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A COMPARATIVE STUDY OF ENVIRONMENTAL CONDITIONS, BEE MANAGEMENT AND THE EPIDEMIOLOGICAL SITUATION IN APIARIES VARYING IN THE LEVEL OF COLONY LOSSES

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Abstract

Explaining the reasons for the increased mortality of the honey bee (*Apis mellifera* L.) in recent years, in Europe and North America, has become a global research priority in apicultural science. Our project was aimed at determining the relationship between environmental conditions, beekeeping techniques, the epidemiological situation of pathogens, and the mortality rate of bee colonies. Dead bee samples were collected by beekeepers from 2421 colonies. The samples were examined for the presence of *V. destructor*, *Nosema* spp. (*Nosema apis* and *Nosema ceranae*), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV), deformed wing virus (DWV), and Israeli acute paralysis virus (IAPV).

Among the environmental and colony management factors under analysis, significant differences between apiaries with high (>10%), low (≤10%) or no losses of the colonies were only found in the case of the methods used by beekeepers to combat varroa mites. However, the epidemiological patterns in the case of *V. destructor* infestation and the DWV and ABPV infections highly differed. The data we obtained indicated that co-infections play a decisive role in the etiology of the significant collapse of colonies in apiaries in Poland. The main reason for this phenomenon can be described as strong infestation with *V. destructor*, followed by an intensive development of viral infections caused by DWV and (much less frequently) by ABPV. Despite a high prevalence of *Nosema* spp. microsporidia (with a dominant incidence of *N. ceranae*), a direct relationship between these parasites and the mortality rate of colonies was not proved.

Keywords: beekeeping management, colony collapse, environmental conditions, *Nosema* spp., *Varroa destructor*, viruses.

INTRODUCTION

Honeybees, acting as principal pollinators of crops and wild-growing plants constitute an exceptionally important element in numerous ecosystems, indispensable for their correct functioning (Costanza et al.,1997). Almost one third of the global food of plant origin production is possible thanks to the pollination service rendered by these insects and is a measurable effect of apian work for the sake of humankind (Klein et al., 2007).

Bearing this in mind, the significant rise in the mortality of managed honeybee colonies, persisting in many regions of Europe and North America for a number of years, gives rise to justified concern in the affected communities (vanEngelsdorp et al., 2007, 2008; Neumann and Carreck, 2010; Potts et al., 2010; vanEngelsdorp et al., 2011, 2012; van der Zee et al., 2012, 2014). The numerous scientific research undertaken to explain the reasons of this phenomenon is one of the planes on which intensive efforts aimed at improving the situation in the global beekeeping sector are being made (Brown and Paxton, 2010; Moritz et al., 2010).

Honey bee health and lifespan are affected by a number of biotic and abiotic factors, and the extent of their impact depends on environmental conditions and apiary management methods (Chen et al., 2006; Chauzat et al., 2009; Alaux et al., 2010b; Aumeier et al., 2010; Bernal et al., 2010; Brodschneider and Crailsheim K., 2010; vanEngelsdorp and Meixner, 2010). The results of COLOSS Genotype-Environment Interactions experiment has also proved the existence of significant interactions between honey bee genotypes and the environment which affect the survival of *Apis mellifera* L. colonies in Europe (Büchler et al., 2014; Meixner et al., 2014). Due to the genetic variability of honey bee populations and the wide variety of honey bee breeding conditions it is impossible to indicate the global causes for the mass dying out of bee colonies. Newly published study results, however, show that pathogenic microorganisms (viruses, bacteria and fungi), parasites and pesticides play a central role among the many potential stressors. The dominant view is that high colony losses result from the synergistic effect of different pathogenic sets in the case of co-infection (e.g. *Varroa destructor* and viruses, *Nosema ceranae* and viruses) (Dainat et al., 2012a, b; Nazzi et al., 2012), individual pathogens and pesticides (e.g. *N. ceranae* and neonicotinoids) (vanEngelsdorp et al., 2009; Alaux et al., 2010a; Vidau et al., 2011; Pettis et al., 2012; Retschnig et al., 2014) or combinations of pesticides (e.g. insecticides and fungicides) (Mullin et al., 2010; Gill et al., 2012). Around the world *Varroa destructor*, the external parasite affects both, the adult and developmental bee stages, posing a particular threat to the Western honey bee (*Apis mellifera* L.). An introduction of the varroa mite from *Apis cerana* species to *Apis mellifera* species took place in the fifties. Despite such a long passage of time, the problem of varroosis has still not been definitively resolved (Rosenkranz et al., 2010). A shortened lifespan of the parasitised bees and suppression of their immune resistance are the result of the morphological and physiological changes due to the loss of nutrients contained in hemolymph (Ball, 1993; Gregory et al., 2005). Moreover, the disease is usually accompanied by viral infection. Viruses transmitted via *V. destructor* penetrate directly into the hemocele, thereby rapid multiplication of the viruses and transition of covert form of the infection to the symptomatic form is possible. Among a few varroa transmissible viruses, the deformed wing virus (DWV) and the acute bee paralysis virus (ABPV) cause the most serious effects leading to the death of bee colonies. Recent studies have found that both infections coexisting with severe *V. destructor* infestation caused high winter colony losses in European apiaries (Genersch et al., 2010; Guzman-Novoa et al., 2010; Le Conte et al., 2010; Dainat et al., 2012b). While, the Israeli acute bee paralysis virus (IAPV) was implicated with massive declines of managed colonies in US as a one of the main reasons of the syndrome named Colony Collapse Disorder (CCD) (Cox-Foster et al., 2007; vanEngelsdorp et al., 2009; Hou et al., 2014). Although a link between the chronic bee paralysis virus (CBPV) and *V. destructor* has not yet been documented (Ball and Allen, 1988; Tentcheva et al., 2004) it is known that the virus can also be highly pathogenic and may cause serious mortalities among the honey bee colonies (Allen and Ball, 1996).

Nosema ceranae and *Nosema apis* microspridia are fungal endoparasites of adult honey bees. *Nosema apis* as a parasite of the Western honey bee *Apis mellifera* has been known for more than 100 years while *N. ceranae* was detected for the first time in this bee species only ten years ago (Huang et al., 2007). Despite this fact, a high prevalence of *N. ceranae* infection has already been reported in many regions of the world (Klee et al., 2007). The spores of the both *Nosema* species germinate in the midgut epithelial cells causing disorders in the digestive processes (Bailey and Ball, 1991; Amdam et al., 2004; Mayack and Naug, 2009; Fries, 2010). *Nosema apis* shortens the lifespan of the infected bees and adversely affects colony strength and productivity (Farrar, 1947; Moeller, 1962; Fries, 1995). In cases of severe nosemosis the sick colonies can die in the spring. There is still not enough information to explain the impact of *N. ceranae* on honey bee colonies and the links with high colony losses (Forsgren and Fries, 2010; Martin-Hernandez et al., 2012; Botías et al., 2013).

In Poland, the nationwide problem of a prominent rise in bee colony mortality appeared in the winter of 2007/2008 and has persisted until now. Between 15 and 20% of colonies (from about 1.2 million managed colonies) have died out in the successive years, except for the 2008/2009 season (approximately 12%) (van der Zee et al., 2012; van der Zee et al., 2014). We performed the first studies which shed some light on the background of this phenomenon in 2008 and 2009 (Topolska et al., 2010; Pohorecka et al., 2011a). The downside of these studies was that we assessed the presence of only one potentially noxious factor (pathogens) and only in those apiaries in which the percentage of the dead colonies in winter exceeded 30%. Apart

from that prevalence of *Nosema* microsporidia was determined without differentiation between the species of *N. apis* and *N. ceranae*. That is why, within the scope of the present project, we carried out comprehensive analyses of numerous factors important for the state of bee health at apiaries with different winter colony loss rates. The analyses were done to indicate those factors that account for the colony decline in Poland. This paper presents an analysis of the natural food resources, beekeeping techniques and epidemiological status of pathogens and parasites posing the greatest threat for the life of honey bees.

MATERIAL AND METHODS

Research organisation

The present analyses constituted one of the tasks in an international non-co-financed project under the auspices of the Ministry of Science and Higher Education, COST ACTION FA0803, grant number 527/N-COST/2009/0: "Definition of the Role of Environmental, Genetic and Pathogenic Factors in Mass Bee Colony Losses" carried out between 2009 and 2012.

According to the programme's assumption, this task was carried out in cooperation with beekeepers who voluntarily decided to take part in the project. The program was primarily aimed at beekeepers who observed greater mortality in their bee colonies in winter but the project also included the participation of beekeepers who did not face this problem. Through beekeeping organisations, apiarists were informed about the purpose of the research and its rules. Each of the participants was obliged to collect and transfer specific types of samples to our laboratory together with a completed questionnaire form. The survey questions referred to, among others, the number of colonies before and after wintering, habitat conditions around the apiaries (including the type of wild-growing plants and crops), issues connected with apiary management methods including, among others, the varroa mite treatment. a detailed list of the survey data is set out in Table 2 and 3.

Climatic conditions

A large part of Poland is lowland country (75% northern and central area) with uplands and mountain ranges in the south. Poland is situated in a temperate zone of mixed, continental and oceanic influences. The average annual air temperatures are lowest in the north-east (from 6 to 7°C) and the highest in the south-western region (from 8 to 9°C). Weather conditions during the project were characteristic for recent years (shorter and less frosty winters), the four-year average winter temperature ranged between -4 and 0°C and between 17 and 19°C in the summer.

Materials for laboratory analyses

The participants (beekeepers) had to comply with a uniform sample collection and dispatch system. In the apiaries which suffered the loss of at least 10 colonies, the material had to be collected separately from 10 randomly selected dead colonies. In the apiaries in which fewer than 10 colonies fell to the bottom board, samples had to be collected from each dead colony. In the apiaries with low losses or those in which all the colonies survived the winter, the samples had to also be collected from the surviving colonies (altogether 10 samples per apiary at the maximum). No matter what the state of the colony was (living or dead), the material for epidemiological analyses had to be made up of a maximum of 0.5 L of dead bees and debris collected from the bottom of the hive. The same colonies also provided samples of wax, honey and bee bread for toxicological analyses (the results will be presented in a separate paper). Prior to the laboratory analyses, the samples were stored at below -15°C.

