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Monitoring of nosemosis in the Lublin region and preliminary morphometric studies of Nosema spp. spores

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Summary

Nosemosis is serious and widespread bee disease connected with Collony Colapse Disorder (CCD). Monitoring of this disease is crucial to a better understanding of the effect of this disease on the health in both individual bees and at the whole colony level. There is little information about nosemosis in different parts of Poland, which have distinct beekeeping management and climate conditions. It is also important to have quick and easy methods of differentiation of the type of nosemosis (type A – caused by Nosema apis and C – by N. ceranae). Therefore the aims of the study was to determine the degree of Nosema spp. infection amount in the Lublin region during the course of 7 years and to find morphometric parameters which could help to distinguish the differences between spores of N. apis and N. ceranae.

In the Lublin region the amount of nosemosis infection has risen from the year 2001 to 2008, with the highest infection scores in 2008. This decreasing number of healthy colonies probably provides evidence of the impact of the greater frequency of N. ceranae infection, which could be the one of the causes of CCD.

Scanning Electron Microscopy (SEM) reveals that N. apis and N. ceranae spores differ in their surface structure. Generally, spores of N. ceranae seemed to be more sculptured with deeper ornamentation than those of N. apis. Therefore ornamentation of the spores cell walls with special reference to their area can be considered as a taxonomic criterion for separating these two Nosema taxa.

Keywords: nosemosis, Nosema apis, Nosema ceranae, distribution, SEM

Nosemosis is a serious and widespread bee disease. There is still little known about the symptoms and its course caused by two species *Nosema apis* and *N. ceranae* (Fungi: *Microsporidia*). The illness is associated with honeybee Colony Collapse Disorder (CCD), which is responsible for massive loss of bees outside the hive, and consequently the extinction of bee colonies around the world (12). This results in a deficit of pollinators, without whose help there would be only fruit of anemophilous plants (*Phaneuf S.*: Importance of honey bees for sustaining life on earth. 2007, http://findarticles.com/p/articles, downloaded in April 2012). The first description of this disease in honeybees (*Apis mellifera*) comes from almost 100 years ago (26 after 23). Initially it was thought that nosemosis is exclusively attributed to *Nosema apis*. At the end of the twentieth century, a new species of this microsporidium has been described in Asia: *Nosema ceranae* (6) – attacking the eastern bee (*Apis cerana*). At present, there has been widespread nosema infection caused by these two *Nosema* species (13), also in Poland (23, 24).

Nosemosis causes many changes both at the level of individual bees and colonies. This results in economic losses, which can be felt around the world. The life expectancy of infected bees is reduced by one third. In families affected by *Nosema* spp. worker bees become lethargic and unwilling to work. The honey yield of colonies decreases by 40%, and the secretion of wax falls by 25% (14, 16, 17).

Until now it was thought that *N. apis* can be found only in the bee gut, while *N. ceranae* is also found in other tissues and glands (4, 5, 8). Recent studies have shown that both *Nosema* species are not tissue-specific and besides the ventricular epithelium, their spores can also be found in the Malpighian tubule system, hypopharyngeal glands, salivary glands, and venom sacs. This may interfere with the function of the glands, which is related to the production of royal jelly, honey, wax and bee bread (3). So far there is no evidence that *Nosema* spp. can pass the full life cycle beyond the intestine. Life stages of this parasite are found only in intestinal epithelial cells. Some spores can be found in various tissues and glands, including the hypopharyngeal glands (3). In addition, spores remaining in the glands are potential reservoirs of infection (3). This way of bee disease transmission has been confirmed by numerous studies (2, 20).

Infection occurs after eating nosema spores that are found in large numbers in excrement excreted by diseased bees. Feces of ill bee contain many undigested sugars, therefore are readily licked by healthy bees (19, 25). Germination and release of invasive stages progress in the intestine. *Nosema* spp. multiplies rapidly and infects epithelial cells, leading to lesions, and eventually to the death of bees. The spores are extremely resistant to external factors and can survive several years without losing their ability for further infection within the body of insects. For this reason, combating nosemosis is difficult.

Nosema spp. are obligate intracellular parasites. The spore germinates in the midgut, extrudes polar tubule, inserts the tubule into the epithelial cell and injects the infective sporoplasm, which after that becomes a meront. The *Nosema* spp. intracellular life cycle has two phases: merogony and sporogony. The merogony is the proliferactive stage, in which pairs of spindle--shaped meronts are replicated. Firstly, injected sporoplasms develop into spindle-shaped meronts, then after approximately 4 h the merogonial replication starts. Subsequently, meronts are produced and next are multiplied. *N. apis* merogonial stages are more pleiomorphic than *N. ceranae* and in addition to the more frequently occurring spindle-shaped meronts there are also round to oval ones (7). Then meronts develop into sporonts, which are characterized by a dense surface coat. Sporonts multiply and the sporogonic phase ends with spores formation. The primary spores have a rather round shape and environmental spores are ovocylindrical (6, 7). Mature spores germinate and can infect new cells thus continuing the cycle or are excreted with the feces.

