

“Nozevit patties” Treatment of Honey Bees (*Apis mellifera*) for the Control of *Nosema ceranae* Disease

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ABSTRACT

Nosema disease affects adult honey bees and due to its mostly inconspicuous signs and the need for eradication by exchange of frames with brood from a disinfected hive and often use of new wax, beekeepers devote insufficient attention or often neglect the disease. Also, there is a problem of controlling nosemosis, especially caused with *N. ceranae* because of its asymptomatic duration and prohibition of using antibiotics in the treatment of apian diseases in the European Union, as well as in Croatian regulations. We have predicted great results for use of protein pollen patties with “Nozevit” herbal preparation, as a feed supplement for bee colonies, where it can have an effect on brood rearing (colony strength) and at the same time reduce the number of *Nosema ceranae* spores. The aim of this study was to assess the effectiveness of the “Nozevit” phyto-pharmacological preparation in protein/pollen substitute patties for treatment of nosema disease in comparison with patties without “Nozevit” and sugar solution in a similar control group.

INTRODUCTION

Nosema disease is a parasitic disease of adult honey bees (*Apis mellifera*) caused by two described species of microsporidia, *Nosema apis* (Zander, 1909) and *Nosema ceranae* (Fries et al., 1996), which in adverse living conditions forms spores. This disease affects adult bees and due to its inconspicuous signs and the need for eradication by exchange of frames with brood from a disinfected hive and often use of new wax, beekeepers devote insufficient attention or often neglect the disease. Honey bees afflicted with nosemosis start to forage earlier (Fries, 1995), while pathological changes of their mid-gut epithelial cells, as well as digestive and metabolic disorders (Hassanein, 1951), cause malnutrition (Muresan et al., 1975), lack of population build up and consequentially decrease of population size of honey bee colonies (Malone et al., 1995) leading to premature deaths (Morse and Shimanuki, 1990).

New *Nosema ceranae* is highly pathogenic and there are usually no visible symptoms of diarrhea or adult bee deaths and there is total lack of seasonality in the diagnosis (Martin – Hernandez et al., 2007), and little is known about pathogenicity (Oldroyd, 2007). Infections with *N. ceranae* induce a nutritional stress, suppression of the bee’s immune functions and cause changes in behavior where

infected bees tend to forage at cooler temperatures (Mayak, 2009). Bees infected with new parasitic pathogen starve to death due to lack of digestive function and this leads to increased number of honey bee colony losses, destruction of plant communities and low production in the same areas which consequently cause significant loss of beekeeper’s income (Stefanidou et al., 2003).

There is a problem of controlling nosemosis, especially caused with *N. ceranae* because of its asymptomatic duration (Martin – Hernandez, 2007) and prohibition of using antibiotics in the treatment of apian diseases in EU, as well as in Croatian regulations. Recently, we have published results of experimental nosema disease treatment with the natural phyto-pharmacological preparation “Nozevit” in sugar solution (Tlak Gajger et al., 2009) which shows that a large number of spores were considerably reduced upon preventive (70.91%) and curative (78.37%) treatment. But, bees need more than just carbohydrates from honey or sugar syrup to survive, especially proteins. The most significant source of proteins in nutrition of honey bee colonies is pollen or pollen substitutes. Proteins are mainly needed for reproduction and brood rearing (Herbert, 1999); to produce protein-rich brood food to feed larvae, but also the queen needs a steady supply with protein-rich royal jelly, to have enough protein to lay up to 2000+ eggs a day. Also, there are a lot of reasons for additionally feeding bees with pollen substitutes like: early spring build up before appearance of first vegetation; build up in preparation for pollination; to force building in preparation for a strong nectar flow, to encourage early drone rearing; to maintain drone and brood rearing through a strong dearth (Day et al., 1990), and ensure wintering survival. Less brood rearing eventually reduces the number of adult bees, including foragers, and may consequently affect pollination efficiency and honey yields (Herbert, 1999) and if we draw a comparison with nosemosis, it has the same consequences. So, the pollen patties composition is important both for its nutritional value and for its effect on how readily bees consume it (Keller et al., 2005a). Because of that we have predicted great results for use of pollen patties with “Nozevit” as a feed supplement for bee colonies in early spring and in autumn, where it can have an effect on brood rearing (colony strength) and at the same time reduce the number of *Nosema* spores, thereby preventing the spread of disease inside the colony. The aim of this study was to assess the effectiveness of the “Nozevit” phyto-pharmacological preparation added to “Brood Builder” - protein/pollen substitute patties for treatment of nosema disease in comparison with patties without “Nozevit”, and sugar solution in a similar control group. Also, we have checked the strength (number of populated and brood frames) of treated and untreated honey bee colonies during the clinical examination in the field conditions.