Laboratory analytical methods

The bee samples were individually examined for the following pathogens:

Varroa destructor

The sampled bees were transferred into plastic containers with water and detergent, and mechanically shaken for 10 minutes. The bees were separated from the mites by filtration using a convenient sieve to collect the mites. The bees were washed in a stream of water for several minutes to wash out all the mites. The mites and bees in the sample were calculated and infestation levels were expressed as the number of mites per 100 bees.

Nosema spp.

A single sample consisting of 30 abdomens of worker bees was crushed with a mortar and pestle in 30 mL of distilled water. The homogeneous suspension was placed under a cover-slip of a Bürker's haemocytometer. The spores in each square were counted under light microscope (400x).

Nosema intensity (the number of spores per bee in the sample) was evaluated according to the formula reported in the OIE Terrestrial Manual (2013).
A polymerase chain reaction (PCR) analysis was performed on the *Nosema* spp.- positive samples in the light microscopy examination. Ten bees from each sample were homogenized in liquid nitrogen and placed in a 1.5 mL tube. Afterwards, 360 µL of ATL buffer (Qiagen) and 40 µL of Proteinase K (Qiagen) were added. The samples were incubated overnight at 56°C. An extraction of the DNA was then done using a DNeasy Blood&Tissue Kit (Qiagen). The positive and negative controls were used in the DNA preparation. In the end, the tubes containing DNA were refrigerated at below -15°C. The extracted DNA was analysed to identify the *Nosema* species using specific 218MITOC F/R and 321APIS F/R primers previously described by Martín-Hernández et al. (2007). The polymerase chain reaction (PCR) was performed in a total volume of 50 µL containing 25 µL of Fast Start PCR Master (Roche), 0.4 µM of each pair of primers, 0.01 mg/mL BSA (Roche), 0.1% Triton X-100 (Roche) and 5 µL of the DNA template. The PCR conditions were as follows: 10 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 55.8°C, 45 s at 72°C; final extension of 7 min at 72°C. The positive and negative PCR controls were used in each run. The amplicons were analysed by means of gel electrophoresis and UV light visualisation. The specificity of the PCR products was verified by sequencing (Institute Biochemistry and Biophysics Polish Academy of Sciences) and comparing the obtained isolates with *Nosema ceranae* and *Nosema apis* isolates available in the GenBank database.

Viruses
The incidence of four bee viruses: deformed wing virus (DWV), acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV) and Israeli acute paralysis virus (IAPV) was investigated. Ten dead bees from each sample were homogenized in the presence of liquid nitrogen and used for total RNA extraction with a Total RNA Mini kit (A&A BIOTECHNOLOGY, Poland) and afterwards for reverse transcription - PCR with a OneStep RT-PCR Kit (Qiagen). Each virus was targeted with a single diagnostic primer pair (Tab. 1). Reverse transcription polymerase chain reaction (RT-PCR) was performed using the following thermal profile: reverse transcription for 30 min at 50°C, followed by a polymerase chain reaction: 15 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by 10 min at 72°C for the final extension. The positive and negative controls were included in each run of RT-PCR. The amplicons were analysed by means of gel electrophoresis and UV light visualisation. The specificity of the PCR products was verified by sequencing (Institute Biochemistry and Biophysics Polish Academy of Sciences) and comparing the obtained isolates with ABPV, CBPV, DWV and IAPV sequences available in the GenBank database.

Data evaluation and statistical analyses
Although there were 525 beekeepers who reported for the program, some of them did not comply with our conditions. For example, the samples they sent were either too small or did not enable all the analyses to be done or the collected material was of poor quality. That is why only the data obtained for 2421 colonies from 476 apiaries were used for the statistical analyses. All the statistical analyses were carried out using Statistica 10 StatSoft®.

Table 1.

Primers used for the detection of the viruses

Target	Sequence of primer (5'-3')	Length of product (bp)	Reference
ABPV	GCT CCT ATT GCT CGG TTT TTC GGT TTA TGT GTC CAG AGA CTG TAT CCA	900	Benjeddou et al., 2001 Chen et al., 2006
CBPV	TCA GAC ACC GAA TCT GAT TAT TG ACT ACT AGA AAC TCG TCG CTT CG	569	Blanchard et al., 2008a
DWV	TCG ACA ATT TTC GGA CAT CA ATC AGC GCT TAG TGG AGG AA	702	Chen et al., 2004 Chen et al., 2006
IAPV	ATC GGC TAA GGG GTT TGT TT CGA TGA ACA ACG GAA GGT TT	767	Cox-Foster et al., 2007 Blanchard et al., 2008b

DWV - deformed wing virus; ABPV - acute bee paralysis virus; CBPV - chronic bee paralysis virus; IAPV - Israeli acute paralysis virus.

The values obtained for the investigated parameters were compared between the following groups: Apiaries with High Losses (>10%) of bee colonies (named in the text as AHL), Apiaries with Low Losses (≤10%) of bee colonies (named in the text as ALL), and Apiaries with No Losses of bee colonies (named in the text as ANL). Some of the analyses were performed to compare the group created out of the colonies from all the AHL, ALL, and ANL groups, in which the investigated factor was identified and those colonies in which it was absent. Due to a lack of a normal distribution of variables we used nonparametric tests: Kruskal-Wallis median-test, Mann-Whitney U-test.

The relationship between the intensity of *V. destructor* infestation and *Nosema* spp. infection was evaluated using Spearman's rank correlation coefficient (r_s). Multiple regression was used to define the influence of quantitative parameters (*V. destructor* infestation level and *Nosema* spp. infection level, and the numbers of pathogens, in the case of co-infections) on colony loss rates. The relationships between the investigated qualitative characteristics were evaluated with the Chi-square test. P-values ≤0.05 were considered significant.

RESULTS

Analysis of questionnaire data

Apiary location and structure

Beekeepers from all the 16 voivodeships in Poland joined the study. Apiarists from southern and south-eastern Poland were the most numerous group (Fig. 1) The apiaries involved in the study contained between 5 and 800 bee colonies during the full summer period. Amateur apiaries dominated (altogether, 15.7% were apiaries containing between 5 and 10 colonies, and 45.4% apiaries with 11 to 30 colonies). In the small-scale and large-scale apiary categories, 17.6% of the apiaries contained between 31 and 50 colonies, 13.6% between 51 and 80, and 7.6% more than 80 colonies.

Bee colony losses

In 401 apiaries, the colony loss rates during the winter period (from October to April) were higher than the 10% loss threshold acceptable in Poland. These apiaries were included in the group called AHL. Approximately 8400 colonies (54.8%) were lost in these apiaries during the overwintering period, with a mean of 21 ± 27.7 collapsed colonies per apiary.

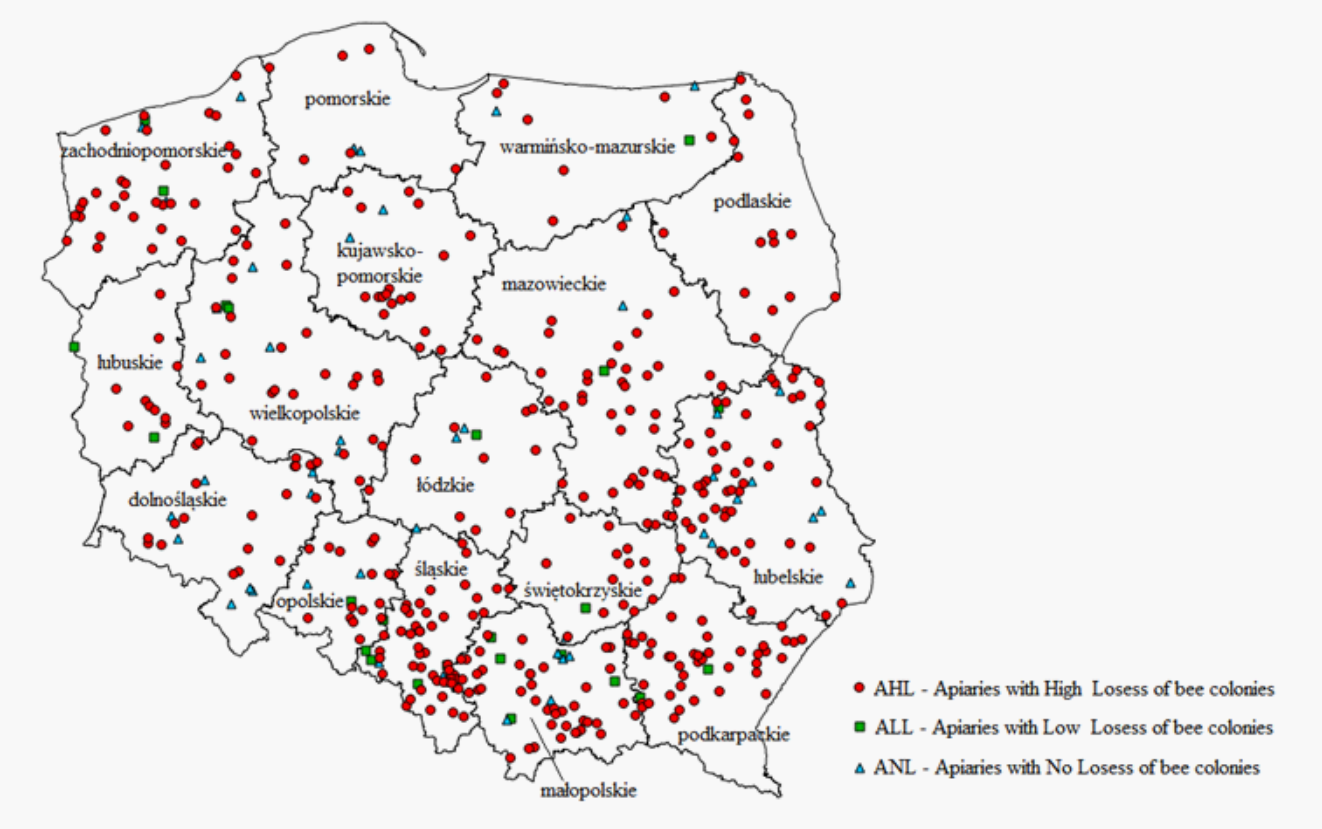

Fig. 1. Map of Poland with geographical locations of the apiaries (n = 476) assessed during the project (2009 - 2012). AHL - Apiaries with High Losses (>10%) of bee colonies (n = 401), ALL - Apiaries with Low Losses (≤10%) of bee colonies (n = 48), ANL - Apiaries with No Losses of bee colonies (n = 27).

