SURVEY OF THE PREVALENCE OF *NOSEMA* SPECIES IN HUNGARIAN HONEY BEES, AND DEVELOPMENT OF THE METHOD OF DETECTION

Theses

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Gödöllő

2022

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1 Background of the thesis

Of the two unicellular species belonging to the *Microsporea* (DELPHY 1936), *Nosema apis* (ZANDER 1909) was previously thought to infect only the western honey bees, *Apis mellifera* (LINNAEUS 1758), and *Nosema ceranae* (FRIES et al. 1996) to infect only the eastern honey bees, *Apis cerana*, (FABRICIUS et al. 1792). For nearly a hundred years, nosemosis was brought about in parallel with the onset of dysentery symptoms (brown fecal dots on frameworks, spleens, and outlets) in the spring and then usually spontaneously terminating during the summer (BAILEY 1955; BAILEY 1967). By the early 2000s, more and more beekeepers were reporting summer stock decline, but in the absence of seasonal cyclicality and classic symptoms, they did not suspect nosemosis. However, there have been recent reports that *Nosema ceranae* also infects western honey bees (CHAUZAT et al. 2007; CHEN et al. 2008; DAINAT et al. 2012; FRIES et al. 2006; GIERSCH et al. 2009; HIGES et al. 2006; HUANG et al. 2007; INVERNIZZI et al. 2009; KLEE et al. 2007; NABIAN et al. 2011; PAXTON J. et al. 2007; STEVANOVIC et al. 2011; TAPASZTI et al. 2009; WILLIAMS et al. 2008b). From an epidemiological point of view, it has been shown that the symptoms and pathology of *Nosema apis* and *Nosema ceranae* infection show a different picture (HIGES et al. 2010). In 2009, at the COLOSS workshop in Guadalajara, the proposal to differentiate the disease according to the pathogenic species was adopted (HIGES et al. 2009): infection by *Nosema apis* assessed as nosemosis type “A” and infection by *Nosema ceranae* assessed as nosemosis type “C”. The chronic state of type “A” nosemosis typically occurs in the spring, before the onset of foraging, which is a symptom of flightless bees crawling or already dead bees near the hive and traction development is a symptom in addition to dysentery. There are no clear clinical signs of type “C” nosemosis, there is no seasonality in the

Spanish records, and the colonies left untreated collapse over time (MARTÍN-HERNÁNDEZ et al. 2007; HIGES et al. 2008; HIGES et al. 2009; HIGES et al. 2010). However, a survey in Germany did not show persistent disease of *Nosema ceranae* (GISDER et al. 2010).

The presence of *Nosema ceranae* in Hungary was first detected by TAPASZTI et al. (2009). Of the 38 Nosema-infected bee samples collected in 2007, only *Nosema ceranae* was detected by PCR-RFLP in 37 bee samples. CHEN et al. (2008) from U.S. bee samples collected in 1995 and PAXTON et al. (2007) from Finnish bee samples collected in 1998 showed that *Nosema ceranae* infects western honey bees. At the level of the bee colony, the calculation of the average number of spores per bee has traditionally been used to determine the severity of nosemosis (FURGALA and HYSER, 1969), which later became the method for detecting *Nosema apis* infection. According to the study of FINGLER et al. (1982), the percentage of infected bee individuals within a bee sample correlates with the average number of spores calculated for bee individuals.

Studies in Spain (HIGES et al. 2008b; MEANA et al. 2010) for *Nosema ceranae* did not show a direct correlation between the average number of spores per honeybee and the level of infection in the bee colony. The spores of the two Nosema species are almost impossible to distinguish on the basis of standard microscopic examination (FRIES 1997). A clear distinction requires molecular testing, but it is costly, especially to extract the DNA stock, from the highly resistant spore form without the commercial kit and the automated technology that serves it (HIGES et al. 2009a).

The overall goal of my work is to investigate the spatial and seasonal occurrence of Nosema species in Hungary, which cause the nosemosis of honey bees, and to develop the diagnosis of the infection, paying special attention to the following hypotheses that are regularly formulated among the producing apiaries:

1. It is assumed that *Nosema ceranae* was present in Hungary before 2007. My objective is the examination of bee sample collected earlier than 2007, and the determination of the type of infection by PCR on samples that are confirmed *Nosema spp.* positive by microscopic.
2. According to independent, contradictory results reported within Europe, *Nosema ceranae* is the most common of the *Nosema* species. My aim is to study the distribution of *Nosema* species in Hungary by processing sample collection over several periods and over a large area.
3. It is assumed that for the same bee colony, the results of sample processing methods for *Nosema* species are different based on sampling to distinguish between interior worker and forager bees. My goal in the diagnosis of nosemosis is to develop a practical method that can more accurately determine the infection at the bee colony level, taking into account the method of sample collection.
4. It is hypothesized that the molecular test used to detect *Nosema* species and differentiate their type of infection can be performed without the commercial kit and the automated technology that serves it. My goal is to perform a triplex PCR assay to differentiate infection types without a commercial kit and the automated technology that serves it.

