Effect of the herbal preparation Nozevit on the mid-gut structure of honeybees (*Apis mellifera*) infected with *Nosema* sp. spores

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ABSTRACT: The histopathological structure as well as content and distribution of mucosubstances in the mid-gut mucosa of the honeybee (*Apis mellifera*) treated with the phyto-pharmacological preparation Nozevit was studied. For the purpose of describing neutral, acid and sulphate mucopolysaccharides we used the Periodic Acid-Schiff Reaction (PAS), Alcian blue-specific (pH = 1.0 and 2.5) and Toluidine blue-specific staining. Based on our results we have concluded that the herbal preparation Nozevit induces the production and secretion of mucous from the epithelial layer of treated bees, and additionally coats the peritrophic membrane to form a firm and resilient envelope. Thus, the preparation may ensure protection from new invasion with *Nosema* sp. spores and also from normal physiological processes.

Keywords: nosema disease; Nozevit; mid-gut mucosa; mucosubstances

Nosema disease is a parasitic disease of adult honey bees (*A. 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. The disease occurs throughout the world and causes significant detriment to honey production and results in economic losses. The losses are manifested as reduced yields of honey and other apian products (Anderson and Giacon, 1992), and as poor quality and reduced yields in agriculture (Goodwin et al., 1990). Honey bees afflicted with nosema disease start to forage earlier (Hassanein, 1953). Pathological changes in their mid-gut epithelial cells cause digestive and metabolic disorders, as well as malnutrition leading to the premature deaths of bees (Morse and Shimanuki, 1990) and decreases in population sizes of honey bee colonies (Malone et al., 1995). The disease is often referred to as the "Silent Killer" (Hornitzky, 2005), because the absence of obvious signs means the disease is often not noticed and beacuse affected honey bees tend to die of exhaustion away from the hive.

Honey bees are an extremely important part of natural ecosystems because they enhance agricultural productivity and help maintain biodiversity by engaging in pollination, a process based on the ecological principal of mutual interactions between the pollinated-plants and pollinator (Williams, 1994; Delaplane and Mayer, 2000; Pham-Delegue et al., 2002). Previously, *Nosema* sp. infections in Europe were attributed just to *N. apis*, but it appears that *N. ceranae* is an emerging pathogen (Fries et al., 2006) that has increased its distribution and it may be displacing *N. apis* in Europe (Klee et al., 2007). *N. ceranae* is highly pathogenic, especially for new hosts like *A. mellifera* (Higes et al., 2007). Nevertheless, Cornman et al. (2009) described a widespread distribution of *N. ceranae* in both healthy and declining honey bee colonies, and its overall contribution to honey bee losses is debatable. There are usually no visible symptoms of diarrhoea or adult bee deaths and there is total lack of seasonality in the diagnosis (Martin-Hernandez et al., 2007), while little is known about pathogenicity (Oldroyd, 2007). The spread of a parasitic

pathogen to new geographic areas in combination with Colony Collapse Disorder (CCD), leads to increased numbers of honeybee colony losses, destruction of plant communities and low production in affected areas leading to significant losses in the incomes of beekeepers (Stefanidou et al., 2003).The asymptomatic nature of nosema disease means that controlling the condition, especially when caused by *N. ceranae*, is fraught with difficulty (Martin-Hernandez et al., 2007). The European Union as well as Croatian regulations prohibit the use of antibiotics in the treatment of apian diseases (EU 3/01/081) because of the potential development of resistance to used chemotherapeuticals, masking of the disease, possible relapses, as well as harmful residua of antibiotics and their secondary metabolites in apian products. For that reason, the need arises for the production and utilization of natural phyto-pharmacological preparations in the treatment of nosema disease. Nozevit is natural preparation produced as a water solution of plant polyphenols, and is marketed as a "partner for nosema disease repression". The results of field experiments treating nosema disease with Nozevit (Tlak Gajger et al., 2009a) showed considerable reductions in the numbers of spores upon preventive (70.92%) and curative (78.35%) treatment (Table 1). Also, the mid-gut structure was histologically analyzed in order to determine the mechanism of action of the tested preparation. The aim of this study was to perform histochemical examination of the mucous layer of the mid-gut in healthy bees, bees with nosema disease and diseased bees treated with Nozevit. The mucous cover formed by mucosubstances in the digestive tract acts to increase slickness and protects from proteolytic degeneration and pathogenic microorganisms (Reid et al., 1988), so we envisioned that Nozevit stimulates production of mucosa and in that way protects epithelial gut cells from *Nosema* sp. invasion. Therefore, we studied the histopathological structure, content and distribution of mucosubstances in the mid-gut mucosa of honeybees treated with Nozevit.