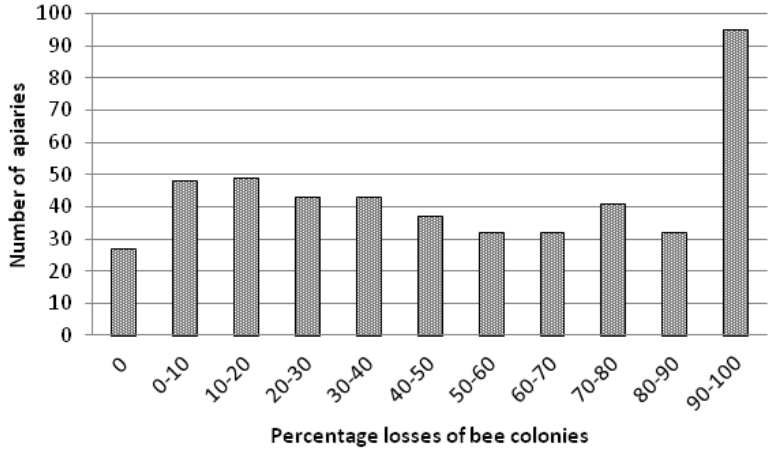

Fig. 2. Distribution of mortality rate of bee colonies during the winter period in the apiaries of the beekeepers participating in the project (2009 - 2012).

The share of dead colonies in the particular apiaries ranged from 11% to 100% (Fig. 2).
The colony mortality rate did not exceed 10% in the apiaries called ALL (n = 48). Altogether, 124 colonies (4.9%) died in them, with a mean debris rate of 2.6 ± 1.8 colonies / apiary.
No winter losses were identified in 27 apiaries ANL. The percentages of small-, medium- and large-scale apiaries were similar in the AHL, ALL, and ANL groups (Chi^2, $p>0.05$).

Environmental conditions

Apart from wild-growing plants, a vast majority of the apiaries in all the groups were surrounded by agricultural and/or garden crops. Among the plants cultivated in Poland and serving as a source of nectar and pollen for bees were mostly oilseed rape fields, orchards and fruit-bearing bush (mostly raspberry) fields located in the vicinity of the apiaries (Tab. 2).

Beekeeping management

Apis mellifera carnica was the dominant breed of bees kept at the apiaries in all the groups. About 8% of beekeepers in the AHL and ALL groups also declared to have *Apis mellifera caucasica* bees. Buckfast bees were kept by approximately 4% of apiarists in the AHL and ALL groups, and 7% of beekeepers in the ANL group.
Stationary apiaries prevailed in all the groups. The highest percentage of apiaries (32%) from which part of the colonies were transferred to distant nectar flow areas to increase their honey production efficiency, was identified in the ALL group (Tab. 2).
For almost half of the AHL apiaries the last honey harvest was finished in July. The other beekeepers who obtained commercial honey from buckwheat, autumn raspberry, phacelia, mustard, goldenrod, heather or coniferous honeydew performed the last honey centrifugation in August and September. Similar tendencies were observed in the other groups, although the highest percentage of beekeepers (27%) who performed the last honey harvest in September was identified in the ANL group (Tab. 2).
The methods of supplementing the winter resources of the bee colonies were also similar in all the groups. Almost half the beekeepers used sugar syrup prepared by themselves from beet sugar and water. The percentage of beekeepers using commercially produced sugar and starch invert syrups ranged from 25% to 37%. Few beekeepers used both types of syrups (home-made and commercial) to feed the colonies.
In the opinion of 45% of the AHL to 62% of the ANL beekeepers, the pollen reserves gathered by the bees were sufficient to satisfy the colony demand in the autumn and winter period. Although the percentage of apiaries in which the colonies definitely had insufficient bee bread reserves was the highest in the AHL group (8%), the differences were not significant in comparison to the other groups (Tab. 2).
The assessment of the condition of the colonies prepared for the overwintering period (August-September) revealed the best results in the AHL group. As many as 68% of the beekeepers said they had only strong or medium-strong colonies at the time, whereas such an opinion was voiced by 46% of apiarists in the case of the ALL group, and 60% in the ANL group. As regards the other apiaries, colonies with bee populations which were too small were also identified separately from those in satisfactory condition (Tab. 2).

Table 2.

Analysis of questionnaire data on environmental conditions and management of apiaries of the beekeepers participating in the project (2009 - 2012)

		The proportion of the apiaries (%)		
		AHL	**ALL**	**ANL**
Plants surrounding the apiaries	Agricultural and/or horticultural crops (in total):	87.8ab	95.8b	74.1a
	- fruit trees and bushes	39.6	41.7	44.4
	- oilseed rape	57.6	58.3	48.1
	- maize	23.4	27.1	14.8
	- cereal	41.7	52.1	36.0
	- buckwheat	21.7	22.9	20.0
	Non-agricultural areas (forests, meadows, wasteland)	9.7	4.2	18.5
	no data	2.5	0	7.4
Type of an apiary	stationary	84.5b	68.1a	77.8ab
	migratory	15.5a	31.9b	22.2ab
Date of the last honey harvest	July	49.6	40.4	54.2
	August	35.1ab	40.4b	16.6a
	September	14.5	19.1	29.2
	honey was not harvested	0.8	0.1	0
Type of syrup used for supplementary winter feeding	sucrose solution	68.4	56.2	51.8
	commercial syrup	24.2	35.4	33.3
	both types	4.4	4.2	11.2
	no data	3.0	4.2	3.7
Pre-winter bee bread reserves	small	8.4	4.5	4.2
	medium	36.8	31.1	29.2
	sufficient	45.7	51.1	62.4
	high diversity of provisions in individual colonies (small, medium, and sufficient)	9.1	13.3	4.2
Strength of colonies entering in winter	strong and medium	68.1b	45.9a	60.0ab
	strong, medium and weak	19.9a	45.7b	32.0ab
	medium and weak	12.0	8.4	8.0

AHL - Apiaries with High Losses (>10%) of bee colonies (n = 401); ALL - Apiaries with Low Losses (≤10%) of bee colonies (n = 48); ANL - Apiaries with No Losses of bee colonies (n = 27).
Different letters a and b indicate significant differences determined with the Chi-square test, p≤0.05.

No procedures to eliminate the presence of *V. destructor* mites in the colonies were carried out in almost 2% of the apiaries in the AHL group. The analysis of selected aspects of parasite fighting in the other apiaries has been presented in Table 3. In all the groups of apiaries, the beekeepers most often used veterinary drugs registered in Poland, with amitraz as the active substance. In a vast majority of apiaries amitraz was applied in the form of fumigation tablets. However, organic acids, biotechnical methods, and other veterinary preparations were much more often used in the apiaries in which no colony losses were identified (ANL) or the losses were low (ALL). Moreover, in the ALL and ANL apiaries, significantly higher percentages of beekeepers started fighting *V. destructor* mites already in July. Individual assessments of each beekeeper's varroosis treatment elements (numbers, date and type of procedures, and the dose of the applied substance/preparation) revealed that the therapeutic procedures were correctly carried out only in 16% of apiaries in which the percentage of dead colonies was high (AHL). On the other hand, significantly more, i.e. almost 44%, beekeepers correctly carried out the procedures in the ALL and ANL groups (Chi^2, p = 0.000). Due to a lack

Table 3.

Analysis of questionnaire data on methods of *V. destructor* control used by the beekeepers participating in the project (2009 - 2012)

Fighting of *V. destructor*		The proportion of the apiaries (%)		
		AHL	ALL	ANL
The colonies which were not treated in the last year		1.8	0	0
Authorised veterinary formulations	with amitraz (in total):	64.4	79.1	77.8
	- fumigation (Apiwarol)	56.9	72.9	70.4
	- contact (Biowar 500)	10.0	18.7	11.1
	with flumethrin (Bayvarol)	35.6	25.0	33.3
Not authorised veterinary formulations	with tau-fluvalinate (Gabon PF)	1.0a	8.3b	0a
„Home-made" formulations	with tau-fluvalinate	4.1	0	0
	with amitraz	0.8	0	0
	with coumaphos	0	2.1	0
Natural substances/ preparations	Organic acids (in total):	15.5a	16.7ab	33.3b
	- formic acid	9.0	6.2	18.5
	- oxalic acid	7.2a	16.7b	25.9b
	BeeVital Hive Clean	4.9	4.2	0
	ApiLife Var	3.3	4.2	3.7
	use of only organic substances	6.9	6.2	7.4
Biotechnical methods (in combination with other treatments)	drone brood removal or trapping combs	2.3a	10.4b	11.1b
The number of substances and/or preparations used during the year	one	62.6b	43.7a	44.4ab
	two	30.1a	45.8b	40.7ab
	three	4.4a	8.3ab	14.8b
	four	0.5	0	0
Parasite control season throughout the year	early spring (April) and the late summer/winter (from August to November)	10.1a	20.8b	0a
	the whole year (from April to November)	2.3a	10.6b	11.1b
	in late summer/winter only	85.8b	70.2a	88.9ab
Starting point (month) of varroa control procedures after last honey harvest	July	11.6a	25.0b	48.1b
	August	35.6b	27.1ab	18.6a
	September	27.6	25.0	22.2
	October	4.9	2.1	0
	not specified	20.3	20.8	11.1

AHL - Apiaries with High Losses (>10%) of bee colonies (n = 401); ALL - Apiaries with Low Losses (≤10%) of bee colonies (n = 48); ANL - Apiaries with No Losses of bee colonies (n = 27).
Different letters a and b indicate significant differences determined with the Chi-square test, p≤0.05.

of complete data, it was impossible to evaluate the correctness of fighting varroa mites by 17%, 16%, and 7% of the beekeepers in the AHL, ANL and ALL groups, respectively.

