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Original article

RESISTANCE OF NATIVE HONEY BEES FROM RHODOPE MOUNTAINS AND LOWLAND REGIONS OF BULGARIA TO *NOSEMA CERANAE* AND VIRAL PATHOGENS

R. SHUMKOVA[1], B. NEOV[2], A. GEORGIEVA[3], D. TEOFANOVA[4], G. RADOSLAVOV[2] & P. HRISTOV[2]

[1]Agricultural and Stockbreeding Experimental Station, Agricultural Academy, Smolyan, Bulgaria; [2]Department of Animal Diversity and Resources, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, Bulgaria; [3]Department of Pathology, Institute of Experimental Morphology, Pathology and Morphology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria; [4]Department of Biochemistry, Faculty of Biology, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria

Summary

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The Western honey bee (*Apis mellifera* L., Hymenoptera: Apidae) is a species of fundamental economic, agricultural and environmental importance. The aim of this study was to compare the prevalence of some parasitic and viral pathogens in local honey bees from the Rodope Mountains and plain regions. To achieve this goal, molecular screening for two of the most distributed *Nosema* spp. and molecular identification of six honey bee viruses – Deformed wing virus (DWV), Acute bee paralysis virus (ABPV), Chronic bee paralysis virus (CBPV), Sacbrood virus (SBV), Kashmir bee virus (KBV), and Black queen cell virus (BQCV) was performed. Molecular analysis was carried out on 168 honey bee samples from apiaries situated in three different parts of the country where a mix of different honey bee subspecies were reared. In South Bulgaria (the Rhodope Mountains), a local honey bee called *Apis mellifera rodopica* (a local ecotype of *A. m. macedonica*) was bred, while in the other two regions (plains) different introduced subspecies existed. The results showed that the samples from the lowland regions in the country were outlined with the highest prevalence (70.5%) of *N. ceranae*, while those from the mountainous parts had the lowest rate (5.2%). Four of the honey bee viruses were identified – DWV (10/5.9%), followed by SBV (6/3.6%) and ABPV (2/1.2%), and one case of BQCV. In conclusion, the local honey bee *A. m. rodopica* (despite the higher number of samples) has shown lower prevalence of both nosemosis and viral infections. Therefore, this honey bee has to be preserved as a part of the national biodiversity.

Key words: *Apis mellifera*, Bulgaria, honey bee diseases, molecular detection

INTRODUCTION

Honey bees are among the main pollinators of both agricultural and non-agricultural plants (Gallai *et al.,* 2009; McMenamin *et al.,* 2016). Consequently, honey bee colony losses have a negative impact on the agricultural production, the ecosystem diversity of many plants (including endemic ones) as well as the production of many bee products such as honey, royal jelly, bee propolis, etc. (Pasupuleti *et al.,* 2017; Sforcin *et al.,* 2017). Numerous factors lead to reduction of the number of honey bee colonies but some of them are of major significance. These include various pathogens, pests, wide use of insecticides and herbicides (in particular, neonicotinoid insecticides) in crop protection (Pettis & Delaplane, 2010; van der Zee *et al.,* 2012; Nazzi & Pennacchio, 2014; Goulson *et al.,* 2015). Last but not least, anthropogenic activity such as complete replacement of local bees with non-natives (a gene flow between native and introduced subspecies), mobile communications have also reduced many natural bee habitats and exerted a negative impact on bee colonies (Dahal, 2013). Among these negative factors, pathogens seem to have the most significant effects. A variety of pathogens infect bees, including fungi, mites, protists, bacteria, and viruses (the majority of which RNA viruses). In the last decades, two pathogens have raised a considerable scientific interest because they are clearly essential for honey bee health – namely, the ectoparasitic mite *Varroa destructor* and the microsporidian fungus *Nosema ceranae* (Kurze *et al.,* 2016). In addition to mite's consumption of hemolymph in both the adult bee and the larva, *Varroa* mite was proven as a vector in transmitting many honey bee viruses (Sumpter & Martin, 2004; Shen *et al.,* 2005; Gisder *et al.,* 2009).

There are a numerous studies focused on honey bee genotypes, the environmental factors and pathogens infestation levels (Costa *et al.,* 2012; Francis *et al.,* 2014; Buchler *et al.,* 2014; Meixner *et al.,* 2014a; Hatjina *et al.,* 2014a; Uzunov *et al.,* 2014a). Some of them confirm the higher resistance and vitality of the local honey bees compared to the introduced, indicating that a more economical, effective and sustainable beekeeping is possible by using and breeding bees from the local populations (Meixner *et al.,* 2014b).

