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RESEARCH REPORT

Nosema ceranae changes semen characteristics and damages sperm DNA in honeybee drones

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Abstract

In this study, we aimed to determine how infection with *Nosema ceranae* spores affected semen volume, sperm concentration, and fragmentation of sperm DNA in honeybees. A total of 120 one-day-old drones were marked with queen bee marking numbers, and equally divided into two groups. Drones in the infected group were individually fed with honey syrup containing 200 000 *N. ceranae* spores per 1 µL, while those in the uninfected (control) group were fed honey syrup without spores. The groups were then placed in two separate colonies. Fourteen days later, ejaculate was collected from the drones and was analyzed for semen volume, sperm concentration per 1 µL semen, and sperm DNA fragmentation. Compared to uninfected controls, the *N. ceranae* spore-infected drones showed significantly decreased semen volume and sperm concentration, as well as a higher percentage of sperm DNA fragmentation.

Key Words: *Nosema ceranae; Apis mellifera;* sperm; drone; DNA fragmentation; chromatin dispersion

Introduction

One cause of *Apis mellifera* colony loss or depopulation is the intestinal parasite *Nosema ceranae*, which infects honeybees with the same intensity year-round (Martin-Hernandez *et al.*, 2007; Giersch *et al.*, 2009; Tapaszti *et al.*, 2009). The parasite causes immune system suppression in honeybees (Antúnez *et al.*, 2009). *Nosema* spp. destroys intestinal epithelial cells, thereby limiting nutrient absorption and increasing energy requirements (Fries *et al.*, 1996; Mayack and Naug, 2009). Microsporidia lack functional mitochondria, and are thus fully dependent on the energy produced by the host (Agnew and Koella, 1997). Infection with *Nosema* spp. spores leads to declining protein levels in infected workers, resulting in hypopharyngeal gland atrophy and changes in the fatty acid composition of the hemolymph (Roberts, 1968; Wang and Moeller, 1970; Malone *et al.*, 1995, 1998). This limits the development of young bees and leads to honeybee colony weakening, depopulation, and collapse (Higes *et al.*, 2008, 2011, 2013; Botías *et al.*, 2012).

Honeybee colonies in Poland commonly exhibit infection with *N. ceranae* spores (Michalczyk *et al.*, 2013). *N. ceranae* spores infect bees and queens as well as juvenile and adult drones, which transmit the infection (Traver and Fell, 2011). *Nosema apis* infection does not impact the acceleration of maturation or the undertaking of earlier mating flights by infected individuals (Tofilski and Kopel, 1996). Older drones infected with *N. apis* exhibit spores in their reproductive tissues and ejaculate, which may cause vertical/sexual transmission of the infection and deterioration of reproduction performance and reflects sperm damage caused by *Nosema* spp. (Peng *et al.*, 2015). *N. apis* and *N. ceranae* represent microsporidia from the kingdom of fungi (Adl *et al*., 2005; Huang *et al*., 2013). Both *Nosema* species are obligatory parasites of adult bees causing similar severe bee infections worldwide (Klee *et al*., 2007). Therefore, results obtained for *N. apis* can be extrapolated to those for *N. cerana*, but *N. ceranae* is more virulent towards *Apis mellifera* honey bees (Huang, 2011).

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In the present study, we investigated the hypothesis that *N. ceranae* damages sperm DNA in honeybees. Drones were individually infected with *N. ceranae* spores. We then examined the effects of infection on semen volume, sperm concentration per 1 µL semen, and sperm DNA fragmentation relative to uninfected drones.

Material and Methods

Nosema ceranae spore collection

We obtained 30 dead bees from each of the 50 winter-killed colonies that originated from the apiary of the University of Life Sciences in Lublin. These bee samples were homogenized in 30 mL of distilled water, and the homogenates were applied to a Bürker hemocytometer. We counted the spores within each square under a light microscope at 400× magnification. Infection intensity was assessed as the number of spores per honeybee. Winter deaths characterized by the marked presence of *Nosema* spp. spores were subsequently subjected to polymerase chain reaction (PCR) analysis using specific primers for *N. ceranae* 218MITOC and *N. apis* 321APIS.

