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Original papers

Preliminary studies on the occurrence of honeybee pathogens in the national bumblebee population

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ABSTRACT. Dangerous pathogens that affect honeybees, including microsporidia of the genus *Nosema*, the acute bee paralysis virus (ABPV) and the deformed wing virus (DWV), also contribute to the global decline in bumblebee populations. In this study, a few insects with the bumblebee population present in country was checked for the presence of *Nosema apis*, *Nosema bombi* and *Nosema ceranae* microsporidia, ABPV, the chronic bee paralysis virus (CBPV) and DWV. Thirteen adult bumblebees (*Bombus*) representing 4 species were analyzed: *Bombus lapidarius* (2 workers, 2 males), *Bombus lucorum* (1 worker), *Bombus pascuorum* (5 workers, 1 male) and *Bombus terrestris* (2 workers). These specimens were found dead in two urban botanical gardens and four landscape parks in Lower Silesia. Microsporidia of the genus *Nosema* and CBPV were not detected in any of the examined samples. ABPV was identified in 2 insects of *B. pascuorum* and *B. terrestris* (2 workers). The presence of DWV was detected in 12 bumblebees (10 workers, 2 males).

Key words: invertebrate, *Nosema* sp., pathogens, PCR

Introduction

There are around 300 bumblebee species in the world, 37 of them have been identified in Poland [1]. The most common species include the white-tailed bumblebee (*Bombus lucorum*), red-tailed bumblebee (*B. lapidarius*), common carder bee (*B. pascuorum*) and buff-tailed bumblebee (*B. terrestris*). All of the above species are protected, but despite those efforts, their populations continue to decline steadily. The decrease in bumblebee populations is attributed mainly to increased pesticide use, lower availability of food resources resulting from the loss of natural habitats, and the expansion of cereal monocultures [2]. Pathogens also substantially contribute to bumblebee mortality. *Nosema bombi*, a pathogen that was described by Fantham and Porter, is the most widespread cause of the decline in bumblebee populations [3]. Bumblebees are also infected by pathogens that are specific to honey bees (*Apis mellifera*), including the *N. ceranae* microsporidian, the acute bee paralysis virus (ABPV) and the deformed wing virus (DWV) [4–6]. There are research on bee pathogens like *N. ceranae* in bumblebees, but the association with the increase on mortality in these insects has not been fully demonstrated [5,6]. In recent years, losses of bumblebee species have been reported worldwide [2].

Vast research efforts have been made to identify the unexplained causes of decline in bumblebee and honey bee populations. Bumblebees and honey bees play very important roles in the ecosystem and the economy. They pollinate flowering plants, and insect pollination is essential in food production, which is why effective measures for conserving the populations of these useful insects are urgently required [7].

The aim of the present pilot study was to identify microsporidia of the genus *Nosema* (*N. apis, N.*

bombi, N. ceranae), the acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV) and deformed wing virus (DWV) and *V. destructor* in selected bumblebee species in Poland.

Materials and Methods

The experiment was performed on 13 adult bumblebees belonging to 4 species: *B. lapidarius* (2 workers, 2 males), *B. lucorum* (1 worker), *B. pascuorum* (5 workers, 1 male) and *B. terrestris* (2 workers). The insects were found dead in July 2015 in the Medicinal Plants Botanical Garden in Wrocław (MP), the Botanical Garden in Wrocław (BG) and in the following landscape parks in Lower Silesia: Bystrzyca River Valley (A), Barycz River Valley (B), Jezierzyca River Valley (C), Ślęża Mountain (S). These study were conducted, with the permission issued by Minister of the Environment. Genomic DNA and RNA were isolated from whole insects that were homogenized individually under sterile conditions in a mortar.

DNA isolation. Genomic DNA was isolated with the Genomic Mini kit (A&A Biotechnology, Gdynia, Polska) for DNA isolation which relies on genomic DNA's ability to bind to silica at high concentrations of chaotropic salts. The isolation process was conducted according to the manufacturer's recommendations, and purified DNA was stored in test tubes at a temperature of -20°C until further analysis.