MATERIAL AND METHODS

Samples of honeybee mid-gut were taken from three groups of colonies: a healthy control group, a test group infected with *N. ceranae* and curatively treated with Nozevit, and a test group infected with *N. ceranae* without treatment. Each test groups was composed of 12 honeybee colonies to assess the effectiveness of one-time basis treatment with Nozevit. A blend of half a litre of a 1 : 1 sugar solution and 20 drops of Nozevit was given to each treated colony. The control group received just the sugar solution. The number of spores was determined using a haemocytometer, according to Bürker-Türk (Cantwell, 1970). For determination of *Nosema* species molecular analysis was performed (Tlak Gajger et al., 2009b).Twenty bees were removed from each testing colony on the 10th day after treatment, and the intestines of each bee were pulled out after brief exposure to a low temperature (10 min, 4 °C). For extraction purposes, a larger pair of forceps was used to hold the head and chest of each bee, and a smaller pair of forceps to hold the top of the last abdominal segment and carefully pull out the intestines. The

Table 1. Mean spore counts (million spores per bee) upon preventive and curative treatment, and one-time treatment of Nosema disease with Nozevit

	Treatment of Nosema disease with Nozevit					
	preventive			curative		
	day after artificial infection			day after Nozevit treatment		
	10	15	22	15	20	25
A	1.45	5.40	18.78	14.30	6.92	1.52
B	2.98	10.70	26.48	18.25	9.17	6.38
One-time basis treatment						
A	day 10 after Nozevit treatment				12.90	
B					16.40	

A = sugar solution + Nozevit (test group); B = sugar solution (control group)

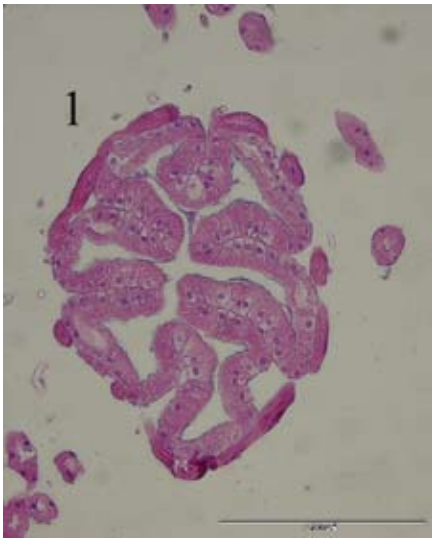


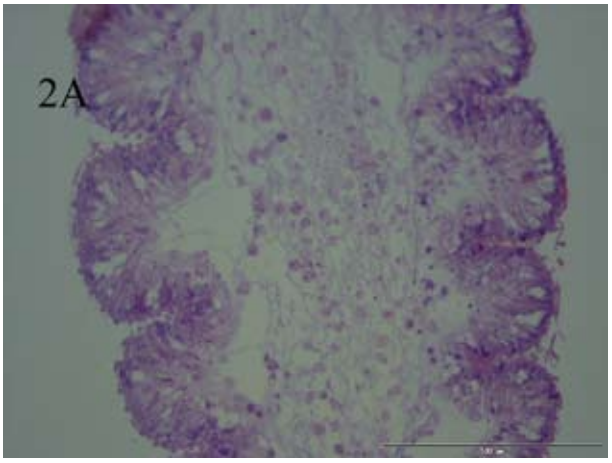
Figure 1. Mid-gut, non-infected honeybee; HE, 100× magnification; scale bar = 200 μm

intestines were fixed in a 4% formaldehyde solution, inserted into paraffin blocks and cut with a microtome to 6 to 7 μm thick sections. Dewaxed sections were stained for general morphological purposes according to the Hemalaon-Eozinic method (HE; Roulet, 1948), and for the purposes of describing neutral (hexose-containing) mucosubstances, acid and sulphate mucosubstances and metachromasia we used the Periodic Acid-Schiff Reaction (PAS; McManus, 1948), Alcian blue (pH = 2.5; Mowry, 1956), Alcian blue (pH = 1.0; Lev and Spicer, 1964) and Toluidine blue (TB)-specific staining (Pearse, 1968). For analysis of stained preparations we employed a bright field microscope Olympus BX41 while photographs were taken using an Olympus DP12 U-TVO camera.