Bulgaria has a long tradition in beekeeping. According to Ruttner's morphometric analysis (Ruttner, 1988) *A. m. macedonica* is considered as a native honey bee subspecies for the country. In Bulgaria, there also exists a local honey bee called *A. m. rodopica* (Petrov, 1990). This honey bee is reared only in the mountainous part of the country (the Rhodope Mountains). Numerous investigations (morpho-ethological, mtDNA, RAPD, microsatellite DNA, SNP analysis) have been carried out to distinguish this honey bee from other subspecies (Martimianakis *et al.,* 2011; Nikolova, 2011; Radoslavov *et al.,* 2017). Although the results of these studies have revealed some differences, currently the taxonomic status of *A. m. rodopica* is that of a local ecotype of *A. m. macedonica* (Uzunov *et al.,* 2014b). Over the last decades, different subspecies like *A. m. ligustica*, A. *m. carnica* and *A. m. caucasica* have been imported, mostly with the aim to increase honey bee productivity (Bouga *et al.,* 2011). These races are reared mainly in the lowland regions in the country.

The main focus of this study was to compare the prevalence of some viral and parasitic pathogens in the local honey

bees *A. m. rodopica* and honey bees in some plain regions.

MATERIALS AND METHODS

Nosema infection in native and introduced honey bee subspecies

Honey bee samples were collected between April and June 2017 from 100 apiaries, comprising a total of 168 colonies in three different parts of the country – South Bulgaria (the Rhodope mountains, n=96); North Bulgaria (Rousse district, n=44) and West Bulgaria (Sofia district, n=28). There was no bias concerning the obtained honey bee samples. The last two regions comprise generally flat plains where different subspecies of *A. mellifera* are reared. The first region is situated in the Rhodope Mountains. Sampling was done according to the guidelines of the Office International des Epizooties (OIE, 2008). None of the honey bee colonies had been treated against *Nosema* infection for at least 6 months. In each hive, sixty adult worker honey bees were randomly selected at the entrance of the hive or on frames away from the brood nest. The honey bees were placed in a falcon tube, put in a cooler bag and stored at – 20 °C prior to analysis.

DNA extraction and PCR amplification. Prior to DNA extraction, the abdomen of a single bee was cut off with scissors, mechanically homogenised with a cell lysis buffer and centrifuged for 1 min at 12,000×*g*. Total DNA was isolated by using a GeneMATRIX Tissue and Bacterial DNA purification Kit (Cat. No. E3551-01, EURx Ltd., Poland) according to the manufacturer's instructions. After that, the pellet was resuspended in a cell lysis buffer (a component of the DNA purification kit); proteinase K was added and incubated overnight at 56 °C. The extracted DNA was resuspended in 50 µL of an elution buffer. The DNA concentration was determined spectrophotometri-

Table 1. Primers used for molecular detection of six honey bee viruses and *Nosema* spp.

Primer	Forward and reverse sequences 5'-3'	Product size (bp)	Reference
321APIS	F:GGGGGCATGTCTTTGACGTACTATGTA R:GGGGGGCGTTTAAAATGGAAACAACTATG	321	Martín-Hernández *et al.* (2007)
218MITOC	F:CGGCGACGATGTGATATGAAAATATTAA R:CCCGGTCATTCTCAAACAAAAAACCG	218	Martín-Hernández *et al.* (2007)
DWV	F:TTTGCAAGATGCTGTATGTGG R:GTCGTGCAGCTCGATAGGAT	395	Stoltz *et al.* (1995)
SBV	F:GGATGAAAGGAAATTACCAG R:CCACTAGGTGATCCACACT	426	Tentcheva *et al.* (2004)
CBPV	F:AGTTGTCATGGTTAACAGGATACGAG R:TCTAATCTTAGCACGAAAGCCGAG	455	Ribiere *et al.,* (2000)
ABPV	F:TGAGAACACCTGTAATGTGG R:ACCAGAGGGTTGACTGTGTG	452	Tentcheva *et al.* (2004)
BQCV	F:GGACGAAAGGAAGCCTAAAC R:ACTAGGAAGAGACTTGCACC	424	Tentcheva *et al.* (2004)
KBV	F:GATGAACGTCGACCTATTGA R:TGTGGGTTGGCTATGAGTCA	393	Shimanuki *et al*. (1994)

cally, and the quality of the DNA samples was examined on 1% agarose gel electrophoresis stained with Greensafe premium (Cat. No. MB13201, Nzytech, Portugal). The purified DNA was stored at –20 °C until PCR assay.