In addition to examining the above hypotheses, my aim is to evaluate the results and to make recommendations for *Nosema* species collection and sample processing methods for recognition of its development at an early stage, assessment and treatment of the disease, and to simplify and make more economical the molecular testing method.

2 Materials and methods

2.1 Collection of archived, preserved samples

Exploring the frozen bee samples of KATKI, we found bee samples from 2004. According to the designation of the samples, during the sampling in 2004 a total of 26 samples were collected from weakened colonies in front of the hives and from the bottom boards. Samples consisted of 10 and 20 bees from 11 different counties.

2.2 Field sampling in 2010

In April, spring, July, summer and October, autumn 2010. we organized a national bee sampling campaign with the participation of the network of consultants of the National Hungarian Beekeeping Association, the Gödöllő Animal Husbandry Research Institute (now the National Center for Biodiversity and Gene Conservation) and voluntary apiaries. In the sampling campaign, we sought to ensure that the sampling sites were derived from all major geographical units in the country for a representative assessment of the prevalence of *Nosema apis* and *Nosema ceranae*. The sampling sites were in operating production apiaries. Sampling sites were selected based on personal acquaintance and the willingness of beekeepers to participate, so there was a minimal variance between sites and sample numbers per season. Throughout the year, the overlap of samples was 42 sites and 126 colonies.

We randomly selected three bee colonies per site for sampling. Two types of bee samples were taken from these bee colonies. Indoor worker bees were picked from the peripheral frames of the nest boxes that did not contain uncapped brood. To sample the forager bees, the hive exits were blocked for 20 minutes, and after the returning bees had settled on the entrance board, we swept them together. Sampling of forager bees was performed before 9 a.m. or after 3 p.m. so as not to interfere with the young bees performing the orientation flight during the day. For each sample, at

least 60-60 individual bees were collected and later divided into two 30-bee sections.

2.3 Sample processing in "CAR"

The bee samples collected in the spring and summer of 2010 were processed at the Beekeeping Research Center in Spain (Centro Apicola Regional, Marchamalo). To purify the spores, 30 whole bee samples were placed simultaneously in BA6040/STR (Seward) type filter bags and 5 ml of molecular biological grade H2O (MilliQ) was added. The filter bags were stomached with the Stomacher 80 Biomaster (Seward) at maximum speed for 2 min. In the bags, the inner mesh served as a filter. The filtrate was centrifuged in 15 ml centrifuge tubes at 800 x g for 6 minutes at 20 °C. After centrifugation, the contents of the tubes were decanted and the pellet adhering to the bottom of the centrifuge tubes was resuspended in 1 ml of H_2O with a vortex mixer for 3 minutes. All slides processed from the suspension were inspected for the presence of *Nosema spp*. occurrence with a phase contrast microscope at 400 X magnification according to OIE (2008) guidelines

To determine the presence of *Nosema* species, triplex PCR analysis was performed on the suspensions using the QIAGEN commercial kit and instrumentation. To extract spores, I used a TissueLyser II (Qiagen, Inc.) and dispensed 150 microliters per suspension into 96-well plates (Qiagen, Hilden, DE) in pre-filled tubes with glass beads (2 mm, Sigma) for 30 min at 30 hertz. I filled the last tube of the plate with water for a blank sample to have a negative control sample as well. For protein degradation, 30 microliters of ATL buffer (Qiagen 19076), 20 microliters of proteinase K (Qiagen 19131) were measured for the suspension and incubated at 56 ° C for 12 hours. For DNA isolation, I ran the BS96 DNA tissue extraction protocol on a BioSprint 96 (Qiagen) capable of isolating 96 DNA samples at a time. Plates containing isolated DNA were stored at -20 ° C until use.

The extracted DNA was analyzed by PCR with *Nosema* species-specific primers 218MITOC F / R and 321APIS F / R. During the study, I set up the positive control by checking the presence of the cytochrome c-oxidase (COI) gene of western honey bee DNA with the species-specific 118COI F/R primers (MARTÍN-HERNÁNDEZ et al. 2007). The PCR conditions were as follows: 12 microliters of 12.5 microliters Fast Start Master (No. 04710452001 Roche Diagnostic, Basel, CH), 0.4 micromol of the 218MITOC F/R and 321APIS F/R primers and 0.2 micromol of the 118COI-F/R primers were used, 0.2 milligrams / ml BSA, 0.1% Triton X-100 and 2.5 microliters DNA template. The thermocycle program is set to 95° C for 10 minutes, 35 cycles to 95° C for 30 seconds, 61.8° C for 30 seconds, and 72°C 45 seconds, final extension 72 ° C 7 minutes. The sensitivity levels of this technique are 2.5 spores of *Nosema ceranae* or 25 spores of *Nosema apis* from 150 microliters of the sample suspension. Negative and positive controls were processed in parallel during the DNA isolation and PCR analysis phases to detect potential contamination and to assess the reliability of the sample processing. The PCR program was performed on an Eppendorf Mastercycler Ep gradient S 7601 device. All PCR products were analyzed in a high-resolution capillary electrophoresis system using a Qiagen QIAxcel using the QIAxcel DNA Resolution Kit (QIAgen, No. 929002).