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Table 1 Spore counts (per 0.04 mm) on initial day and on 12, 28, 40th and 60th day after initial treatment. Divide counts by 4 to obtain millions of spores per bee.

Treatment of Nosema disease with “Nozevit patties” and sugar solution with “Nozevit” applied using drench method						
Test No. 1		Initial sampling before treatment	12 th day after first treatment	28 th day after first treatment	40 th day after first treatment	60 th day after first treatment
A	Mean	54,70	27,00	21,80	17,20	22,00
	Min	27,00	18,00	18,00	13,00	13,00
	Max	79,00	48,00	26,00	26,00	42,00
	Std. Dev.	16,78	9,41	2,93	4,04	11,36
B	Mean	59,20	30,60	26,10	15,30	10,70
	Min	42,00	20,00	22,00	8,00	6,00
	Max	79,00	46,00	28,00	20,00	16,00
	Std. Dev.	13,10	7,67	2,13	4,27	2,86
C	Mean	32,40	41,80	65,30	51,00	52,80
	Min	20,00	38,00	37,00	28,00	32,00
	Max	41,00	48,00	79,00	83,00	89,00
	Std. Dev.	6,75	3,82	13,26	18,49	19,32
D	Mean	50,50	44,60	65,00	64,20	74,90
	Min	36,00	29,00	46,00	37,00	69,00
	Max	60,00	57,00	73,00	74,00	84,00
	Std. Dev.	6,99	9,52	8,89	10,90	5,68

(A = “Nozevit patties”; B = sugar solution + “Nozevit” phyto–pharmacological preparation; C = pollen patties; D = sugar solution)

MATERIALS AND METHODS

The feeding phase of the experiment was conducted during eight weeks beginning June 1, 2009 in the apiary situated in the continental part of Croatia.

Bee colonies and assessment

We selected 48 hives and before testing took 60 bees per colony from the hive entrance and examined them under microscope for the presence of *Nosema* spores. Also, from part of those bee samples we have isolated genomic DNA for molecular analysis with purpose of determination of *Nosema* species. The queens were two months old; daughters of the same mother queen obtained from the local queen breeder. We assessed the hives and frames following a standard clinical observation and measurement procedure for determination of strength of bee colonies that included an estimate of the number of populated frames, the comb area with sealed brood, open brood and stored food. After that we divided the hives into two testing group having similar means of sealed brood area (the test groups were simultaneously treated with pollen patties (A), sugar solution (B) with addition of phyto–pharmacological preparation “Nozevit”), and two control groups without “Nozevit” stimulative fed with pollen patties (C) and 1:1 sugar solution (D). Each group included 12 bee hives.

Field treatments and feeding

The treatment of the colonies was provided every 12 – 14 days, with exception for last treatment which was provided after 20 days because of bad weather. There were five treatments. All patties weighed 200 grams (commercially available Dadant “Brood Builder” patties with “Nozevit” and commercially available Dadant “Brood Builder” control patties without “Nozevit”), each and 1:1 sugar solution in amount of 200 ml with addition of 20

drops of “Nozevit” for testing colonies. The testing pollen supplement patties contained 63.41% carbohydrates and 13.69% proteins. At each feeding we placed patties directly on the frames and sugar solution was applied using the “drench” method.

During the clinical examination of bee hives in the field conditions, we have checked the strength of bee colonies on the 12th, 40th and 60th day, by counting a total number of populated and brood frames.

Determination of infective dose

Each time before the next treatment we took 60 bees per colony from the hive entrance and examined them under a microscope for the presence of *Nosema* spores, and determined the number of spores by counting in a haemocytometer according to Bürker – Türk (Cantwell, 1970). Samples were taken from about 60 adult bees at the hive entrance on the 12th, 28th, 40th and 60th day after initial sampling. Bee samples were collected into clean plastic receptacles around 12 o'clock noon. Bees were counted in each sample, their abdomens were separated and 1 ml of water per bee was added. The abdomens were thoroughly crushed. 10 spore samples were counted in each sample using a haemocytometer according to Bürker – Türk, and the infective dose was calculated according to Cantwell (1970). We used 400 x magnifications under a bright field microscope Olympus Bx41 and took photographs with Olympus DP12 U–TVO camera. The counting equipment was carefully washed after each sample counting in order to avoid contamination with spores from the previous sample.

