Action FA0803

Proceedings of WG2 Workshop "*Nosema*, from knowledge to experimental setup"

Prevention of Honey Bee COlonyLOSSes

Hacettepe University/Warsaw University of Life Sciences
Istanbul, Turkey

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A. Agenda

TIME	PROGRAMME
2nd March 2012	
19:00 -	Welcome
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9:40 – 10:00	Plenary talk by Judy Chen (Invited Speaker)
10:00 – 10:30	*Coffee break with snacks*
10:30 – 12:00	**10min talks by participants** Anna Gajda Ales Gregorc Andrzey Bober Aygün Yalçınkaya Beata Bąk Cansu Özge Tozkar Claudia Dussaubat Geoffrey Williams Gina Tanner
12:30-14:00	*Lunch*
14:00-15:00	**10min talks by participants** İrfan Kandemir Kamyar Ahmadi Martin Kamler Mary Frances Coffey Meral Kence Muhammad Forsi Mustafa Muz Tamas Csaki Vincent Doublet
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16:30-16:45	*End of workshop meeting*

Registration on site

Registration fee: 50 €

Please fill the hotel reservation form send back them to Dr. Aslı Özkırım (ozkirim@hacettepe.edu.tr) . Deadline: 27th Feb. 2012

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B. List of Abstracts

Epidemiological changes in appearance of Nosemosis in Iran

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Nosemosis of European honey bee (Apis *mellifera*) is present in bee colonies worldwide. Until recently, *Nosema apis* had been regarded as the causative agent of the disease, that causes heavy economic losses in apicultures. *Nosema ceranae* is an emerging microsporidian parasite of European honeybees, *A. mellifera*, but its distribution is not well known. Previously, nosemosis in honeybees in Iran was attributed exclusively to *N. apis*.

Active Surveillance of Honeybee diseases in Iran carried out in 4 ecozones (Caspian , Mountain , Warm and dry , Moderate).
Selected Provinces were :

1- Mountainous ecozone:West Azarbayejan,Lorestan,Ardebil
2- Caspian ecozone :North Khorasan ,Golestan
3- Warm and dry ecozone:Kerman ,Isfehan,Yazd
4- Moderate ecozone: Fars

Sampels collected from 2273 colonies (1130 Apiaries).In each zone some provinces selected as representative of each ecozone.Examinations carried out according to OIE protocols. As resalts , from 2273 colonies , there were 163 positive & 1933 negative cases and 38 damaged samples.(Without information). Prevalence rate of *Nosemosis* in our study was 7%. Respectively Ardebil and Kerman had most and less prevalence rates.Against our expectations prevalence rate of *Nosemosis* in dry and warm ecozone. Was more than humid ecozone. But according to the scientific texts prevalence of *Nosema Apis* in humid areas is more than dry areas. So Because of new reportation of *Nosema ceranae* (Nabian & Ahmadi,2011) for The first time in Iran , it is assumed *N. Ceranae* can be the cause of epidemiological changes in appearance of the disease.

Infection of bees from winter hive debris with *Nosema sp.* spores in bee colonies treated for varroosis with various methods

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In Poland various treatment patterns for varroosis are used. The aim of this study was to assess whether medications and substances used in controlling *Varroa destructor* mites affect the degree of winter hive debris' infection with *Nosema sp.* .

In the spring 2010, four experimental groups (each consisting of 25 colonies) were randomly created out of 100 bee colonies not treated for varroosis in 2009. The bees were divided into the groups based on the varroosis treatment pattern to be applied in the study: group I (CH) – the main summer treatment only, with a use of chemotherapy; group II (IT) – integrated treatment for varroosis; group III (N) – only natural ways of controlling the mite with a use of essential oils and organic acids; group IV (C) – control group, not treated for varroosis.

The varroosis treatment applied in individual groups was based on substances available and registered in Poland. In the spring 2011, hive debris samples were collected to be examined for their infection with *Nosema sp*.

In the debris coming from bee colonies of all experimental groups, *N. apis* and *N. ceranae* spores were found. In the colonies where natural methods were applied, none of the samples exhibited a high degree of infection (Table). The study is in progress.

