Original Article

DYNAMICS OF *NOSEMA APIS* AND *NOSEMA CERANAE* CO-INFECTION SEASONALLY IN HONEY BEE (*APIS MELLIFERA* L.) COLONIES

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Abstract

***Nosema apis* is a pathogen spesific for the European honeybee, *Apis mellifera* L., while *Nosema ceranae* is specific for the Asian honeybee, *Apis cerana*. Turkey provides different environmental and host conditions for both *Nosema* species. The aim of the study is to determine the dynamic of *N. cerenae* and *N. apis* seasonal infection. A number of samples were collected from different apiaries between 2009-2016 years. The samples were kept at -20°C in the laboratory. Light microscopy was used for spore counting and molecular techniques were used to identify the *Nosema* species. The results showed that winter season had an impact on the type of *Nosema* as well as on infection rates. The number of *N. ceranae* spores decreases significantly at low temperatures (≤ 5°C). The winter period was found to be the main factor affecting nosema infection level and dominancy of *Nosema ceranae*. Furthermore, co-infection of both species is an indicator of the dynamics of *N. apis* and *N. ceranae.* This study suggests, that there is a dynamic prevalence among the *Nosema* species depending of the average winter temperature and not a replacement of *N. apis* by *N. ceranae*.**

Keywords: *Nosema apis, Nosema ceranae,* co-infection, survey, Turkey

INTRODUCTION

Nosemosis is a common worldwide disease of adult honey bees (*Apis mellifera* L.) that is caused by microsporidia (OIE, 2008). *Nosema apis* was the only agent known to produce this disease in *A. mellifera* L. until *Nosema ceranae* was identified in this host in 2005 in Europe (Higes, Martín, & Meana, 2006), and Taiwan (Huang et al., 2007). Originally, *N. apis* was assumed to be a pathogen specific for causing nosemosis in the European honeybee, *A. mellifera* L., while *N. ceranae* was specific for the Asian honeybee, *Apis cerana* (Fries et al., 1996). However, early cross-infection experiments demonstrated that *N. apis* could infect *A. cerana* and that *N. ceranae* infected *A. mellifera* L. (Fries, 2010). *N. apis* is considered as a low-prevalence infection of *A. mellifera* even though it occurs world-wide (Hornitzky, 2005; OIE, 2008). However, the *N. ceranae*-caused disease is considered as emergent that is posing a major threat to the health of individiual honey bees and/or whole bee colonies (Higes et al., 2010). *N. ceranae* is reported to be more prevalent than *N. apis* and *N. apis* may be replaced by *N. ceranae* in different regions of Europe (Klee et al., 2007; Paxton et al., 2007).

The virulence of *N. apis* and *N. ceranae* has been examined by means of artificial infection in laboratory conditions or measured through epidemiological surveys in several studies (Bourgeois et al., 2010; Burgher-MacLellan et al., 2010; Chaimanee, Warrit, & Chantawannakul, 2010; Traver & Fell, 2011). Gisder et al. (2010) and (Traver, Williams, & Fell, 2012) referred to how climatic conditions affected the virulence of *Nosema* spp. and compared the hive sampling and seasonal activity of *N. ceranae* in honey bee colonies. However, those studies focused on the infection route and management of *N. ceranae* in hives rather than the comparison of *N. cerenae* and *N. apis* infections under different climatic and geographical conditions. The aim of the current study is to determine the dynamics of *N. cerenae* and *N. apis* seasonal infection and

if there is of *N. apis* replaced by *N. ceranae* in Turkey.

MATERIAL AND METHODS

Sampling Area

Turkey is a convenient sampling area for the observation of different climatic and geographical conditions. It has characteristics of a small continent with a high level of such biodiversity richness as agriculture, forests, mountains, steppes, wetlands, coastal areas and seas. Turkey is situated within the Holoarctic floral kingdom. Only on the Anatolian Peninsula there are about 11000 plant species, whereas in the whole of Europe there are 12500 plant species. Beekeepers raise five different races of honeybees and their three ecotypes, *A. m. anatoliaca* (three ecotypes: *adami, thrace* and *mugla), A. m. meda, A. m. caucasica, A. m. syriaca,* and *A. m. carnica* (Kandemir & Kence, 1995; Palmer, Smith, & Kaftanoglu, 2000). Migratory beekeeping is common and may influence the transfer of *N. apis* and *N. cerenea* spores between different locations, but as this study aimed to determine the effect of different weather and geographical conditions on *Nosema* spp. spores, stationary apiaries were chosen for sampling in each provinces. During the past eight years, bee samples were collected from the different provinces (Fig. 1).