Determination of *Nosema* species

Extraction of genomic DNA and further molecular analysis was performed as follows: For each of selected suspensions of isolated *Nosema* spores, an aliquot of 50 µl was transferred to a fresh tube,

boiled at 100°C for 30 minutes and centrifuged at 14,000 x g for 10 minutes. 30 µl of supernatant was removed and supplemented with 10x TE buffer to a final concentration of 10mM Tris and 5mM EDTA, pH8. This supernatant served as source of template DNA and was stored at -20°C, or, used immediately for multiplex polymerase chain reaction. Primers used for specific amplification of *N. apis* DNA were 321APIS-FOR (5'-GGGGGCATGTCTTTGACGTACTATGTA-3') and 321APIS-REV (5'-GGGGGGCGTTTAAAATGTGAAACAACACTATG-3') and expected size of amplicon was 321 bp. Primers for *N. ceranae* were 218MITOC-FOR (5'-CGGCGACGATGTGATATGAAA-ATATTAA-3') and 218MITOC-REV (5'-CCCGGTCATTCTCAAACAAAA-AACCG-3') and amplicon size is expected 218 – 219 bp. Primers were selected taking into account that primer sequences were specific to each of the two species, and that both amplicons could be simultaneously amplified and separated using agarose gel electrophoresis for visualisation of results. The PCR conditions were following instructions of the manual of the manufacturer of Taq polymerase (Sigma, USA). The molecular size of PCR products were determined by electrophoresis in a 2% agarose TAE (Tris-acetate-ethylene diamine tetra-acetic acid) gel in standard TAE buffer, stained with SYBR green, and visualised using UviTec gel documentation system.

RESULTS

The results of microscopic examination of spore presence in field testing of "Nozevit patties" treatment before and on 12, 28, 40 and 60 day after its introduction are provided in Table 1 and Figure 1. Strength of honey bee colonies is provided in Figure 2.

The results of PCR amplification with generic *Nosema* primer pair perfectly matched the results of amplification with specific *N. ceranae* primer pair. PCR amplifications of representative bee samples, positive and negative controls are presented in Figure 3.

DISCUSSION

This experiment was designed to test the effectiveness of repeated treatments with "Nozevit" phyto-pharmacological preparation to control nosema disease, in field conditions. The study involved four groups of bee colonies fed with sugar solution and pollen patties with and without "Nozevit", and treatment was applied via drench method for sugar solution and patties were put directly on top of comb frames. In the first part of the experiment, concerned with activity of "Nozevit patties", results demonstrated that the disease was not cured, but a considerable reduction in spores number was achieved: 50.63% on 12 day; 19.25% on 28 day and 21.10% on 40day after initial treatment if reduction is calculated in relation with result in previous treatment; and

Figure 1
Mean spore counts (million spores per bee) on 1st, 12th, 28th, 40th and 60th day after initial treatment

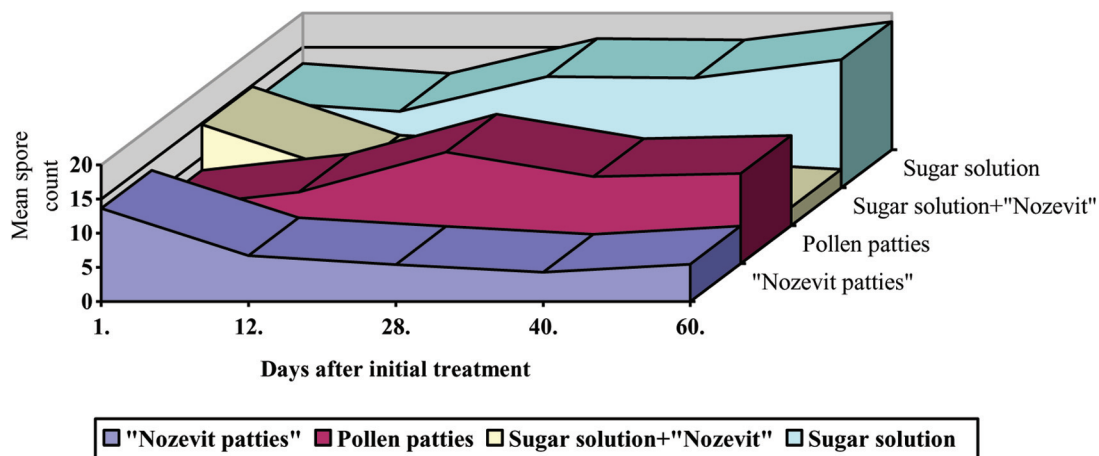
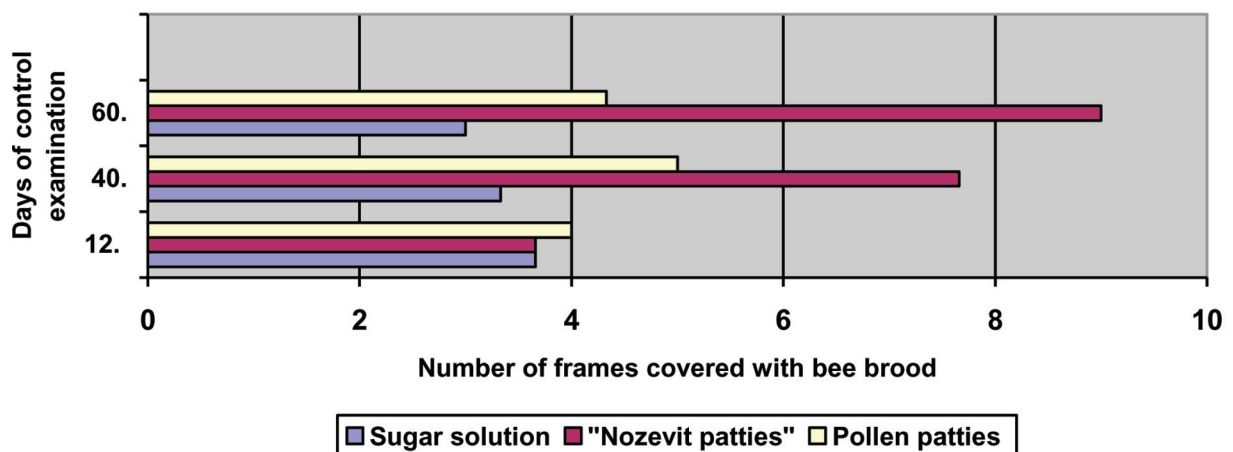