Table. Infection of hive debris with *Nosema sp*. spores in individual groups

group	Average number of spores in the field of vision	Percentage of sampples infected with *Nosema sp.*	Percentage of samples highly infected with *Nosema sp.*
CH	34	43.8	12.5^{Aa}
IT	23	50.0	7.0^{b}
N	4	55.0	$0.0B^{c}$
C	38	57.1	14.3^{Aa}

Legend: CH – main summer treatment only, with a use of chemotherapy; IT – integrated treatment for varroosis, N – natural ways of controlling the mite, with a use of essential oils and organic acids, C – control group, not treated for varroosis.

Capital letters denote statistical differences for p=0.01, and lower-case letters: for p=0.05

The role of nosemosis (*Nosema apis* / *Nosema ceranae*) in increased bee mortality in Poland.

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From the autumn of 2009 we are conducting examinations the aim of which is the parallel analysis of the occurrence of factors involving potential threats to the proper functioning of colonies. Detection and estimation of the influence of *Nosema* spp. on increased mortality is one of the tasks of undertaken examinations. Bees for laboratory tests were collected from autumn 2009 to spring 2010 and from autumn 2010 to spring 2011. In each apiary samples were collected separately from several colonies. Until now in our studies we have examined 1948 samples (1688 from apiaries with losses >10% and 260 from apiaries with losses ≤10%) from 418 apiaries (345 - losses >10% and 73 - losses ≤10%) from all 16 Polish voivodeships. In order to check presence of *Nosema* spp. spores and for estimation the level of infestation Cantwell method was used. For our analysis we establish 3 ranges (below 1, from 1 to 5 and above 5 million of spores per bee), which were used to compare intensity of the invasion (low <1, medium 1-5, high >5) in colonies with distinct mortality. Differentiation between *Nosema apis* and *Nosema ceranae* was performed by multiplex PCR previously described by Martin-Hernandez et all (2007). *Nosema* spp. spores were detected in 68,3% of samples from apiaries with losses >10% and in 64,2% of samples from apiaries with losses ≤10%. Low level of invasion was detected in 16,5%, medium in 25,9% and a high in 25,9% of samples from apiaries with losses >10%. In samples from apiaries with losses ≤10% the low level of invasion was in 13,5%, medium in 20,8% and high in 30,0%. Data concerning presence of *Nosema apis* and *Nosema ceranae* in apiaries with different level of mortality are presented in table below.

Period	Apiary with colony losses (%)	Colonies with *Nosema* spp. infestation % (number)	Colonies with pure *N.apis* infestation among positive samples (%)	Colonies with pure *N.ceranae* infestation among positive samples (%)	Colonies with mixed Infestation among positive samples (%)
2009/ 2010	≤10	63,2 (139)	3,8	65,4	30,8
	>10	69,1 (792)	11,3	55,9	32,8
2010/ 2011	≤10	70,0 (28)	3,6	71,4	25,0
	>10	66,6 (361)	0,8	56,2	42,9

Research performed under COST ACTION FA0803: PREVENTION OF HONEYBEE COLONY LOSSES (COLOSS) funded by Ministry of Science and Higher Education of Poland (Decision Nr 527/N-COST/2009/0 of 10 July 2009).

Sequencing, annotation, and comparative genomic analysis of honey bee microsporidian parasite, Nosema

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Nosemosis, a disease caused by microporidian parasite *Nosema*, is regarded as one of the most prevalent and destructive adult honey bee diseases and has been implicated in worldwide colony declines. *N. ceranae* and *N. apis* are two species of the Nosema that are reported to cause *Nosema* disease in honey bees so far. Using genomic approaches, we conducted study to investigate the biological, molecular and genomic feature of two Nosema species. The molecular and biological studies have yield evidence that *N. ceranae* is the more common and predominant infection of two *Nosema* species in honey bees. Sequencing and annotation of the *Nosema* genomes provide a comprehensive overview of the genetic content, structure, and organization of the parasites and give some interesting insights into the complex biological and molecular processes of the parasites. The comparative genomic analysis led to the identification of genes that are conserved between *N. apis* and *N. ceranae*, and genes that are unique characteristics of the individual species, thereby providing a list of virulence factors that are associated with virulence of the parasites in honey bees. There genes are potential targets for innovative therapeutics to break down the life cycle of the parasite, thereby improving honey bee health.