Symptoms observed in the apiaries

The beekeepers noticed the symptoms connected with the appearance or behaviour of the bees in 68% of the AHL apiaries. The most frequent phenomenon (reported by 60% of apiarists) was a decline in the bee populations in the colonies leading to extinction. Some of the beekeepers already started to observe that bees were dying in September, during the supplementation of the winter reserves. Other apiarists identified this phenomenon in the spring. The dominant reported symptom was the presence

of dead or crawling bees in front of the hives (20%). Some of the insects were also found to have wing deformations or no wings at all. Some beekeepers also noticed disoriented bees, low activity of forages, considerable winter debris volumes, diarrhoea or a pathological appearance of the brood. A reported 40% of beekeepers also noted the appearance of alarming symptoms in the ALL group. The most frequently reported observations were losses of the bees (30%), the presence of dead or crawling bees next to beehives (15%) or traces of diarrhoea (10%). In the ANL group, two beekeepers reported huge winter debris in some colonies, one apiarist reported diarrhoea, and one of them informed us about the presence of bees crawling over the apiary ground.

Epidemiological status of the apiaries and bee colonies

In the AHL group, the epidemiological situation of selected pathogens was examined on the basis of samples collected from 2054 colonies constituting 25% of all the colonies which died out in the winter period in these apiaries. In the group of 48 ALL apiaries, the incidence of pathogenic organisms was identified in almost all the dead colonies (n = 107) and in a similar number of the surviving colonies (n = 114). Diagnostic examinations were performed for 146 colonies that survived the winter (almost 25% of all overwintered of the colonies) in the 27 apiaries in which neither of the colonies fell to the bottom board of the hive (ANL). On average, five colonies were sampled in each apiary.

Varroa destructor

The varroa mite was identified in more than 90% of the samples (colonies) from the AHL group. Considerably fewer parasitised bee samples were identified in the ALL and ANL groups (Chi^2, p = 0.000). Their respective percentages were 78.7% and 69.9%, without significant differences between them (Chi^2, p = 0.054). The rate of mite infestation was also very high in the AHL group (Fig. 3). The average parasitisation rate of the samples was 81 ± 136.9 mites/100 bees.

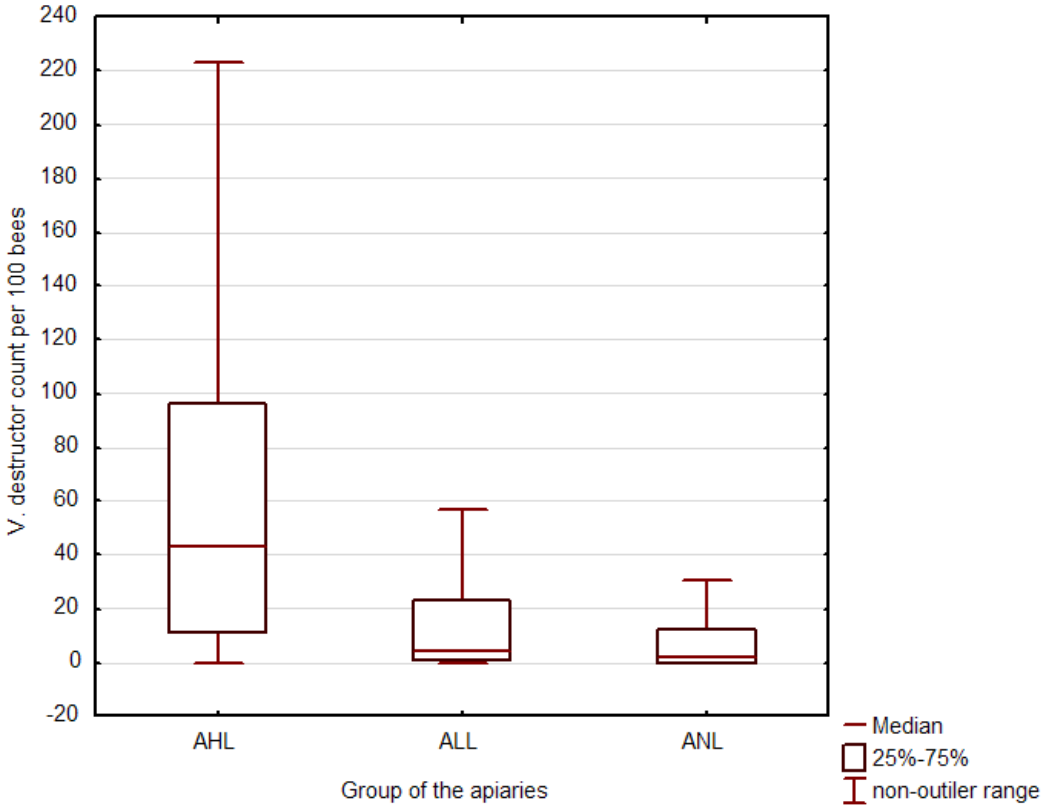

Fig. 3. Intensity of *V. destructor* infestation of the bee samples collected from apiaries with different rates of winter honey bee colony losses in the four year study (2009 - 2012). AHL - Apiaries with High Losses (>10%) of bee colonies (n = 2054), ALL - Apiaries with Low Losses (≤10%) of bee colonies (n = 221), ANL - Apiaries with No Losses of bee colonies (n = 146). The presence of the varroa mite was determined in individual samples of dead bees collected from the bottom of hives (on average, five per apiary). The level of *V. destructor* infestation in the samples from the AHL group was significantly higher than in the ALL and ANL, and did not differ between ALL and ANL groups (Kruskal-Wallis median test, p≤0.05).

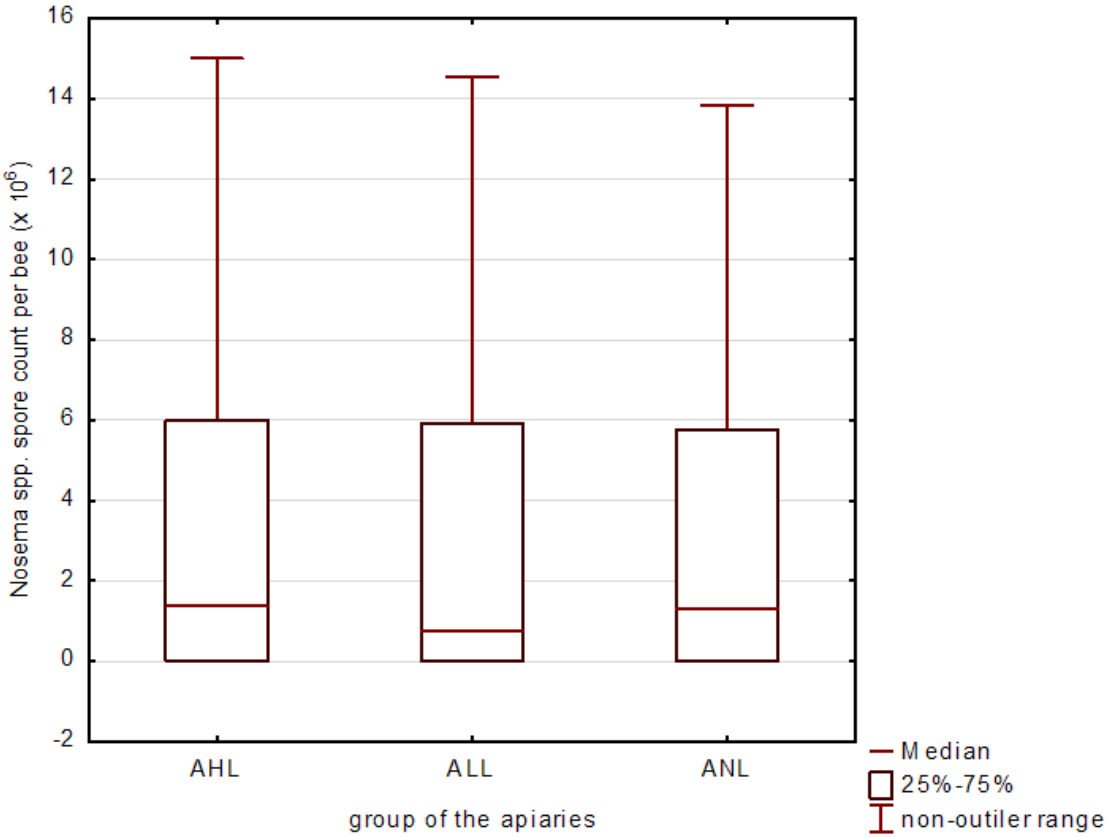

Fig. 4. Intensity of *Nosema* spp. infection in the bee samples collected from apiaries with different rates of winter honey bee colony losses in the four year study (2009 - 2012). AHL - Apiaries with High Losses (>10%) of bee colonies (n = 2054), ALL - Apiaries with Low Losses (≤10%) of bee colonies (n = 221), ANL - Apiaries with No Losses of bee colonies (n = 146). The diagnosis of *Nosema* spp. was performed microscopically in individual samples of dead bees collected from the bottom of hives (on average, five per apiary). The levels of *Nosema* spp. infection in the samples were similar in all the groups of apiaries (Kruskal-Wallis median test, p>0.05).