Isolation of total RNA. Total RNA was isolated with the Total RNA Mini Kit (A&A Biotechnology, Gdynia, Polska) according to the manufacturer's recommendations. The isolated RNA was portioned into aliquots and stored in test tubes at a temperature of -70°C until further analysis.

Primers and conditions of duplex PCR and single PCR for the identification of *Nosema* sp. The small subunit 16S rRNA of *N. apis*, *N. ceranae* and *N. bombi* was amplified by PCR. The primers for the synthesis of *N. apis* and *N. ceranae* DNA were synthesized in accordance with the Manual of Standards for Diagnostic Tests and Vaccines [8] by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences (Oligo, Warsaw, Poland). The primers for the synthesis of *N. bombi* DNA [6] were synthesized by Genomed (Warsaw Poland). The primer sequences used in PCR are presented in Table 1.

Primers and conditions of RT-PCR for the identification of ABPV, CBPV and DWV. The total RNA of ABPV, CBPV and DWV was isolated by reverse transcription PCR (RT-PCR). The primers for total RNA extraction were synthesized by Genomed (Warsaw, Poland). The primer sequences [9–11] used in RT-PCR are presented in Table 2.

Duplex PCR (*N. apis*, *N. ceranae*) and single PCR (*N. bombi*) were carried out with the use of HotStarTaq *Plus* Polymerase (Qiagen) and the HotStarTaq *Plus* Master Mix Kit (Qiagen). The reaction mixtures of 20 µl each had the following composition: around 120 ng of isolated DNA (1 to 3

Table 1. Primer sequences used in PCR

Target	Primer sequence	Product (bp)	Reference
N. apis	5'-GGGGGCATGTCTTTGACGTACTATGTA-3' 5'-GGGGGGCGTTTAAAATGTGAAACAACTATG-3'	321	OIE 2013 [8]
N. bombi	5'-GGCCCATGCATGTTTTTGAAGATTATTAT-3' 5'- CTACACTTTAACGTAGTTATCTGCGG-3'	101	Plischuk 2010 [6]
N. ceranae	5'-CGGCGACGATGTGATATGAAAATATTAA-3' 5'-CCCGGTCATTCTCAAACAAAAAACCG-3'	218	OIE 2013 [8]

Table 2. Primer sequences used in RT-PCR

Target	Primer sequence	Product (bp)	Reference
ABPV	5`-CATATTGGCGAGCCACTATG-3` 5`-CCACTTCCACCACAACTATCG-3`	398	Genersch et al. 2010 [10]
CBPV	5`-TCAGACACCGAATCTGATTATTG-3` 5`-ACTACTAGAAACTCGTCGCTTCG-3`	570	Pohorecka et al. 2014 [11]
DWV	5`-TCGACAATTTTCGGACATCA-3` 5`-ATCAGCGCTTAGTGGAGGAA-3`	702	Chen et al. 2006 [9]

Table 3. Pathogens detected in the analyzed bumblebee species and genders

Sample	Bumblebee species	Sex	Harvest site	*Nosema*			Virus		
				apis	*bombi*	*ceranae*	ABPV	CBPV	DWV
1.	*B. lapidarius*	worker	A*	–	–	–	–	–	+
2.	*B. lapidarius*	worker	B*	–	–	–	–	–	+
3.	*B. lapidarius*	male	C*	–	–	–	–	–	+
4.	*B. lapidarius*	male	MP*	–	–	–	–	–	+
5.	*B. lucorum*	worker	S*	–	–	–	–	–	+
6.	*B. pascuorum*	worker	S	–	–	–	+	–	+
7.	*B. pascuorum*	worker	B	–	–	–	–	–	+
8.	*B. pascuorum*	worker	C	–	–	–	–	–	+
9.	*B. pascuorum*	worker	MP	–	–	–	–	–	+
10.	*B. pascuorum*	worker	A	–	–	–	–	–	+
11.	*B. pascuorum*	male	MP	–	–	–	–	–	–
12.	*B. terrestris*	worker	B	–	–	–	+	–	+
13.	*B. terrestris*	worker	BG*	–	–	–	–	–	+