We mixed 300 µL honeybee homogenate with 180 µL lysis buffer and 20 µL proteinase K, and then isolated total DNA using the DNeasy Blood and Tissue Kit (Qiagen) in accordance with the manufacturer's instructions. To identify the *Nosema* species, total DNA was analyzed using the primers 218MITOC and 321APIS, as described by Martin-Hernandez *et al.* (Martin-Hernandez *et al.*, 2007). PCR was performed using the Taq PCR Core Kit (Qiagen). In a total volume of 30 µL, the reaction mixture included 6 µL of the DNA sample, 9.85 µL H_2O, 3.3 µL 10× PCR buffer, 6.6 µL Q solution, 2.95 µL Mg^{+2}, 0.66 µL dNTP mix, 0.125 µL forward primer, 0.125 µL reverse primer, and 0.39 µL Taq. The PCR conditions were as follows: 10 min at 95 °C; followed by 35 cycles of 30 s at 95 °C, 30 s at 55.8 °C, and 45 s at 72 °C; and then a final extension of 7 min at 72 °C. The PCR product was analyzed in 5% agarose gel under UV light.

We selected the winter-killed colonies that were infected solely with *N. ceranae* spores. Homogenates including only *N. ceranae* spores were used to prepare honey-water syrup (1:1 ratio) containing 2×10^6 spores/µL. This syrup was administered to 120 one-day-old honeybee workers for 5 days, and was then replaced with spore-free syrup. For the next 14 days, the workers were kept in wooden cages of 12.5 × 12.5 × 4 cm, maintained under laboratory conditions (25 °C; H= 65%). After this period, they were euthanized with CO_2 for 10 min. Once again, the hemocytometric method was applied to determine the number of spores. These spores were used to prepare a honey-water syrup (1:1) containing 200 000 *N. ceranae* spores per 1 µL to be administered to one-day-old drones. This procedure ensured good viability of the *N. ceranae* spores, such that they would have high ability to infect immediately and effectively the honeybee drones in the further experiments.

Drone breeding and infection

The investigations were conducted in June 2017, using drones originating from one *Apis mellifera carnica* queen. To obtain drones of the same age, the queen was caged for two days on a single drone comb using a queen excluder. Then the queen was released, and the comb remained caged to protect it from further oviposition. On post-oviposition day 23, the comb was transferred to a 34 °C incubator to induce drone emergence. The emerging drones were individually marked using queen bee marking numbers.

A total of 120 drones were evenly divided into two groups: uninfected and infected with *N. ceranae*. Using a micropipette, the control/uninfected drones were individually fed 2 µL of honey:water (1:1) solution, whereas drones in the infected group were fed 2 µL of honey:water (1:1) solution containing 200 000 *N. ceranae* spores per 1 µL. Then, the drones were placed in separate colonies, with 60 uninfected drones in three colonies and 60 *N. ceranae* spore-infected drones in another three colon (20 drones per colony were free living). All colonies were *Nosema* spp. free, as confirmed by PCR tests. The exit were barred by queen excluders to prevent the drones from leaving the colonies.

On day 14 after emergence, the drones were captured and their semen was collected using a 1 µL calibrated micropipette. The semen volume was measured using an electronic caliper following the method of Czekońska *et al.* (2013b). Then the semen collected from each drone was divided into two samples of equal volume. One sample was used to determine the sperm concentration, and the other sample was used for DNA fragmentation analysis.

Sperm concentration determination with a Muse flow cytometer

Sperm concentration was determined using a flow cytometer and a Muse Count and Viability Kit from Merck. The sperm was diluted 20× in phosphate-buffered saline (PBS), and then mixed with 380 µL of Muse Count and Viability reagent. The mixture was vortexed for approximately 30 s, incubated at room temperature for 5 min, and then vortexed again for 10 s. Samples prepared in this manner were then measured three times using the Muse flow cytometer.

Sperm DNA fragmentation (SDF)

To quantify the sperm DNA fragmentation, we used the Sperm DNA Fragmentation (SDF) test from Halosperm® (Halotech DNA SL, ISO 13485), in accordance with the manufacturer's instructions. This assay is based on sperm chromatin dispersion.

The sperm samples were diluted with a 400× PBS solution. Agarose was dissolved at a temperature of 95 °C for 5 min, and then cooled to 37 °C. We then combined 50 µL diluted sperm with 100 µL of agarose. Next, 8 µL of the mixture of sperm and agarose was mounted on horizontal microscopic slides provided in the kit, and covered with a cover glass. The preparations were transferred to a refrigerator and kept at 5 °C for 5 min

Table 1 Semen volume and sperm concentration of infected and uninfected drones (Mann-Whitney tests)

Trait	Number	Uninfected drones	*Nosema ceranae*-infected drones	Statistics	
		mean	mean	Z	p
Semen volume (µL)	60	1.18	0.37	9.48	0.00001
Sperm concentration (× 10^6/µL)	60	10.06	4.93	9.49	0.00001

until the agarose set. Then the cover glasses were removed, and the slides were submerged in "Solution 1" (Denaturant Agent) and incubated at room temperature for 7 min. Next, Solution 1 was removed, and the slides were dried, submerged in "Solution 2" (Lysis Solution), and incubated at room temperature for 20 min. The slides were then rinsed in distilled water, dried, placed in a horizontal position, and dehydrated by submersion in 70% ethanol for 2 min. After removal of the 70% ethanol, 100% ethanol was applied for 2 min. Next, the slides were dried and stained with "Solution 3" (Eosin Staining Solution) at room temperature for 7 min. Finally, the slides were dried, and then stained with "Solution 4" (Thiazine Staining Solution) at room temperature for 7 min.