Environmental spores of two *Nosema* species differ very slightly while observed under the optical microscope. The fresh mature spores of *N. apis* measure 4-6 μm in length and 2-4 μm in width, while *N. ceranae* 3.3-5.5 μm and 2.3-3.0 μm respectively (6, 10). Therefore the largest *N. ceranae* spores have the same dimensions of the smallest *N. apis* ones. *N. ceranae* spores are less symmetrical and rather rod shaped (9).

Nosema studies are very important because of the heavily and widespread infection that have been caused worldwide and in Poland. Especially the fact that *Nosema* spp. infection is correlated with Colony Colapse Disorder (CCD) has a strong economical connotation, e.g. in Poland during the winter of 2007/2008 the loss of about 15.3% of bee colonies was observed. Heavy *Nosema* spp. infection was detected in 32% of them. During the next winter a 8.7% colonies loss was recorded, but *Nosema* spp. was detected in 60% of the apiaries (24). There is little information about nosemosis with regards to different parts of Poland, which have distinct beekeeping management and climate conditions (19, 20).

Traditionally nosemosis is treated as an infection of ventricular cells caused by *N. apis* and classified as nosemosis type A (10). Conversely, dry nosemosis or nosemosis type C is exclusively assigned to the *N. ceranae* bee infection and is now considered to be one of the main causes of CCD (10). It is crucial to be able to quickly and easily determine the type of nosemosis. Techniques based on DNA analysis are clear but time-consuming and expensive. Due to the *Nosema* spp. life cycle the only easily accessible stage which could be examined is the germinated spore, but the differentiation between spores of *N. apis* and *N. ceranae* is very difficult. Scanning electron microscopy showed excellent morphological and structural details of the microsporidian spores and remain as gold standards for the diagnosis of human and animal infection caused by these organisms. Its value is both as a confirmatory tool and for determining the amount of infection.

Therefore the aims of this study was to determine the degree of *Nosema* spp. infection in the Lublin region during the course of 7 years and to find morphometric parameters which could help to distinguish the differences between spores of *N. apis* and *N. ceranae*.

Material and methods

Monitoring of nosemosis in the Lublin region. Bees were collected from apiaries in the Lublin region during seven years (2001, 2002, 2003, 2004, 2005, 2007 and 2008). A total number of 1495 bee colonies from 78 apiaries was examined for *Nosema* spp. infection. From each apiary dead bees were collected before the first spring cleansing flight. To estimate the *Nosema* spp. spores numbers, 20 whole bees (WB) were grounded in 20 ml of sterile distilled water and smeared on a microscope slide for examination. Three independent samples (per 20 dead bee) for each colony were conducted. The estimation of *Nosema* spores per bee was accomplished using Olympus BX 61 light microscope and a haemocytometer (1, 11). For each spore suspension, averages of 2 estimates of intensity were used.

Morphometric studies of *Nosema* spp. spores. In 2012 dead bees from the winter loss were collected to study morphometric differences between *Nosema* spp. spores. Three independent experiments were conducted and each consisted of 50 whole bees (WB), 50 ventriculus (VE) and 50 hypopharyngeal glands (HG). The alimentary tracts were removed individually, ventriculus (VE) were gently washed in distilled water to prevent contamination by a haemolymph and crushed in distilled water. Hypopharyngeal glands (HG) were carefully separated under Olympus SZX 16 stereomicroscope, gently washed in distilled water to prevent contamination by the haemolymph and ground in sterile distilled water.

Tab. 1. The percentage of colonies infected by *Nosema* spp. in apiaries from the Lublin area

Year	Number of colonies tested	Percentage of colonies at each infection amount level				
		120 mln.spores/bee	50 mln.spores/bee	10 mln.spores/bee	4 mln.spores/bee	0 mln.spores/bee
2001	116	0.0	0.0	3.0	4.5	92.4
2002	100	6.0	8.6	6.9	8.6	69.8
2003	336	5.2	6.6	14.8	3.3	70.2
2004	553	3.3	14.1	23.1	22.1	37.4
2005	160	6.9	14.4	7.5	5.6	65.6
2007	120	9.0	28.0	14.0	8.0	43.0
2008	110	32.2	28.9	7.8	0.0	31.1
Mean		6.4	13.0	15.5	11.3	53.9

Scanning Electron Microscopy. Samples were fixed in 5% gluteraldehyde (v/v) in 0.1 M phosphate buffer pH 7.3 for 24 hours and then were washed in a phosphate buffer prior to post fixation in 1% osmium tetraoxide in 0.1 M-phosphate buffer for 24 hours followed by washing in the same buffer. SEM specimens were dehydrated by immersion for 15 min each in fresh solutions of 30%, 50%, 75%, 90%, and 100% acetone and critical point dried. The dried samples were mounted on specimen stubs using double side adhesive tape and coated with gold. Coated samples were viewed in a VEGA LMU scanning microscope at 30 KV, measured and photographed. In addition, the surfaces of individual spores were calculated through the formula: Area = Π * length * width.

Statistical analysis. The results were statistically analyzed with the SAS software (SAS Institute 2002-2003 SAS/STAT User's Guide Version 9.13, Cary, NC, Statistical Analysis System Institute) using the one-way ANOVA (a group effect was the experimental factor) and the HSD (honestly significant difference) test and correlation (20).

