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Original article

Nosema apis infection in worker and queen *Apis mellifera*

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Abstract – Worker and queen honey bees were fed individually with *Nosema apis* spores in sucrose solution and then returned to cages containing several hundred of their worker bee nestmates. After 3 to 7 days, the workers and queens that had been fed spores were sacrificed. Worker and queen ventriculi were removed and examined for spores by light microscopy, and DNA was extracted. The DNA was subjected to amplification with polymerase chain reaction, using primer sequences specific to *N. apis* DNA. The PCR analysis was more sensitive than examination for spores by light microscopy, in detecting *N. apis* infection. Worker bees and queen bees were infected at similar rates by the inoculation procedure.

***Apis mellifera* / *Nosema apis* / PCR / queen / worker**

1. INTRODUCTION

The microsporidian *Nosema apis* Zander causes a destructive disease of honey bees, *Apis mellifera* L., worldwide (Bailey and Ball, 1991; Fries, 1997). By infecting the ventriculus of adult bees (White, 1919; Bailey, 1955) this organism shortens bee life span (Kleinschmidt and Furguson, 1989) and causes greater colony mortality in winter (Nitschmann, 1957). Earlier observations that the disease is distributed widely across the US by the mailing and trucking of honey bees (Farrar, 1947, 1954; Jay, 1966) probably hold true today.

A bee becomes infected when it ingests spores while cleaning feces from the comb, left by other infected bees (Fries, 1988). Contaminated food and water may also be sources of spores. Beekeepers routinely control the disease by culling older comb and by treating their hives with fumagillin (Fries, 1997).

The effects of *N. apis* on queen bees are particularly serious for practical beekeeping. Infected queens are often superseded (Farrar, 1947; Furgala, 1962). Nurse worker bees, which may feed their queen, suffer reduced hypopharyngeal gland activity when infected (Wang and Moeller, 1969, 1971).

Our present study was a test of the polymerase chain reaction for the amplification and detection of *N. apis* DNA extracted from honey bee ventriculi. We also wished to compare worker bees to queen bees for sensitivity to this pathogen. Perhaps the queen is less sensitive than the workers, an adaptation that would minimize the overall effects of the disease on the colony.

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2. METHODS

2.1. Inoculum preparation

Spores were collected from diseased bees by adding water to the abdomens, macerating the tissues in a blender, filtering through Whatman 4 filter paper, and centrifuging the filtrate. The pellet was resuspended in sucrose solution, at a final concentration of 50% sucrose and 3.7×10^6 spores per mL. One ml of this diet weighed 1.28 g, so that each mg of diet contained 2.89×10^3 spores.

2.2. Preparation of experimental hives

Thirteen small honey bee hives were established with mature queen cells on 22 and 23 August 2001. The queen cells had been reared from larvae taken from the same hive. Each colony was established in a box made to hold 5 deep Langstroth frames, and kept at an apiary site in Franklin Co., KY.

2.3. Inoculation with *Nosema apis* spores

The queen and approximately 200 workers from each hive were caged, during 19–30 October 2001. At this time brood rearing had nearly ended and workers were relatively old. Shortly after collection each cage was filled with CO_2 for several seconds to immobilize the bees. The queen and 20 workers were then removed, restrained on paraffin blocks with insect pins, and painted with distinctive colored marks on their thoraces. When the pinned bees revived, they were each fed with a microcapillary tube containing the above diet of *N. apis* spores, a method similar to that of Furgala and Maunder (1961). The microcapillary tube was weighed before and after feeding to determine the weight of diet and number of spores consumed. The worker bees consumed $(73.1 \pm 3.5) \times 10^3$ spores (mean $\pm$ S.E.). The queen bees consumed $(66.8 \pm 10.9) \times 10^3$ spores (mean $\pm$ S.E.). After inoculation, each bee was returned to its cage through a hole at the bottom. Each cage of bees was then provided with a feeder containing 50% sucrose in water, and kept in an incubator at 25 °C for 3 to 7 days.