A survey of the incidence of Nosema spp. in Irish honeybee colonies

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Nosemosis is an adult bee disease, which is caused by two distinct microsporida, which are single celled parasites of the honeybee gut. The two species identified to date are *Nosema apis* Zander in 1907 and more recently *Nosema ceranae* Fries 1994. Infection is similar in both species and the negative impact on the health status of the honeybee include the inability of the adult bee to digest food properly, to reduce the activity of the hypopharyngeal gland and to decrease the life span on the adult bee. The virulence of *Nosema apis* in similar in temperate and warmer climates, showing a decrease during the Summer months, however, it appears for *Nosema ceranae* the virulence remains constant throughout the Summer having serious implications on colony survival especially in warmer climates. Recent studies have shown that *Nosema ceranae* is becoming the dominant species in Europe and has increased in frequency over the past decade. In Ireland, both species occur, but the frequency of occurrence of either species has not been well documented. Data received for the Irish Bee Disease Diagnostic Service (Teagasc Research Centre) indicates that during 2007 – 2011, the incidence of occurrence of Nosema spp. was approximately 18%, reaching a maximum of 27.4% in 2009. The possible cause for the increase in incidence during 2009 was the poor weather during the active season and consequently bees were confined to their hives for many days. Although this data gives preliminary information on the status of Nosema spp. in Irish honeybee colonies, it lacks a research-level of sampling and thus the data collected only provides confirmatory information rather than the frequency occurrence of Nosema spp. Furthermore, positive samples in this dataset were not analysed to species level. Therefore, the aim of the future study is to carry out a comprehensible study of the incidences of *Nosema apis* and *Nosema ceranae* in Irish honeybee colonies. Sampling will be carried out using a randomised approaching and analysis will be carried out according to OIE standard

The reliability of diagnosing and a theory of reducing *Nosema* infections in the Honey bees

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Infective Nosema spores in corbicular pollen from forager honeybees were detected in Spain. Individual honey bee pollen foragers have a high fidelity for a plant species in one flight and they enter into their hive and unload their cargo. Besides, fresh pollen is consumed by nurse bees during the brood rearing period, the stored pollen are mixed and transformed to beebread for winter food. Despite of the separate handling of the pollen species by the pollen foragers, analyzing the rectum of the nurse bees and the winter bees they consume a mix of the incoming pollen species. This means, that a pollen source from one forager bee is not consumed by only one bee. So there should be a statistical relationship between the odds of the swallowing spores and the concentration of spores in the pollen storage cells. With replacing the pollen frames from the hive with empty frames we could take out a main proportion of consumable spores from the hive. But this would make high pollen need in the hive and the division of labor like pollen foraging is influenced by the availability of food inside and outside of hive and on the actual needs of the colony. This means that we must restore the colony's pollen reserve with - spore free - pollen substitutes. It has been demonstrated various substitutes to remedy the nutritious needs that are recommended in the literature.

Impact of the interaction between *Nosema*, viruses and pesticides on honey bee health

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Many drivers of honey bee decline have been identified, but no single factor seems to be the main one explaining colony losses. In recent studies, focus has been placed on the interactions among several drivers of honey bee diseases, and particularly on the interaction between the microsporidian *Nosema* and sublethal doses of pesticides (Alaux et al. 2010, Vidau et al. 2011, Pettis et al. 2012). Our research on adult honey bees aims to test how interactions among pathogens and pesticides affect individual bees. We use *Nosema ceranae* as our model parasite, two common viruses (Black Queen Cell Virus and Deformed Wing Virus) and two widely used pesticides (thiacloprid, τ-fluvalinate), that we feed to bees in sub-lethal doses. Different responses to these multiple infections are recorded as honey bee mortality, change in behavior and response of the bees' immune system. We shall discuss in this workshop how we prepare *Nosema* spores for individual infection of bees in cages experiments.

Comparative study of artificial infections in the european honey bee with *Nosema ceranae* spores from two different geographic origins

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The microsporidia *Nosema ceranae* is an obligate intracellular parasite that develops in the midgut epithelium of the honey bee. Since 2005, when it was first isolated from the European honey bee, it has been the focus of an increasing number of laboratory assays to study its impact on honey bee health. Concern has been arisen since experimental conditions differ between laboratories, making comparisons of results difficult. Consequently, we carried out artificial infections of honey bees with two groups of *N. ceranae* spores, each one from a different geographical origin (South of France and Central Spain) that in previous independent experiments induced different honey bee survival rates. We performed artificial infections under identical experimental conditions to evaluate the development of infection. Preliminary results show that infection development was similar between both infected groups but differed significantly from controls. We discuss how different experimental factors can influence results in laboratory trials.