Figure 2
Strength of bee colonies treated with "Nozevit patties"



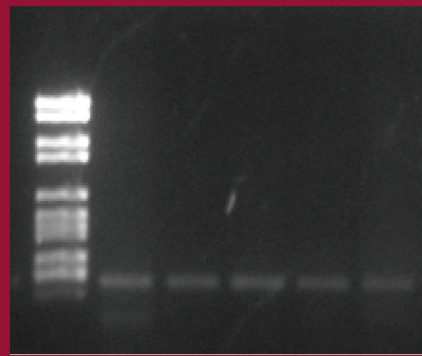


Figure 3.
PCR amplification of representative bee samples infected with *N. ceranae*

Lane 1: DNA ladder (DNA molecular weight marker VI; Roche, Germany)

Lane 2: Positive control

Lane 3-6: PCR reactions

50.63% on 12 day; 96.70% on 28 day; 68.55% on 40 day in relation with results of initial sampling time, respectively. On last sampling time we detected an increase in spore number (21.81% in relation with previous sampling time, but there was however, 59.78% spore reduction in relation to initial infection level) and we think that is a consequence of bad weather because we had a delay in the last sampling. The test group treated with sugar solution plus “Nozevit” showed very good results in reducing number of *Nosema* spores (48.31% on 12 day; 55.91% on 28 day; 74.15% on 40 day and 81.92% on 60 day after initial bee sampling). In the pollen patty control group it was determined to have a increase in spore numbers of 14.28% at last spore counting in comparison with the infection dose beginning the test. Also, we have alterations in spore number in control group fed with sugar solution and at last counting we determined an increase of 32.57%.

Despite failure to achieve complete cure, it needs to be stressed that both test groups treated with “Nozevit” had a reduced number of spores compared to the control groups. Also, we can conclude that “Nozevit” preparations (in sugar solution or in patties) works in field conditions, if they are applied precisely according to label instructions.

During the clinical examination of tested colonies we have determined that colonies treated with “Nozevit patties” show a significant increase in number of frames covered with sealed bee brood. Pollen absence may have an effect on the strength of colonies and honey production (Keller et al., 2005a; 2005b), but our colonies had additional pollen in nature and this effect of reinforcement-tested colonies is probably a consequence of “Nozevit” activity against *Nosema*. “Nozevit patties” contained just 13.69% protein in its composition and tannins from “Nozevit” can’t have any significant negative effect (like squeezing or coagulating of proteins) on additional feeding functions of pollen substitutes and development of colonies. Also, additionally feeding with proteins is very important in cases where colonies are diseased, because of stimulation and furtherance of the bee’s immune functions and population build up of infected colonies.

Previously, *nosema* infections in Europe were attributed just to *N. apis* (Ellis and Munn, 2005), but it appears that *N. ceranae* is an emerging pathogen that has increased its distribution to European honey bees (Klee et al., 2007) and it may be displacing *N. apis* in this area. *N. ceranae* has not been confirmed in Croatia to date, but we have predicted its presence in pure or mixed infections with *N. apis*, because a high percentage of the *Nosema* spores were also detected during summer and because it has been diagnosed in some neighboring countries. Our results of examination using molecular methods showed that *N. ceranae* is the only *Nosema* species found to infect honey bees (Tlak Gajger et al., unpublished data) in our tested colonies, so we can conclude that “Nozevit” herbal preparations are effective for this new *nosema* disease management.

ACKNOWLEDGEMENTS

The authors express their sincere gratitude to beekeeper Goran Dužaić, student Danijela Grilec and Mr. Predrag Manger for technical assistance and encouragement during the whole period of study. Also, we thank Dadant and Sons, Inc. for supplying “Brood Builder” patties with and without Nozevit.

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