2.4. Examination of tissues for spores

After either 3 (2 cages), 4 (4 cages), 6 (2 cages) or 7 days (5 cages) the bees in each cage were immobilized with CO_2. Surviving, marked bees were removed for dissection. The ventriculus was removed from each bee, placed in a 1.5 mL plastic microcentrifuge tube, and macerated with a small plastic pestle. A small drop of the preparation was removed and examined on a microscope slide for the presence of spores at 400× by light microscopy using phase contrast optics (Cantwell, 1970). The tube containing the remainder of each macerated ventriculus was then frozen at –70 °C until the DNA extraction procedure.

The microcentrifuge tubes were autoclaved before use. To prevent cross contamination, the fine forceps used for dissection were cleaned carefully before removing the ventriculus from a bee. No ventriculi ruptured during the dissection procedure. Each was rinsed with distilled water immediately before placing it in the tube.

2.5. Primer for *N. apis* PCR analyses

The sequences for the small subunit of the ribosomal RNA gene of a number of *Nosema* species and close relatives were obtained from internet searches of Genbank and aligned with the Clustal program. Most of the sequences were highly conserved but two polymorphic regions were found that allowed us to design specific primers specific to *N. apis* (Fig. 1). Primers were designed so that the 3' terminus of each primer would end in this polymorphic region, as shown in the figure. Primers were checked with Amplify Software version 1.2 (Engels, 1993) to predict whether they would amplify one product from the gene.

The *Nosema apis* sequence was tested for similarity against the Genbank non-redundant database with a standard nucleotide BLAST. This database contains 1.77 million sequences. There were many significant "hits" with other microsporidia RNA genes. However, none of the twenty most significant alignments nor the sequences we compared in our table showed a PCR product when tested with the Amplify Software. Therefore, there is no reason to believe that we would get a product amplified from these species with our standard PCR procedure.

2.6. *N. apis* DNA extraction

The following procedure was used for the isolation and identification of *Nosema apis* DNA in honey bee tissues. Honey bee ventriculi known to be infected or free of *N. apis* were collected and stored at –70 °C until their use as positive and negative controls. Approximately 0.05 g of tissue was used for DNA extraction using a modified protocol for the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI). Tissue was ground in a mortar and then transferred to a 1.5 mL microfuge tube. Nuclei Lysis Solution (NLS, 200 μL) was added to each tube and it was then vortexed for 3 s in order to wet the tissue. The sample was then incubated at 65 °C for 60 min, after which 2 μL RNAse (10 mg/mL; Sigma Chemical Co., Saint Louis, MO) solution was added and mixed by