Significantly fewer parasites were detected in the samples from the ALL and ANL groups (Kruskal-Wallis test, df = 2, n = 2421, p = 0.000). The level of *V. destructor* infestation in these two groups was similar, with a mean number of 26 ± 74.3 and 15 ± 37.5 mites per 100 bees, respectively (Kruskal-Wallis test, df = 2, n = 2421, p = 0.120). Similar results were obtained when assessing the prevalence of *V. destructor* in the apiaries. The parasites were detected in 96.3% of the AHL apiaries, whereas their numbers were significantly lower in the other groups (Chi^2, p = 0.000) (Fig. 6).

***Nosema* spp.**

The epidemiological situation connected with *Nosema* spp. infection was similar in all three apiary groups. The share of infected samples in the AHL group (70.7%) was significantly higher (Chi^2, p = 0.003) in comparison to the ALL group (61.1%) but did not differ (Chi^2, p = 0.276) from the percentage of infected samples in the ANL group (65.7%). No significant differences were identified between the percentages of infected colonies in the ALL and ANL groups (Chi^2, p = 0.364).

The intensity of infection with *Nosema* spp. in the colonies did not significantly differ in any group (Kruskal-Wallis test, df = 2, n = 2421, p = 0.204), with the mean levels of 5.89 ± 12.28; 11.05 ± 31.63 and 7.04 ± 16.67 million spores per bee in the AHL, ALL, and ANL resepctively (Fig. 4). The percentages of the colonies in which the spore infection levels of the bees were equal or higher than the level considered as harmful (5 million spores/bee) were also similar in the case of all the groups (Chi^2, AHL and ALL p = 0.682; AHL and ANL p = 0.704; ALL and ANL p = 0.564): 27.9%, 26.7%, and 29.4%, respectively.

The incidence of *Nosema* spp. in these apiaries (Fig. 6) was also high and similar (between 77 and 86%) in all the groups (Chi^2, AHL and ALL p = 0.100; AHL and ANL p = 0.902; ALL and ANL p = 0.399).

The differential identification of *Nosema* spp. by

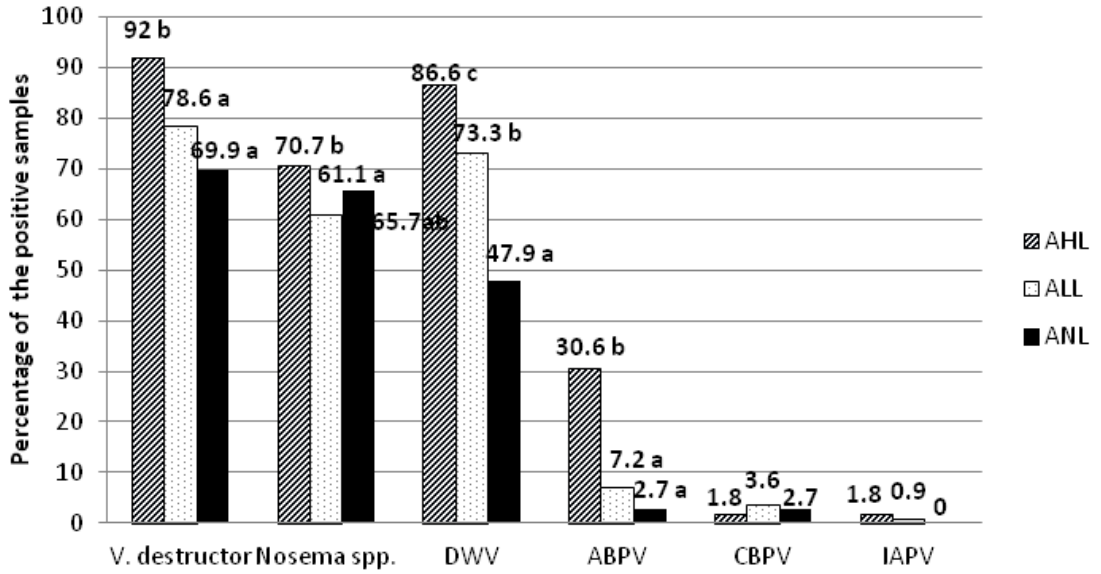

Fig. 5. Prevalence of the pathogens in the bee samples collected from apiaries with different rates of winter honey bee colony losses in the four year study (2009 - 2012).
DWV - deformed wing virus; ABPV - acute bee paralysis virus; CBPV - chronic bee paralysis virus; IAPV - Israeli acute paralysis virus.
AHL - Apiaries with High Losses (>10%) of bee colonies (n = 2054), ALL - Apiaries with Low Losses (≤10%) of bee colonies (n = 221), ANL - Apiaries with No Losses of bee colonies (n = 146). The presence of the pathogens was determined in individual samples of dead bees collected from the bottom of hives (on average, five per apiary). Different letters a, b, and c indicate significant differences determined with the Chi-square test, p≤0.05.

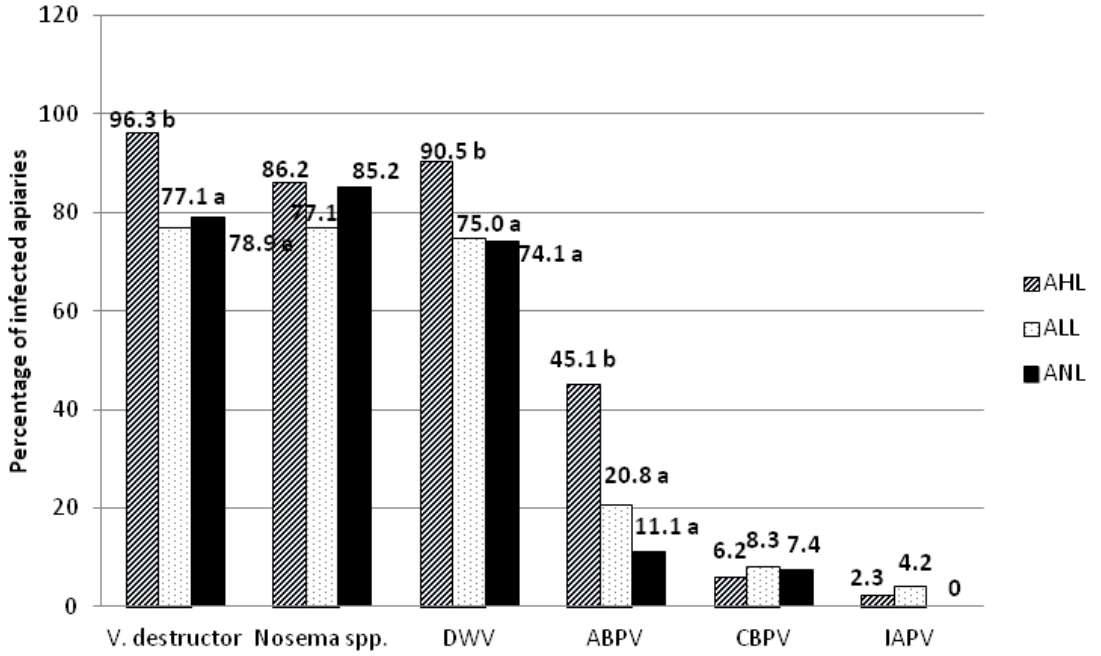

Fig. 6. Prevalence of the pathogens in the apiaries with different rates of winter honey bee colony losses in the four year study (2009 - 2012).
DWV - deformed wing virus; ABPV - acute bee paralysis virus; CBPV - chronic bee paralysis virus; IAPV - Israeli acute paralysis virus.
AHL - Apiaries with High Losses (>10%) of bee colonies (n = 401), ALL - Apiaries with Low Losses (≤ 10%) of bee colonies (n = 48), ANL - Apiaries with No Losses of bee colonies (n = 27). The presence of the pathogens was determined in individual samples of dead bees collected from the bottom of hives (on average, five per apiary). Different letters a and b indicate significant differences determined with the Chi-square test, p≤0.05.

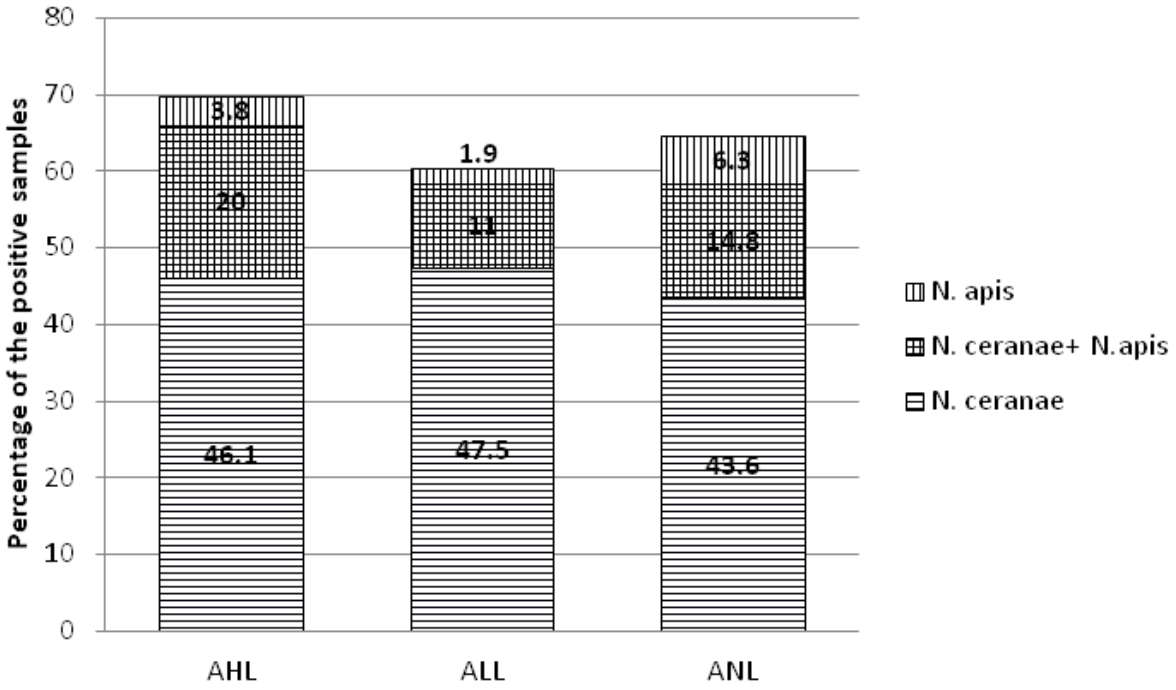

Fig. 7. Prevalence of the *N. ceranae* and *N. apis* in the bee samples collected from apiaries with different rates of winter honey bee colony losses in the four year study (2009 - 2012). AHL - Apiaries with High Losses (>10%) of bee colonies (n = 1998), ALL - Apiaries with Low Losses (≤ 10%) of bee colonies (n = 217), ANL - Apiaries with No Losses of bee colonies (n = 142). The presence of the pathogens was determined in individual samples of dead bees collected from the bottom of hives (on average, five per apiary). *Nosema* differentiation was performed via multiplex analysis of a PCR-amplified partial sequence of the 16S rRNA gene. No differences were found in the prevalence of *Nosema apis, Nosema ceranae*, and both species (mixed infections) between the AHL and ANL groups. Significantly higher prevalence of both, pure and mixed infection was reported in AHL, as compared with ALL. Differences were determined with the Chi-square test $p \leq 0.05$.