2.4 Sample processing in “RET”

The retrospective examination of the archive, preserved bee samples from 2004 and the bee samples collected in October 2010 and the examination of some of the bee samples from the summer 2010 collection and the autumn 2010 collection at the Szent István University Gödöllő Regional University Knowledge Center. The archive bee samples consisted of 10-20 bees, so I used as many bees as were available per sample to process the archive bee samples.

From the bee samples collected in 2010, I used 30 bees per sample. The bees belonging to each sample were placed in an LDPE sealed sachet (M6080B, Labsystem) and 5 ml of molecular biological grade H_2O (MilliQ system) was added. I rubbed the sachets in my hand until their contents became homogeneous. I did not close the opening of the bags so that the excess air could escape and the bags would not tear. The procedure lasted at least 1 minute per sample.

The contents of the sachets were filtered through 15 micron nylon filter mesh into 15 ml centrifuge tubes, then the tubes were centrifuged at 800 x g for 6 min at 20 °C. After centrifugation, the contents of the tubes were decanted and the pellet adhering to the bottom of the centrifuge tubes was resuspended in 1 ml of H_2O for 3 minutes using a vortex mixer. All slides processed from the suspension were inspected for the presence of Nosema spp. occurrence with a light microscope at 400 X magnification, than I counted the average number of spores per bee (discussed in detail in the section “Calculating the proportion of spores and infected bees”). The 1 ml suspension was centrifuged again, this time in 1.5 ml Eppendorf tubes for 15 minutes at 4 °C at 13,000 rpm. The contents of the tubes were decanted and the pellets adhering to the bottom of the centrifuge tubes were mixed with 300 µl of CTAB buffer (CORNMAN et al. 2009) and 200 milligram glass beads (425–600 microns, Sigma-Aldrich). With tens of the Eppendorf tubes were attached with adhesive tape to the vibrating head of the vortex mixer (ZX3m, Velp Scientific) and operated at maximum rotation for 5 min. The suspension was incubated for 12 hours after the addition of 1000 milligrams of proteinase K with shaking at 200 rpm.

To recover the DNA, I used 4 cycles of centrifugation for 15 minutes per cycle at 4 °C, and each time the supernatant (300 microliters) was transferred to a new Eppendorf tube as follows:

in the first cycle 1: 1 suspension phenol mixture in equal amounts, in the second cycle 1: 1: 1 supernatant phenol-chloroform mixture, in the

third cycle 1: 1 supernatant chloroform mixture in equal amounts, in the fourth cycle was centrifuged 1: 2.5 supernatant - -20 °C ethanol mixture.

After the fourth cycle, the ethanol was removed, the pellet adhering to the bottom of the Eppendorf tube was dried, and then was dissolved in 30 microliters of H_2O. Eppendorf tubes containing isolated DNA were stored at -20 °C until use. The quantity, quality and purity of the DNA were analyzed spectrophotometrically with an Implen nanophotometer (Implen GmbH, Germany) and the concentration was adjusted to 20 nanograms/microliter with water. Extracted DNA was analyzed by PCR with *Nosema* species-specific primers 218MITOC F/R and 321APIS F/R (MARTÍN-HERNÁNDEZ et al. 2007). During the study, I set up the positive control for the presence of the western honey bee DNA by checking cytochrome c-oxidase (COI) gene. The sequences of the COI primers used were the same as those of the primers used in “CAR”. The components of the 25 microliter PCR reaction mixture were as follows:

1 unit AmpliTaq Gold Polymerase buffer (Applied Biosystems), 1.5 microliters MgCl (25 mM), 2 microliters dNTP (2.5 mM), 2.5 microliters BSA (250 mg/ml), 3.3 microliters Triton X-100, 0.9 microliters each 218MITOC (10 mM) and 321 APIS (10 mM) primers, 0.2 microliters of COI-F/R (10 mM) primers, and 9 microliters of DNA template (20 nanograms/microliter).

The setting sequences of the PCR thermocyclic program were the same as those used in the “CAR. PCR products were visualized by agarose gel electrophoresis.

2.5 Calculation of the number of spores and the proportion of infected bees

The average spore count was calculated with a haemocytometer when checking the suspensions prepared from the October 2010 sample collection. (Bürker, Fein - Optik Jena, Tiefe 1/100mm, 1/400 és

1/25 qmm). The standard haemocytometer I used consists of 3 x 3 large squares separated by triple lines. Above the middle large square is a layer of 0.1 microliters of liquid. The middle quadrature consists of an additional 25 smaller squares, also separated by a triple line, above which are 4 nanoliter liquid layers. The 25 squares consist of an additional 16 smaller squares separated by a single line over which there is a 0.25 nanoliter liquid layer. If the number of spores in the case of squares below 4 nanoliter volume was clearly more than 100 spores at first glance, then I calculated the number of squares below 0.25 nanoliter volume. Counting the spores from those above the line, I counted only those above the left line and the top line.