In this manner, we prepared 20 samples total from the infected drones and 20 samples total from the uninfected drones (40 samples total). We then counted the sperm cells in five fields of vision from each sample using a Nikon Eclipse Ni bright-field microscope at 400× magnification. In each field of vision, we counted 10 sperm cells with fragmented and degraded DNA and normal spermatozoa. We calculated the percentage of sperm with fragmented DNA using the following formula:

$$SDF(\%) = \frac{\text{Fragmentation + degradation}}{\text{Total cell count}} \times 100$$

Statistical analysis

Statistical analysis was performed using SAS software version 9.5 (Statistical Analysis System Institute, Cary, NC). Comparisons between the uninfected drones and *N. ceranae*-infected drones were performed using Mann-Whitney tests. Percentage data were arcsine-transformed (Sokal and Rohlf, 1981).

Results

A significantly higher volume of semen was collected from the uninfected drones than from the *N. ceranae*-infected drones ($p \leq 0.05$; Table 1).

The concentration of sperm in semen was also significantly higher in the uninfected drones (10.06 × 10^6/µL) than in the *N. ceranae*-infected drones (4.93 × 10^6/µL) (Table 1).

In the present study, we analyzed 1000 spermatozoa from each uninfected and infected drone. Sperm cells with undamaged DNA were characterized by a round central core with a large circular aureole/halo along the perimeter of the sperm head (Fig. 1a). In spermatozoa with damaged DNA and nucleotides, we observed a central core with a small elongated aureole/halo that tightly adhered to the sperm head, which was formed by dispersed DNA fragments (Fig. 1b). Degraded sperm exhibited no aureole/halo (Fig. 1c).

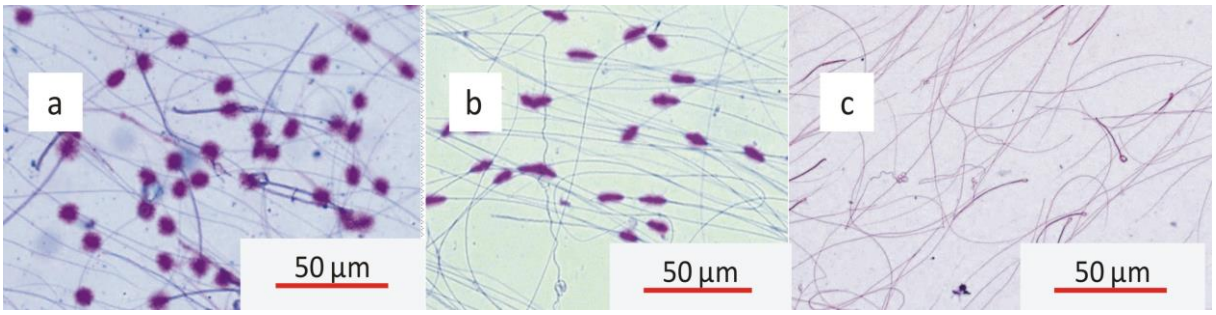

Fig. 1 Representative photographs of sperm after the sperm DNA fragmentation (SDF) test. (a) Healthy sperm with no DNA fragmentation. (b) Sperm with dispersion of DNA fragments. (c) Degraded sperm

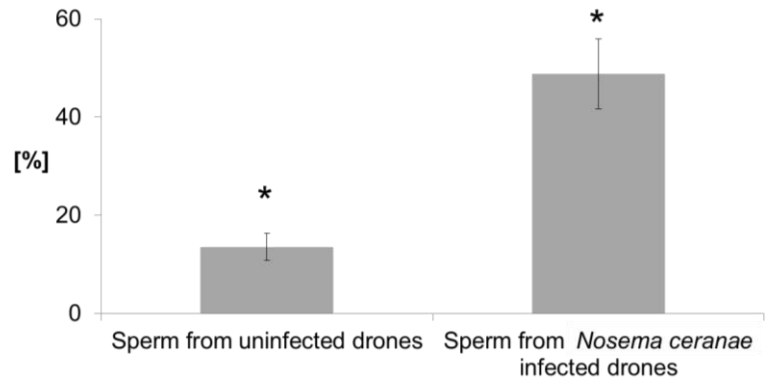

Fig. 2 Percentage of sperm DNA fragmentation. *p < 0.05 for the difference between sperm from uninfected drones and *N. ceranae*-infected drones (Mann-Whitney test; Z= 13.38 p= 0.00001). Error bars represent standard deviation of the data

