The effect of Nozevit on leucine aminopeptidase and esterase activity in the midgut of honey bees (*Apis mellifera*)

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ABSTRACT: The histochemical activity of aminopeptidase and non-specific esterase, both important enzymes of intermediate metabolism in the mid gut of honeybees (*Apis mellifera*), was investigated. Nosema disease control poses a major challenge, and thus, the treatment of this serious parasitic disease using natural phyto-pharmacological preparations could be of great besnefit. Additionally, the effects of residues and their by-products in honey and wax represent an environmental concern and are another reason for reducing the use of conventional chemical control methods in beekeeping. Nozevit is a naturally prepared water solution of plant polyphenols, and is distributed throughout the market as "the partner for Nosema disease repression". Samples of the midgut tissue were used for the description of enzymatic activity of infected honey bees before treatment and 1, 2, 3, 5, 8 and 12 days after a one-time treatment with Nozevit. The possible effects of Nozevit treatment on the activity of the investigated enzymes and digestion are discussed.

Keywords: honey bee (*Apis mellifera*); Nosema disease; Nozevit; LAP; esterase

Honeybees are economically and ecologically important insects because they enhance agricultural productivity and help maintain biodiversity by engaging in valuable pollination. Nosemosis is a serious parasitic disease of adult honeybees caused by the two described species of microsporidia, *Nosema apis* (Zander 1909) and *Nosema ceranae* (Fries et al. 1996). Pathological changes of the mid gut epithelial cells cause digestive and metabolic disorders (Bailey and Ball 1991), reduced hypopharyngeal gland (Malone and Gatehouse 1998), altered fatty acid composition of the hemolymph (Roberts 1968), as well as malnutrition, all of these leading to the premature death of diseased honeybees and a decrease in the population size of honey bee colonies. Energetic stress is the probable cause of shortened life span observed in infected bees (Mayack and Naug 2009); since microsporidia lack mitochondria and therefore have little metabolic ability themselves (Agnew and Koella 1997) they steal energy directly from the infected bees. *N. ceranae* has better mechanisms to evade host immunity which targets its growth and reproductive capacity when compared to *N. apis* (Chen et al. 2009). Also, it is found that *N. ceranae* has different immune response genes and is able to thrive over a wider range of temperatures (Martin-Hernandez et al. 2009) so it exerts its negative influence over a longer period of the year. Those differences make it a more virulent pathogen for honey bees. Foragers have the biggest energetic demand and are the ones with the highest *Nosema* sp. spore load (Higes et al. 2008). Even early proliferation of the vegetative stages of *Nosema* sp. spores in bee gut cells may cause disruption by placing high metabolic demands on the bee. Also, by subsequent replacement of the cytoplasm of gut epithelial cells with spores and the ensuing cell lysis they can disrupt the entire structure and presumably the function of the gut epithelium (Malone and Gatehouse 1998).

Food digestion and absorption take place in the mid-gut of bees (Chapman 1978; Cruz-Landim et al. 1996; Snodgrass and Erickson 2003). The midgut wall consists of three layers: the inner epithelium, the median basement membrane and the outer muscular coat (Chapman 1978). The mid-

gut epithelium is composed of different cell types such as cylindrical, regenerative, endocrine and goblet cells. Cylindrical cells are the predominant cell type, forming a transversely stripped edge – rabdorium on their upper surface (Cruz-Landim and Cavalcanate 2003). In order to grow and develop normally, honeybees must obtain a sufficient amount of protein through food. Nosema infection of the gut cells has an insidious effect because it reduces the ability of the gut to digest pollen and absorb nutrients and diverts the proteins that would normally be used for royal jelly production into the replacement of damaged gut cells.