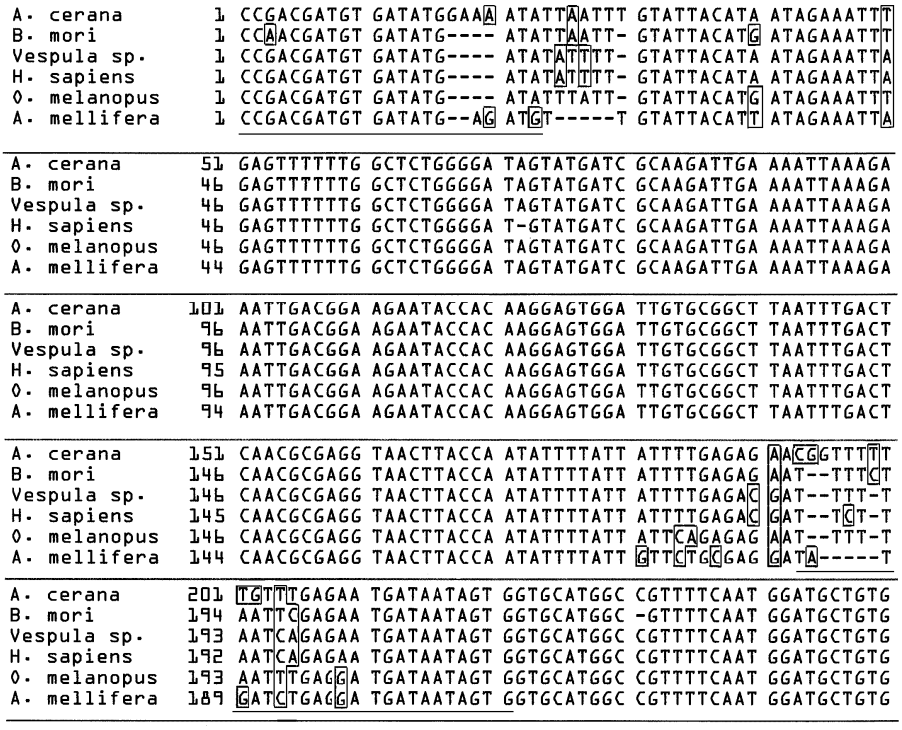

Figure 1. Host species and alignment of DNA sequence for small subunit RNA from *Nosema* species used for primer design. Genbank (http://www.ncbi.nlm.nih.gov/Genbank/) accession numbers: *N. ceranae*, 857489; *N*. spp. from silk worm 1339944 (now revised to 1838930); *N. vespula*, 507913, *Vairimorpha* sp., from human, 954829; N. *Oulemae* from *Oulema melanopus* L. (Coleoptera), 849158; *N. apis* from *Apis mellifera*, 857487. The primer sequences are underlined.

inverting, and the tube incubated at 37 °C for another 15 min. The sample was then allowed to cool to room temperature for approximately 5 min prior to adding 67 µL protein precipitation solution. The sample was then vortexed vigorously for about 20 s and then centrifuged (15 800 *g*) for 5 min. The supernatant was removed without disturbing the pellet and transferred to a new tube containing 250 µL isopropanol. The sample was mixed by inversion and centrifuged for 5 min. The supernatant was then discarded and the DNA pellet washed with 200 µL of 70% ethanol and centrifuged for 1 min. The supernatant was discarded and the pellet was washed again with 200 µL of 95% ethanol and allowed to air dry for 15 min. The pellet was resuspended in 150 µL of 1X TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and then incubated for 60 min at 65 °C. The tube was stored overnight at 4 °C to dissolve the pellet. The DNA concentration and 260/280 ratio was determined using a GeneQuant RNA/DNA calculator spectrophotometer (Pharmacia Biotech, Cambridge, England). All samples were stored at 4 °C until needed.

2.7. PCR amplification conditions

A 20 µL reaction volume was used that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µM each of dNTP; 0.25 µM of each primer; 5.0 ng of genomic DNA template, and 2.0 U of *Taq* polymerase (Promega Co., Madison, WI). The primers NosA-F (CCG ACG ATG TGA TAT GAG ATG) and NosA-R (CAC TAT TAT CAT CCT CAG ATC ATA) were used to amplify a 209 bp product of the 16S ribosome of *N. apis* (Greg Hunt, Purdue University, unpublished data). Amplifications were carried out using a GeneAmp PCR System 9700 (Perkin Elmer Applied Biosystems, Foster City, CA) programmed for an initial 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 62 °C, 2 min at 72 °C, followed by a

Table I. *Nosema apis* infection in worker and queen bee ventriculi. The 86 worker and 8 queen samples are tabulated according to whether no infection was observed (NI), spores were observed but *N. apis* DNA was not detected (Sp), *N. apis* DNA was detected by PCR but spores were not observed (PCR), or spores were observed and *N. apis* DNA detected (Sp+PCR).