RESULTS

On the 10th day after the treatment of Nosema disease with Nozevit, we observed very similar percentage reductions in the numbers of spores in the treated colonies (78.65%) as in our previous testing (Table 1) (Tlak Gajger et al., 2009a,b). The results of PCR amplification with a generic Nosema primer pair perfectly matched the results of amplification with a specific *N. ceranae* primer pair (data not shown). All sections obtained from the sampled material of healthy bees were characterised by an outer muscular coat (circular inwards, longitudinal outwards) and median basement membrane coated with a stratum of soaring cylindrical epithelium cells with small regenator cells between them, and rabdorium with perithrophic membrane (Figure 1). In addition, the nuclei of epithelial cells had a normal appearance, while the cytoplasm was filled with fine and dense homogeneous inclusions with intact and regular cell boundaries.

In some microscopic preparations from the midguts of bees infected with *N. ceranae* spores, we observed degenerative and lytic processes occuring within invaded cells, and due to an increase in osmotic pressure, cell membranes were burst and destroyed (Figure 2). Analysis of the mid-gut epithelium revealed that some cells were with invisible nuclei, while the nuclei of other cells appeared to be scattered; the cytoplasm of these cells was densely granulated with vacuoles of various sizes, while cell boundaries were not clearly delineated and most cell membranes had been degraded. The results of the general histological examination showed that the lumen of treated bees was coated with a firm layer (Figure 3), while untreated



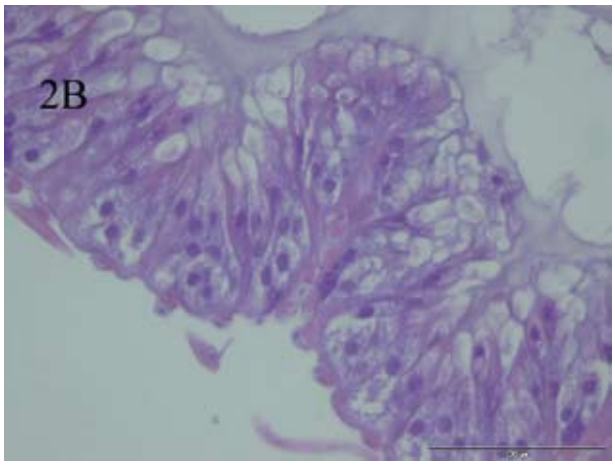


Figure 2. A, B – Mid-gut, bee infected with *N. ceranae* spores, untreated; HE, scale bar A = 500 μm, B = 100 μm

