Original Article

HEALTH STATUS OF HONEYBEE COLONIES DIFFERING IN GENETIC INTRA-COLONIAL DIVERSITY

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Abstract

Two different levels of diversity within a colony were compared for the prevalence of pathogens and diseases. Lower genetic diversity was obtained in the colonies in which the queens were inseminated with semen collected from drones originating from a single colony, while greater was obtained in the colonies with queens inseminated with semen from drones of thirty different colonies. Bees were tested for *Varroa destructor* infestation, microsporidia *Vairimorpha* spp. infection, acute bee paralysis virus (ABPV) and deformed wing virus (DWV). Colonies with a greater genetic diversity of workers in colonies were more infested with *Varroa* mites than genetically uniform colonies. *Varroa* infestation was not found to be associated directly with the weakening of bee colonies after winter. The two experimental groups had a similar number of colonies infected with *Vairimorpha*, and viruses. Intensity of *Varroa* infestation and *Vairimorpha* infection did not significantly affect the overwintering of bee colonies. Colonies in which DWV was detected significantly weakened during overwintering.

Keywords: ABPV, DWV, genetic diversity, polyandry, *Vairimorpha*, *Varroa destructor*

INTRODUCTION

Bees' health status is one of the most crucial factors determining the economic effectiveness of beekeeping. Bee diseases are responsible for the sudden depopulation of bee colonies in the northern hemisphere over the past several years (Cox-Foster et al., 2007; Higes et al., 2008; Berthoud et al., 2010; Carreck et al., 2010; Paxton, 2010). The main cause of CCD syndrome as well as winter losses is the ectoparasite *Varroa destructor*. In Europe, parasites are additionally accompanied by two types of virus acute bee paralysis virus (ABPV) and deformed wing virus (DWV) (Schroeder & Martin, 2012). Another pathogen which affects bee losses are internal parasites *Vairimorpha* spp. (formerly *Nosema* redefined by Tokarev et al., 2020). Depending on latitude, *Vairimorpha ceranae* in the south in warm climates (Higes et al., 2008; Hatjina et al., 2011) and *Vairimorpha apis* in Scandinavia (Forsgren & Fries 2013).

In Poland, the incidences of bee losses are observed mostly in the autumn and winter and in the period 2006-2021 ranged from 8.2 to 21.8% (Mazur & Gajda, 2022). They are generally caused by parasites *Varroa destructor* and the accompanying viral infections and microsporidia *Vairimorpha* spp. (Topolska et al., 2008, 2010; Pohorecka et al., 2011). According to Pohorecka et al. (2011), in 2008-2009, 74.5% of colonies with both species of *Vairimorpha* were infected. Recently, the percentage of infected colonies was similar at 68% but they were only infected with *V. ceranae* (Pohorecka et al., 2019), mixed infections were always dominated by *V. ceranae* in apiary (Mullholland et al., 2012). Incidence of viruses in Polish apiaries was widespread between 2014 and 2018 (Pohorecka et al., 2019) and were detected more often in samples taken in spring than in summer. The most frequently detected viruses were DWV and black queen cell virus (BQCV), 61% and 63% of samples respectively, and slightly rarer was the sack brood virus (SBV) 52%.

The least detected were ABPV and chronic bee paralyse virus (CBPV), 30 and 15% respectively (Pohorecka et al., 2019).

Bees susceptibility to disease is genetically determined. Different genotypes of bees within races or lines characterize with varying degrees of susceptibility to pathogens as *Ascosphera apis* (Gilliam et al., 1988), American foulbrood (Rothenbühler & Thompson, 1956), and parasites: *Varroa destructor* (Guzman et al., 1996), *Acarapis woodi* (Gary & Page, 1987) *Vairimorpha apis* (Woyciechowski et al., 1994). One of the mechanisms of bee resistance to disease is behavioral resistance, e.g., hygienic behavior, VSH (varroa sensitive hygiene) and grooming behavior. Arathi & Spivak (2001) showed that only individual worker bees in the bee colony were involved in performing the desirable activity on which the resistance of the entire colony depends. Genetic composition and genetic variability of the bee colony ensures evolutionarily developed multiple mating (polyandry) and increases disease resistance (Sherman et al., 1988, Shykoff & Schmid-Hempel, 1991, Schmid-Hempel, 1998; Baer & Schmid-Hempel, 1999; Tarpy, 2003; Tarpy & Seeley 2006; Seeley & Tarpy 2007). Polyandry is important for natural selection, and without it, colonies would not adopt easily to changing environmental conditions (Page & Robinson, 1991). The current intensive selection of bees reduces genetic variability, especially when artificial insemination is applied. Lower genetic variabilityof individuals with a colony may decrease the viability of bee colonies and therefore reduce the adaptability of bees.