means of PCR revealed the presence of genetic material of *N. ceranae* in a vast majority of the positive samples. During the 3 years of the study, this microsporidian species was identified in 66% of samples, with the share of positive samples amounting to 62.2%, 63.5% and 78.4% in the successive years. Samples containing *N. apis,* in turn, constituted 24.7%, 27.3% and 12.5%.

The assessment of *N. ceranae* incidence in the samples from the apiaries with different colony mortality rates (Fig. 7) revealed that although the share of infected colonies was significantly higher in AHL than in ALL (Chi^2, $p = 0.025$), it did not differ from the share in the ANL group (Chi^2, $p = 0.063$).

In comparison to the extent of *N. ceranae* infection, the development of *N. apis* infection was detected in a definitely lower percentage of samples (colonies) (Fig. 7). It was similar in the AHL and ANL groups but significantly higher in comparison with the ALL group (Chi^2, AHL and ALL $p = 0.000$; AHL and ANL $p = 0.472$; ALL and ANL $p = 0.038$).

The share of samples in which a combined infection with both *Nosema* microsporidia was diagnosed, ranged from 10% to 20%.

The degree of sample infection with only *N. ceranae* or *N. apis* or both microsporidian species, did not significantly differ between the groups with different colony loss rates. The mean infection levels in the samples in which only *N. ceranae* was detected in the AHL, ALL, and ANL groups were 7.73 ± 13.2, 17.59 ± 38.76, and 12.75 ± 21.52 million spores/bee, respectively (Kruskal-Wallis test, $df = 2$, $n = 1086$, $p = 0.103$). In the case of a combined infection, the mean numbers of spores in the samples amounted to 9.86 ± 15.49, 25.72 ± 45.15, and 8.45 ± 18.96 million per bee (Kruskal-Wallis test, $df = 2$, $n = 445$, $p = 0.195$). On average, 5.16 ± 8.62; 8.54 ± 7.05, and 2.81 ± 2.80 million spores/bee were found in the *N. apis*-positive samples from the AHL, ALL, and ANL groups (Kruskal-Wallis test, $df = 2$, $n = 88$, $p = 0.176$).

All in all, the mean infection intensities were similar in all the samples infected only with *N. ceranae* or only with *N. apis* (8.95 ± 18.01 and 5.07 ± 8.17 million

spores/bee, respectively). On the other hand, the level of infection was significantly higher in the case of samples containing both microsporidian species (10.68 ± 18.68 million spores/bee) (Kruskal-Wallis test, df = 2, n = 1619, p-values for multiple comparisons: pure *N. ceranae* with pure *N. apis* p = 0.111, pure *N. ceranae* with co-infection p = 0.002, pure *N. apis* with co-infection p = 0.001).

The presence of colonies with *N. ceranae* was identified in 82.1%, 72.3% and 80% of AHL, ALL, and ANL apiary groups, while the prevalence of *N. apis* concerned 44.6%, 36.2% and 52.0% of the apiaries. The mean colony losses (51.8%) in the apiaries in which *N. ceranae* was found were similar (50.9%) as in the apiaries in which this infection was not identified in any of the samples (Mann-Whitney U test, df = 1, n = 464, p = 0.828).

Viruses

The presence of DWV was diagnosed in the majority of the samples collected from all the groups but its incidence was significantly higher in both the AHL colonies and apiaries (Chi^2, p = 0.000, p = 0.001), amounting to 87% and 91%, respectively (Fig. 5 and 6). Statistically significant differences (Chi^2, p = 0.000) were also identified in the proportions of colonies infected with ABPV. The share of positive samples in the AHL group was 31%, and in ALL and ANL 7% and 3%, respectively. These two groups also included low numbers of apiaries in which colonies with ABPV were detected. The incidence of CBPV and IAPV was detected in very low percentages of the samples, which was similar in all the groups (Fig. 5 and 6).

The mean colony losses (52.4%) in the apiaries in which DWV was identified (in all the groups collectively) were significantly higher in comparison to the colony mortality (43%, on average) in all the other apiaries (Mann-Whitney U test df = 1, n = 476, p = 0.039). a similar correlation was found between the scales of collapsed colonies (57.3% of colonies, on average) in the apiaries infected with ABPV and in those in which this virus was not detected (47.2% of colonies, on average) (Mann-Whitney U test df = 1, n = 476, p = 0.001).

Coinfections and interactions

Coinfections with two, three or four pathogens were identified in a significantly higher percentage (92%) of bee samples from the AHL group in comparison to the ALL (83%) and ANL (74%) samples (Tab. 4). Co-infections caused by at least three types of pathogens/parasites were found in almost 67% of bee samples collected from the AHL colonies, as compared with 41% and 21% in the ALL and ANL groups. On average, the presence of 2.8 ± 0.9 pathogens was detected in the AHL samples, 2.2 ± 0.8 in the ALL samples, and 1.9 ± 0.8 pathogenic organisms in the ANL samples (Kruskal-Wallis test, df = 2, n = 2421, p = 0.000).

In the AHL group, the mean intensity of *V. destructor* infestation of colonies infected with DWV was 85.0 ± 140.4 mites/100 bees and was significantly higher than in the ALL and ANL groups, in which DWV was also detected (27.9 ± 75.4 mites on average) (Mann-Whitney U test, df = 1, n = 2010, p = 0.000). The rates of varroa infestation were also significantly higher in all the DWV-positive colonies (78.4 ± 135.8 parasite, on average) in comparison to the levels of

Table 4.

Proportion of the bee samples with mixed-infection collected from apiaries with different rates of the winter honey bee colony losses in the four year study (2009 - 2012)

Number of pathogenic organisms per sample of bees	The group of the apiaries		
	AHL	ALL	ANL
0[a]	0.4a	2.7b	3.4b
1[a]	6.3a	14.1b	22.6c
2[a]	26.0a	42.5b	52.7b
3[a]	46.1c	37.1b	19.9a
4[a]	20.4b	3.6a	1.4a
5[a]	0.8	0	0
6[a]	0	0	0
Average[b]	2.8c	2.2b	1.9a

AHL - Apiaries with High Losses (>10%) of bee colonies (n = 2054); ALL - Apiaries with Low Losses (≤10%) of bee colonies (n = 221); ANL - Apiaries with No Losses of bee colonies (n = 146).
Different letters a, b, and c indicate significant differences determined with:
[a] Chi square test, [b] Kruskal-Wallis median test, p≤0.05.

infestation of all the colonies free from this virus (40.4 ± 92.3 mites, on average) (Mann-Whitney U test, df = 1, n = 2421, p = 0.000).
Similar correlations were identified in the case of coinfections with varroa and the ABPV virus. The average level of infestation of the colonies from the AHL group in which we detected ABPV was 107.0 ± 176.8 mites, and 37.6 ± 78.8 parasites in the ALL and ANL colonies infected with the ABPV virus (Mann-Whitney U test, df = 1, n = 648, p = 0.000).
The mean level of *V. destructor* infestation of all the colonies infected with ABPV was 105 ± 175.0 mites/100 bees, and 61.6 ± 108.0 parasites in the case of the other colonies (Mann-Whitney U test, df = 1, n = 2421, p = 0.000).
On the other hand, no significant correlations were identified between the intensity of infestation of the colonies with *V. destructor* and *Nosema* spp. (r_s = -0.137).
Among the three analysed factors, i.e. the level of *V. destructor* infestation and the level *Nosema* spp. infection, and the number of coinciding pathogens, the greatest effect on the mortality rates of colonies in the analysed apiaries was exerted by the load of varroa mite and the number of pathogenic organisms (multiple regression, R = 0.274 R2 = 0.0755 corrections. R2 = 0.069 F(3.476) = 12.849 p, number of pathogens p = 0.000, intensity of *V. destructor* infestation p = 0.002, intensity of *Nosema* spp. infection p = 0.286).

DISCUSSION

Analysis of questionnaire data (environmental conditions and beekeeping management)

The problem of severe overwintering losses of colonies had been reported by the beekeepers from all over the country but nearly half of them had their apiaries in the south-eastern area. We consider that it is largely due to the fact that the region is characterised by a significantly greater beekeeping activity, in which number of apiaries (above 100 thousand per province) and density of colonies (7 per square kilometer) is highest. The second factor which we cannot exclude is the possibility additional weakening of the colonies by bacterial diseases. This hypothesis is based on the previous research (Pohorecka et al., 2012) that showed a high prevalence of *Paenibacillus larvae* spores in apiaries, in the south-eastern area which creates the risk of an outbreak of American foulbrood.