For duplex PCR amplification, a region of a small subunit (16S) ribosomal RNA gene was chosen for molecular identification of *Nosema ceranae* and *Nosema apis*. A fragment of this gene was amplified in both *Nosema* species, using primers designed by Martín-Hernández *et al.* (2007) (Table 1).

In addition, a negative control was included for all PCR reactions. As a positive control, cytochrome c-oxidase gene (CoI2) of *Apis mellifera* was used in all studied samples. The sequence of primers used for positive control was CoI2-F (5'-CCTGATATAGCATTTCCTCG-3') and CoI2-R (5'-TGTGAATGATCTAAAGG TGG-3') designed on the base of the known mitochondrial genome of *A. m. ligustica* (Acc. No. L06178, Crozier & Crozier, 1993). The PCR mixtures contained 25 μL of NZYTaq 2× Colourless Master Mix (Cat. No. MB04002, Nzytech, Portugal), 0.4 μM of each species-specific primer (FOR/REV), 1 μL of template DNA and PCR water (Cat. No. E0211-01, EURx Ltd., Poland) in a total volume of 50 μL. All PCR reactions were carried out using a Little Genius thermocycler (BIOER Technology Co., Ltd) under the following conditions: initial denaturation at 94 ºC for 5 min; 30 cycles (denaturation at 94 ºC for 30 s; primer annealing at 50 ºC for 30 s; extension at 72 ºC for 1 min) and final extension at 72 ºC for 10 min. PCR products were visualized on a 2 % agarose gel with Greensafe premium (Cat. No. MB13201, Nzytech, Portugal). The fragment size was determined using GeneRuler™ 100 bp Ladder Plus (Cat. No. SM0323, ThermoFisher Scientific Inc.).

The successfully amplified products were purified by a PCR purification kit (Gene Matrix, PCR clean-up kit, EURx, Poland) and sequenced in both directions by a PlateSeq kit (Eurofins Genomics Ebersberg, Germany).

Honey bee sampling for viruses' detection

Approximately 100 individuals were collected from each colony, following the method described by Chen *et al.* (2004). All colonies were checked for clinical signs. The obtained samples were immediately sent to the laboratory in a cooler bag where they were frozen at –20 °C.

Total RNA extraction and RT-PCR amplification. The frozen samples were crushed in a mortar and were homogenised in a RL lysis buffer (GeneMATRIX Universal RNA Purification Kit, Cat. No. E3598, EURx Ltd., Poland). After homogenisation, the samples were centrifuged for 3 min at 15 000×*g* to remove unhomogenised particles. An aliquot of supernatant was used for extraction of total RNA according to the manufacturer's recommendations. The quality of the extracted total RNA was checked by electrophoresis and spectrophotometry. An average of 2 μg of the total RNA was used for copy DNA (cDNA) synthesis using $Oligo(dT)_{20}$ primers (NG dART RT-PCR kit, E0802, EURx Ltd., Poland) according to the manufacturer's instructions.

The primers used for detection of the viruses (DWV, ABPV, CBPV, SBV, KBV and BQCV) are shown on Table 2. They cover the 3` end of the ORF region of the viral genome. The PCR mixture contained 25 μL of NZYTaq 2× Colourless Master Mix (Cat. No. MB04002, Nzytech, Portugal), 0.4 μM of each virus

specific primer (FOR/REV), and 1 μL of template cDNA in a total volume of 50 μL. In each RT-PCR reaction, positive and negative controls were included. All RT-PCR amplifications were carried out using a Little Genius thermocycler (BIOER Technology Co., Ltd) under the following conditions: initial denaturation at 94 ºC for 5 min; 35 cycles (denaturation at 94 ºC for 30 s; primer annealing at 56 ºC for 30 s; extension at 72 ºC for 1 min) and final extension at 72 ºC for 10 min. PCR products were visualized on 1% agarose gel with GreenSafe Premium (Cat. No. MB13201, Nzytech, Portugal). The fragment size was determined using GeneRuler™ 100 bp Ladder Plus (Cat. No. SM0323, ThermoFisher Scientific Inc.). The successfully amplified products were purified by a PCR purification kit (Gene Matrix, PCR clean-up kit, EURx, Poland) and sequenced in both directions by a PlateSeq kit (Eurofins Genomics Ebersberg, Germany).

RESULTS

Molecular detection of Nosema apis and Nosema ceranae

Duplex PCR produced PCR products in 57 samples out of 168 analysed (33.9% successful amplifications), while 111 samples failed to produce a PCR product (66.1%). There were no PCR products in the negative controls. The results from the obtained sequences confirmed only the identity of *Nosema ceranae*.