Incubation time	Worker ventriculi				Queen ventriculi			
	NI	Sp	PCR	Sp+PCR	NI	Sp	PCR	Sp+PCR
3 days	12	1	13	7	0	0	1	1
4 days	22	1	6	1	2	0	0	0
6 days	0	0	2	4	0	1	0	1
7 days	0	0	3	14	0	2	0	0
TOTAL 34	2	24	26		2	3	1	2

final extension period of 10 min at 72 °C. PCR products were separated by electrophoresis on replicate 1.4% agarose gels and a digital image of each gel was analyzed using a software program by Kodak Scientific Imaging Systems (New Haven, CT).

2.8. Examination of spores for *N. apis* DNA

We wished to determine whether DNA could be released from *N. apis* spores prior to treatment by the above procedure. Ten ventriculi from infected worker bees were removed from the bees, each placed in 1 mL of sterile water, and homogenized to release spores from the tissue. The homogenates were combined, mixed well, and a drop was examined in with a hemacytometer to determine the spore concentration (5.1×10^7 spores / mL). Fifteen 200 µL samples of the homogenate were each placed in microcentrifuge tubes and centrifuged 60 s. The supernatant was removed, and 0.1g of glass beads (1.0 mm dia) and 200 µL of NLS was added to each tube. The tubes were then vortexed at 3000 rpm (Maxi Mix 1, Thermolyne Corp., Dubuque IA) for either: no time (control), 5 s, 10 s, 20 s, or 60 s. The extraction of each sample proceeded as above (Sect. 2.6).

Serial dilutions were prepared from each of the 15 extracts: 5.0 µg/mL (corresponding to 5.0×10^4 spores), 2.0 µg/mL (2.0×10^4 spores), 1.0 µg/mL (1.0×10^4 spores), 0.50 µg/mL (5.0×10^3 spores), 0.25 µg/mL (2.5×10^3 spores), 1.2 µg/mL (1.2×10^3 spores), and 0.062 µg/mL (6.2×10^2 spores). The DNA amplification procedure above (Sect. 2.7) was followed for each.

3. RESULTS

The presence of *N. apis* spores and DNA in worker and queen ventriculi are summarized in Table I. For worker bees, the extraction and amplification of *N. apis* DNA revealed more infections than the examination of tissues for spores by light microscopy. This difference is highly significant ($P < 0.001$; $Q = 21.94$) by the McNemar test for significance of changes (Sokal & Rohlf, 1995). Of the worker ventriculi examined, two were found to contain spores but no *N. apis* DNA while 24 were without spores but did contain *N. apis* DNA.

For queen ventriculi, the test for *N. apis* DNA was not clearly more sensitive than examination for spores ($P > 0.10$; $Q = 1.05$) by the McNemar test. Three of the eight queen ventriculi were found to contain spores but no *N. apis* DNA.

Overall, a similar fraction of the inoculated workers were found to be infected (60%) compared to the inoculated queens (75%). This difference is not significant ($X^2 = 0.65$).

DNA was more readily recovered from spores agitated with glass beads than from undisturbed spores. DNA was detected in the smallest samples (6.2×10^2 spores) when the spores were agitated for 10, 20 or 60 s. DNA was detected from as few as 2.5×10^3 spores when agitated for 5 s. DNA from undisturbed spores was detected only in preparations of 5.0×10^3 or more spores.

4. DISCUSSION

It is not surprising that the amplification of *N. apis* DNA from infected tissue provided a more sensitive test for the disease than an examination for spores by light microscopy. However, in some ventriculi (two workers and

three queens) *N. apis* DNA was not detected even though spores were observed (Tab. I). These spores were probably ungerminated, and remained in the ventriculi from the time that they were ingested. Perhaps the spore's resilient wall (Undeen, 1997) prevents the extraction of DNA. This possibility is supported by our finding that DNA is released from spores agitated with glass beads for at least 10 s.