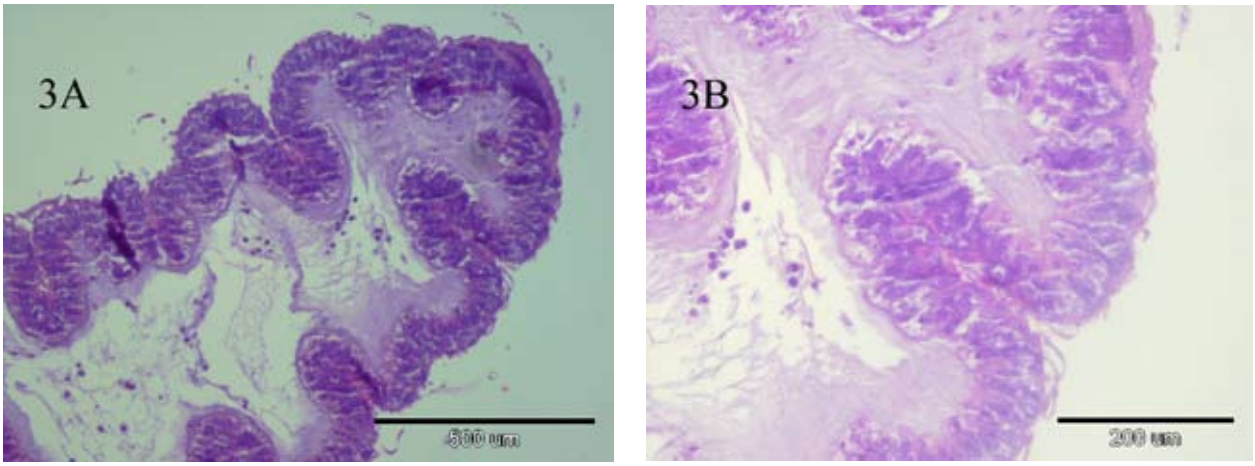


Figure 3. A, B – Mid-gut, bee infected with *N. ceranae* spores treated with Nozevit; HE, scale bar A = 500 μm, B = 200 μm

bees had a much looser and not clearly demarcated area of peritrophic membrane. In addition, it was observed that in the presence of numerous Nosema spores the intestinal contents tended to be squeezed into the centre of the intestinal lumen (Figure 4), probably impeding the germination of spores.

Histochemical analysis showed that the mid-gut mucosa content included glycogen and/or oxidable dioles (PAS+), but treating diseased bees with Nozevit did not lead to increasing amounts of neutral mucosubstances in comparison with diseased non-treated bees (Figure 5). Nozevit treatment of diseased bees led to visible and significant increases in AB (pH = 2.5) positive mucosubstances (Figure 6), or mucosubstances with carboxyl groups, sialic acid or uranic acid and/or with sulphate esters. At the same time, the O-sulfated esters of mucosubstances (AB, PH = 1.0) in the mucous coat of the intestine did not show significant changes in structure (Figure 7), in comparison with non-treated bees. Metachromatic stained (TB) non-sulfated mucosubstances contain sialic acid and appear red-purple. These were visible in the apical part of epithelial cells and in the apical mucosa coat. In the diseased group treated with Nozevit increasing amounts of non-sulfated mucosubstances were clearly visible in the upper mucosa stratum (Figure 8). The histochemical method employed showed high AB (pH = 2.5) positivity, as well as a high number of AB (pH = 1.0) positive reactions and weak methachromatic reactions (TB) in the superficial mucosa mid-gut of honeybees from the healthy group.

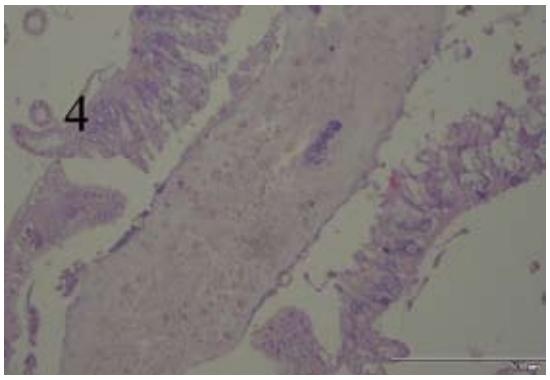


Figure 4. Mid-gut, bee infected with *N. ceranae* spores treated with Nozevit. Intestinal content with numerous spores tended to be squeezed in the centre of the lumen; HE, scale bar = 500 μm