Proteolytic enzymes are secreted by midgut epithelial cells of honey bees (Malone and Gatehouse 1998), and are extremely important for the digestion of pollen which is the main source of protein in the honeybee diet (Brodschneider and Crailsheim 2010). These enzymes preferentially catalyse the hydrolysis of peptides and proteins. Leucine-aminopeptidase (LAP) belongs to a group of proteolytic enzymes whose activity leads to the degradation of proteins to amino acids. Aminopeptidases belong to the group of exopeptidase enzymes and they separate N-terminal amino acid from the peptide chain. Esterases are hydrolysing enzymes that degrade esters into acids and alcohol during the chemical reaction of hydrolysis. Crailsheim and Stolberg (1989) found that in healthy bees proteolytic enzyme activity is highest in the first seven days of life, after which it decreases to a certain value maintained throughout their life (Grogan and Hunt 1980). Carbohydrate esterases catalyse the degradation of saccharides. Two groups of substrates for degradation are distinguished: the one in which sugars represent "acid", such as pectin-methyl ester and the other in which the sugars behave like alcohols such as xylan (Bitondi et al. 1983; Mackert et al. 2008).

Since Nosema disease control is a major problem, and since both European Union and Croatian regulations prohibit the use of antibiotics in the treatment of apian diseases, the need for the production and utilisation of natural phyto-pharmacological preparations in the treatment of Nosema disease arises. Nozevit is a naturally prepared water solution of plant polyphenols, and is distributed and marketed as "the partner for Nosema disease repression". The aim of this study was to determine the effect of Nozevit on enzymatic LAP and esterase activity in the mid-gut of honeybees before treatment and 1, 2, 3, 5, 8 and 12 days after treatment.

MATERIAL AND METHODS

Treating bee colonies with the Nozevit phytopharmacologic preparation. The study was conducted on two groups of colonies: an experimental group infected with spores of *N. ceranae* and simultaneously treated with Nozevit and an experimental group infected with *N. ceranae* spores which did not receive treatment. Each experimental group consisted of 12 bee colonies. Honeybee colonies were individually once fed a mixture of 0.5 l of sugar syrup in a 1 : 1 ratio with the addition of 20 drops of Nozevit. The control group of colonies received only sugar syrup prepared in the same way.

Sampling. 1 , 2 , 3, 5, 8 and 12 days after a one-time treatment with Nozevit, 30 gatherer bees from each colony were caught on the return flight to the hive using long tweezers and then sampled. Bees were placed in labelled plastic cups with firm lids and delivered to the laboratory. Ten bees from each sample were used for the laboratory determination of the number of *Nosema* sp. spores and for molecular differential diagnosis of species specificity. The midgut of the remaining 20 bees from the sample was prepared for the histological part of the research.

Laboratory tests for Nosema. The bee samples were microscopically examined for the presence of *Nosema* sp. Spores. To this end, the 10 bees from each sample were crushed with a plastic stick in a pot to which 10 ml of distilled water were later added. These native smears of intestinal contents were examined using an Olympus BX41 light microscope under 400 × magnification and phase contrast. The number of *Nosema* sp. spores was determined by counting spores using a Bürker-Türk haemocytometer (Cantwell 1970), and to identify the species of *Nosema* sp. molecular diagnostic methods were used (Tlak Gajger et al. 2009a). The same samples of crushed bees were used to extract *Nosema* sp. spores. To induce the mechanical breakage of spores from each suspension of an individual sample we transferred 50 µl to a new Eppendorf tube and heated it in a thermal block at 100 °C for 30 min. To isolate genomic DNA the boiled spore samples were centrifuged at 14 000 g for ten min. Then, 30 µl of supernatant were separated and 10× TE buffer to a final concentration of 10mM and 5mM EDTA, at pH 8 was added. This supernatant was used as a source of DNA for further analysis. Samples were stored at a temperature of –20 °C or were directly used for PCR. The PCR procedure

was performed according to the Taq polymerase manufacturer›s instructions (Sigma, USA). The PCR reaction mixture contained 200μM of each dNTP, 3mM $MgCl_2$, 0.5μM forward and reverse primers and one unit of Taq DNA polymerase. To this PCR mix were added 4 μl of extracted DNA (Tlak Gajger et al. 2010a). The molecular size of PCR products was determined using electrophoresis in 2% agarose TAE (Tris-acetate-ethylene diamine tetra acetic acid) gels with standard TAE buffer stained with SYBR green. For the purpose of visualization a UviTec gel documentation system was used.