Nosema and stress

*Mohammad Forsi**

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Nosema spores are very resistant in the colony or in the body of honeybees. In normal conditions the spores do not germinate in the mid gut but in the conditions that the immunity is suppressed Nosema can germinate quickly.

The factors which are involved in reduction of the immunity of colony and the bees are:

- Agricultural pesticides
- Poor ventilation of the hives
- Long transportation of colony
- Improper use of chemicals and drugs
- Food Shortage

The setup for experiments with *Nosema ceranae*.

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To date, we have gathered a great deal of information on *Nosema ceranae* biology, infection course and many other aspects of type C nosemosis in general. Nevertheless, there are many areas of this subject which need to be further examined. For instance, the difference in the course of infection under different climatic conditions, the responsiveness to various treatments, resilience of different *Apis mellifera* subspecies, etc. Some institutes are actively running cage tests, and some are experimenting on entire apiaries. The CoLoss network creates a great opportunity to initiate ring tests which would hopefully give answers to remaining questions concerning *Nosema ceranae*. The ring tests however need to be run in possibly identical conditions, which will require unification of many factors. The cage tests for instance are carried out in different laboratories using sometimes very different guidelines. For example the age of the bees most suitable for experimental infection. Some researchers take 24 hour old bees, some - 5 day old bees (brood frames kept in an incubator), but some scientists use adults of average age of 2 weeks (directly from the colony). The method of counting the spores to prepare spore solutions varies, so it may again produce bias in research. Usually the Rosenthal-Fuchs chamber is used, but the number of squares in which spores are counted is very different. The number of spores used to infect individual bees is similar in all published papers (approximately 10^4 spores for each bee). Whereas, the act of feeding the bees is again different. Some laboratories use carbon dioxide to anesthetize the bees before handling, when most other laboratories don't do it. Also the method of handling/feeding the bees should be unified, which will exclude the manual error as a cause of huge differences in mortality in different laboratories. The temperature in which the bees are kept varies from 36 to 30 centigrade. Considering the temperature impact on *Nosema* development and also on the wellbeing of the bee, this topic should be discussed. Also the time of the

experiment should be taken into consideration. Should it last 2 weeks or maybe it should be terminated after most of the bees die? To make the conditions for bee's most comfortable additional pollen and vitamin feeding may be required. Also the presence of an empty comb and artificial queen hormone may create the conditions more similar to the natural ones. The cage test is followed by the examination step. There are several (at least 3 most common) DNA extraction methods used within CoLoss member laboratories. Also PCR or qPCR protocols differ slightly amongst laboratories, which may produce bias in data comparisons.

Experimental infection with *Nosema ceranae* spores and Chronic Bee Paralysis Virus in caged workers

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Experimental infection with *Nosema ceranae* spores and additionally with Chronic Bee Paralysis Virus (CBPV) was performed in winter worker bees (*Apis mellifera carnica*). Workers used for *Nosema* spores inoculum were obtained from colony and molecular characterization of spores was determined using PCR method and numbers of spores were counted with help of a Bürker haemocytometer. Spore inoculums consisted of 61,000 spores/inoculated bee. We used 50 caged winter workers originating from two colonies. Workers were individually inoculated. Live and dead bees were sampled and mortality rate of *Nosema* inoculated workers was evaluated. Additionally we have performed experiments where workers were simultaneous inoculated with CBPV strain (p/c or p/o) and *Nosema* spores (p/o).

The highest bee mortality was observed in workers inoculated with CBPV *p/o*, followed with workers simultaneously inoculated with CBPV (p/o or p/c) and *N. ceranae* (*p/o)*, and lower bee mortality was observed in workers received only *N. ceranae* spores, followed with bee mortality in control uninfected bees. The synergistic effects of two pathogens inoculated in experimentally caged bees have been successfully simulated.

Can we microscopically distinguish *Nosema apis* and *Nosema ceranae*? Experience in population dynamics of *Nosema* spp. in the Czech Republic.

