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Original Article

HONEY BEE INFECTION CAUSED BY *NOSEMA* SPP. IN LITHUANIA

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Abstract

The infection of Lithuanian honey bee colonies by *Nosema apis* and *N. ceranae* and the consequences were analysed over a four-year (2011 - 2014) period. Both mono-infection either by *N. apis* or *N. ceranae*, and co-infection by both *Nosema* species, were found. There was a decrease in the percentage of *Nosema* infected colonies during the four-year study period. There were fewer colonies infected by *N. ceranae*, whereas the number of colonies with *N. apis* remained approximately at the same level during the study. The prevalence of both types of *Nosema* in honey bee colonies varied seasonally, i.e. there was a higher percentage of colonies infected in spring and summer but very rarely was *Nosema* detected in autumn. Mono-infection by *N. apis*, and co-infection by both *Nosema* species, were significantly more often recorded in weak and moderate colonies than in strong colonies. Mono-infection by *N. ceranae* was more often detected in weak colonies than in moderate and strong colonies, but more often detected in strong than in moderate colonies. A moderate link between a high prevalence of *N. ceranae* infection and an increased risk for winter colony mortality was observed.

Keywords: colony losses, *Nosema apis, Nosema ceranae, Apis mellifera*, seasonality

INTRODUCTION

Nosemosis is a disease of honey bees caused by two species of microsporidia; *Nosema apis* (Zander, 1909) and *N. ceranae* (Fries et al., 1996). For a time, it was assumed that *N. apis* was a pathogen specific to the European honey bee, *Apis mellifera* (Ellis & Munn, 2005), and *N. ceranae* specific to the Asian honey bee, *A. cerana* (Fries et al., 1996). However, many recent reports have revealed that *N. ceranae* has successfully infected *A. mellifera* worldwide (Higes, Martín, & Meana, 2006; Klee et al., 2007; Martín-Hernández et al., 2007; Paxton et al., 2007; Chen et al., 2008; Invernizzi et al., 2009; Tapaszty et al., 2009; Stevanovic et al., 2013; Blažytė-Čereškienė, Skrodenytė-Arbačiauskienė, & Būda, 2014). *Nosema ceranae* was considered an emergent pathogen suspected to replace *N. apis* in *A. mellifera* (Klee et al., 2007). There is increasing evidence that the pathogen has been present in the European honey bee for longer than 20 years (Paxton et al., 2007; Chen et al., 2008; Invernizzi et al., 2009). During the last 10 years, nosemosis in European honey bees caused by *N. ceranae* has been considerably more prevalent than that caused by *N. apis* in warmer climates (Tapaszty et al., 2009; Stevanovic et al., 2011), whereas *N. apis* remains more prevalent in colder climate regions (Budge et al., 2010; Gisder et al., 2010). There was a difference in the prevalence of *N. ceranae* in Scandinavian countries. In Finland, *N. ceranae* is more prevalent compared to Sweden and Norway although the climate conditions are similar (Klee et al., 2007; Paxton et al., 2007). Little is known of the current situation of nosemosis caused by *N. ceranae* in the Baltic countries. During the 2012 - 2013 time period, the clinical prevalence of nosemosis was not observed in Latvia and, though the species of *Nosema* was not identified, it barely exceeded 3.1% in Estonian honey bee colonies (Chauzat et al., 2014). In Lithuania, *N. ceranae* was detected in 2011, with a pronounced prevalence (up to 59%) in the south and west part of the country,

while *N. apis* prevailed (up to 50%) in the rest of the country (Blažytė-Čereškienė, Skrodenytė-Arbačiauskienė, & Būda, 2014).