Determination of enzyme activity. After the honeybee samples were submitted to the laboratory, they were subjected to low temperature refrigeration (4 °C) for 10 min. Then we slowly and carefully removed the gut from 20 bees from each sample. During this procedure we carefully held the bees' chest and abdomen with big forceps and with small anatomical forceps the last seen abdomen scale and pulled the gut out. After this we immediately cut off the front (honey bladder) and rear (rectum) part of the gut. Samples of the midgut of bees were fixated in glass tubes with a chilled (4 °C) solution of formol-calcium for 24 h in the refrigerator. This solution was then taken out and cooled sucrose was poured in. Prepared samples were kept in the refrigerator until further processing. Samples of the midgut of the bees were then embedded in paraffin blocks and cut using a microtome into 10 μm sections. Degreased cuts of midgut were stained according to the the HE method (Roulet 1948) to determine the general morphological characteristics of the tissue, and stained with special stains for determination of LAP activity (Hrapchak and Shennan 1980) and esterase activity (Burstone 1962).

The level of enzyme activity was determined using qualitative microscopic examination (Olympus BX41), under 10–40× magnification, and histological preparations were photographed using the Olympus DP12 U-TVO camera. The areas of LAP enzymatic activity in the sample stain red-purple. Areas with stronger LAP activity stain darker, while areas of weaker activity stain lighter shades of red-purple. Areas with stronger esterase activity in the samples stain grey-blue. Areas with stronger esterase activity stain darker, while areas with weaker activity stain a lighter shade of the same colour. The strength of enzymatic activity was described as: no enzymatic reaction, weak (barely noticeable), enzymatic reaction, variable enzymatic reaction and strong enzymatic reaction.

RESULTS

Ten days after the one-time treatment of bee colonies with Nozevit, a 78.65% reduction in the number of *Nosema* sp. spores was determined in comparison with samples from control groups (Tlak Gajger et al. 2011).

The results of PCR amplification using a generic pairs of Nosema primers perfectly matched the results of the *N. ceranae* multiplication with specific pair primers. The results also show that after multiplication with specific pair primers all examined samples of bees were negative for the presence of the parasite *N. apis* (data not shown).

The histological examination of the midgut preparations of bees in which *Nosema* sp. spores were not coprologically found, revealed the presence of all layers of the intestine wall- outer longitudinal and transverse muscle layer, median basement membrane coated with a layer of highly cylindrical epithelial cells among which individual regeneratory cells were visible, and rabdorium and a partly peel perithrophic membrane next to the intestinal lumen. In the histological preparations of the midgut of bees infected with *N. ceranae* spores, we found degenerative changes and lytic processes within the epithelial cells, and depending on the severity of the invasion and the consequent high osmotic pressure due to the presence of a large number of spores, destroyed epithelial cells.

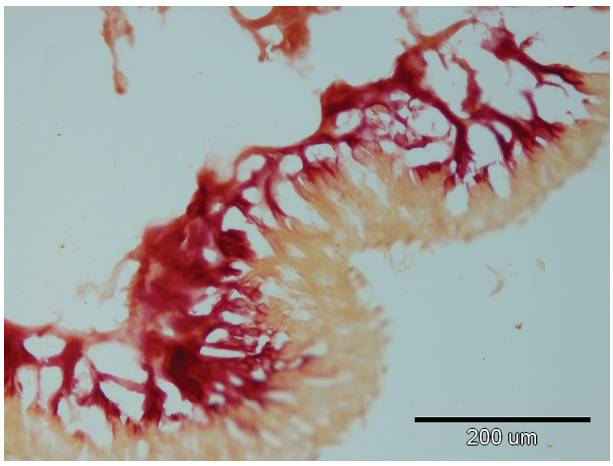

Figure 1. LAP activity in the mid gut of honeybees one day after Nozevit treatment; magnification 20×