The aim of the research was to determine how bee worker diversity within a colony affects bee health. Two groups of bee colonies with different levels of genetic diversity of individuals were tested. The appropriate levels of diversity within a colony were obtained by the selection of drones for the insemination of the queens. Bees infested by *Varroa destructor*, infected by microsporidia *Vairimorpha* spp. and varroa-related viruses ABPV, DWV were studied.

MATERIAL AND METHODS

Experimental set-up

The study was performed at the Research Institute of Horticulture, the Apiculture Division in Puławy, Poland. Sister queens originating from a Carnica commercial line (M) were reared and then instrumentally inseminated to obtain two levels of genetic diversity in the offspring within colonies. The queens from one group (SCS- single colony semen) were inseminated with semen collected from drones from one of thirty paternal colonies belonging to the same strain as the queen (M) and additionally from two unrelated strains (N) and (G), ten colonies each strain. This gave three sub-groups indicated below as SCS-M (n=17), SCS-N (n=16) and SCS-G (n=18), and each combination was represented by one or two queens. The queens from the second group were inseminated with semen collected and mixed from drones coming from each of the thirty colonies (the MCS - mixed colony semen, n=51). Preparation of the semen used for insemination is described in detail in the publication by Gerula et al. (2014). When oviposition started, the queens of the two groups were introduced into the newly created colonies. A total of 102 colonies were set in Dadant hives with wax foundation frames. The colonies of the two experimental groups were randomly placed in two apiaries (est. 2009) located in Wola Bukowska (W) 51°40’09” N 22°21’22” E and Sielce (S) 51°26’23” N 22°04’46” E, 50 km apart.

In late summer at the beginning of experiment in 2009 and in 2010-2011 *Varroa destructor* mites were controlled with amitraz or flumethrin strips for about eight weeks and oxalic acid was applied during the broodless period usually in the middle of November. Throughout the *Varroa* control period, dead mites on the bottom board were counted. Bee colonies were grouped into three classes according to mite infestation: 1- up to 200 mites, 2- from 200 to 400 mites and 3- over 400 mites found on the bottom boards during *Varroa* treatment. Data from 2009 is not included in the paper because later assessed queens were introduced to the colonies in late July 2009.

In the spring of each year from 2010 to 2012, the degree of infestation of bees by microsporidia

Vairimorpha spp. was examined microscopically. Samples were taken from winter debris and the dead bees were counted. Then, in order to estimate the numbers of *Vairimorpha* spp. spores for every colony, by twenty whole bees were grounded in 20 ml of sterile distilled water and microscope samples for examination were prepared (Pohorecka et al., 2011). Three independent samples (twenty bees) for each experimental colony were prepared. The number of *Vairimorpha* spp. spores per bee was estimated through the use of Olympus BX 51 light microscopy and a haemocytometer (Cantwell, 1970; Human et al., 2013). For each spore suspension, the averages of three estimates of intensity were used. Based on the number of spores, bee infection in each colony was determined as low <1 mil/bee, medium 1-5 mil/bee and high above 5 mil/bee.

In the autumns of 2010 and 2011, bee samples (ca. 60) were collected for testing to identify bee viruses accompanying *Varroa destructor* infestations. The tests considered the presence of ABPV and DWV genetic material. The study was conducted by RT-PCR qualitative analysis using bee heads. For each colony, the heads of all workers were pooled, crushed in liquid nitrogen and further submitted to RNA isolation with Total RNA Mini Kit (A & A Biotechnology, Gdansk, Poland). According to manufacturer's protocol, the powder was suspended in phenosol. RNA was reverse transcribed to produce cDNA using Thermo scientific, Revert Aid First Strand cDNA Synthesis Kit. PCR analysis for the presence of viruses was done using Taq PCR Core Kit (Qiuagen) The thermal cycling profiles were set as follows: the mixture was heated for 3 min at 94°C, 40 PCR cycles at 94°C for min, 55°C for 1 min and 72°C for 1 min. The reactions were completed by a final elongation step at 72°C for 2 min and the next step was cooling down to 4°C. The PCR products were electrophoresed in a 1.5% agarose gel stained with ethidium bromide.