Currently, the nosemosis caused by these two microsporidia species occurs in honey bee colonies worldwide and has been shown to have a number of negative impacts on honey bee colonies, depending on the geographic location and the *Nosema* species involved. Damage may cause a shortened worker lifespan (Fries, Ekbohm, & Villumstad, 1984; Higes et al., 2007), reduced colony productivity (Anderson & Giacon, 1992), and altered pheromone production by honey bee workers and queens that could induce queen supersedure (Dussaubat et al., 2010; Alaux et al., 2011). Reports on the impact of *Nosema* infection on colony survival are contradictory. Thus, *Nosema* infection's role in colony losses is not fully understood (Paxton, 2010). In Spain, heavy colony losses have been attributed to *N. ceranae* infection (Higes et al., 2008). Studies in some other European countries (Poland, Austria, and France) have suggested that *N. ceranae* infection could be related to the mortality of honey bee colonies (Derakhshifar et al., 2009; Gajda &Topolska, 2009; Chauzat et al., 2010). Several investigations from different geographical regions were made. It was found that though colony losses did not correlate with *N. apis* or *N. ceranae* infection, the infection combined with other synergistic factors might be involved in colony losses (Cox-Foster et al., 2007; Topolska, Gajda, & Hartwig, 2008; Ratnieks & Carreck, 2010). Among such factors, differences in climatic conditions in geographical regions may be important. In most areas, the *N. apis* infection level typically peaks in spring and decreases during summer and autumn (Pickard & El-Shemy, 1989; Martín-Hernández et al., 2007; Gisder et al., 2010; Mariani et al., 2012), whereas seasonal variation of *N. ceranae* infection depends on geographical region. Martín-Hernández et al. (2007) reported that no seasonal differences were found in the prevalence of *N. ceranae* in honey bee samples during 2005 year in Spain. However, Traver, Williams, & Fell (2012) found seasonal variation in *N. ceranae* infection over a 13-month sampling period in the USA. There was a similar seasonal variation in infection observed in Germany (Gisder et al., 2010) and Canada (Copley & Jabaji, 2012). Insufficient data on the impact of *Nosema* infection (especially that caused by *N. ceranae*) on *A. mellifera* in different areas in Europe, suggest a need for such an investigation in different geographical areas and climatic conditions (Fries, 2010). The Baltic countries, including Lithuania, are in the temperate continental climate zone with pronounced seasonality *i.e.* warm, dry summers and fairly severe winters. Thus, in our four-year (2011 - 2014) investigation, we studied the prevalence of *Nosema* infection in honey bee colonies in Lithuania. We tried to define if *N. ceranae* and *N. apis* infections in honey bee colonies varied seasonally, how the infections were related to colony strength, and if the infections were related to winter colony losses.

MATERIAL AND METHODS

Honey bee samples

In the present study, the prevalence of two microsporidia species, i.e. *N. apis* and *N. ceranae,* in Lithuanian apiaries, was investigated. Live worker honey bees (*Apis mellifera* L.) were collected at 44 apiaries from 130 colonies in 2011 (April – July), at 36 apiaries from 174 colonies in 2012 (July – October), at 30 apiaries from 129 colonies in 2013 (June – July), and at 22 apiaries from 110 colonies in 2014 (May – September) (Tab. 1). For sampling, two to six colonies per apiary were selected randomly, irrespective of signs of any disease. Yearly sampling was performed in the same apiaries and in the same colonies. However, the number of tested apiaries decreased as some beekeepers refused to participate during the course of the survey. Moreover, many beekeepers were moving their colonies from one apiary to other. This meant that during the four-year study, just 15 colonies were sampled four times, 29 colonies were sampled three times, 94 colonies twice, and 209 colonies were sampled once. In total, 347 honey bee colonies were tested.

Bees were collected at the hive entrance or

Table 1
Number of honey bee colonies sampled in the three seasons during the four-year study period (2011 - 2014)

	Year			
	2011	2012	2013	2014
Spring (April – May)	67	-	-	29
Summer (June – July)	63	40	129	44
Autumn (September – October)	-	134	-	37
Total number of colonies tested	130	174	129	110

on frames away from the brood nest following the OIE guidelines (OIE, 2008). Each sample contained bees from a single colony only. Bee samples were frozen and stored at –20 °C until used for suspension preparation.