From all investigated samples, only *Nosema ceranae* infection was detected. The highest level of infection was observed in North Bulgaria. From all 44 investigated samples, 34 (77.2%) were *Nosema* positive (Table 2; Fig. 1). In the west part of the country (Sofia district), *Nosema*-positive samples were detected in 18 of all 28 studied samples (64.2%). The lowest level of infections was found in the local honey bee samples from the mountainous part of the country (Smolyan district, the Rhodope Mountains).

From all 96 investigated samples, only 5 (5.2%) were *Nosema* positive. Neither presence of *Nosema apis*, nor *N. apis/N. ceranae* co-infections were detected (Table 2). Moreover, the honey bee samples from the lowland parts (Sofia and Rousse districts) exhibited a higher prevalence of *N. ceranae* infection compared with samples obtained from the mountainous part (Smolyan district).

Table 2. Distribution of viruses and microsporidian parasites in honey bee colonies in three regions in Bulgaria (number and % of positive colonies)

	Region					
	Smolyan (South Bulgaria)		Sofia (West Bulgaria)		Rousse (North Bulgaria)	
	n=96		n=28		n=44	
Deforming wing virus (DWV)	+	2 (2.1)	+	3 (10.7)	+	5 (11.4)
Black queen cell virus (BQCV)	–		+	1 (3.6)	–	
Sacbrood virus (SBV)	–		+	6 (21.4)	–	
Acute bee paralysis virus (ABPV)	–		–		+	2 (4.5)
N. ceranae	+	5 (5.2)	+	18 (64.2)	+	34 (77.2)
N. apis	–		–		–	

Fig. 1. Sampling location and distributions of honey bee pathogens. Abbreviations: N. c. – *Nosema ceranae*; DWV – deforming wing virus; ABPV – acute bee paralysis virus; SBV – sacbrood virus; BQCV – black queen cell virus.

Molecular detection and virus prevalence in honey bee colonies.

Four of all six bee viruses were successfully identified by RT-PCR analysis (Table 2). CBPV and KBV were not detected, while the 4 other viruses were found with different frequencies in the investigated regions of the country. DWV was found in 10 honey bee colonies and was with the highest frequency (5.9%). It was detected in all three parts of the country – South Bulgaria (Smolyan; 2, 2.1%), West Bulgaria (Sofia; 3, 10.7%) and North Bulgaria (Rousse; 5, 11.4%) (Fig. 1). SBV was the second most prevalent viral pathogen (6, 3.6%). This virus was found only in West Bulgaria (Sofia; 6, 21.4%) and nowhere in the other regions. In contrast to SBV, ABPV was detected only in North Bulgaria (Rousse; 2, 1.2%), but had a low prevalence (2, 4.5%) in all 44 investigated colonies. This virus appeared to show higher prevalence in the lowland regions of the country. BQCV was with the lowest prevalence, recorded only in West Bulgaria (Sofia; 1, 3.6%) in only 0.6 % from all samples.

As with *Nosema* infection, the honey bee viruses are distributed predominantly in the two lowland regions of the country.

Sequence analysis

The successfully amplified products for *Nosema ceranae* (20 samples) were deposited in GenBank under accession number MG657260. The obtained sequences (DWV – 388 bp, SBV – 417 bp, ABPV – 435 bp and BQCV – 486 bp) were deposited in the GenBank database National Biotechnology Information Center (NCBI) under accession numbers MG599458–MG599464 and MG649495–MG649502.

DISCUSSION

According to many studies, the main causes of honey bee colony losses are associated with the parasitic mite *Varroa*

destructor, some viruses, and nosemosis (Boecking & Genersch, 2008; Botias *et al.,* 2013). In the current study, we investigated the last two factors in the native Bulgarian honey bee *A. m. rodopica* and introduced subspecies reared mainly in the lowland regions of the country. Our results suggest that the local honey bee showed lower prevalence of parasitic and viral diseases compared to other honey bee races.

There are several possible explanations for this possible resistance. One reason for the differences in *Nosema* infection rates between the flat and in the mountainous regions of the country may be differences in the climatic conditions. It is well known that *N. ceranae* infection is associated with colony losses in the Mediterranean regions where the climate is usually characterised by rainy winters and dry, warm to hot summers (Bacandritsos *et al.,* 2010; Soroker *et al.,* 2011). This suggests that climate may influence its virulence (Gisder *et al.,* 2011).