Bee Samples

Bee samples consisting of thirty adult bees were collected from different colonies in the seventy-two provinces of Turkey (Fig. 1). Four apiaries from province were visited to collect samples during 2009-2016. 4 Samplings were repeated four times each season, so an average of 1041 bee samples were collected randomly from the same provinces over the eight years. For a sample, thirty bees were collected from outer frames. Clinical signs were not considered for sampling. The different levels of spores were measured under the different conditions by using the same colonies in each provinces during the study.

Nosema Analyses

The samples were kept at -20°C in the laboratory. Two diagnostic methods were applied at Hacettepe University/Bee Health Laboratory respectively. First a light microscopy method was used for detection infestation level (spore counting), and then a molecular method was used to identify the species. Fifteen bees were carefully dissected and homogenized in a mortar and mixed with 1 mL of water for each bee (15 mL totally). The homogenate was filtered by Whatman paper No.1. The filtered solution was centrifugated at 300 rpm 15 min. The supernatant was discarded and 30 mL of water was mixed with pellet, and. 0,1 mL of

Fig. 1. Seventy-two Provinces from which bee samples were collected for the past eight years in Turkey.

the solution was inoculated to the heamacytometer (Improved Neubauer) for counting the *Nosema* spores as described by Human et al., (2013).

Nosema DNA extraction was performed on fifteen bee homogenates with the DNeasy® Plant Mini Kit (Qiagen) following the Mini protocol for plant tissue (Fries et al., 2013). For multiplex Polymerase Chain Reaction (PCR), the primer combination suitable for the amplification of *N. apis* and *N. ceranae* was used as described by Fries et al. (2013). PCR products were resolved in a 1-2% agarose gel with suitable size marker and then visualised through staining with ethidium bromide and photographing on a u/v transilluminator.

Statistical Analyses

The Pearson's Correlation Test (SPSS software programme v. 21) was used to compare the weather conditions and spore levels of *Nosema* species in each province during 2009-2016.

RESULTS

Monitoring lasted eight years and showed some interesting results concerning the distribution of *Nosema ceranae* and *Nosema apis* in Turkey. Three types of infection were found: single *N. apis* infection, single *N. ceranae* infection and co-infection with both species (mixed infection) in the samples. The minimum, maximum and average numbers of spores for both species between 2009 and 2016 are summarized in Tab. 1. The positive rates (%) of *N. apis*, *N. ceranae* and *N. apis+N. ceranae* in all samples between 2009 and 2016 are shown at Fig. 2.

The correlation between weather conditions and the infection levels of *N. apis, N. ceranae*, *N. apis+N. ceranae* was also found statistically positive by Pearson correlation coefficient Test (Tab. 2). The winter period was found to be the main factor affecting *Nosema* pathogens and dominancy of one over the other species. This correlation between winter temperatures from the lowest to the highest values and types of *Nosema* infection can be seen in Fig. 3-4.

There was a positive strong relation between the *N. ceranae* infection rate and winter temperatures, as the rate increased as temperatures. However, *N. apis* infection rate decreased as the winter temperatures increased. The distribution of different types of *Nosema* infection between 2009 and 2016 are shown on the map of Turkey (Fig. 5) according to weather conditions.

Table 1.

The minimum, maximum and average numbers of *Nosema* spores (x10^6) for both species in the years 2009-2016

Years	*N. ceranae*			*N. apis*			*N.ceranae+N.apis*		
	Min.	*Max.*	***Average***	*Min.*	*Max.*	***Average***	*Min.*	*Max.*	***Average***
2009	1.6	14	**7.8**	1.2	21	**11.7**	1.6	43.8	**23.5**
2010	2.1	7.8	**4.9**	1.8	9.8	**6.7**	2.1	21.6	**12.9**
2011	2.7	6.6	**6.0**	3.4	9.2	**8.0**	2.4	19.8	**12.3**
2012	1.0	5.4	**3.7**	1.3	11	**6.8**	1.8	31.4	**17.5**
2013	0.2	1.6	**1.0**	7.8	69.3	**42.4**	0.3	8.5	**4.5**
2014	2.4	4.2	**4.6**	1.6	7.2	**5.2**	1.4	12.7	**7.7**
2015	3.5	21.3	**14.1**	3.1	9.4	**7.8**	1.6	38.9	**21.0**
2016	0.3	1.9	**1.2**	6.7	44	**28.7**	1.4	32.3	**17.5**