Based on the reports of CANTWELL (1970) and HUMAN et al. (2013), I followed the origianl dilution ratio of 5 milliliters of water added to 30 bees. From the number of spores per square of 4 nanoliter volume, I calculated the dilution factor and the average number of spores per bee based on the formula in Figure 21 of my dissertation. The proportion of infected bees was calculated using the remaining bees (n=30) of the samples from the October 2010 sample collection.

From the samples, each bee was grinded separately in a mortar by adding 1 ml of distilled water.

On the slides made of homogeneous rubs I checked for *Nosema spp.* occurrence.with a light microscope at a magnification of 400 X. For each sample, I used multiple field-of-view checks, and if I did not see spores when positioned on three different fields of view on the slide, I classified it as negative.

2.6 Defining infection categories and risk levels

When processing the data measured during my study, I coordinated the traditional, qualitative and quantitative categorization of the extent of infection. To determine the infection categories and risk levels, I did not find a risk-level semiquantitative assessment method in the diagnosis of

nosemosis, so I could only partially rely on the literature. I found the largest number of elements in Fingler's paper, so I started from the values he measured. I categorized the highest values in the "we are already late" category. For average spore counts, the value of the square root of the highest value of Fingler et al. (1982) was taken as +++, the lowest measured value was taken as the value of +, and the value of ++ was determined by the mean of the two.

For the percentage of infected bees, the highest value of Fingler, which was 100%, half of that, 50%, I defined as extremely risky. For the medium risk value, 30% was subsequently determined according to the measurement results already obtained (CSÁKI et al. 2015), when I had already counted all the samples. I discuss this in the results.

2.7 Statistical analysis

To compare the independent probabilities, I performed a Z-test using MS Excel. To examine the homogeneity of the distributions, I also used a Chi-square test using MS Excel. Linear quantile regression using MS Excel was used to analyze the correlation between the average number of spores and the proportion of infected bees.Eredmények és azok megbeszélése

2.8 Retrospective test results of archived, preserved samples

In all 26 samples the occurrence of *Nosema spp.*. were confirmed by light microscopy. However, PCR assays confirmed the occurrence of *Nosema spp*. in only 6 (23%) samples. Of the 6 samples, I confirmed the occurrence of both Nosema species in 5 samples, and I confirmed the independent occurrence of *Nosema ceranae* in only one sample.

2.9 Test results of bee samples collected in 2010

Based on data from testing of bee samples collected in 2010, the proportion of *Nosema spp.* infected bee colonies ranged from 95% to 98%. Neither the number nor the proportion of infected bee colonies showed a significant difference in the seasonal comparison (Z-test: P> 0,05).

In terms of seasons the distribution of *Nosema ceranae* and *Nosema apis* infection rates is homogeneous (Chi-square test: $\chi^2(2) = 1,11$; $P = 0,57$). The prevalence of *Nosema ceranae* in all seasons was significantly higher than *Nosema apis* or the co-occurrence of both species (in all three seasons: $Z>15,3$; $P<0,001$). The rate of *Nosema ceranae* infections in the infected samples ranged from 95% to 98%.

In the number of spores per individual in indoor bee samples I calculated the 5%; 10%; 50%; 90% and 95% infection quantiles. I fitted a linear model depending on the number of spores per individual to the quantiles according to the proportion of infected bees. According to my calculations, in the case of indoor bees, if the number of spores per individual is below 1,2 million, the risk level of infection in the bee colony is approximately 95% probability to be moderate (this is when less than 30% of the bee individuals are infected). If the number of spores per individual is between 1,2 million and 3,6 million, the risk level for infection of the colony remains only approximately 50% probability to be moderate. If the number of spores per individual exceeds 3,6 million, the infection of the bee colony is only approximately 10% probability to remain moderate, 90% probability for high risk, and we have to deal with a probability of more than 10% probability for an extremely high risk (this is when more than 50% of the bee individuals are infected).

In the case of samples of forager bees, I also calculated the 5%; 10%; 50%; 90% and 95% infection quantiles for the number of spores per individual. I also fitted a linear model depending on the number of spores per individual to the quantiles according to the proportion of infected bees. According to my calculations, in the case of forager bees, if the number of spores per individual is below 3,6 million, the risk level of infection in the bee colony is approximately 10% probability to be moderate (this is when less than 30% of the bee individuals are infected) If the number of spores per individual is between 3,6 million and 6 million, the risk level for infection of the colony remains only approximately 5% probability to be

moderate and we have to deal with a probability of more than 50% probability for an extremely high risk (this is when more than 50% of the bee individuals are infected). If the number of spores per individual exceeds 6 million, the infection of the bee colony is more than 95% probability for high risk (when 30-50% of the bee individuals are infected), and a probability of more than 50% probability for an extremely high risk (this is when more than 50% of the bee individuals are infected).

2.10 Comparison of forager bee and indoor bee samples

Regression analysis shows a significant correlation between the number of spores per individual bees and the percentage of infected bees ($R_{indor} = 0,65$; N = 130; $R_{orager} = 0,43$; N = 138; P<0,001 in both cases). The proportion of infected bees in the samples of forager bees was higher in all seasons than in the samples of indoor bees, however, no significant differences were detected (Z-test: P>0,05).