A* – Bystrzyca River Valley (south Poland); B* – Barycz River Valley (south Poland); C* – Jezierzyca River Valley (south Poland); MP*– Medicinal Herb Garden in Wrocław (south Poland); BG* – the Botanical Garden in Wroclaw (south Poland)

µg), 10 µl of HotStarTaq *Plus* Master Mix 2x, 2 µl of CoralLoad Concentrate 10x, 0.1 µl of each primer (with a final concentration of 0.5 µM), made up to 20 µl with RNase-Free Water. Every duplex PCR reaction had three controls: two positive controls with *N. apis* and *N. ceranae* DNA (Centro Apicola Regional, Dirección Genera de la Producción Agropecuaria, Consejería de Agricultura, Junta de Comunidades de Castilla-La Mancha, Spain). Single PCR had two controls: a positive control with *N. bombi* DNA and a negative control where DNA was replaced with water. The reactions were carried out in the Eppendorf Mastercycler thermocycler under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 45 s, primer annealing at 55°C for 45 s (*N. apis* and *N. ceranae*) or 52°C for 40 s (*N. bombi*) and elongation at 72°C for 1 min. The last reaction was followed by final chain synthesis at 72°C for 10 min. The products of duplex PCR (*N. apis* and *N. ceranae*) and single PCR (*N. bombi*) were separated by electrophoresis on 2% agarose gel with 6 µl of the Midori green stain for visualizing the DNA fragments of *Nosema* sp. Product size was determined based on the GeneRuler$_{TM}$ 100bp 36 Ladder Plus (Fermentas) molecular weight standard. Electrophoresis results were documented in the GelDoc (Bio-Rad) imaging system.

Viruses (ABPV, CBPV, DWV) were detected by RT-PCR with the use of the OneStep RT-PCR Kit (Qiagen). Reverse transcription was carried out at 50°C for 30 min. It was followed by PCR with the following reaction profile: initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 50 s, primer annealing at 55°C for 50 s (ABPV, CBPV, DWV) and elongation at 72°C for 1 min. The last reaction was followed by final chain synthesis at 72°C for 10 min. Every RT-PCR reaction had a positive control with the RNA of the analyzed virus and a negative control where viral RNA was replaced with water. The reaction products (ABPV, CBPV, DWV) were separated by electrophoresis on 2% agarose gel with 6 µl of the Midori green stain for visualizing the RNA fragments of ABPV, DWV and CBPV. Product size was determined based on the GeneRuler$_{TM}$ 100bp 36 Ladder Plus (Fermentas) molecular weight standard. Electrophoresis results were documented in the GelDoc (Bio-Rad) imaging system.

The presence of *Varroa destructor* mites was analyzed in every bumblebee under a stereoscopic microscope at ×40 magnification.

Results

A total of 13 insects (samples), including 10 ♀ and 3 ♂, were analyzed. ABPV was detected in 2

individuals (2 ♀ *B. pascuorum and B. terrestris*), and DWV was identified in 12 bumblebees (10 ♀ and 2 ♂). Microsporidia of the genus *Nosema* and CBPV were not detected in any of the samples. The examined insects were free of *V. destructor* mites. Pathogens were identified in bumblebees harvested in the landscape parks of Bystrzyca River Valley (A), Barycz River Valley (B), Jezierzyca River Valley (C), Medicinal Herb Garden in Wrocław (ORL), Ślęża Mountain (S) and the Botanical Garden in Wrocław (OB). The results of the analysis are presented in Table 3.

