_{23,48} = 2.38$; $P<0.0056$). Control bees had significantly higher hypopharyngeal gland protein concentration (426.58 ± 46.46 μg/μl, average 21.47 ± 0.17 mg/bee) compared to all other treatments ($F_{23,48} = 2.38$, $P<0.0056$), while bees treated with 40,000 spores/bee had the lowest protein concentration (282.40 ± 36.51 μg/μl, average 14.12 ± 1.82 mg/bee).