The results of the microscopic evaluation of the samples collected in 2010 were confirmed by the results of the PCR analysis. Data obtained with a phase contrast microscope can be compared with data obtained with a light microscope. According to Table 7, the results of PCR tests show a slightly higher level of infection compared to the results of any of the microscopic examinations. In contrast to 89% of the microscopic examinations, 95% of the PCR examinations were positive for samples from the spring collection. In contrast to 97% of the microscopic examinations, 98% of the PCR examinations were positive for samples from the summer collection. In contrast to 95% of the microscopic examinations, 97% of the PCR examinations were positive for samples from the autumn collection. However, according to the Z-test, there is no significant difference between the rate of infection detected by microscopy and PCR in any season (P>0,05).

Using a cheaper DNA isolation method and multiplex PCR analysis, I detected and identified the two *Nosema* species from the western honey bees. Using this method, I have detected the presence of *Nosema*

spp. also from bee samples that could not be confirmed by light microscopy.

2.11 Novel scientific results

1. Based on archived, preserved bee samples, I determined the infection and presence of *Nosema ceranae* in Hungary in 2004. I accept Hypothesis 1.
2. Based on my PCR analyzes of bee samples collected in the spring, summer and autumn of 2010 in Hungary, I determined the territorial distribution of *Nosema* species in Hungary in terms of seasons. A *Nosema sp*. it can be detected in all seasons and in all areas, and *Nosema ceranae* is more common. I determined the prevalence of "C" type nosemosis disease. I accept Hypothesis 2.
3. In the diagnosis of nosemosis, I introduced a risk-level semiquantitative evaluation method, with which infection can be more accurately determined at the bee colony level. Samples of indor workers bees and forager bees should be evaluated differently. I accept Hypothesis 3.
4. To the best of my knowledge, I performed the first test for domestic *Nosema spp*. successful triplex PCR with 218MITOC F / R, 321APIS F / R and 118COI F / R primers, replacing a commercial tissue lyser and cell breaker with a conventional vortex mixer and conventional phenol-chloroform mixture centrifugation to recover and purify DNA, and the electrophoresis was also run on a conventional agarose gel. Using this method, a molecular test for the detection of *Nosema* species has become simple and economical to reliably differentiate between *Nosema spp*. types of infections. I confirm Hypothesis 4.

3 Discussion and proposals

By retrospective examination of the archived, preserved samples I proved that *Nosema ceranae* has existed in Hungary at least since 2004. The presence of both Nosema species in five of the six samples found to be positive may also suggest that the introduction of *Nosema ceranae* may have occurred at about this time (not long before), and has not yet displaced

Nosema apis. In studies by TAPASZTI et al. (2009) and in my own studies in 2010, we found a much higher proportion of *Nosema ceranae* than in 2004. According to the latest studies, *Nosema apis* has almost disappeared from apiaries. In the case of archived, preserved samples, the confirmation of a positive result for nosema infection was less than in PCR studies compared to the results of light microscopy, which I think was caused by the storage conditions. These samples have been unfreeze and refreeze several times over the years.

Since I found an extremely low prevalence of *Nosema apis* in all seasons, I think the cyclical occurrence theory of *Nosema apis* reported by BAILEY (1955) could be revised. In contrast, the occurrence of *Nosema ceranae* is continuous. TAPASZTI et al. (2009) showed that *Nosema ceranae* was dominant during the summer season, and data from my studies confirm that *Nosema ceranae* is dominant throughout the year.

Knowing that *Nosema ceranae* infection persists throughout the year, the standard seasonal treatment strategy originally adapted for *Nosema apis* would not be appropriate. This is supported by terminology based on the type of infection, i.e., type “C” nosemosis other than type “A” nosemosis. Previously, nosemosis was treated when the classic symptoms appeared in early spring and late fall. During the summer months, many beekeepers in all parts of Hungary observed unexpected weakening and collapses of bee colonies. As *Nosema ceranae* was not expected to occur, no intervention was applied against it. According to the work of FORSGREN and FRIES (2010), there should be no significant differences in pathogenicity between the two *Nosema* species, however, a treatment strategy adapted to a single type allowed the spread of *Nosema ceranae*. The latter has been confirmed by other communications (MARTÍN-HERNÁNDEZ et al. 2011), (HIGES et al. 2013), (WILLIAMS et al. 2014) és (VAN DER ZEE et al. 2014), therefore, I recommend regular checking for nosema infection during the summer as well.

Based on the results of my study, I confirm the proposal of MEANA et al. (2010) to assess the health status of bee colonies, that the proportion of infected bees may be a more reliable method of determining the level of infection in a bee colony. This is especially important because *Nosema ceranae* is a year-round problem. Proactive monitoring by sampling forager bees is a better inspection method for controlling nosemosis than the inspection method by sampling indoor workers. In terms of endangering and disturbing bee colonies, I recommend the inspection to be thirty individuals in size. In my opinion, this is the sample size that can be taken from virtually any colonies with a population appropriate for the actual season without major losses. However, during my study, I did not schedule sampling for the winter month either. On the one hand, because there are usually no forager bees in winter, and on the other hand, in winter, the sampling of indoor bees would lead to hive opening, which would be such a drastic disruption for a colony that it could have irreversible consequences.