Discussion

Molecular methods support the detection and analysis of pathogens that colonize various species of insects, including in asymptomatic infections. Microsporidia of the genus *Nosema* commonly infect various species of honey bees and other insects, including bumblebees [12]. To date, *N. bombi* microsporidia have been the most frequently identified pathogens in bumblebees [3,13]. The pathogen exerts a negative effect on individuals as well as entire colonies by stunting their development [14,15]. Bumblebees are also colonized by microsporidia specific to honey bees, including *N. apis* and *N. ceranae* [6,16]. At present, *N. bombi* is the most prevalent pathogen in bumblebees. Arbulo et al. [17] identified the above microsporidian in the bumblebee species *B. atratus* and *B. bellicosus*. They analyzed 526 bumblebees, including 317 *B. atratus* and 209 *B. bellicosus*, and detected *N. ceranae* in 364 samples (72% *B. stratus* and 63% *B. bellicosus*). None of the evaluated samples were colonized by *N. apis* or *N. bombi*. Plischuk et al. [6] analyzed the prevalence of *N. ceranae* in 6 bumblebee species (a total of 455 individuals) and identified only *N. cerane* in 3 species: *B. atratus, B. morio* and *B. bellicosus* (49 samples). Vavilova et al. [18] evaluated the prevalence and diversity of *N. bombi* in bumblebees in Western Siberia. They examined 727 individuals belonging to 16 species. The prevalence of *N. bombi* microsporidia ranged from 4 to 20%, and it varied across the examined locations. Bumblebee species *B. agrorum* and *B. equestri* were not colonized by the analyzed pathogen. Cameron et al. [19] studied the prevalence of *N. bombi* in selected bumblebee species. *B. appositus* was free of the pathogen, whereas the prevalence of *N. bombi* was high in *B. occidentalis* and *B. pensylvanicus* (15.2% and 37.2%, respectively). In other studies, *N. bombi* was not detected in bumblebee samples [16,20,21]. Similar results were noted in our experiment where 4 of the examined bumblebee species were free of *N. apis, N. bombi* and *N. ceranae* microsporidia.

Infections caused by ABPV, CBPV and DWV are among the leading causes of the colony collapse disorder (CCD) in honey bees [22]. *V. destructor* mites are also vectors of the above viruses [9,21–24]. Genersch et al. [5] identified the DWV virus in bumblebee which were free of *V. destructor*, and honey bee workers from colonies in the vicinity of the examined bumblebees showed symptoms of DWV infection. The presence of DWV was confirmed in two bumblebee species, *B. terrestris* and *B. pascuorum.* In our experiment, DWV was identified in 12 samples, including in the white-tailed bumblebee (*Bombus lucorum*), red-tailed bumblebee (*B. lapidarius*), common carder bee (*B. pascuorum*) and buff-tailed bumblebee (*B. terrestris*). Our results suggest that DWV infections are prevalent in bumblebees. Reynaldi et al. [25] reported a co-infection with viral pathogens in *B. atratus* in South America. They analyzed 20 samples composed of 40 bumblebee workers from 210 colonies inhabiting a tomato greenhouse in Buenos Aires. The presence of DWV, BQCV and SBV was determined in all evaluated samples. In our study, CBPV was not detected, and DWV was the most prevalent viral pathogen which was detected in 92.3% of the analyzed samples. A co-infection with ABPV and DWV was observed in 2 samples which accounted for 15.38% of all analyzed samples.

The prevalence of pathogens in *B. stratus* bumblebees was studied extensively by Gamboa et al. [26] who screened their samples for the presence of ABPV, BQCV (Black Quenn Cell Virus), DWV, LSV (Lake Sinai Virus), SBV (Sacbrood Virus), *N. ceranae, Critidia bombi, Apicystis bombi* and *Spiroplasma apis. N. ceranae* was omnipresent (both in captive-bred and wild samples), *A. bombi* was detected in 12 out of the 19 analyzed samples, and LSV – in 13 samples. The above results point to the high prevalence of pathogens in bumblebees.

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