The different climatic conditions may explain the lower prevalence of the local honey bee *A. m. rodopica* compared with bees in flat regions. First, the climatic conditions in the Rhodope Mountains are rather severe in comparison with the flat regions, where the climatic conditions are mild. Moreover, the honey bee colonies from the mountainous part of the country begin to develop rather late (May-June) compared with those from the flat regions (March-April). This is a prerequisite for a more continuous period of *Nosema* exposure.

The issue of selecting honey bee colonies resistant to *Nosema* spp. infection has been successfully addressed in the Rhodope mountains where honey bees undergo selection control as part of the national biodiversity. Beekeepers are encouraged to rear only the local honey bee, and crossbreeding with other races is not allowed. Conversely, in the lowland regions, breeding between drones and honey bee queens from various subspecies is uncontrolled. The latter is likely to make honey bee colonies more susceptible to *Nosema* infection.

It is acknowledged that honey bee viruses are transmitted both vertically and horizontally (Chen *et al.,* 2006), including between and among co-foraging wild and managed bee populations (Furst *et al.,* 2014; Mazzei *et al.,* 2014). The main vector of the most viruses is the parasitic mite *Varroa destructor*. Honey bee virus infections may cause different illnesses or remain asymptomatic (Chen & Siede, 2007).

Bees obtain nutrients supply from nectar and pollen, and adequate nutrition is important for proper immune system function. There is evidence that nutritional status is related to pathogen resistance and/or tolerance (Alaux *et al.,* 2010). Several studies suggest that insufficient protein and low-diversity diets influence negatively bees' ability to defend against pathogens (DeGrandi-Hoffman *et al.,* 2010; Locke *et al.,* 2014; Wheeler & Robinson, 2014). Moreover, it has been suggested that a diverse pollen diet, as opposed to monofloral pollen or additional protein, enhance adult bee immunocompetence (i.e. haemocyte concentration, fat body mass, and phenoloxidase and glucose oxidase activities) (Alaux *et al.,* 2010).

While in the mountainous part of the country the pollen diet is very diverse (consisting mainly of meadow flora), in the lowlands pollen is monofloral (rapeseed, sunflower, lime tree). The latter 3 plants flower one after the other, that is why the flora in this regions is mainly monofloral, which is crucial to the innate immune system function.

Bee health is influenced by a variety of environmental factors, including exposure to agrochemicals. Agrochemicals, including pesticides, herbicides, and fungicides are used widely across a range of crops. Agrochemical exposure sometimes results in acute bee losses as well as sublethal toxicity; therefore there is a lot of concern regarding the role of pesticides, particularly neonicotinoids, in bee declines (Johnson *et al.,* 2010; Krupke & Long, 2015). The majority of studies investigating the effects of agrochemicals on bee health have focused on neonicotinoids. Several studies suggest that exposure to these chemicals increases pathogen abundance (Di Prisco *et al.,* 2013; Doublet *et al.,* 2014). In fact, neonicotinoids are among the most widely used insecticides in the world. Neonicotinoids represent insecticides of a similar mode of action that affect the central nervous system of insects, causing paralysis and death. Besides neonicotinoids, there are many other (non-neonicotinoid – organophosphate, fungicide, etc.) agrochemicals utilised in both agricultural and non-agricultural settings that have received less attention and scientific investigation although they may affect pathogen abundance and bee health (Pohanish, 2014).

In the present study, there is irrefutable proof for the use of agrochemicals in the investigated regions. While in the mountainous part of country (the Rhodope mountains) agriculture is organic and agrochemicals are not used, in the lowland regions they are widely applied. Moreover, there are many more crops in the plain regions, in contrast with the mountainous parts of the country. Thus, it is plausible that, the innate immune system of honey bees is rather weakened in the lowlands, which could be associated with a much higher pathogens rate.

CONCLUSION

In conclusion, there are many factors that influence honey bee colonies health. In this study, the local honey bee *A. m. rodopica* showed an lower prevalence of some viral and parasitic diseases compared with honey bees from areas with mixes of subspecies. Therefore, the climatic conditions, the pollen diet as well as the use of agrochemicals against crops and of chemicals for treating honey bee colonies have a great impact on the immune system of bees. It seems that this native honey bee *A. m. rodopica* in its natural conditions is much more healthy than non-native bees in other regions.

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Correspondence:

Peter Hristov
Department of Animal Diversity
and Resources, Institute of Biodiversity and
Ecosystem Research,
Bulgarian Academy of Sciences,
Sofia, Bulgaria
e-mail: peter_hristoff@abv.bg