The similar sensitivity of queens to infection, compared to workers, suggests that queens have no inherent means to protect themselves. Perhaps worker bees protect the queen by limiting her exposure to spores. Such a mechanism would have great adaptive value, because the queen bee is much more critical to colony vitality than any one worker bee (Schmidt-Hempel, 1998). Nearly a century has elapsed since the discovery of this microbe, and it is possible that *N. apis* has been associated with the honey bee for a much longer time.

We chose to study older, late-autumn worker bees. This is the cohort that winters with the queen, when *N. apis* infection is especially prevalent and serious (Bailey and Ball, 1991). However, this choice led to heavy mortality of both inoculated and uninoculated bees when caged, and a reduction in sample size. Several queens also died when insufficient workers remained to support them. However, our results from the surviving bees provide us with data that pertain directly to wintering in a way that young bees could not.

A large fraction of the workers (40%) and queens (25%) were uninfected in spite of the large doses of spores they had received. This is not unusual (Greg Hunt, unpublished data). Possibly, older bees are less vulnerable to infection than younger bees. Also, we suspect that the method of feeding spores in sucrose solution inhibits spore germination in the ventriculus, because the osmotic shock required for germination (Olsen, 1986; De Graaf et al., 1993) cannot happen reliably. A dry preparation of spores would probably be a more effective inoculum because liquid food already in the ventriculus would then provide the osmotic shock. However a diet of dried spores is difficult to feed in measured amounts to individual bees.

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Résumé – Infection des ouvrières et des reines d'*Apis mellifera* par *Nosema apis*. La nosémose, due au protozoaire *Nosema apis* Zander, cause des dégâts aux colonies d'abeilles dans le monde entier et les effets sur les reines sont particulièrement importants pour l'apiculture pratique. Notre étude visait à détecter l'ADN de *N. apis* par la réaction en chaîne de la polymérase (PCR) et à comparer la sensibilité des ouvrières et des reines à cet agent pathogène. Des ouvrières et des abeilles ont reçu individuellement des spores de *N. apis* en nourrissement dans un sirop de saccharose puis ont été encagées avec leurs consœurs durant 3 à 7 j. On a ensuite recherché au microscope optique (×400) la présence de spores dans le tissu des ventricules des ouvrières et des reines. L'ADN a été extrait des tissus pour être amplifié par la PCR à l'aide de séquences d'amorces spécifiques à *N. apis* (Fig. 1). La PCR a été plus sensible que la microscopie optique pour détecter le pathogène (Tab. I). Les ouvrières et les reines avaient une même sensibilité à l'infection ; les reines ne semblent donc pas avoir des moyens propres pour se protéger après ingestion de spores.

***Apis mellifera* / *Nosema apis* / sensibilité / reine / ouvrière / PCR**

Zusammenfassung – *Nosema apis* Infektion von Arbeiterinnen und Königinnen (*Apis mellifera*). Das Microsporidium *Nosema apis* Zander ist weltweit Ursache für eine vernichtende Krankheit der Honigbiene, *Apis mellifera* L., und seine Auswirkung auf Königinnen sind besonders schlimm für die praktische Bienenhaltung. Hier soll der Nachweis von *N. apis* DNA durch eine Polymerase Kettenreaktion (PCR) Analyse untersucht und die Empfindlichkeit von Arbeiterinnen und Königinnen auf dieses Pathogen verglichen werden. Arbeiterinnen und Königinnen der Honigbienen wurden einzeln mit *N. apis* Sporen in Zuckerlösung gefüttert, und danach mit Arbeiterinnen aus ihrem Nest für 3 bis 7 Tage gekäfigt. Anschließend wurde der Hinterleib der Arbeiterinnen und Königinnen auf dieses Pathogen überprüft. Gewebe wurden auf *N. apis S*poren mit dem Lichtmikroskop bei einer Vergrößerung von 400× untersucht. DNA wurde aus den Geweben für PCR mit *N. apis* spezifischen Primer Sequenzen extrahiert (Abb. 1). PCR erwies sich als ein Test, der empfindlicher für das Pathogen ist als das Lichtmikroskop (Tab. I). Arbeiterinnen und Königinnen zeigten eine ähnliche Empfindlichkeit zur Infektion. Damit scheinen Königinnen

keinerlei angeborene Möglichkeiten zu haben sich gegen eine Aufnahme von Sporen zu schützen.