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Nosema spp. is investigated in honeybee colonies of commercial queen breeders in the Czech Republic. A strong contrast of spore morphological and morphometrical differences were observed during microscopical examination. Subsequent analysis revealed their clear difference: the fresh spores *N. apis* were oval- to barrel-shaped in appearance, which varied in size between 4.9-6.9 µm in length and 2.7-3.9 µm in width. Spores of *N. ceranae* were almost cylindrical to rod-shape in appearance, with both ends of the spores pointed and between 3.9-5.4 µm in length and 2.0-2.9 µm in width. PCR analysis confirmed both species. Totally, 700 spores were measured. Of this number of spores, only 30 (4.3%) were intermediate in size between those typical for *N. apis* and *N. ceranae*, respectively. Moreover, field samples were confronted with PCR analysis and replacing of *N. apis* with *N. ceranae* species was confirmed during 3 year survey.

Prevalance of *Nosema ceranae* infections in a wintering region.

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Hatay city is located close to traditional honeybee colony wintering region. Beekeepers send nosemosis suspected samples to “Laboratory of Beeomics”. RT-QT-PCR was performed to analyse samples. Infection prevalence determined as %28 among the colonies.

Distribution and seasonal intensity of *Nosema* spp. in Switzerland

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The Swiss *Nosema* Monitoring is part of the FP7 project BEE DOC and constitutes a national survey of microsporidian midgut parasites *Nosema* spp. in Swiss honey bee colonies. The two-year survey started in October 2010 and involves a total of 60 colonies from 15 apiaries in different regions throughout the country. The primary purpose of this study is to better understand the distribution and the seasonal development of *Nosema* spp. in honeybee colonies, as well as potentially related effects of geographical conditions. Bee sampling occurs monthly between April and October, and once or twice during winters. All collected samples are analysed quantitatively for *Nosema* spores using light microscopy, and subsamples are molecularly analyzed using classical PCR to distinguish between *Nosema ceranae* and *Nosema apis*. Preliminary results revealed a high prevalence of *Nosema* spores in the surveyed colonies. Molecular analysis of pooled samples showed the presence of both *N. ceranae* and *N. apis*. Further details of the analytical methods, as well as the preliminary results of the first year of the survey, will be discussed.

The Future Perspectives of WG2 for Nosemosis

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COLOSS network has many researches about honey bees and their pathogens. WG2 is the biggest group interested in pests and pathogens and Nosema species (N. apis and N. ceranae). Both species have been investigated for their pathology by different scientists from different countries. Each of them is using different methods for diagnosis, treatment, surveys etc.

The previous workshop about Nosema spp. was carried out in Spain. In this workshop, we refreshed our knowledge and filled the gaps as much as possible. Besides, we tried to standardize the methods especially for diagnosis. For evident reasons, the geographical differences of Nosema distribution receive much less attention than medicine and veterinary science. Whereas it is very important to diagnose and treat the

Nosema diseases, determining the effect of physical conditions (different climate, geography, altitudes, moisture etc.) are as important as diagnosing and treatment in all over the world. In order to organize the effective experimental set up, I will classify the titles which could be used for different experiments and propose some possible experimental set ups. I will also present the previous outputs of the Nosema workshop and relate with the experiments on the international basis.

Distributions of *Nosema ceranea* and other pathogens in honey bees of Turkey

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Nosema ceranae is a microsporidian parasite of honey bees. Recent reports indicate that *Nosema ceranae* is becoming more widespread throughout the world and shown to have immunosuppressive effect on honey bees. Other investigations are shown that the presence of *Nosema ceranae* in interaction with other pests and pathogens may be more destructive. Accordingly, honey bee colonies that are found positive for *Nosema ceranae* should also be screened for the presence of viruses. In our survey, honey bee samples from different regions of Turkey representing different races were screened for the incidence of Nosema spp and honey bee viruses and found that *Nosema ceranae*, Acute bee paralysis virus, Black queen cell virus, Deformed wing virus were present together in most of the regions.

An internationally coordinated research involving the control of distribution of Nosema spp and prevention of further infections would be crucial to improve the honey bee health.