Both, hobbyist and professional beekeepers have suffered from high winter mortality of colonies. The proportion of small, medium and large-size operations was compatible with the size structure of national apiaries (in Poland 63% of apiarists kept just 1 - 20 colonies, 26% keep 21 - 50 colonies, 8% keep 51 - 80 colonies and only about 3% keep more than 80 colonies) (Semkiw, 2012). The colony loss rate identified by those holders with 1 - 50 colonies was significantly higher (54%) than those experienced by beekeepers possessing more than 50 colonies (42.8%). A similar trend showed the COLOSS working group for "Monitoring and Diagnosis" (van der Zee et al., 2014). However, it would seem that in small apiaries beekeepers can devote more attention to the better protection of bee health.
Survey data show that in Poland, a stationary management model still predominates (approximately 80%). The main goal of the migration is improving the productivity of apiaries. Beekeepers did not provide payable commercial crop pollination services. On the one hand, more diversity forage is provided for the colonies in the migratory apiaries but on the other hand, the colonies are exposed to additional stressors such as transport and intensive exploitation. In our study the colony mortality was lower in the migratory apiaries compared to the stationary ones, and amounted to 39% and 54%, respectively. It may therefore be concluded that the element had no effect on the course of a colony's wintering.
An appropriate amount and quality of carbohydrate and protein nourishment is crucial for the proper development and functioning of honey bee colonies (Alqarni, 2006; Skubida et al., 2008; Naug, 2009; Brodschneider and Crailsheim, 2010). In our study the plant food resource and supplementary nutrition of colonies from all three groups were similar. We consider that these factors had no direct impact on the death of the bees. The majority of the apiaries were located in the vicinity of agricultural crops (prevailed oilseed rape, buckwheat) and orchards, which were important source of pollen and nectar. The bee foraging plant species listed by beekeepers in the questionnaire testifies to the variety of wild and cultivated plants providing sufficient and diverse nutrition during their growing season. It should be noted that this conclusion is confirmed by the stores of bee bread collected by bees (sufficient in about half of colonies from each group).
After honey harvesting, the three types of carbohydrate food (homemade sucrose solution

of beet sugar, commercial invert sucrose syrup and starch syrup) were used for replenishment of winter supplies. The opinion of the users on which type of syrup is better for bees are differentiate. It is believed that risk of crystallisation and formation of hydroxymethylfurfural (HMF) in invert syrups may entail the overwintering mortality of colonies (LeBlanc et al., 2009; Zirbes et al., 2013). Toxic to bees, HMF occurs in bad production batches of invert syrups or when invert has been stored for a long time. More than half of the surveyed beekeepers in the AHL, ALL, and ANL groups traditionally fed colonies with sucrose syrup. Commercial syrups were used primarily in apiaries benefiting from late nectar flow including migratory apiaries. Overall, only five beekeepers suspected that starvation was the cause of the collapse of the colonies.

For successful winter survival not only the food stores need to be adequate for the dietary requirements of the colonies, but it is also necessary to treat *V. destructor* (Boecking and Genersch, 2008). Varroa control is usually done after the harvest of the last honey. Until recently, for the majority of our beekeepers this date was from mid-July to mid-August. However, there has been rise in the number of apiaries which use late flows from wild plants like heather or goldenrod. These are plants which start to flourish in late summer (September). This means that, beekeepers extended the deadline preparation of colonies for a further period, which was often too late. The bees have to survive a few months and if they will be severely infested by parasites during their larval development, their life expectancy will be shortened (Kovac and Crailsheim, 1988; Amdam et al., 2004; Le Conte et al., 2010). Colonies treated earlier in the season had a lower level of mite infestation over the rearing of winter bees. Consequently, the bees had a longer lifespan and there was a higher colony survival after winter (Delaplane end Hood, 1997; Martin et al., 2010; van Dooremalen et al., 2012). In our study, in the group called AHL, just 12% of the beekeepers began varroa treatment in July, which had an impact on varroa infestation level and survival of the colonies. In July treatment was started in nearly 50% of apiaries, in which all colonies overwintered (ALL group). Combined treatments (acaricides with natural compounds and biotechnical methods) is recommended to effectively control varroa mite (Rice et al., 2004). Significantly more apiarist which did not have large colony losses (ALL and ANL group) applied these rules. Models of varroa treatment in nineteen, mainly European, countries compiled by van der Zee and coworkers (2014) differs somewhat from that used by Polish apiarists. To fight the parasite, chemical methods (mainly with amitraz) are most frequently used by national beekeepers which could create the risk of a mite resistant population and reduction of preparation efficacy. Although, the evaluation of the varroacide activity of amitraz performed last year, did not reveal decrease the effectiveness of the veterinary formulations after long-term use of this chemical compound (Semkiw et al., 2013). In other European states the oxalic acid and formic acid applies a substantially greater number of beekeepers.

Epidemiological situation

The data obtained by us showed diametrically different epidemiological situation of *V. destructor* infestation, and DWV and ABPV infection in the evaluated apiaries. Based on these results, we found that above mentioned pathogens were the direct cause of high mortality of colonies in Poland. In the apiaries where an average 60% of the colonies died during the winter (AHL), varroa mites were present in almost every collapsed colony. The mean varroa infestation level was four fold higher (80 mites/100 bees) in comparison to other colonies.

According to Liebig (2001), over 7% of the *V. destructor* infestation rate of the winter bees is critical and threatens to destroy overwintering colonies under central Europe conditions. In AHL, the infestation rate exceeded 7% in as many as 80% of the samples, which clearly shows that they had no chance of winter survival. The samples with more than 7 parasites per 100 bees constituted 43.4% in the ALL group and 31.5% in the ANL group. The share of samples with threshold infestation levels in the ANL apiaries was also significantly lower in comparison to the ALL group (Chi^2, $p = 0.026$). However, it should be noted that some of the colonies with high infestation rates of more than 7% (see ANL) were able to survive the winter which is consistent with the suggestion of researchers from the USA and Canada (Delaplane and Hood, 1999; Currie and Gatien, 2006). We have demonstrated that the loss rate of the colonies was also dependent on the number of co-occurring pathogens. This may indicate one of the reason for the differences in survival of the colonies highly infested with varroa. The winter colony losses monitored in Canada (Ontario) and Germany over the last few years were also caused by these mites. But mite distribution,

mite infestation rates, and an intensification of colony mortality were lower (Genersch et al., 2010; Guzman-Novoa et al., 2010). However, we are aware that we examined dead bees and it should be mentioned in the comparison of our results with the above cited.

We have found that high colony mortality was not merely the adverse effects of *V. destructor*. In 80% of the collapsed colonies of AHL the parasite infestation were accompanied by the deformed wing virus. The prevalence of DWV demonstrated by us in the colonies of AHL was almost twice as higher as the proportion of infected colonies in the properly functioning apiaries (ALL). Recently, in several studies it has also been proven that co-infection of DWV and *V. destructor* reduces the lifespan of overwintering workers and leads to colony losses (Highfield et al., 2009; Berthoud et al., 2010; Genersch et al., 2010; Dainat et al., 2012a, b; Nazzi et al., 2012). It is generally considered that DWV is harmless to bee health on the colony level, provided that the colonies are free from varroa mite infestation. Varroa as the mechanical and biological vector causes rapid spread of DWV and the development of covert or overt infection (Gisder et al., 2009; de Miranda and Genersch, 2010; Mockel et al., 2011). In Hawaii, for example, during the last few years after *V. destructor* settled, the incidence of DWV increased from approximately 10 to 100% within honey bee colonies. An increase in the varroa mite population in the colony, results in a higher transmission of DWV between the bees. Furthermore, it has been shown that such high parasite load significantly raises viral titres (Martin et al., 2012; Francis et al., 2013). In untreated bee colonies, both the intensity of varroa mite infection and DWV copies grow from spring to autumn, and viral copies are correlated with the parasite infestation rate. In the autumn most bees may be infected with DWV in the colony with severe varroa infestation. The honey bee colonies which collapsed during winter have both a DWV prevalence and virus loads significantly higher than the colonies which survived (Dainat et al., 2012a, b; Francis et al., 2013). In our opinion, these findings coincide with the results presented in this work. Although we did not mark the virus titers, we have shown that the varroa mite load was significantly higher in DWV - positive collapsed colonies of AHL compared to DWV - positive of ALL and ANL, and that parasite infestation level was around two times higher in overall DWV-positive bee samples compared to DWV-negative.

In our study, as in the research of Genersch and collaborators (2010), ABPV occurs as a second virus which in conjunction with *V. destructor* is the cause of severe colony losses. Though the participation of ABPV in this phenomenon was much less. Distribution of ABPV was significantly lower than DWV nonetheless ABPV was predominantly detected in apiaries with high colony mortality (in one third). In other regions of the world, the cases of ABPV infection are also much less frequent (Allen and Ball, 1996). The model varroa-virus developed by Martin (2001) assumes that destruction of a colony due to ABPV occurs, when a very strong mite population is present when the virus enters. The observations made by us confirm this premise because the mean level of *V. destructor* infestation of all the colonies infected with ABPV was highest (105 mites/100 bees, on average). In the AHL group, among 628 colonies infected with ABPV, 97% were simultaneously infected with DWV, and 93 % with DWV and *V. destructor*. In a study, conducted in Denmark, a link was found between the intensity of varroa infestation and viral titres for ABPV, KBV or IAPV (primers "AKI"), and the rate of colony mortality (Francis et al., 2013). In Hawaii and Switzerland, however, such a relationship was noted only for DWV (Dainat et al., 2012a, b,; Martin et al., 2012).

A prevalence of the *V. destructor*, ABPV and DWV determined in the collapsed colonies of our beekeepers was considerably higher compared to the frequency of their occurrence proven by the authors listed above. We think, that the type of tested samples was the main reason for these differences (dead bees from hive bottoms versus live bees from the brood chamber). Based on a prevalence of IAPV and CBPV, we considered that they do not constitute a high threat to the health of the winter bees in our conditions. In recent years, the IAPV associated with the mass destruction of colonies (CCD-syndrome) was observed in the US (Cox-Foster et al., 2007; vanEngelsdorp et al., 2009). In 2012, high titers of IAPV were detected in colonies collapsed with symptoms characteristic of CCD in Israel (Hou et al., 2014). In Poland, the first cases of IAPV incidence (in a few colonies) took place in 2009 (Pohorecka et al., 2011b). Over the following years, the extensiveness of infection remained low (2%). Similarly, low the prevalence of CBPV in our country is observed since 2008, means from the time when the first studies were conducted (Topolska et al., 2009; Pohorecka et al., 2011a). The results regarding incidence of IAPV and CBPV are accordance with the incidence of these viruses observed in the other European

countries (Tentcheva et al., 2004, Chen et al., 2006; Morimoto et al., 2012).