Statistical analysis

The multi-factor ANOVA model was used to evaluate interaction between the groups of colonies, locations, year and *Varroa* infestation. Number of mites in each colony was transformed according to (log10) formula. The LSD test was used to identify the homogeneous groups, and ANOVA was used to determine how *Varroa* infestation, *Vairimorpha* and viruses' infection influenced the number of dead bees in winter debris. Kruskal-Wallis's test was used to determine how *Varroa* infestation, *Vairimorpha* spp. and viruses' infection affected nest adjustment in the spring. Spearman's rank correlation coefficients were calculated to find the relationship between *Varroa* infestation and indicators of weakened colonies in the spring (number of dead bees, nest adjustment). Differences in the frequency of *Vairimorpha* infected colonies (scale 0-3) and colonies infected by the viruses in experimental group of colonies were analysed based on Pearson's χ^2 test. All statistical calculations figures were performed using Statistica 13 (TIBCO Software Inc. (2017).

RESULTS

Effect of genetic diversity on *Varroa destructor* levels

An average of 410 dead mites were found on the bottom boards during the entire period of parasite control in bee colonies n=122 (Tab. 1). In the SCS group colonies, there were on average 352 mites, while in the MCS group colonies significantly more, 456 mites, were found ($F_{(1,\ 114)}$=4.151, p=0.043). Bee infestation was significantly lower in apiary W, as evidenced by the number of dead mites on the bottom boards than in apiary S ($F_{(1,\ 114)}$=12,10, p<0.001). In 2010, infestation was significantly higher than in 2011 ($F_{(1,\ 114)}$=4.64, p=0.033) and on average there were respectively 455 and 345 mites. ANOVA was used to note the interaction effect of the factors of the year of study and the location of the apiary on the bee infestation ($F_{(1,\ 114)}$=48.852, p=0.035) (Fig. 1).

When we divided the SCS group, with homogeneous mating, into SCS-M, SCS-N and SCS-G according to the origin of the paternal colonies, similar numbers of *Varroa* mites were found on the bottom boards after late summer - autumn treatments in each experimental subgroup and MCS group in 2010 and 2011 ($F_{(3,\ 118)}$=1.559, p=0.2) (Tab. 2).

Table 1.

Number of *Varroa* mites found on the bottom boards after late summer-autumn treatments in each experimental group in 2010 and 2011

Groups*	Apiary	Years of research	Number of *Varroa* mites				
			N	Min	Max	Mean**	SD
SCS	**Both apiaries**	Years together	54	16	1806	352 a	316.1
MCS			68	17	1975	456 b	364.6
Both groups	**Apiary (W)**	Years together	70	16	1975	372 a	346.4
	Apiary (S)		52	121	1806	412 b	343.3
Both groups	**Both apiaries**	2010	72	16	1806	455 b	335.5
		2011	50	27	1975	345 a	355.2
Total				16	1975	410	346.4

*SCS colonies with queens instrumentally inseminated using semen from drones of a single colony, MCS colonies with queens instrumentally inseminated using mixed semen from drones of thirty colonies

**different letters in columns separated by blank row indicate significant differences at α=0.05 based on multifactor Anova

The table shows only the main effects. Data were transformed with the Log10 formula.