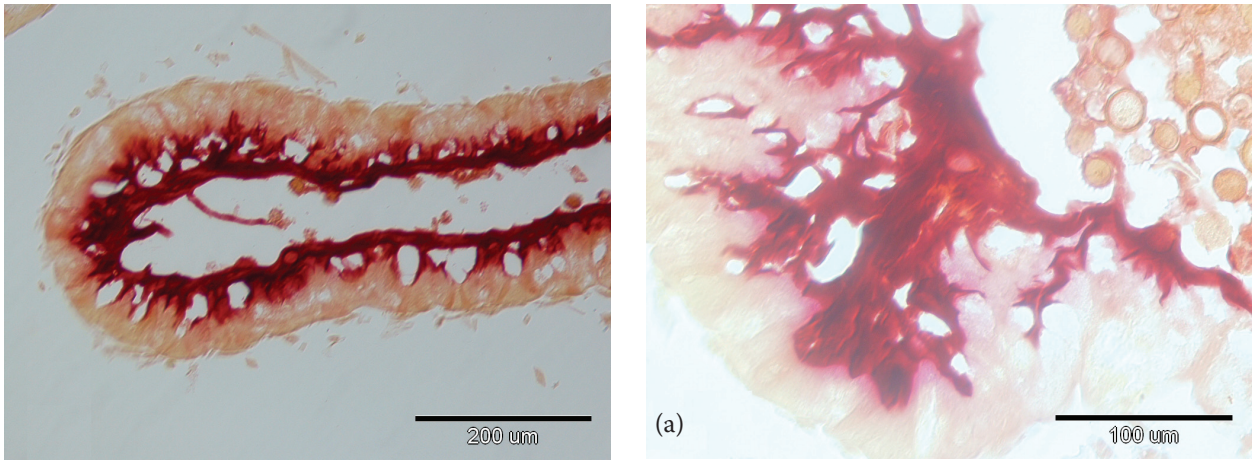

Figure 2. LAP activity in the mid gut of honeybees two days after Nozevit treatment; magnification 20×; (a) = magnification 40×

In all samples of the midgut of sampled bees originating from the experimental group, we found present and visible LAP activity, seen especially in the apical parts of enterocytes. A strong reaction was detected in midgut samples extracted two and three days after the one-time treatment of colonies with Nozevit, while on the 1st , 5th , 8th and 12th day LAP activity was weaker or variable. In samples of the mid-gut of bees sampled from the control group LAP activity was weak throughout the entire experimental period, and there were no differences between individual days. The described results are shown in Figures 1, 2, 2a, 3, 4, 5 and 5a.

In histological mid-gut preparations of bees originating from experimental groups, we found weak esterase activity, more visible in the area of the rabdorium and partly in the lumen of the intestine itself. The strength of reaction was similar throughout the entire experimental period, and we did not note any differences in activity between individual days. In the midgut samples of bees from control groups, esterase activity was negative to barely visible throughout the entire experimental period, and there were no established differences between individual days. The described results are shown in Figures 6 and 7.

DISCUSSION

Nosemosis caused by *N. ceranae* microsporidia is a disease of adult bees hardly noticeable by clinical examination of colonies (Hornitzky 2005). The pathogenesis of the disease is currently under-researched and poorly understood. The prevalence of the disease shows no seasonality (Martin-

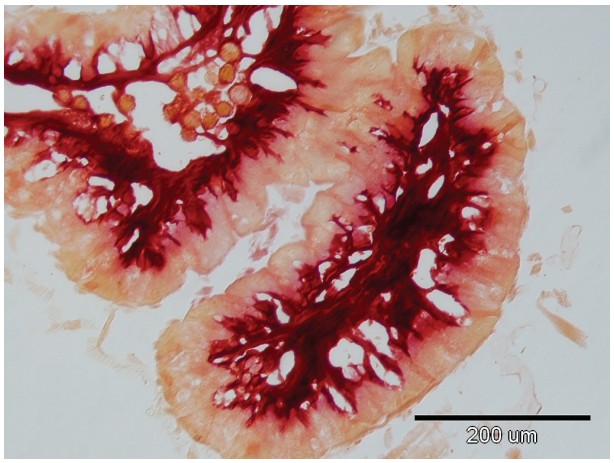

Figure 3. LAP activity in the mid gut of honeybees three days after Nozevit treatment; magnification 20×

Figure 4. LAP activity in the mid gut of honeybees eight days after Nozevit treatment; magnification 20×