The data concerning *Nosema* spp. prevalence in 2001-2009, revealed the spread of this infection in Polish apiaries (Pohorecka et al., 2011a; Ptaszyńska et al., 2012). Our current study also confirmed this tendency and showed that *Nosema* microsporidia are one of the most common pathogens. The incidence of the *Nosema* spores have been confirmed in 85% of apiaries, and the share of infected colonies reached 70%. Moreover, *Nosema* typing revealed that *N. ceranae* is currently the dominant parasite species.

The incidence of *N. ceranae* in Poland was first observed in 2007 (Topolska and Kasprzak, 2007). a subsequent analysis of several archived bee samples from 1994 proved that there was previous presence of this species (Gajda et al., 2013). In recent years, the incidence of *N. ceranae* has also been confirmed in north-eastern Poland (Michalczyk et al., 2013). Our analyses made it possible to evaluate the epidemiological situation of *N. ceranae* and *N. apis* infections on the scale of the entire country. Overall, a *N. ceranae* presence was observed in 81% of apiaries and 65% of colonies, whereas *N. apis* infection was identified in 44% of apiaries and 23% of colonies. Distribution of *N. ceranae* in the particular regions of Poland was similar, regardless of the geographical location of the apiaries (proportions of infected apiaries were between 60% and 90%). The highest percentage of samples containing *N. ceranae* and the lowest percentage of samples with *N. apis* were identified in the last year of our study. However, to determine whether, according to the hypothesis put forward by some scientists (Klee et al., 2007; Martin-Hernandez et al., 2007; Paxton et al., 2007), it is the result of *N. ceranae* displacement *N. apis*, it is necessary to monitor the prevalence of both parasite species over the period of several years in a row. It may be that the such common incidence of *Nosema* spp. has not only arisen from a higher expansiveness (virulence) of *N. ceranae* (Higes et al., 2007; Paxton et al., 2007) but also from a discontinued fighting off of nosemosis as a result of the ban on the use of fumagillin in the EU.

A considerably higher extent of *N. ceranae* than of *N. apis*, has already been reported from other European countries, such as France (Chauzat et al., 2007), Hungary (Tapaszti et al., 2009), Spain (Botias et al., 2012), the Balkan countries (Tlak Gajger et al., 2010; Stevanovic et al., 2011), as well as from North America (the US and Canada) (Chen et al., 2008; Traver and Fell, 2011; Copley et al., 2012; Martin et al., 2013). In Hawaii as well as Croatia, *N. ceranae* is the only species of *Nosema* found to infect bee colonies. Yet, in some regions of the world, *N. apis* is still the dominant microsporidian infection of honey bees. In Australia (Giersch et al., 2009), *N. apis* was detected in samples from all the states (46% positive), while *N. ceranae* was not detected in Western Australia and Tasmania. The proportion of positive outcomes in samples collected from the other states amounted to 16%. A similar situation was observed in Germany during a five-year cohort study (2005 - 2009), when, except for the spring 2007, *N. apis* was more prevalent than *N. ceranae* (Gisder et al., 2010). In a nationwide Swedish survey, in the spring of 2007, only 6% of samples contained *N. ceranae* spores, while approximately 60% had *N. apis.* During the subsequent four years, no tendency of an increased rate of *N. ceranae*-infected samples was noted (Forsgren and Fries, 2013).

On the other hand, the prevalence of both parasite species in bee colonies was similar in England and Wales (a survey of 4600 apiaries and 13,000 colonies between 2009 and 2011), ranging from approximately 30% to 50% depending on the region. The parasites were often simultaneously present in the apiaries (DEFRA, 2013).

The development of *N. ceranae* is highly determined by the temperature. This species is less resistant to low temperatures than *N. apis* (Fenoy et al., 2009; Fries, 2010; Gisder et al., 2010; Chen et al., 2012). Considering this fact, we expected the distribution of *N. ceranae* in our climate to be similar to those levels identified for cooler regions (Gisder et al., 2010; Forsgren and Fries, 2013; Meixner et al., 2014). It turned out, though, that the prevalence of *N. ceranae* in Poland is more similar to South-European areas, with a simultaneous relatively high incidence of *N. apis*.

Despite the fact that the distribution of *N. ceranae* was higher compared to *N. apis* the intensity of both pure infections was similar and we observed no differences in the level of *N. apis*, and *N. ceranae* spores. Whereas, the load of spores in the mixed infections was significantly greater than in pure with *N. apis* and *N. ceranae* infections. Different result were obtained in several other laboratory studies. In the laboratory conditions the infection intensity was significantly greater for *N. ceranae* than for *N. apis* or mixed infections (Huang and Solter, 2013;

Williams et al., 2014). Consequently, we think that our data provided another reason for assuming that the epidemiological pattern of *N. ceranae* infection is also significantly affected by other factors. These factors include the genetic diversity of *N. ceranae* strains (variation in virulence) (Forsgren and Fries, 2010; Genersch et al., 2010; Huang et al., 2012; Medici et al., 2012), different individual and colony-wide host susceptibility (Chaimanee et al., 2010), beekeeping management, and honey bee trade (Higes et al., 2013).

Several detrimental effects exerted by *N. ceranae* on the physiology and behavior of honey bees have been identified (Martín-Hernández et al., 2011; Chaimanee et al., 2012; Dussaubat et al., 2012; Goblirsch et al., 2013). Yet the role of this parasite in the globally occurring phenomenon of increased honeybee colony mortality is still ambiguous.

Our data did not reveal a significant relationship between *N. ceranae* and the high mortality rates of colonies. Both the prevalence and loads of *N. ceranae* spores in managed colonies in which serious losses were suffered and in correctly functioning apiaries, were similar. Moreover, no significant differences could be observed between the mortality rates of uninfected colonies and those of colonies infected by *N. ceranae*. These results are consistent with observations of other authors (Genersch et al., 2010; Gisder et al., 2010; vanEngelsdorp et al., 2010; Stevanovic et al., 2011; Traver and Fell, 2011; Martin et al., 2012; Traver et al., 2012) who also did not identify negative effects of *N. ceranae* at the colony level either.

On the other hand, in Spain, the relationship between *N. ceranae* and intensive colony depopulation leading to extinction, has been unquestionably proved (Higes et al., 2006; Martín-Hernández et al., 2007; Higes et al., 2009, 2010a, b). Colleagues from Israel obtained consistent results because they found a significant negative correlation between worker population in the hive and the presence of viral and *Nosema* infections (Soroker et al., 2010).

Despite a high prevalence of *N. ceranae* in the apiaries we analysed, approximately half the colonies contained not more than 1 million *Nosema* spores (in the case of AHL, ALL, and ANL, the median amounted to 1.37; 0.75; 1.32 x 10^6 spores per bee). We found that the numbers of spores were equal or higher than 5 million only in one third of the colonies. In our study, infection intensity turned out to be much lower than the intensity identified in collapsing colonies in Spain or CCD colonies in the US (Cox-Foster et al., 2007; Higes et al., 2008). The infection intensity in our study was similar to levels identified in the majority of studies in which no relationship between infection intensity and high colony losses was determined.

The hypothesis that, through active suppression of the immune response in honeybees and through damaging midgut epithelial cells, *N. ceranae* creates propitious conditions for viral infection was not been proven in our study. As in other studies (Costa et al., 2011; Martin et al., 2013), the mean number of *N. ceranae* spores in the colonies in which DWV was detected did not differ from the scale of *N. ceranae* infection in the colonies free from DWV (Mann-Withney U test, $n = 1681$, $p = 0.467$). Additionally, the percentage of *N. ceranae*-positive colonies with DWV did not significantly differ from the percentage of colonies without *N. ceranae* in which DWV was also found (Chi^2, $p = 0.278$).

While analysing the level of infestation by *V. destructor* and *N. ceranae* in the same colonies, we did not identify a significant correlation between the development of both species. However, as in the previous studies (Pohorecka et al., 2011a), we observed a similar tendency in relation to the negative value of the correlation coefficient (r). This is confirmed by observations made by Mariani et al. (2012) concerning the modification of the development of *Nosema* spp. by *V. destructor* in the course of a year. Mite and microsporidia parasitising the host, cause the host to lose nutrients and undergo energetic stress. In the case of co-infection, a mutual deterioration of alimentary conditions and development inhibition can take place. It is conceivable that *Nosema* loads are lower in colonies with high varroa loads because life span bees from these colonies is significantly shorter. Moreover, the simultaneous presence of these parasites will also lead to overall combined noxious effects on the particular infected insects. Consequently, the negative effect of *Nosema* spp. infection accompanying the infestation by varroa mites cannot be entirely disregarded. The microsporidia should be considered as an additional factor indirectly responsible for the rise in the bee mortality rate (Manzoor et al., 2013).

Our laboratory findings have confirmed that outbreak of several diseases was the reason for the observed symptoms by beekeepers.

CONCLUSION

In conclusion, there is no doubt that the main cause of the global overwintering bee colony losses in nationwide apiaries is the huge ectoparasitic mite *Varroa destructor* infestation followed by viral infections with DWV and ABPV.
Despite the fact that currently *N. ceranae* is also a very widespread and dominant pathogen of *Nosema* spp. microsporidia, this infection was not the direct reason for massive colony death. However, it should be noted that the presence of *Nosema* spp. in colonies strongly infested by the varroa mite is an additional factor limiting bee vitality and their life expectancy.
The epidemiological situation of apiaries requires verification methods of treatment and the implementation of preventive measures by beekeepers to improve the health status of bee colonies.

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