Colony strength estimation

Each of the beekeepers was asked to answer some questions about the strength of the colonies sampled and the survival of the colonies in the apiary during the winters of the study (2010/2011, 2011/2012, 2012/2013, and 2013/2014). Beekeepers rated the strength of their colonies as weak, moderate or strong according to the number of frames of broods in each colony, the signs of nosemosis and the amount of honey production. The information on 335 colonies sampled for *Nosema* detection during summer over the four-year study period was obtained.

DNA extraction and identification of *Nosema* species

Abdomens of ten adult bees from each colony (according Martín-Hernández et al., 2007) were homogenised in a mortar with 4 mL of deionised water. For DNA extraction, 1 mL of spore suspension was centrifuged (5 min, 16 100 *g*) and supernatant discarded. Pellets were frozen in liquid nitrogen and crushed using sterile pellet pestles. DNA was extracted with the use of a DNeasy Plant Mini Extraction Kit (Qiagen). The extracts were stored at –20 °C until PCR.

For *Nosema* detection and determination of *Nosema* species, a duplex-PCR with species-specific primers (321APIS-FOR/REV for detection of *N. apis* and 218 MIROC-FOR/REV for detection of *N. ceranae*) was used (Martin-Hernandez et al., 2007). The PCR mixtures contained DreamTaq™ Green PCR Master Mix (Thermo Scientific) 25 µL, 0.4 µM of each species-specific primer (FOR/REV), 1 µL of template DNA and water nuclease-free (Thermo Scientific) in a total volume of 50 µL. Amplifications were performed in Mastercycler® nexus (Eppendorf) thermal cycler using a cycling program as previously described in detail (Stevanovic et al., 2011). The PCR products were electrophoresed in 1 % agarose gel (1xTBE, Thermo Scientific) containing 1 ng/mL Midori Green (NIPPON Genetics EUROPE), and visualised under UV light. Fragment sizes were determined with reference to GeneRuler™ Low Range DNA Ladder (Thermo Scientific).

The PCR products were extracted from the agarose gel using a GeneJET Gel Extraction kit (Thermo Scientific). The PCR fragments were sequenced by automated DNA sequencing. The 16S rRNA gene sequences of *N. apis* and *N. ceranae* were similar to sequences from Lithuania deposited in the DDBJ/EMBL/GenBank databases earlier (Blažytė-Čereškienė, Skrodenytė-Arbačiauskienė, & Būda, 2014).

Statistical analysis

The analysis was performed using Statistic 6.0 software package (StatSoft, Inc., Tulsa, OK, USA). The chi-square test was used to compare the proportion of *Nosema*-positive colonies among weak, moderate, and strong colonies. The Kruskal-Wallis test was used to compare colony