We observed a markedly higher percentage of DNA fragmentation among sperm cells from *N. ceranae*-infected drones (48.82%) than from the uninfected drones (13.56%) (Fig. 2).

Discussion

The volume of semen collected from the uninfected drones in this study was similar to the collected volume of 0.9 to 1.1 µL reported by Czekońska *et al.* (2013b, 2015). Strikingly, only 0.37 µL of this volume was collected from the *N. ceranae*-infected drones in this study.

In fact, the sperm concentration in the infected drones was the lowest ever reported from *A. m. carnica* drones. The concentration of sperm per 1 µL of semen generally ranges from 6.76 to 11.95 × 10^6/µL, depending on the size of the drones and study (Woyke, 1960; Moritz, 1981, 1984; Rinderer *et al.*, 1985, 1999; Berg and Koeniger, 1990; Duay *et al.*, 2002; Czekońska *et al.*, 2015).

We assessed sperm chromatin dispersion using a sperm DNA fragmentation (SDF) test, which is widely used for examinations of *Daphnia*, bull, wild boar, and even human sperm (Fernández *et al.*, 2003, 2005; Enciso *et al.*, 2006; Gómez *et al.*, 2016). The SDF test is a simple, accurate, and reproducible method for analyzing sperm DNA fragmentation (Breznik *et al.*, 2016), which is routinely used for sperm analysis in medical laboratories and is an important tool for diagnosing male infertility (Jay, 1963).

Spermatozoa in drones are produced during the preimaginal period (Bishop, 1920; Fukuda and Ohtani, 1977; Czekońska *et al.*, 2013a), beginning at the larval stage and ending at the pupal stage (Snodgrass, 1956). During the first week of adult life, spermatozoa are transferred from the testes to seminal vesicles, where they are stored until copulation (Snodgrass, 1956; Woyke, 1958; De Graaf and Jacobs, 1991). The reduced sperm volume and concentration observed in the *N. ceranae*-infected drones (Table 1) may have been caused by undernourishment of the infected drones during the transfer of sperm from the testes to seminal vesicles, which lasts approximately one week.

Prior studies demonstrate that *N. ceranae* spreads into many tissues and affects the sexual function of infected drones (Roberts and Hughes, 2015; Ciereszko *et al.*, 2017). *N. ceranae*-infected hosts also suffer intestinal epithelial cell damage, limited nutrient uptake, and increased energy requirements (Fries *et al.*, 1996; Mayack and Naug, 2009). *Nosema* infection reduces protein levels in infected honeybees, leading to hypopharyngeal gland atrophy (Wang and Moeller, 1970; Malone *et al.*, 1995), and changing the fatty acid composition of hemolymph (Roberts, 1968). This condition of energy deficiency, limited protein availability, and disturbed fatty acid metabolism leads to undernourishment, which likely resulted in the production of sperm with DNA fragmentation in the *N. ceranae*-infected drones in our present study (Fig. 2).

Ciereszko *et al.* (2017) demonstrated that honeybee spermatozoa are susceptible to the toxic effects of imidacloprid, which aggravates sperm parameters. Moreover, Peng *et al.* (2015) reported that *Nosema apis* infection reduced drone fertility and lifespan, and detected spores in the ejaculates of infected drones. These authors questioned whether infected drones actually leave their colonies to undertake mating flights. If so, they could additionally pose a threat to the mating success of females, since drones can transmit the infection vertically. Our findings suggest that if infected drones undertake the mating flight, their reproductive success will only be achieved by approximately 51% of spermatozoa, as approximately 49% will be unable to fertilize eggs due to the phenomenon of sperm chromatin dispersion and semen volume.

Our present results confirmed the hypothesis that *N. ceranae* infection causes damage to sperm DNA and decreases both the semen volume and sperm concentration in honeybee drones. This is the first demonstration that these phenomena may cause the poorer reproduction performance of *N. ceranae*-infected drones. Sperm with damaged DNA are incapable of fertilizing eggs, which may decrease queen fecundity and contribute to honeybee colony depopulation.

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