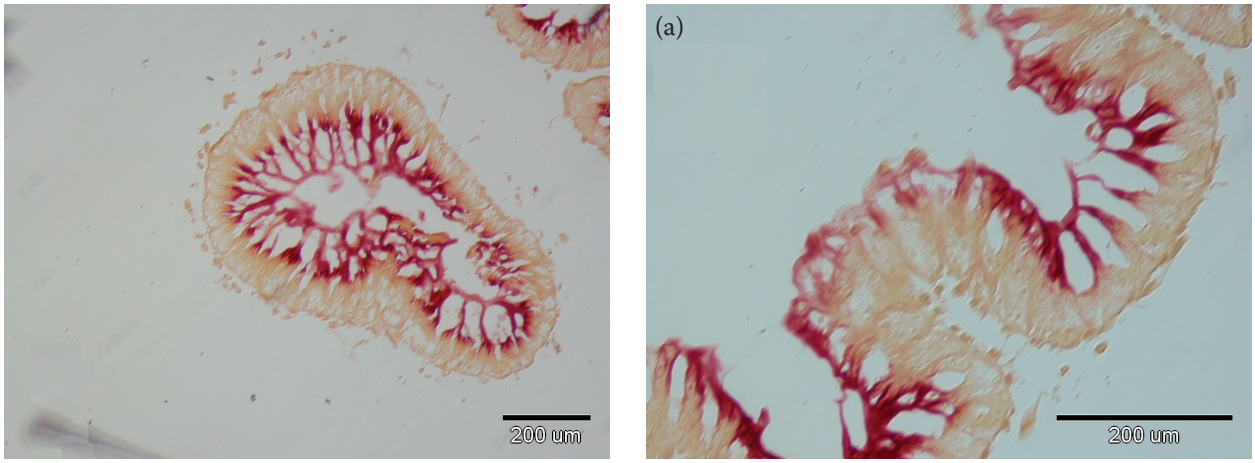

Figure 5. LAP activity in the mid gut of untreated honeybees two days after initial sampling; magnification 10×; (a) = magnification 20×

Hernandez et al. 2007) and is considered to cause the sudden death of colonies without prior visible signs of illness (Morse and Shimanuki 1990; Malone et al. 1995). Results associated with the differential diagnostic distinction of species of *Nosema* sp. Spores only confirmed the presence of "a new pest of European honey bees", *N. ceranae*, which correlates with the results of previously conducted studies related to determining the prevalence of *N. ceranae* in parts of Croatia (Grilec 2010; Tlak Gajger et al. 2010a, b).

The activity of the digestive enzymes of the honey bee is closely associated with the processes of the digestion of food and the absorption of digested nutrients, and depends on feeding habits (Brodschneider and Crailsheim 2010). Since the basis of a bee's diet is honey, pollen and water, LAP and esterase enzymes secreted by midgut epithelial cells of bees have a particularly critical role among digestive enzymes important for the intermediate metabolism (Malone and Gatehouse 1998). The activity of these enzymes is strongest in young bees, and these enzymes are necessary for the degradation of proteins and esters, which are essential for building an entire bee's organism, especially the development of the glandular tissue, and consequently their physiological functions. In order to complete the proper development of hypopharyngeal glands and the body fat, bees must digest large amounts of protein (Winston 1987).

Gatehouse and Malone (1998) quantitatively assessed the level of the LAP enzyme using the Christeller and Shaw method (1989), and we assessed it qualitatively, according to the intensity of colour in stained histological preparations. In the available literature, the different LAP activities

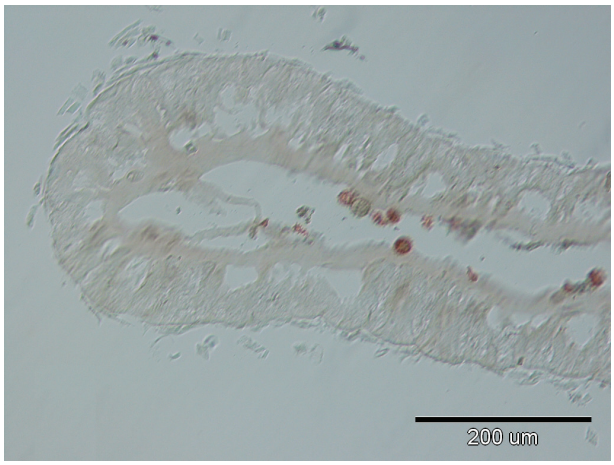

Figure 6. Esterase's activity in the mid gut of honeybees two days after Nozevit treatment; magnification 20×