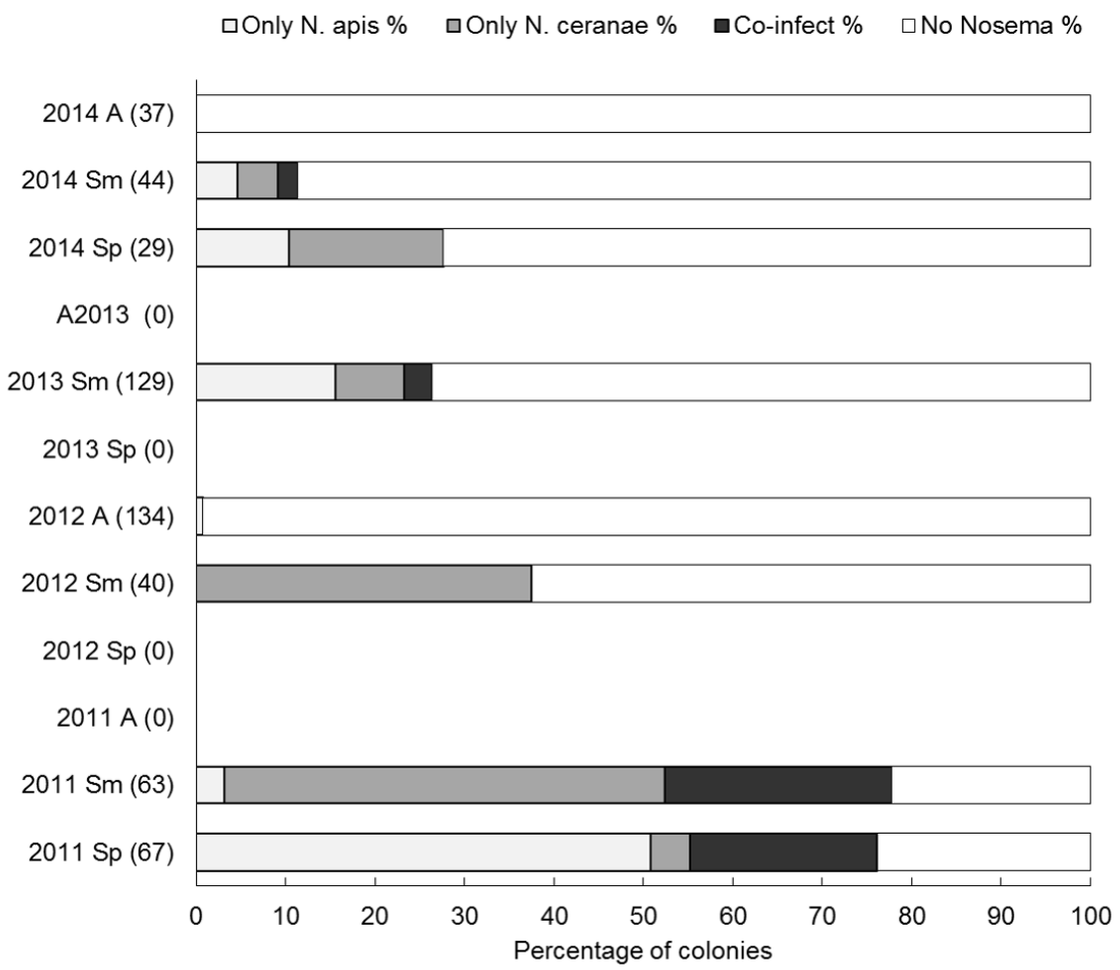

Fig.1
Prevalence of *Nosema apis* and *N. ceranae* in honey bee colonies during the four-year (2011 - 2014) study period in Lithuania.

losses in different years. The Spearman rank order correlation analysis was used to estimate the relationship between *Nosema* infection and colony losses in the following winter season. A p-value of < 0.05 was considered significant.

RESULTS

Prevalence of *Nosema* spp.

A total of 347 honey bee colonies were tested for the presence of *Nosema* infection over a four-year (2011 - 2014) period. *Nosema* was detected in 153 colonies (44%) at least once throughout the study. Mono-infection by *N. apis* or *N. ceranae*, and co-infection by *N. apis* and *N. ceranae*, were detected. Infection by *N. ceranae* was detected in 27.7% of colonies and by *N. apis* in 27.1% of colonies, i.e. infection by each parasite species was at a very similar level. Among infected colonies, 38.6 % contained *N. ceranae* only, and 37.2% contained *N. apis* only, while 24.2% of the colonies were infected by both *Nosema* species.

In colonies which were tested at least twice during the four-year period, the recurrence of the *Nosema* infection was detected in five of them (3.27%): *N. apis* was detected twice in three colonies (1.96%), *N. ceranae* was detected twice in a single colony (0.65%), and in a single colony (0.65%) *N. cerane* (present for two consecutive years) was replaced by *N. apis* (in the third year).