ANTÚNEZ (2009) found that *Nosema spp*. infection results a decrease in vitellogenin levels in bees, especially case of *Nosema ceranae* infection. NELSON (2007) found that vitellogenin in bees influences the exiting and foraging behavior of bees. According to her research, the lower the level of vitellogenin in bees, the earlier they become forager bees. From the first two publications, it can be concluded that vitellogenin levels in bees infected with *Nosema ceranae* are reduced, resulting in earlier exiting and foraging. If this conclusion is true, it also explains why the proportion of infected bees in the bee samples from the forager bees is higher than in the case of the indoor working bees. If, within the bee colony, *N. ceranae* infection shifts the physiological ratio between the indoor working bees and the forager bees (in favor of the latter), this may cause a problem in nursing brood. In my opinion, the problem first arises in the care of the older, already capped brood, because if the youngest nursing bees become infected with *Nosema* spore pollen, also for those, according to ANTÚNEZ

(2009), vitellogenin levels will be in significantly lower levels by the seventh day after infection. At the age of seven days, the indoor worker bees are no longer busy feeding the brood, but they play an important role in covering and warming the older brood. The brood that are left without the cover will catch a cold and die. If the colony's aptitude to clean up does not eliminate this, additional bee diseases may develop in the colony. Those bees thet turned forager earlier as a result of *Nosema ceranae* infection, in my opinion, they will have shorter lives for several reasons, like their companions free of nosema infection, that are at normal age at the age of 21 days become forager bees. One of the main reasons for the decrease in vitellogenin levels due to nosema infection is due to defective digestion. Such an individual is presumably suffering from quality starvation and energy deficit, while as a "collecting beetle" it is much more intensely active than its indoor companions. In addition to more intense activity, due to defective digestion the regeneration is also more defective. These individuals wear faster and fly weaker over time. Either they do not return home from the flight during depletion, or because of their weaker, slower flight, they fall prey to their predators sooner. This assumption is supported by HIGHES et al. (2008a), in which *Nosema ceranae* is typically found in the sputum of the bee-eater (*Merops apiaster*).

In my opinion, if the Nosema infection of the bee colony is noticed in time, then peritrophic membrane thickening resulted from the tannic acid treatment mentioned as an example above in my dissertation, can help with recovery not at the individual level but at the bee colony level. However, this requires a good cleaning aptitude in the colony. Cleaner worker bees will get sick during cleaning, but infection will be delayed. If the intensity of cleaning is greater than the spread of spores within the hive, the colony can recover.

For DNA recovery and purification, I replaced the commercial tissue lysator and cell breaker with an conventional vortex mixer and centrifugation of the phenol-chloroform mixture. The capacity of the

substitution method is low compared to the large number of samples tested in the experiment, but it is sufficient for occasional tests for laboratories with a more modest instrumentation. For the PCR-RFLP method used by TAPASZTI et al. (2009), one PCR product can be run simultaneously, and two more steps are required between PCR and electrophoresis, one purification step and one digestion step. In the case of the triplex PCR I used, three PCR products could be run in one PCR program, and the electrophoresis could be run immediately after the PCR program.

Investigations of nosemosis in Hungary should be supplemented by an examination of the correlation between the percentage of bee colony stock infestation and the number of collapsed bee colonies, and collecting data on its evolution. I also recommend monitoring the mortality of the bee colony caused by Nosema ceranae and the loss of honey production.

4 Publications related tot he topic of the dissertation

4.1 Publications in peer-reviewed refereed journals

1. GRAY A. BRODSCHNEIDER R. ADJLANE N. BALLIS A. BRUSBARDIS V. CHARRIÈRE J.-D. CHLEBO R. F. COFFEY M. CORNELISSEN B. AMARO DA COSTA C. **CSÁKI T.** DAHLE B. DANIHLÍK J. DRAŽIĆ M. M. EVANS G. FEDORIAK M. FORSYTHE I. DE GRAAF D. GREGORC A. JOHANNESEN J. KAUKO L. KRISTIANSEN P. MARTIKKALA M. MARTÍN-HERNÁNDEZ R. MEDINA-FLORES C. A. MUTINELLI F. PATALANO S. PETROV P. RAUDMETS A. RYZHIKOV V. A. SIMON-DELSO N. STEVANOVIC J. TOPOLSKA G. UZUNOV A. VEJSNAES F. WILLIAMS A. ZAMMIT-MANGION M. és SOROKER V. (2019): Loss rates of honey bee colonies during winter 2017/18 in 36 countries participating in the COLOSS survey, including effects of forage sources. Journal of Apicultural Research **58**. (szám 4). o. 479–485.