Results and discussion

Generally, in the Lublin region the amount of *Nosema* spp. infection has risen from year 2001 to 2008 (Tab. 1). Data from 2001 indicated the weakest degree of infestation – none of the colonies was struck in the range above 10 mln./spores/bee. The most serious infection amount incidence was in year 2008, with the highest scores in the range of 50 mln. and 120 mln./spores/bee. These results confirm the data from the whole of Poland (24), where during the winter of 2007/2008 a huge loss of bee colonies was observed. Next year the loss was lower but *Nosema* spp. infection was found to be more frequent (24). Terrifyingly, the quantity of healthy (without nosemosis) bee colonies decreased in the studied period of time very rapidly, except the peak from 2005. This decreasing number of healthy colonies probably provides evidence of the impact of *N. ceranae* infection. *N. ceranae* had time to adapt to the conditions of the temperateness and to result in more efficient bee colonies infection, which could be the one of the causes of CCD. This presumption was confirmed in the preliminary research conducted by Michalczyk and Sokół in the area of north-eastern Poland (15).

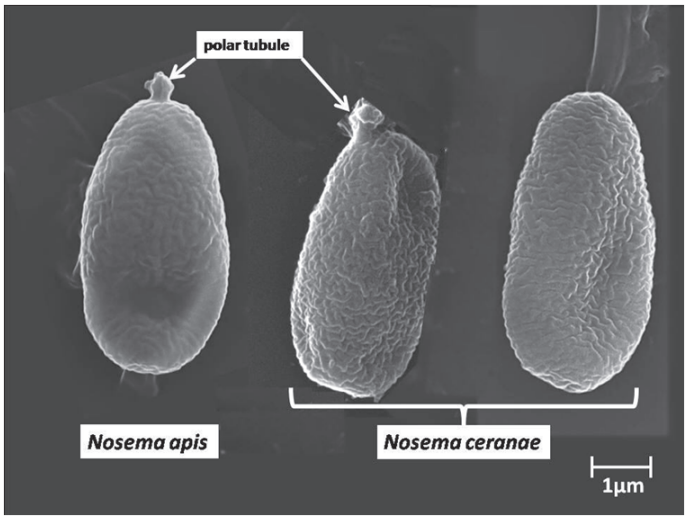

Fig. 1. *Nosema apis* and *N. ceranae* spores observed under SEM. Arrows indicate the start of extrusion of polar tubules. Scale bar = 1 µm

Scanning Electron Microscopy (SEM) revealed that *N. apis* and *N. ceranae* spores differed in surface structure. Spores of *N. ceranae* seemed to be more sculptured with deeper ornamentation than those of *N. apis* (Fig. 1). Generally, spores from HG had a bigger area than from other bee body parts (Tab. 2, 3). These data implied that in HG environmental spores of *N. ceranae* can be primarily observed, which have a more rectangular shape than that of *N. apis*. Similar data were obtained from duplex qPCR studies conducted by Coplay and Jabaji (3). Therefore ornamentation of the spores cell wall with special reference to their area can be considered as a taxonomic criterion for separating these two *Nosema* taxa.

Tab. 2. Morphometric traits of *Nosema* spp.

Part of bees body	*Nosema* spp.														
	lenght					width					area				
	Mean	CV	SE	Min.	Max.	Mean	CV	SE	Min.	Max.	Mean	CV	SE	Min.	Max.
WB	3.90	11	0.10	3.17	4.81	2.00	12	0.06	1.55	2.36	24.62	20	1.21	17.26	34.32
VE	3.93	8	0.07	3.53	4.87	2.09	11	0.05	1.45	2.45	25.85	16	0.91	16.83	37.50
HG	4.16	12	0.13	3.21	4.97	2.11	11	0.06	1.66	2.47	27.74	21	1.51	19.45	37.97

Explanations: WB – whole bees; VE – ventriculus; HG – hypopharyngeal glands; CV – coefficient of variation; SE – standard error; Min. – minimum value of the features; Max. – maximum value of the features

Tab. 3. Correlation among length, weight and area of *Nosema* spp. spores from different parts of bee's body

	Part of bee's body					
	WB		VE		HG	
	lenght	weight	lenght	weight	lenght	weight
Weight	0.57*		0.41		0.64*	
Area	0.88*	0.89*	0.79*	0.88*	0.91*	0.90*

Explanations: WB – whole bees; VE – ventriculus; HG – hypopharyngeal glands; * – significance at $p \leq 0.05$

Conclusion

Monitoring of bee colonies is essential in the combat against dangerous pathogens, which can lead to major health problems for colonies. Currently monitoring colonies for the presence of *Nosema* spp. helped to determine the extent of the growing invasion. Such studies will clarify the role of these microsporidia on colony health and will increase the knowledge of epidemiological factors of nosemosis. Morphological features differentiating the *Nosema* spp. contribute to their rapid identification and determination of *N. apis* and *N. ceranae* role in Colony Collapse Disorder. All this can limit the extinctions of honey bee colonies in the future.

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