The most *Nosema*-positive colonies were detected in spring and summer of 2011: 76.1% and 77.8%, respectively. In 2014, infection was found in 27.6% of the colonies in spring and in 11.4% of the colonies in summer. In honey bee samples collected in autumn, *Nosema* was detected only in a single colony (from 134) in 2012. Analysing *N. apis* and *N. ceranae* as a single

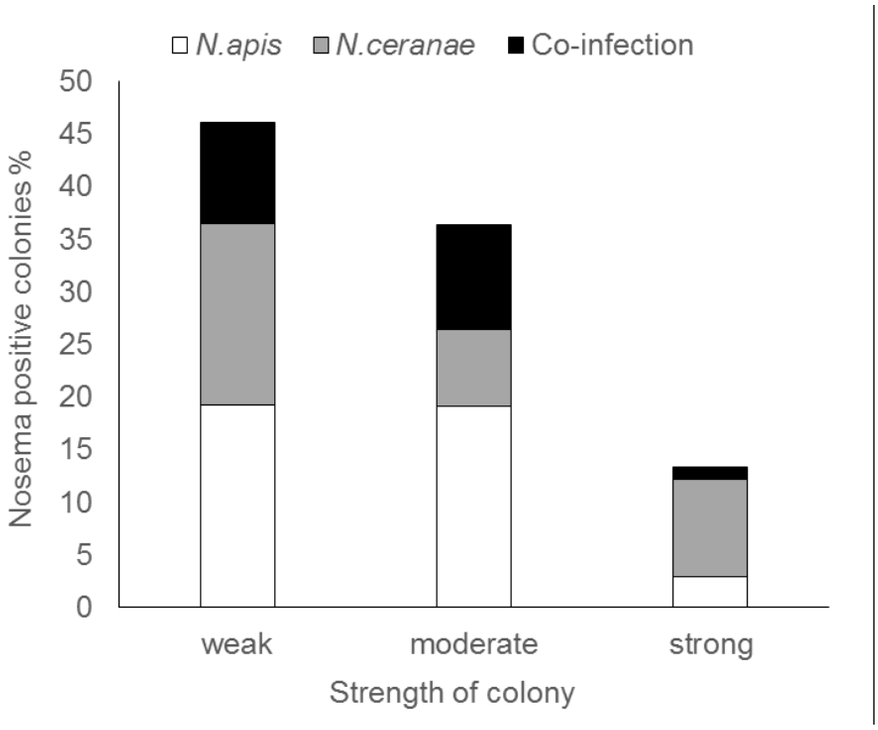

Fig.2
Relationship between the colony strength (weak, moderate, and strong) and the type of *Nosema* spp. infection (mono-infection by either *N. apis* or *N. ceranae*, and co-infection by both *N. apis* and *N. ceranae*). The results are based on the summer samples only.

infection, *N. apis* was found to be more prevalent in spring, whereas *N. ceranae* was most common in spring and summer. Co-infection by both *N. ceranae* and *N. apis* was most common in summer. Thus, the results of the 4-year study suggested that *Nosema* infection in honey bee colonies varied depending on the season.

Based on an analysis of summer samples only, most *Nosema*-positive colonies were detected in 2011 (78%), while infection was found in 11% of the colonies in 2014. In summer, the level of infection by *N. apis* was approximately the same during the 2011 - 2014 period and did not exceed 15% of the colonies. While the infection level by *N. ceranae* decreased during the same period from 49% of the colonies in 2011 to 4.5% of the colonies in 2014 (Fig. 1). Co-infection by *N. ceranae* and *N. apis* varied during this period: the highest level of co-infection was detected in 2011 (25%), and the lowest in 2013 and 2014 (approximately 2 – 3% of the colonies). So, the number of *N. ceranae* infected colonies decreased from 2011 to 2014.

Infection and colony strength

Beekeepers rated the strength of their colonies as weak, moderate or strong. The main signs of colony weakness as noted by the beekeepers: were a low number of worker bees and the slow development of a colony (37% of colonies), low honey production (23%), sick brood (14%), faecal marks and sick adult bees (21%), and queen loss (5%). Based on such data, 52% of the colonies were defined as strong, 33% as moderate, and 15% as weak. With the aim of determining whether colony strength was related to *Nosema* infection and whether colonies rated as weak by beekeepers had significantly higher levels of *Nosema*, statistical analysis was applied. The chi-square test revealed that *Nosema* was significantly more present in weak (46%) and moderate (36%) colonies than in strong (13%) colonies (weak *vs.* strong: $\chi^2 = 26.1$, $P < 0.01$; moderate *vs.* strong: $\chi^2 = 20.7$, $P < 0.01$). The difference was found both for mono-infection by *N. apis* and for co-infection by both *Nosema* species. Whereas mono-infection by *N. ceranae* was more present in weak colonies than in moderate and strong ones (weak *vs.* strong: $\chi^2 = 4.9$, $P = 0.03$; weak *vs.* moderate: $\chi^2 = 8.0$, $P < 0.01$; moderate), and more present in strong colonies compare to moderate ones ($\chi^2 = 15.2$, $P < 0.01$) (Fig. 2).