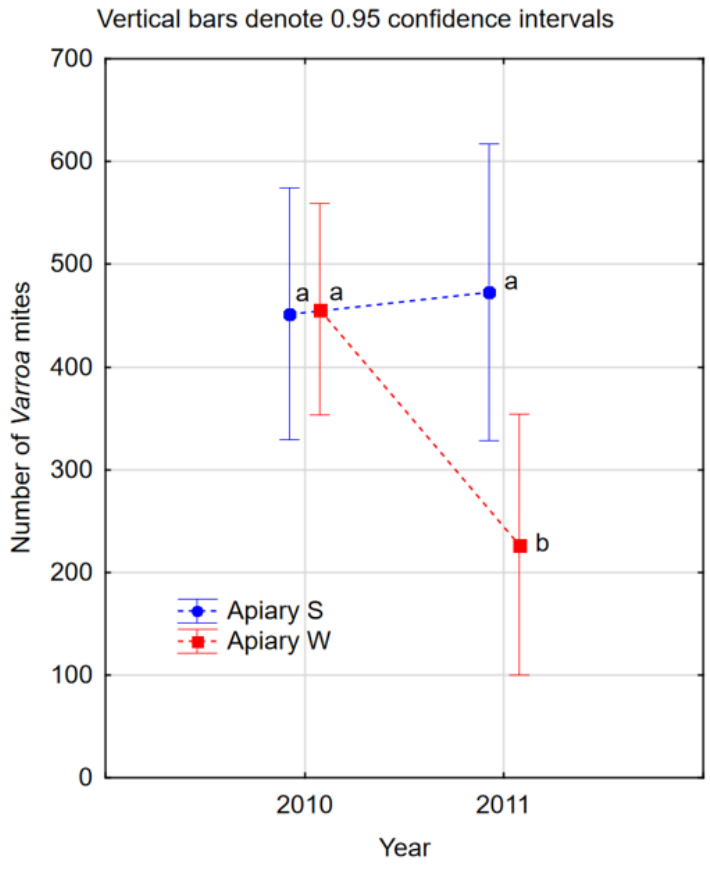


Fig. 1. Number of *Varroa* mites found after treatment on the bottom boards in particular years and apiaries.
Means among seasons indicated with different letters differed significantly from each other (P<0.05).

Varroa infestation was not found to be associated directly with the weakening of bee colonies after winter. Bee colonies from each class was similar, in regard to the number of dead bees with the winter debris ($F_{(2, 91)}$=0.552, p=0.57) as well in terms to the Kruskal-Wallis nest adjustment ($H_{(2, 91)}$=5.36, p=0.68) (Tab. 3). Relationships between *Varroa* infestation and the number of dead bees in spring, defined as Spearman rank correlation coefficients, were not significant, while the relationship between *Varroa* infestation and nest adjustment was significant and unexpectedly amounted to -0.23.

Infection with microsporidium *Vairimorpha* spp.

In the spring of each year, a total of 182 bee samples were tested (Tab. 4) to examine the rate of bees infected with *Vairimorpha* spp. In 2010 and 2011, a lower

Table 2.

The number of *Varroa* mites found on the bottom boards after late summer- autumn treatments in each experimental group in 2010 and 2011. The SCS group was divided according to the origin of the paternal colonies

Groups*	Number of *Varroa* mites				
	N	Min	Max	Mean**	SD
SCS-M	23	27	1806	337 a	388.5
SCS-N	12	16	941	378 a	289.4
SCS-G	19	43	922	354 a	240.4
MCS	68	17	1975	456 a	364.6

*SCS-M, SCS-N and SCS-G colonies with queens instrumentally inseminated with semen from drones of a single colony, MCS colonies with queens instrumentally inseminated with mixed semen from the drones of thirty colonies
**different letters in columns separated by blank row indicate significant differences at α=0.05 based on Anova. Data for analysis were transformed with the Log10 formula.

Table 3.

Number of dead bees with winter debris and nest adjustment in spring 2010 and 2011 in bee colonies according to *Varroa* infestation level expressed on a scale of 1-3

Varroa infestation (1-3)	N	Mean number of dead bees with winter debris*	Change in the size of the nest in the spring (%)		
			Mean**	Median	Range
1	23	1359 a	-14.2 a	-7.1	-50 - 0
2	33	1188 a	-11.0 a	0	-40 - 0
3	38	1356 a	-11.4 a	-14.3	-50 - 33
Total	95	1288	-11.8	-14.2	-50 - 33

*different letters in columns indicate significant differences at α=0.05 based on Anova. Data were transformed with the Log10 formula.
**different letters in columns indicate significant differences at α=0.05 based on Kruskal-Wallis test.

percentage of colonies infected with *Vairimorpha* was found at 40% and 39%, respectively, compared to the year 2012 when the percentage increased dramatically to 80%. In 2010, in the slightly more SCS group colonies were observed to be free of the *Vairimorpha* (71%) than in MCS group colonies (54%). Colonies with high infection (above 5 mil/bee) in both groups accounted for 70%.