Identification of *Nosema spp* among Honey Bees from Different Regions of Turkey

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Health of honey bees in Turkey has importance because of high honey bee biodiversity and high number of managed colonies. In Turkey, excessive honey bee losses were first reported almost simultaneously with USA by 2007. The sudden and excessive unknown colony losses lead to economical losses in terms of honey bee colonies and crops. Nosema infection in honey bees is one of the many disease causing factors that may be contributing to recent extraordinary colony losses (>30%) in Turkey. In earlier studies the presence of *Nosema apis* and *Nosema ceranae* have been detected in some regions. In a recent investigation we determined the regional and seasonal presence of Nosema spp on field collected adult honey bees during 2010 spring and fall from different regions of Turkey. Identification of the pathogen frequency was done by cDNA synthesis and quantitative PCR diagnosis. *Nosema ceranae* was detected but although the analysis for *Nosema apis* was repeated twice, this pathogen could not be found on those samples. Generally in the world, *Nosema ceranae* is more common than *Nosema apis*. Recently, the replacement of *Nosema apis* by *Nosema ceranae* was reported by many researchers in the world. Our results can also be the indication of the replacement of *Nosema apis* by *Nosema ceranae* in many regions in Turkey. Another finding was the presence of *Nosema ceranae* in the samples of migratory beekeepers but not within the samples of stationary beekeepers. With this finding we can conclude that the infectivity of *Nosema ceranae* expands with migratory beekeeping activities. It seems that migratory beekeeping practices takes from and distributes the disease factors to honey bees in places where they visit.

An update on the COLOSS network and the *BEEBOOK*, with special emphasis on *Nosema* and hoarding cage chapters

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The international research network COLOSS (Prevention of Honey Bee Colony Losses) was established to co-ordinate efforts towards improving western honey bee health at a global level. When recent increases in honey bee colony losses were first recognized it became apparent that research performed in various laboratories around the world could not always be easily compared, thus seriously hindering our understanding of the phenomenon. As a result, COLOSS has placed a strong emphasis on the standardization of honey bee research. Here, we will discuss the COLOSS *BEEBOOK*, a manual of honey bee research methodologies, paying particular attention to the *Nosema* and hoarding cage chapters.

Please limit your title to 150 characters. (font 12, Times new Roman, bold)

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In recent years, *Nosema ceranae* has the world's attention by showing much more lethal pathogenity in *Apis mellifera*, apart from its host *Apis ceranae.* This new type of Nosemosis which reported firstly in 2006, has alarmed the world countries and the presence and distribution of *Nosema ceranae* has begun to be investigated. In this study honey bee samples were collected from different climatic geographical regions of Turkey. 2130 honey bee samples were collected from 71 apiaries in 20 provinces (Adana, Ankara, Antalya, Artvin, Balıkesir, Çanakkale, Çankırı, Çorum, Düzce, Elazığ, Erzincan, Hatay, Karabük, Kastamonu, Malatya, Mersin, Muğla, Ordu, Sivas, Tekirdağ). Samples were analyzed with molecular and microscopic diagnostic methods. All adult honey bees were dissected and intestines were homogenized. DNA isolation was done from these homogenates and obtained DNA was replicated by PCR methods with specific *N. ceranae* and *N.apis* primers. The epidemiology of *N. ceranae* was determined in Turkey by this study. Our results showed that *N. ceranae* replaces *N. apis* as compatible with other researches and its prevalence is higher than *N. apis*.

Nosemosis in the Middle East

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Nosemosis has been a threat for honeybee colonies for a long time. However recently there is a big shift from one species Nosema apis to Nosema ceranae. Since then, different methodologies both microscopical and molecular developed in a short time and still advancing. Using both methodologies I surveyed around 500 colonies from 7 countries (4 from Middle east, 2 from Europe and 1 from Africa). Samples were first analyzed with microscopically that each at least 10 bees per colony individually checked for spores. Positive samples than subjected to molecular analysis. For molecular analysis PCR-RFLP first tried and then changed to plexing of both primers to differentially amplify N. ceranae and N. apis. From all analysis I found that N. ceranae is predominated in all of the countries. One double infestation was recorded in İran that both fragments were amplified from the same colony. Later the spores were re-checked and saw that the spores actually different for both species if they co-exist in the same slide. I got two notifiable results from historical samples from Caucasian regions. One is that this region is more susceptible to nosemosis, during the rainy season the infestation is pretty high. The second important finding is that N. apis is seen samples before year 2000. After year 2000, no N. apis is seen, N. ceranae is dominated the area.

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