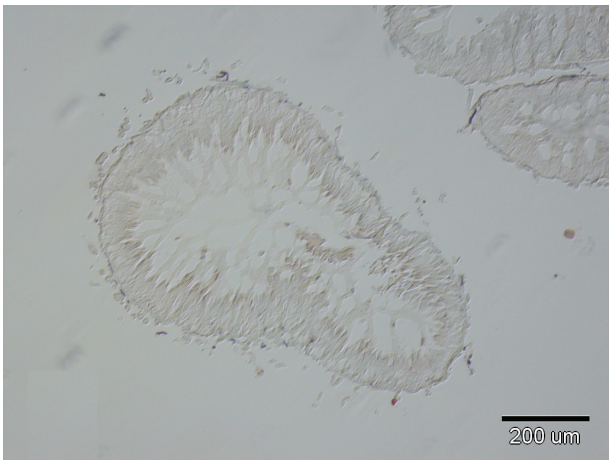

Figure 7. Esterase's activity in the mid gut of untreated honeybees two days after initial sampling; magnification 10×

in healthy and diseased bees have been reported (Malone and Gatehouse 1998), but data is lacking on the extent of enzymatic activity in bees naturally infected with nosema or those treated with natural phytopharmaceutical preparations. In nosema-infected bees protease activity was generally weaker, and it is believed that one of the ways in which *Nosema* sp. disrupt the digestive ability of infected bees is via a reduction in proteolytic capability (Liu 1984).

Normative acts in force in the EU and Croatia prohibit the use of antibiotics and fumagillin in the treatment of nosemosis and other apian diseases, especially because of the potential development of resistance to multiple chemotherapeutics used and possible agent residues in bee products intended for human consumption. Currently, beekeepers in other parts of the world use fumagillin, which was effective for the treatment of nosemosis caused by *N. apis*. However, the effect of fumagillin in the treatment and suppression of nosemosis caused by *N. ceranae* is still being investigated (Williams et al. 2008). Poor results from the use of fumagillin to combat the related parasite *Nosema bombi* in bumble bees (Whittington and Winston 2003) indicates that this antibiotic may not be suitable for the continuous treatment of nosemosis caused by the parasite *N. ceranae*.

The use of plant products as an alternative treatment option for nosemosis must be additionally investigated, but recently published results using Nozevit show its high efficacy as a preventive and curative measure to combat diseases of bees infected with *N. ceranae* (Tlak Gajger et al. 2009a, b, 2011). Based on the results of these studies, it is assumed that Nozevit at once mechanically prevents the germination of *Nosema* sp. spores that have entered the intestinal lumen and partially envelops rabdorium midgut epithelial cell membrane and the peritrophic membrane to form an additional mechanical protection against new invasions. Previously performed histochemical studies also showed that the use of Nozevit in the midgut of treated bees stimulates the production and secretion of several different types of mucopolysaccharide mucus that assist in the processes of digestion, especially digestion of pollen, and the further envelopment of the peritrophic membrane (Tlak Gajger et al. 2011).

Greater LAP and esterase enzymatic activities in midgut samples of honey bees treated with Nozevit compared to control groups were observed, especially on the 2nd and 3rd day after treatment. LAP activity was markedly stronger in the first three days after treatment with Nozevit compared to the control groups indicating that all phases of the development and multiplication of the pathogen, as well as the number of spores, interfere with proteolytic enzyme production. The very weak enzymatic activity in midgut samples of control groups observed on the 8th and 12th day is similar to the results of Malone and Gatehouse (1998), whereas all of the preparations made on Days 8 and 24 show the replacement of bee tissue with Nosema spores which results in the lysis of infected epithelial cells and provides an obvious explanation for the drop in enzyme activity. Esterase activity was determined to be very weak, which is not surprising when taking into consideration that the study was conducted on adult honey gatherer bees infected with *Nosema* sp. spores. These results show that the use of Nozevit in the midgut of treated bees stimulates the activity of the studied proteolytic enzymes. In future research, it will be necessary to determine the effect of Nozevit on the lifespan of bees and whether its administration leads to any adverse effects on the physiological processes in bees. These data might ultimately allow the drawing of a conclusion regarding its effectiveness.

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