Infection and colony losses

In order to evaluate the impact of *Nosema* infection on the mortality of honey bee colonies, we related the overwintering losses to the detection of the two *Nosema* species; *N. apis*, and *N. ceranae*. Based on the beekeepers' information, colony losses varied from 2.8% to 13.1% during the 2011 - 2014 period. No statistically significant differences were revealed in colony losses during the study among the sampled apiaries ($H = 5.1$, $df = 3$, $P > 0.05$). No statistically significant correlation was found between *N. apis* infection and colony losses in the following season ($P > 0.05$). However, correlation analysis revealed a moderate relationship between *N. ceranae* infection in 2011 and colony losses in the following winter (2011/2012) ($r_S = 0.58$, $t = 2.5$, $P = 0.03$).

DISCUSSION

Our four-year study revealed that honey bee colonies in Lithuania were infected by both *Nosema* species. Mono-infections either by *N. apis* or *N. ceranae*, and co-infection by both *Nosema* species were found. The total prevalence of infection during the whole study period was approximately 44%, and infection by each *Nosema* sp. was at a very similar level. The number of infected colonies might be higher as Nosema-free colonies could still bear microsporidia that were undetected since the tested samples only contained ten bees, and this could cause an underestimation of the pathogens' presence. If the estimated prevalence of *Nosema*-species-infection was compared between years, however, we found that the number of infected colonies decreased. The decrease in the level of Nosema infection was due to the decrease in the number of colonies infected by *N. ceranae*, whereas the number of colonies with *N. apis* remained approximately at the same level during the study period. Thus, the study did not confirm our assumption that invasive *N. ceranae* was replacing the local parasite *N. apis* in Lithuanian honey bee colonies (Blažytė-Čereškienė, Skrodenytė-Arbačiauskienė, & Būda, 2014). However, the replacement of *N. apis* by *N. ceranae* seems to have occurred in different regions of Europe and in the US (Botias et al., 2012; Chen et al., 2008). *Nosema ceranae* has become more prevalent than *Nosema apis* in Spain, Denmark, Italy, Poland, Greece, and the Balkan countries (Klee et al., 2007; Tlak Gajger et al., 2010; Michalczyk et al., 2011, 2013; Stevanovic et al., 2011; Botias et al., 2012). *Nosema apis* remains prevalent in Sweden and the United Kingdom (Klee et al., 2007). In Lithuania, infection by each parasite species was at a very similar level, and this situation corresponds to that reported in Germany (Gisder et al., 2010).

Although there were analysed samples collected in 4 summer seasons and those collected in 2 spring and autumn seasons, the main trend became clear: in spring and summer honey bee colonies were infected by both *N. apis* and *N. ceranae*, while in autumn the microsporidia infection level decreased (*Nosema apis* was detected in a single colony only). Our results indicated that the prevalence of *Nosema* in Lithuanian honey bee colonies varied seasonally. In Lithuania, the observed seasonal prevalence of *N. apis* infection was in agreement with that previously reported as characteristic to a temperate climate zone: i.e. a peak in spring and low prevalence during summer and autumn (Fries, 2010; Gisder et al., 2010). The seasonal prevalence of *N. ceranae* infection in Lithuanian honey bee colonies was quite similar to the seasonality observed in the north-eastern part of Germany where the proportion of colonies with *N. ceranae* was always higher in spring than in autumn (Gisder et al., 2010). However, it was different from that reported by Martín-Hernández et al. (2007) in Spain, and by Stevanovic et al. (2013) in Serbia. In Spain, Martín-Hernández et al. (2007) observed a total lack of seasonality of *N. ceranae* infection. In Serbia, the highest prevalence of *N. ceranae* was recorded in spring, with a lower prevalence in summer and then an increase in autumn, but the proportion of infected colonies was always high (never below 73%) (Stevanovic et al., 2013). Hence, our study confirms that the pronounced seasonal variation

of *N. ceranae* prevalence is characteristic of countries with cool climate conditions.