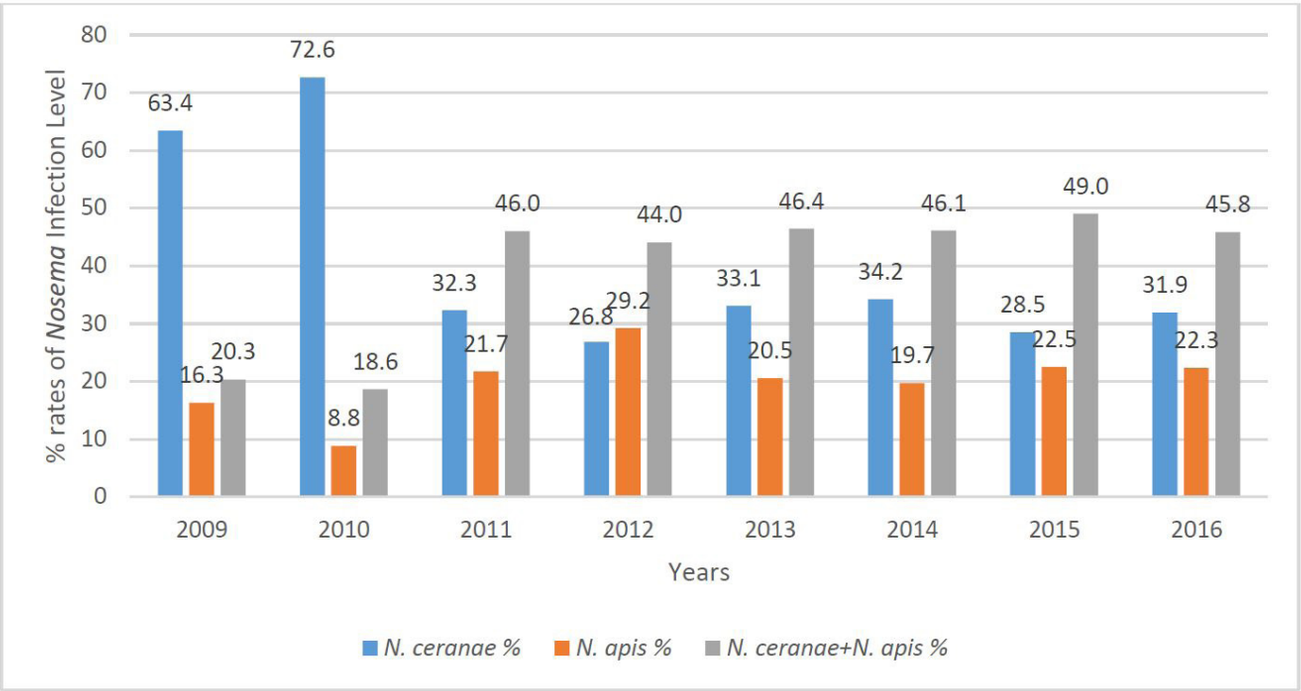

Fig. 2. The rates of *N. apis, N. ceranae* and *N. apis+N. ceranae* (co-infection) in all samples (%) in the years 2009-2016.

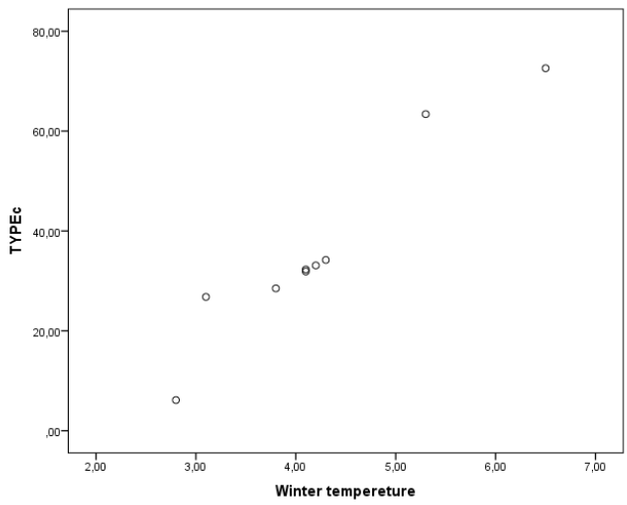

Fig. 3. The correlation between winter temperatures from the lowest to the highest values and *N. ceranae* infection level.

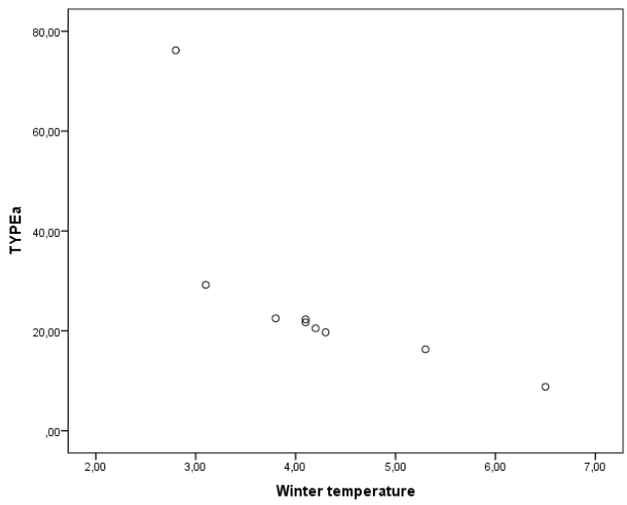

Fig. 4. The correlation between winter temperatures from the lowest to the highest values and *N. apis* infection level.