In the MCS group, only 46% out of thirteen positive samples were determined as highly infected, and in the SCS group, as many as 71% out of fourteen positive samples, were thus identified. In 2012, *Vairimorpha*-free colonies in the MCS group accounted for 18%, while only 9% in the SCS group. The percentages of colonies with a high infection level were 60% and 72%, respectively. Based on data from all years, the number of infected and healthy colonies was found to be similar in both experimental groups (Pearson χ^2=3.581, df=3, p=0.31) (Fig. 2). In colonies, where *Vairimorpha* spp. infestation was tested at least twice, some colonies (n=58) had a recovery rate of 27.6% and new case of disease rate of 39.6%. The number of colonies that were always healthy or always sick were 18.9% and 13.8%, respectively. MCS colonies recovered more frequently than those from the SCS group, but there were no significant differences (Pearson χ^2=3.721, df=3, p=0.29) (Fig. 3).

The winter loss of bees found on the bottom boards in both healthy and infected colonies did not differ significantly and averaged 1174 individuals $F_{(3, 174)}$=0.240, p=0.86) as well in terms of the nest adjustment Kruskal-Wallis ($H_{(2, 91)}$=5.36, p=0.68) (Tab. 5).

Table 4.

Distribution of healthy colonies and those infected with *Vairimorpha* spp. from each experimental group

Groups*	Years of research	N	Number of healthy colonies (0), and infected with *Vairimorpha* spp. spores expressed in a scale of (1-3)			
			0	1	2	3
SCS	2010	89	28	2	1	8
MCS			27	5	4	14
SCS	2011	68	18	1	3	10
MCS			23	6	1	6
SCS	2012	25	1	1	2	7
MCS			4	2	1	7
Total		182	101	17	12	52

*SCS colonies' queens instrumentally inseminated with semen from drones of single colony,
MCS colonies queens instrumentally inseminated with mixed semen from drones of thirty colonies.

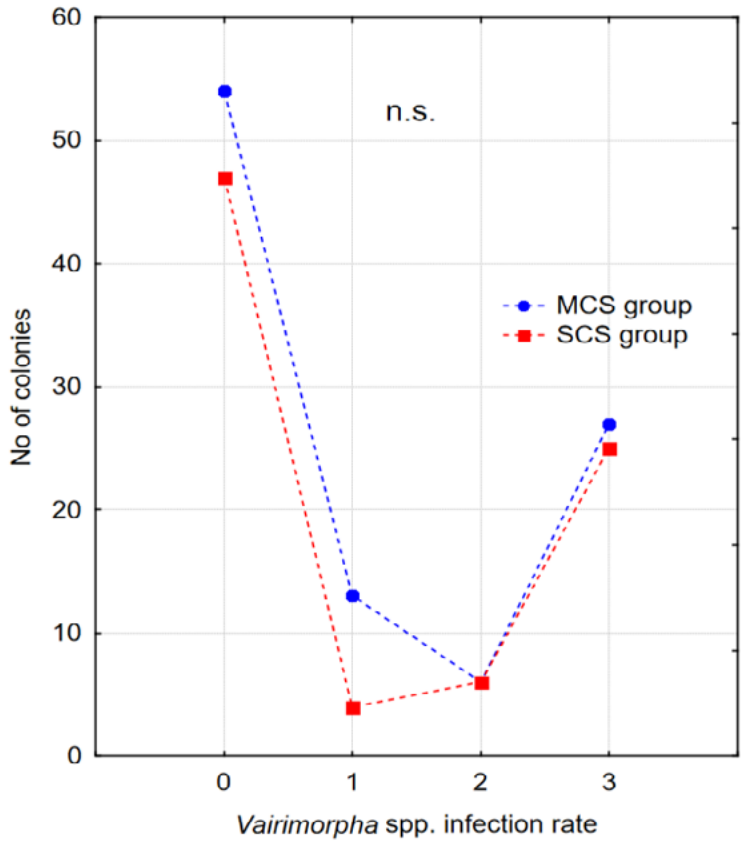


Fig. 2. Number of healthy colonies (0) and infected with *Vairimorpha* spp. (scale 1-3) in the different experimental groups,
n.s. - not significant.