***Apis mellifera* / *Nosema apis* / PCR / Königinnen / Arbeiterinnen**

REFERENCES

Bailey L. (1955) The infection of the ventriculus of the adult honey-bee by *Nosema apis* Zander, Parasitology 45, 86–94.

Bailey L., Ball B.V. (1991) Honey bee pathology, Academic Press, New York.

Cantwell G.E. (1970) Standard method of counting Nosema spores, Am. Bee J. 110, 222–223.

De Graaf D.C., Masschelein G., Vandergeynst F., De Brabander H.F., Jacobs F.J. (1993) In vitro germination of *Nosema apis* (Microsporidia: Nosematidae) spores and its effect on their αα-trehalose/D-glucose ratio, J. Invertebr. Pathol. 62, 220–225.

Engels W.R. (1993) Contributing software to the Internet: the Amplify program, Trends in Biochem. Sci. 18, 448–450.

Farrar C.L. (1947) Nosema losses in package bees as related to queen supersedure and honey yields, J. Econ. Entomol. 40, 333–338.

Farrar C.L. (1954) Fumagillin for Nosema control in package bees, Am. Bee J. 94, 52–53.

Fries I. (1988) Comb replacement and nosema disease (*Nosema apis* Z.) in honey bee colonies, Apidologie 19, 343–354.

Fries I. (1997) Protozoa, 3rd ed., in: Morse R.A., Flottum K. (Eds.), Honey bee pests, predators, and diseases, A.I. Root, Medina, OH.

Furgala B. (1962) The effect of the intensity of *Nosema* inoculum on queen supersedure in the honey bee, *Apis mellifera* Linnaeus, J. Insect Pathol. 4, 429–432.

Furgala B., Maunder M.J. (1961) A simple method of feeding *Nosema apis* inoculum to individual honeybees, Bee World 42, 249–252.

Jay S.C. (1966) A survey of Nosema disease in package bees, queens and attendant bees entering Manitoba (1963–1966), Proc. Entomol. Soc. Manitoba 22, 61–64.

Kleinschmidt G.J., Furguson F. (1989) Honey bee protein fluctuations in the Channel Country of South West Queensland, Australas, Beekeeper 91, 163–165.

Nitschmann J. (1957) Die Füllung der Rektalblase von *Apis mellifera* L. im Winter, Dtsche Entomol. Z. (n.s.) 4, 143–171.

Olsen P.E., Rice W.A., Liu T.P. (1986) In vitro germination of *Nosema apis* spores under conditions favorable for the generation and maintenance of sporoplasms, J. Invertebr. Pathol. 47, 65–73.

Schmidt-Hempel P. (1998) Parasites in social insects, Princeton Univ. Press, Princeton NJ.

Sokal R.R., Rohlf F.J. (1995) Biometry: The principles and practice of statistics in biological research, 3rd ed., W.H. Freeman, New York.

Undeen A.H. (1997) Microsporidia (Protozoa): A handbook of biology and research techniques, http://pearl.agcomm.okstate.edu/scsb387/content.htm (viewed on 23 October 2003).

Wang D.-I., Moeller F.E. (1969) Histological comparisons of the development of hypopharyngeal glands in healthy and *Nosema*-infected worker honey bees, J. Invertebr. Pathol. 14, 135–142.

Wang D.-I., Moeller F.E. (1971) Ultrastructural changes in the hypopharyngeal glands of worker honey bees infected by *Nosema apis*, J. Invertebr. Pathol. 17, 308–320.

White G.F. (1919) Nosema-disease, USDA Bull. 780.