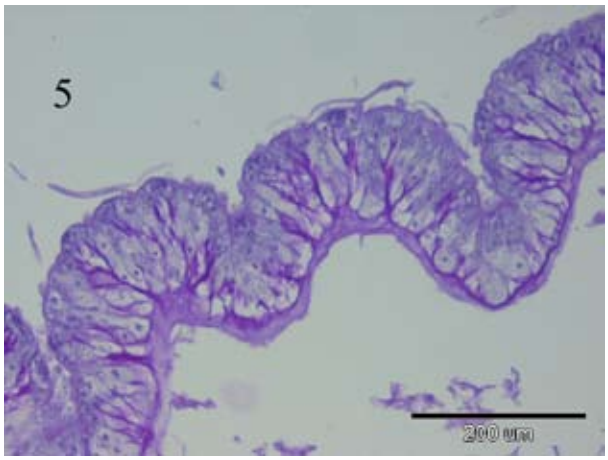


Figure 5. Mid–gut, a non-infected honeybee, PAS; scale bar = 200 μm

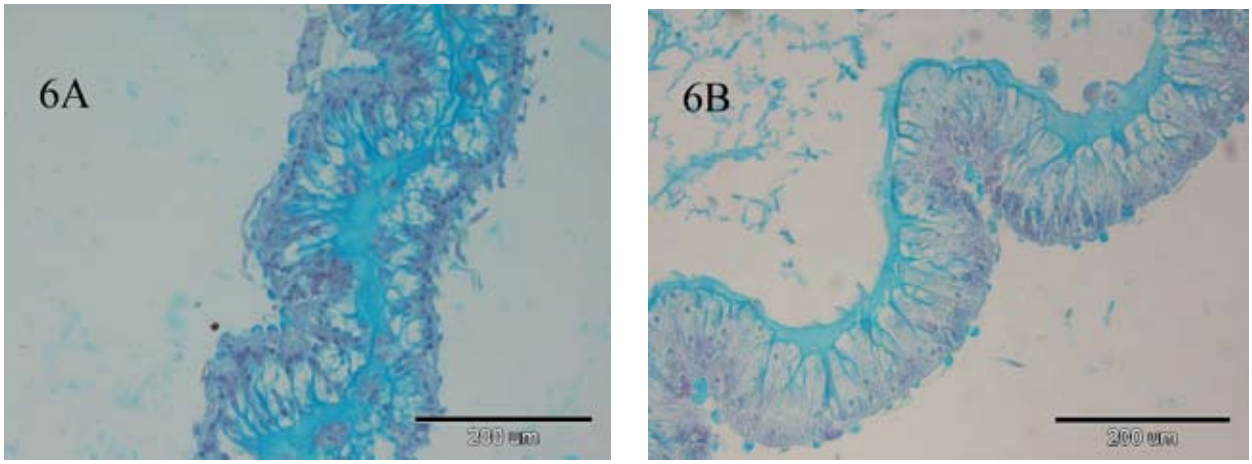


Figure 6. A – Mid-gut, bee infected with *N. ceranae* spores treated with Nozevit; B – Mid-gut, bee infected with *N. ceranae* spores, untreated; A, B – pH = 2.5, scale bar = 200 µm

DISCUSSION

Nosema disease is serious parasitic condition affecting adult honey bees. Infections with *N. ceranae* are especially dangerous because of its uncharacterised pathogenicity, lack of seasonality and contribution to high colony mortality. Due to its inconspicuous signs and the need for eradication by interchange of frames with a brood from a disinfected hive, beekeepers often devote insufficient attention to the disease or neglect it completely. Since EU and Croatian regulations prohibit the use of antibiotics in controlling apian diseases, it appears necessary to introduce herbal preparations into the treatment of Nosema disease. Currently, beekeepers outside of Europe use Fumagillin which is effective against *N. apis* and *N. ceranae* infections (Higes et al., 2008, 2009; Williams et al., 2008). However, it is has been established that it cannot eradicate closely related species like *Nosema bombi* (Whittington and Winston, 2003). Previously, we reported a significant effectiveness of the Nozevit phyto-pharmacological preparation after repetitive preventive and curative treatments of bees afflicted with the disease (Tlak Gajger et al., 2009a). At the time we assumed that Nozevit simultaneously coats both the gut lumen and *Nosema* sp. Spores and probably impedes spore germination.

In this study we studied the histological structure as well as the content and distribution of mucosubstances in the mid-gut mucosa of the honeybee (*A. mellifera*) treated with Nozevit. The mid-gut epithelium of honeybees is constituted of different cell types such as columnar, regenerative, endo-

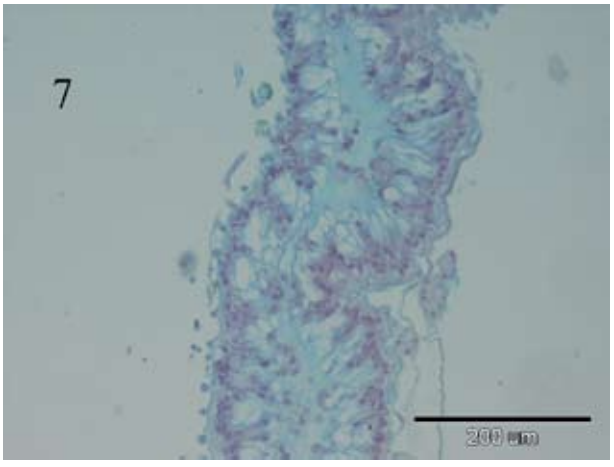


Figure 7. Mid-gut, bee infected with *N. ceranae* spores, untreated; A, B – pH = 1.0, scale bar = 200 µm