The diagnosis of *Nosema*-infected colonies in the field is possible if the honey bees are infected by *N. apis* (such as dysentery accompanied by defecation within the hive and crawling bees). However, the diagnosis of colonies infected by *N. ceranae* is almost impossible due to a lack of infection signs (Fries, 2010). In Serbia, though, the symptoms traditionally attributed to *N. apis* infection were also observed in colonies infected by *N. ceranae* (Stevanovic et al., 2013). In Lithuania, our study revealed that the colonies rated by beekeepers as weak and moderate were more often infected by *N. apis* than strong colonies were. The symptoms of nosemosis (faecal marks and sick adult bees) were noted in 21% of weak colonies. A low number of worker bees and a slow increase in numbers were the main indicators of colony weakness most frequently denoted by beekeepers. Among strong colonies infected by *Nosema*, most contained mono-infection by *N. ceranae*. We assume the reason for that might be recent infection and a still low negative impact. Besides, such colonies were detected in apiaries maintained by professional beekeepers, who take special measures to prevent colony weakening, i.e. merge colonies, change queens etc.

Infections by *N. ceranae* have been suggested to be implicated in severe colony losses in Spain (Higes et al., 2008, 2009). Similar colony losses that could be attributed to *N. ceranae* have been reported in Poland, Finland, Denmark, and Turkey (Gajda & Topolska, 2009; Korpela, 2009; Vejsnæs, Nielsen, & Kryger, 2010; Whitaker, Szalanski, & Kence, 2011). However, in other countries (Germany, France, Serbia, the USA, etc.) no relationship between infection by *Nosema* species and winter losses have been revealed (Chauzat et al., 2007; van Engelsdorp et al., 2009; Genersch et al., 2010; Dainat et al., 2012; Stevanovic et al., 2013). Likewise, the results obtained in our study did not reveal a relation between *N. apis* as mono-infection and losses the following winter. Though the cause of colony losses cannot be conclusively attributed to *N. ceranae* infection, we found a significant but moderate correlation between the presence of the infection in 2011 and the occurrence of losses in the following winter (2011/2012). During the winter of 2011/2012, colony losses amounted to about 10% only, but most of the colonies that did not survive the winter were in apiaries infected by *N. ceranae*. In 2011, *N. ceranae* was widespread in Lithuanian apiaries, possibly due to climatic changes in the country. Based on the data reported by the Lithuanian Hydrometeorological Service for the past decade, the average temperatures were continually increasing: in the first half of 2011, the average temperature was 3°C higher than the temperature averages of previous years (Aplinkos būklė 2011. Tik faktai, 2012). Possibly, there were congenial conditions for the spread of southern-origin parasites, and *N. ceranae* might have been transferred to autumn bees. Insufficient food stores were the usual cause of honey bee death indicted by beekeepers. Knowing the role of *N. ceranae* in honey bee immune suppression (Antúnez et al., 2009; Chaimanee et al., 2012) and induced energetic stress (Mayack & Naug, 2009), we assume that this parasite could be one of the causative factors of colony mortality during the winter of 2011/2012.

In summary, during the four-year period, the percentage of *N. ceranae*-infected colonies decreased. In honey bee colonies in Lithuania, *N. ceranae* has not replaced *N. apis*. Such a situation is likely to be observed in other Baltic countries and in regions with similar climatic conditions. The prevalence of both types of nosemosis in honey bee colonies varied seasonally. A moderate link between a high prevalence of *N. ceranae* infection and an increased risk of colony mortality was established.

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