Viral prevalence

In 2010, out of seventy-one bee samples, ABPV was detected in twenty-three samples (nine in the SCS and fourteen in the MCS group), while DWV was detected in three samples (two in the SCS group and one in the MCS group). Neither virus was detected jointly in any bee sample. In 2011, out of forty-five bee samples tested, ABPV was detected only in one sample (SCS group), while DWV was detected in as many as twelve samples (five in SCS group and seven in MCS group) (Tab. 6). In one sample from a single colony, both viruses were identified, and in the previous year, no viruses were found in this colony. There was no interaction between the presence of the viruses and the experimental group (Pearson χ^2=1.525, df=3, p=0.67) (Fig. 4), and the listed bee viruses were present in the colonies only temporally. None of the colonies which were infected by the viruses in 2010 were still infected in 2011. The virus had persisted only in three colonies over the two years of the study, ABPV infection in the

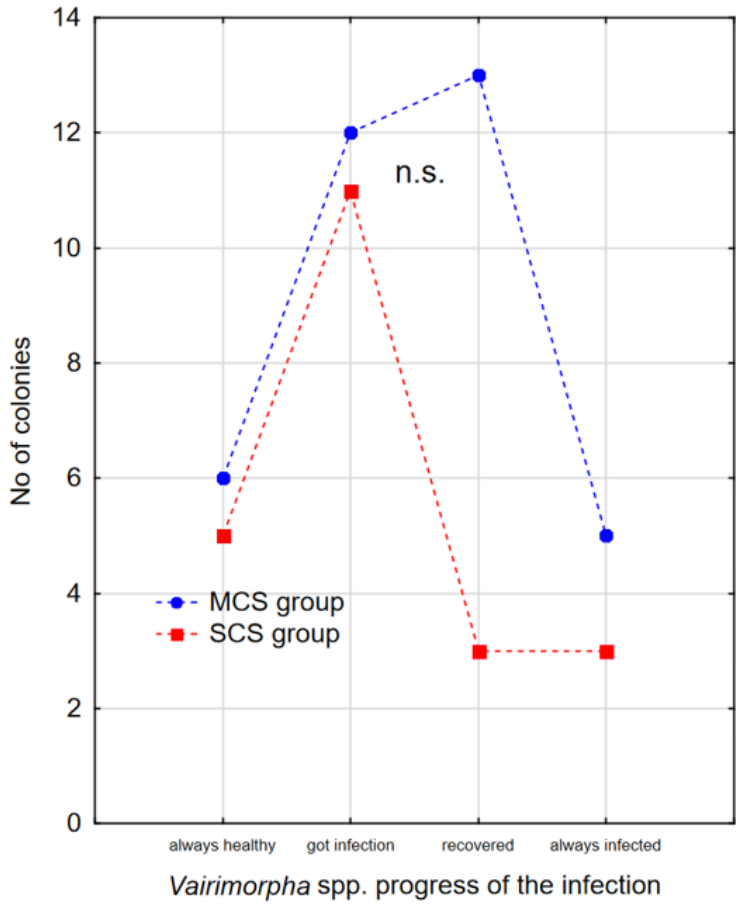


Fig. 3. Number of healthy colonies and those infected with *Vairimorpha* spp. and then recovered changes during the experimental period in each experimental group colonies, n.s. - not significant.

first year while DWV infection in the second year.