Fig. 5. The distribution map of *Nosema* spp. in Turkey

Table 2.
Correlations between temperatures and spore numbers tested by Pearson Correlation Test

	N. ceranae		*N. apis*		*N. ceranae+N. apis*	
	Correlation coefficient	**p**	**Correlation coefficient**	**p**	**Correlation coefficient**	**p**
Winter	0.961	<0.001*	-0.710	0.032*	-0.386	0.305
Spring	0.254	0.509	-0.568	0.111	0.436	0.241
Summer	-0.271	0.517	0.169	0.689	0.299	0.472
Autumn	0.035	0.934	0.210	0.617	-0.147	0.728

*Correlation is significant P≤ 0.05

DISCUSSION

Nosema ceranae is an emergent and potentially virulent pathogen of the honey bee *(Apis mellifera)* that has spread around the world in the last ten or so years (Paxton, 2010). In order to determine if there is a replacement between *Nosema ceranae/Nosema apis*, a long-term survey is needed for evaluating the situation. *N. ceranae* is also coming from Asian source populations, so Turkey is a critical location for transferring *N. ceranae* to Europe and adaptation of the pathogen.

The results show that *N. apis* and *N. ceranae* have been found in Turkey at different rates since 2009. *N. ceranae* presents a different epidemiological pattern compared to *N. apis*. The co-infection was found mainly at high levels during the eight years in Turkey. Chen et al. (2009), Williams et al. (2014) and Charbonneau et al. (2016) revealed that both microsporidia produced single and mixed infections.

Table 3.

The mean values of temperatures (°C) seasonally between in 2009-2016

Years	Winter	Spring	Summer	Autumn
2009	5.3	11.3	22.9	15.1
2010	6.5	13.1	24.7	16.3
2011	4.1	11.5	24.5	13.8
2012	3.1	12.1	24.6	17.6
2013	4.2	13.5	23.3	14.6
2014	4.3	12.9	24.4	14.1
2015	3.8	12.1	24.2	16.6
2016	4.1	13.8	25.3	15.3

In addition, both infection intensity and honey bee mortality have been reported to be significantly greater for *N. ceranae* than for either *N. apis* or mixed infections. The mixed infection resulted in mortality similar to *N. apis* parasitism and reduced spore intensity, possibly due to inter-specific competition. *N. ceranae* is can be detected throughout the year, while *N. apis* is observed mainly in spring and autumn time during rainy days and a high moisture level (Aydin et al., 2005). So, there is a relationship between *N. apis* and *N. ceranae* infections during the year. Several studies have reported the relationship of temperature and viability of spores (Fenoy et al., 2009; Higes, Martín-Hernández, & Meana, 2010; Paxton, 2010; Retschnig et al., 2017; Whitaker, Szalanski, & Kence, 2010). According to the data, especially winter conditions changed the rates of nosema infection level in colonies, so co-infection might indicator that the dominant *Nosema* species depends on the season and climatic conditions. For instance, when the winter temperatures decrease as in 2012 and 2015, *N. apis* has dominancy and cause a high level of infection. The winter period manages the type and levels of *Nosema* infection. So, "replacement" may not be the correct term to describe the change in the spread of *N. apis* and *N. ceranae* distribution around the world.

The co-infection level was low during the first two years (2009-2010) of the study, although it rose in the following years. This indicates that the years 2009 and 2010 years had the first and second highest temperature values of winter seasons (Tab. 3). *N. ceranae* was seen more frequently than either *N. apis* or co-infection. In paralell, the statistical analyses also revealed that there is also significant correlation between warm weather conditions in winter and high spore level and high prevalance of *N. ceranae* in bee colonies.

Location was found to indirectly affect the dynamics of *N. apis*, *N. ceranae* and co-infection, and the difference was again due to climatic conditions. Although the floral diversity and geographical conditions are different, if two provinces have the same climatic conditions, they have a similar results for *Nosema* type and infection level (e.g. Eskişehir and Kars in Fig. 5). This study suggests that a dynamic prevalence among the *Nosema* species depends on the average winter temperature and not the replacement of *N. apis* by *N. ceranae*.

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