2. HUANG S. K. **CSAKI T.** DOUBLET V. DUSSAUBAT C. EVANS J. D. GAJDA A. M. GREGORC A. HAMILTON M. C. KAMLER M. LECOCQ A. MUZ M. N. NEUMANN P. ÖZKIRIM A. SCHIESSER A. SOHR A. R. TANNER G. TOZKAR C. Ö. WILLIAMS G. R. WU L. ZHENG H. és CHEN Y. P. (2014): Evaluation of Cage Designs and Feeding Regimes for Honey Bee (Hymenoptera: Apidae) Laboratory Experiments. Journal of Economic Entomology **107**. (szám 1). o. 54–62.
3. WILLIAMS G. R. ALAUX C. COSTA C. **CSÁKI T.** DOUBLET V. EISENHARDT D. FRIES I. KUHN R. MCMAHON D. P. MEDRZYCKI P. MURRAY T. E. NATSOPOULOU M. E. NEUMANN P. OLIVER R. PAXTON R. J. PERNAL S. F. SHUTLER D. TANNER G. VAN DER STEEN J. J. M. és BRODSCHNEIDER R. (2013): Standard methods for maintaining adult Apis mellifera in cages under in vitro laboratory conditions. Journal of Apicultural Research **52**. (szám 1). o. 1–36.
4. **CSÁKI T.** HELTAI M. MARKOLT F. KOVÁCS B. BÉKÉSI L. LADÁNYI M. PÉNTEK-ZAKAR E. MEANA A. BOTÍAS C. MARTÍN-HERNÁNDEZ R. és HIGES M. (2015): Permanent prevalence of *Nosema ceranae* in honey bees (Apis mellifera) in Hungary. Acta Veterinaria Hungarica Acta Veterinaria Hungarica **63**. (szám 3). o. 358–369.
5. **CSÁKI T.** HELTAI M. és SZABÓ GY. (2011): The opportunities of large scale beekeeping in Hungary. Hungarian Agricultural Research: Environmental Management Land Use Biodiversity **20**. (szám 4). o. 4–8.

4.2 Publications in peer-reviewed Hungarian journals

1. **CSÁKI T.** HELTAI M. és BÉKÉSI L. (2011): Labor- és szabadföldi kísérletek a mézelő méhekkel. Animal Welfare

Etológia És Tartástechnológia **7.** , (szám Klnsz). o. 209–214.

2. **CSÁKI T.** HELTAI M. és SZABÓ GY. (2009): A nagyüzemi méhészkedés lehetőségei Magyarországon. Animal Welfare Etológia És Tartástechnológia **5**. (szám 4). o. 423-430.
3. **CSÁKI T.** és Oreskovic G. (2009): A nagyüzemi méhészkedés feltételei egy Amerikai Egyesült Államokbeli (Wisconsin és Florida) méhészet alapján. Animal Welfare Etológia És Tartástechnológia **5**. (szám 3). o. 209-230.

4.3 Book in Hungarian, as an editor

5. BROSS P. CSUJA L. HEGEDŰS D. RÁDI T. SZABÓ GY. **CSÁKI T. (szerk.)** (2015): Méhegészségügy az ökológiai méhészetben. Ökológiai Mezőgazdasági Kutatóintézet, Budapest, Magyarország. ISBN: 9786158024716
6. BROSS P. CSUJA L. ERDÉLYI T. RÁDI T. SZABÓ GY. **CSÁKI T. (szerk.)** (2015): Szakmai feladatok az ökológiai méhészetben. Ökológiai Mezőgazdasági Kutatóintézet, Budapest, Magyarország. ISBN: 9786158024709

4.4 Book excerpt in Hungarian

1. **CSÁKI T.** (2015): Varroa atka elleni ökológiai védekezési módszerek on-farm vizsgálata. In: DREXLER D. (szerk.) On-farm kutatás 2014. A harmadik év eredményei. Ökológiai Mezőgazdasági Kutatóintézet, Budapest, Magyarország. o. 97-110.
2. DREXLER D. PAPP O. **CSÁKI T.** (2015): Az ÖMKi on-farm kutatási hálózata. In: DREXLER D. (szerk.) On-farm kutatás 2014: A harmadik év eredményei. Ökológiai Mezőgazdasági Kutatóintézet, Budapest, Magyarország. o. 2-3.
3. **CSÁKI T.** DREXLER D. (2014): Varroa atka elleni ökológiai védekezési módszerek on-farm vizsgálata. In: DREXLER D. (szerk.)

On-farm kutatás 2013: A második év eredményei. Ökológiai Mezőgazdasági Kutatóintézet, Budapest, Magyarország. o. 139-146.
4. DREXLER D. PAPP O. **CSÁKI T.** (2014): Az ÖMKi on-farm kutatási hálózata. In: DREXLER D. (szerk.) On-farm kutatás 2013: A második év eredményei. Ökológiai Mezőgazdasági Kutatóintézet, Budapest, Magyarország. o. 2-3.
5. DREXLER D. PAPP O. **CSÁKI T.** (2013): Az ÖMKi on-farm kutatási hálózata. In: DREXLER D. (szerk.) On-farm kutatás 2012: Az első év eredményei. Ökológiai Mezőgazdasági Kutatóintézet, Budapest, Magyarország. o. 2-3.