Varroa infestation of bees in the period prior to sampling for testing did not differ significantly in either healthy colonies or those in which the viruses were detected, $F_{(2,108)}$=2.15, p=0.12 (Tab. 7). No relationship was found between the presence of ABPV virus in the bees' bodies and the loss of bees during the subsequent overwintering period. However, the number of bees died in winter, in colonies with DWV was observed to amount to 2047 bees and to be significantly higher than in healthy and ABPV-infected colonies, 1251 and 1035 bees respectively $F_{(2, 91)}$=6.77, p=0.001 (Tab. 7). By analogy, when there were more bee deaths in winter, more combs were removed from nests in spring after the first inspection. Significantly more combs were removed from colonies with DWV (33.3%) than from healthy and ABPV-infected colonies, (11.2 and 7.7%, respectively) Kruskal-Wallis Test $H_{(2, N= 85)}$=9.05, p=0.011 (Tab. 7).

Table 5.

Distribution of healthy colonies from each experimental group and those infected with *Vairimorpha* spp.

Healthy colonies (0), infected expressed in a scale of (1-3)	Indicators overwintering of healthy colonies and infected by *Vairimorpha* spp. spores			
	N	Mean number of dead bees in winter debris*	Change in the size of the nest in the spring (%)	
			Mean**	Median
0	101	1188 a	-9.7 a	0.0
1	17	1161 a	-12.5 a	-15.4
2	12	1208 a	-16.6 a	-16.6
3	52	1140 a	-14.1 a	-16.6
Total	182	1174	-11.6	-14.2

*different letters in columns indicate significant differences at α=0.05 based on Anova.
Data were transformed with the Log10 formula.
**different letters in columns indicate significant differences at α=0.05 based on Kruskal-Wallis test.

Table 6.

Distribution of viruses in bee samples taken in autumn from colonies in each experimental group in 2010 and 2011

Groups *	Years of research	Number of healthy colonies (0), and infected by viruses				
		N	0	ABPV	DWV	ABPV + DWV
SCS	2010	71	21	9	2	0
MCS			24	14	1	0
SCS	2011	45	13	1	5	1
MCS			19	0	7	0
Total		116	77	24	15	1

*SCS colonies with queens instrumentally inseminated with semen from drones of a single colony,
MCS colonies with queens instrumentally inseminated with mixed semen from drones of thirty colonies.

Table 7.

Number of *Varroa* mites before winter and some indicators of overwintering of healthy colonies and those infected by viruses

Viral presence	N	Number of *Varroa* mites before winter*	Mean number of dead bees in winter debris*	Change in the size of the nest in the spring (%)	
				Mean**	Median
Healthy	75	420 a	1251 a	-11.2 a	-14.0
ABPV	23	485 a	1035 a	-7.7 a	0.0
DWV	13	417 a	2047 b	-31.1 b	-33.3
ABPV + DWV* **	1	321	2621	-16.6	-16.6
Total	112	432	1288	-12.7	-14.2

*different letters in columns indicate significant differences at α=0.05 based on Anova. Data were transformed with the Log10 formula.
**different letters in columns indicate significant differences at α=0.05 based on the Kruskal-Wallis test.
*** group excluded from the test due to the small number of cases.

DISCUSSION

The studies did not provide sufficient evidence that increased genetic diversity among colony members, that results from polyandry, increases colony resistance to pathogens and parasites. An average of 352 *Varroa* mites were found during treatments in late fall in the SCS group. The number of mites ranged from 337 to 378 in specific subgroups created by a different patriline. Unexpectedly, there were significantly more mites, 456, in colonies from the MCS group which consisted of the same genotypes but combined in a single queen. None of the paternal and maternal components were selected for resistance to *Varroa*, and no genotypes were added with VSH behavior that could affect the reduction of infestation. This unexpected data could be explained by the occurrence of heterosis in MCS colonies which was confirmed by more brood throughout the year (Gerula et al., 2014). Typically, in unselected bee populations if there is more brood then there are more mites (Wilkinson & Smith, 2002), and the same results were reached by Neumann & Moritz (2000) and Beaurepaire et al. (2022). Desai & Currie (2015) found that the genetically diverse colonies had a reduced *Varroa* population in comparison with genetically uniform colonies, which could be explained by the use