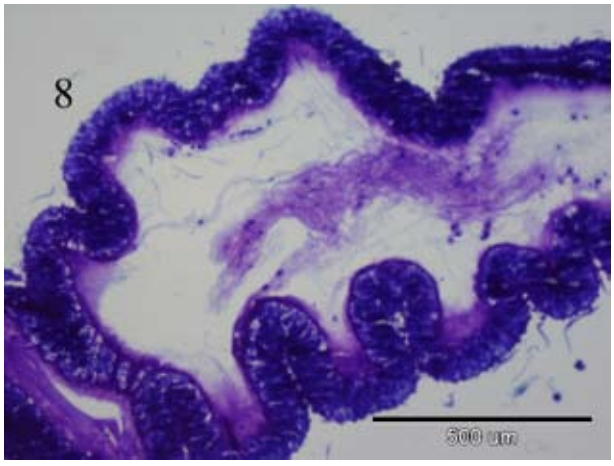


Figure 8. Mid-gut, bee infected with *N. ceranae* spores treated with Nozevit; scale bar = 500 µm

crine and goblet cells. The columnar cells are the most abundant cell type in the honeybee mid-gut and typically have a striated border on their apical surface, consisting of a regular array of cylindrical microvilli (Cruz-Landim and Cavalcanate, 2003). They also function in digestion, while regenerative cells serve to regenerate the damaged functional cells (Cruz-Landim et al., 1996). The peritrophic matrix is a cellular material composed of chitin, proteins and proteoglycans, and its porosity controls the partitioning of digestive enzymes and food as well as protecting the underlying epithelial cells from pathogenic microorganisms and parasitic invasions (Jacobs-Lorena and Oo, 1996), as well as chemicals and acidity (Vegetti et al., 1999).

Histochemical analysis revealed an increase in the production of mucosubstances with carboxylic groups and rich in sialic acid (AB, Ph = 2.5), while the large amount of TB methachromatic substances points to the presence of sialic acid-rich non-sulfated mucosubstances (TB) in the superficial layer of the intestinal lumen in diseased bees after treatment with Nozevit. The secreted mucous layer functions to lubricate undigested materials and plays an important role in osmoregulation (Vegetti et al., 1999), the transfer of proteins or their fragments as well as of ions and fluids (Domeneghini et al., 1998) and protection from mechanical injuries or bacterial invasions. Mucosubstances secreted by the ventriculus (mid-gut) epithelium have been implicated in the absorption of easily digested molecules (Grau et al., 1992) and have a very important role in the intestinal absorption process, especially after starvation when the number of intestinal goblet cells increases (Kakamand et al., 2008). Also, as reported by Tibbetts (1997) mucous cells containing sialo- and sulfoglycoproteins promote an increase in the viscosity of secretion, which is likely to be play a protective role. Nozevit is a natural extract of a specific species of oak bark, derived through a patented process, and has been recognised as a rich source of tannin for many years. Such phenols of high molecular weight contain sufficient numbers of hydroxyl groups to form complexes with proteins, cellulose and some minerals and thus protect against diarrhoea. As diarrhoea is induced by nosema disease, tannins from Nozevit should function to inhibit this and thus substantially reduce the spread of the pathogen within bee colonies. Based on the presented data we can conclude that the herbal preparation Nozevit induces the production and secretion of mucous from the epithelial layer and additionally coats the peritrophic membrane to form a firm and resilient envelope. We envision that this plays a dual role of ensuring protection from new infections with *Nosema* sp. spores and also against normal physiological processes. In future studies it will be useful to see the effect of Nozevit on individual bee survival, and the activity of proteolytic enzymes, in order to demonstrate unequivocally that the preparation protects against infection with *Nosema* sp. spores without altering normal bee physiology.

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