4.5 Publications in conference proceedings

4.5.1 Presentations in English

1. **CSÁKI T**. DREXLER D (2016): Hungarian on - farm research program for varroa control in organic beekeeping. Madarász, B; Tóth, A (szerk.) International Conference on Conservation Agriculture and Sustainable Land Use : Book of Abstracts. Budapest, Magyarország : MTA CSFK Földrajztudományi Intézet o. 27-27.
2. **CSÁKI T**. DREXLER D (2015): Hungarian on-farm research program for varroa control in organic beekeeping. ACTA FYTOTECHNICA ET ZOOTECHNICA 18. o. 157-159.
3. **CSÁKI T**. DREXLER D (2014): Hungarian on-farm research program for varroa control in organic beekeeping. Rahmann, G; Aksoy, U (szerk.) Building Organic Bridges. Proceedings of the 4th ISOFAR Scientific Conference at the Organic World Congress and 18th IFOAM ORGANIC WORLD CONGRESS, Braunschweig, Németország: Johann Heinrich von Thünen-Institut (2014) o. 583-586. , 4 p.
4. **CSÁKI T**. DREXLER D (2013): On-farm research program for varroa control in organic beekeeping. In: Dóra, Drexler (szerk.) 4th

International Conference on Organic Agriculture Sciences (ICOAS) Budapest, Magyarország, Eger, Magyarország o. 32

5. **CSÁKI T**. KRISTÓF É. (2012): The domination of medical treatments' budget on the hungarian national program for beekeeping. REVIEW ON AGRICULTURE AND RURAL DEVELOPMENT 2012 : 1 O. 159-163.
6. **CSÁKI T**. HARKA L. BÉKÉSI L. SÜTŐ J. (2012): Evaluation of protein utilization of a pollen substitute and a natural pollen mixture by measuring total protein content of the honeybee (Apis mellifera). In: Benjamin, Barth; Holger, Scharpenberg; Robin, FA Moritz (szerk.) The Fifth European Conference of Apidology. Bern, Svájc: University of Bern Paper: P.3.26
7. **CSÁKI T**. HARKA L. BÉKÉSI L. (2012): Changes of the two year COLOSS Questionnaire in Hungary. COLOSS Workshop on monitoring of colony losses 2011-2012 - temporal and spatial patterns. o. 7 Paper: ECOST-MEETING-FA0803-011012-014080
8. **CSÁKI T**. BÉKÉSI L. MARKOLT F. (2012): The reliability of diagnosing and a theory of reducing Nosema infections in the Honey bees COLLOS Workshop on Nosema, from knowledge to experimental setup o. 13 Paper: ECOST-MEETING-FA0803-030312-014083
9. **CSÁKI T**. SCHILLER M. BÉKÉSI L. (2012): Treatment efficiency in higher need of protein sources. COLOSS Workshop on honey bee nutrition. Szabadka, Szerbia (2012) O. 29 Paper: ECOST-MEETING-FA0803-151012-014146
10. WILLIAMS GR. ALAUX C. **CSÁKI T.** DOUBLET V. EISENHARDT D. KUHN R. MCMAHON DP. MURRAY TE. NATSOPOULOU ME. NEUMANN P. (2012): Recommendations from the COLOSS BEEBOOK for maintaining adult workers in laboratory cages. Benjamin, Barth; Holger, Scharpenberg; Robin, FA Moritz (szerk.) The Fifth European Conference of Apidology. Bern, Svájc: University of Bern o. 240

11. **CSÁKI T.** SZALAINÉ MÁTRAI E. BÉKÉSI L. (2011): Introduction of some methods and use of lab and field work with honey bees in hoarding cages. COLOSS Workshop on diagnostic surveys o. 20. ECOST-MEETING-FA0803-250811-008757
12. **CSÁKI T.** SZALAINÉ MÁTRAI E. BÉKÉSI L. (2011): Wintering report from Hungaryan apiaries 2009/2010. COLOSS Workshop: Coloss questionnaire from question formulation to data analysis. o. 5 Paper: ECOST-MEETING-FA0803-210311-006292

4.5.2 Presentations in Hungarian

1. **CSÁKI T.** (2016): *Nosema apis* és ceranae kialakulása, kezelése. NÉBiH Méhegészségügyi továbbképzés, Pécs, 2016.02.19.
2. **CSÁKI T.** HELTAI M. BÉKÉSI L. SZALAINÉ MÁTRAI E. HIGES M. MARTÍN-HERNÁNDEZ R. MEANA A. (2011): A *Nosema apis* és a *Nosema ceranae* eloszlása Magyarországon a 2010 során gyűjtött méhminták alapján. Akadémiai beszámolók: Paraziológia, állattan, halkórtan o. 32

4.5.3 Poster presentation in English

1. **CSÁKI T**. DREXLER D (2015): Hungarian on-farm research program for varroa control in organic beekeeping. 5th International Conference on Organic Agriculture Sciences., Bratislava, Szlovákia: 2015.10.14-2015.10.17.
2. **CSÁKI T**. DREXLER D (2014): Hungarian on-farm research program for varroa control in organic beekeeping. 18th IFOAM World Congress, ICC, and 4th ISOFAR Scientific Conference at the Organic World Congress 13-15 October 2014