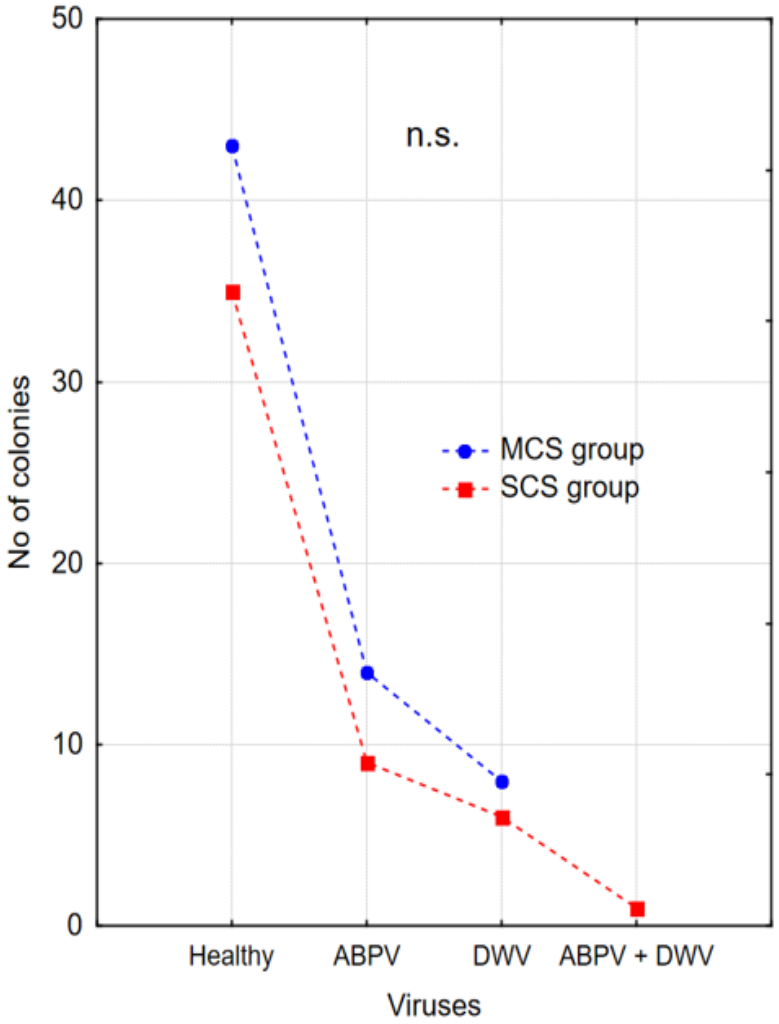


Fig. 4. Number of healthy colonies and infected with ABPV and DWV in the different experimental groups,
n.s. - not significant

of drone and queen from mite-resistant stocks. Polyandry could provide better defence through either social or innate immunity. Social immunity mechanisms include such responses as hygienic brood removal behavior (detection and removal of infected brood) (Wilson-Rich et al., 2009) and grooming of nest mates (Currie & Tahmasbi, 2008; Ugelvig et al., 2010).

Distribution of healthy colonies and those infected with *Vairimorpha* spp. in each experimental group was similar. *Vairimorpha* did not significantly affect the overwintering of bee colonies. Unfortunately, the species of *Vairimorpha* has not been identified however based on research by Topolska et al. (2008, 2010), Pohorecka et al. (2011) and Pohorecka et al. (2019) it can be assumed that bees in current experiment were infected mainly by *V. ceranae,* which does not give typical symptoms in winter.

The lack of effect of intra-colonial diversity on *V. apis* prevalence was confirmed by Wojciechowski & Krol (2001) and Desai & Curie (2015), but authors have found that *V. ceranae* prevalence differed significantly between groups with different diversity. The prevalence of this pathogen appears to depend on an innate response involving both cellular and humoral defences (Gillespie et al., 1997).

Even though there was more *Varroa* in the MCS group, this did not affect the increase in the number of colonies infected with ABPV and DWV, as Martin et al. (2012) suggested. Distribution of viruses in bee samples taken in autumn from colonies in each experimental group was similar. The same had been confirmed by Desai and Curie (2015) in relation to DWV, while ABPV proportion differed in groups of colonies with different diversity. Disregarding intra-colonial diversity, DWV significantly affected the weakening of colonies during wintering. The prevalence of DWV is an omen of possible greater bee losses due to *Varroa* mites, as bees can only tolerate infestation even at higher levels if unaccompanied by DWV infection (Roberts et al., 2020).

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