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Original Scientific Article

SEASONAL VARIATIONS OF FOUR HONEY BEE VIRUSES IN PUPAE, HIVE AND FORAGER BEES OF CARNIOLAN GRAY BEE *(APIS MELLIFERA CARNICA)*

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Summary: The seasonal dynamic of four honey bee viruses in 18 honey bee colonies from six selected apiaries were studied using a specific reverse transcriptase-polymerase chain reaction (RT-PCR) method. In each investigated colony, pupae, hive and forager bees were sampled and tested for acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV) and black queen cell virus (BQCV) once per month during a 14 month-period. In pupae samples, only BQCV and DWV were found in very low percentages, with no major differences throughout the investigating period. In hive bees ABPV, BQCV, CBPV and DWV were detected, but in lower percentages than in foraging bees where the number of all four viruses was the highest. The BQCV was discovered in 100% of foraging bees and in 94% of hive bees. For the other viruses, seasonal differences were observed in hive bees and foraging bees; however, the highest seasonal variations were detected for ABPV. This study suggests that horizontal transmission of ABPV, BQCV and DWV may occur through contacts between social groups in the direction from forager to hive bees.

Key words: honey bee viruses; RT-PCR; pupae; hive bees; forager bees

Introduction

During the last decade, losses in honey bee colonies were observed worldwide and also in Slovenia, which triggered a great interest in the research to find out the causative agent for these losses. Besides already well-known and established honey bee pathogens, the focus of recent research studies is also on honey bee viral infections (1, 2, 3). The most frequently detected bee viruses are acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV) and deformed wing virus (DWV) (2, 4, 5, 6, 7, 8, 9, 10, 11, 12). In nature, ABPV, BQCV and DWV infect larvae and pupae, as well as adult bees, while CBPV produces symptoms only in adult bees (2, 4). The diagnostics of honey bee virus infections is complicated because the infected honey bee colonies usually do not show clinical signs of the viral diseases (13). In addition, honey bee colonies can be infected with more than one virus at the same time (2, 5, 14, 15). In both the apparently healthy and the diseased bee colonies, multiple viral infections have been reported, suggesting that other pathogens and environmental factors can also play an important role in bee pathology (1, 5, 6, 7, 8, 16, 17). The prevalence and seasonal

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variations for 6 bee viruses in 36 apiaries were reported for samples collected in the pupae stage and the adult bee in the spring, summer and autumn of 2002 in France. During a 1-year period the percentage of viruses was lower among pupae samples in comparison to adult bee samples (2). Viruses can be transmitted horizontally and vertically. In a horizontal transmission, viruses are passed among individuals of the same generation, while vertical transmission occurs with infected queens (4, 9).

The presence of the Varroa (*Varroa destructor*) over the decades has had an important impact on the honey bee viral pathology (10, 11, 12, 14, 18, 19). The Varroa mite is considered as an important vector of the viruses within the colony, the apiary and among the apiaries across long distances (20). The transmission of DWV from the infected to the uninfected bees by the Varroa mite has been experimentally demonstrated and a positive relationship has been shown between Varroa infestation and morphological deformities, such as small body size, shortened abdomen and deformed wings, which has also resulted in the death of the heavily infested bees (20). Nevertheless, it is certain that honey bee colonies simultaneously infested with the Varroa mite and infected with viruses could be the reason for colony losses (15).

Nosemosis is the parasitic intestinal disease of adult bees caused by the protozoan *Nosema sp.*, which is present worldwide. This microsporidian pathogen has a significant impact on health and longevity of the individual honey bees as well as on the overall colony survival and productivity (21). Co-infection of adult bees with CBPV and *N. ceranae* using *per os* or *per cutis* virus inoculation showed increased replication ability of the virus as well as bee mortality (22). The average infectious dose is estimated to be from 20 to 90 spores per bee, but in the acute form of the disease, 30 to 50 million spores can be found in the midgut (23).

In Slovenia, breeding of the indigenous Carniolan gray bee *(Apis mellifera carnica*) is only allowed under the protection of the national law. The seasonal variation of viral infections is not known for different age categories in this honey bee race. In this survey, the presence of ABPV, BQCV, CBPV and DWV in samples of pupae, hive and forager bees from 18 colonies was observed monthly over a 14 month-period. Additionally, the number of spores of *Nosema sp.* and the fall of the Varroa mite were monitored for each colony during this period.

Materials and methods

Field work was carried out in 6 professional apiaries located in 3 different geographical regions in Slovenia (Gorenjska, Primorska and Dolenjska). Colonies were placed on the same location during the entire study and were treated once during the summer time with Checkmite (Bayer, Germany) against Varroa. In each apiary, 3 Carniolan gray bee (*Apis mellifera carnica*) colonies were randomly selected at the beginning of the study. Over the 6-month beekeeping period from April to September 2010 and 2 months from April to May 2011, each apiary was visited by a veterinarian specialist for bee diseases once a month. Each investigated hive was opened and honey bee colonies were checked to assess the clinical signs of the diseases. During visits to each investigated colony brood, hive and foraging bees were sampled for laboratory analysis. The brood was sampled in the pupae developing stage; the hive bees were those that were light grey and collected on the brood comb (nursing bees), while the foraging bees were gathered from the beehive entrance as they returned from the pasture with pollen in the baskets. In total, 419 pool samples of pupae (n=131), hive bees (n=144) and foraging bees (n=144) were collected; each sample consisted of 30 individuals. Samples were then stored below minus 60 °C until their use.

The fall of the Varroa mite was counted every month. For this purpose, screened bottom boards were placed in each hive in the beginning of May 2010 and May 2011; thus, for the first month of each sampling year, the data of infestation with the Varroa mite were missing. Every month, the accumulated debris on the screened bottom boards was removed. In total, 108 units of accumulated debris originating from 18 colonies were taken to the laboratory for a Varroa mite count.

In the laboratory, 419 pool samples were homogenised in 30 mL of RPMI (Gibco, UK) in ULTRA-TURRAX® DT-50 dispersing tubes (IKA, Germany). In each bee sample, the spores of *Nosema sp.* were counted with a standard counting chamber (Neubauer hemocytometer). After homogenisation, the suspensions of samples were centrifuged for 15 minutes at 2500 rpm, and supernatant was recovered. The total RNA was extracted with the QIAamp viral RNA mini kit (Qiagen, Germany), using 140 µL

of supernatant, according to the manufacturer's instructions. Individual RNA samples were tested for the presence of nucleic acids of ABPV, BQCV, CBPV and DWV, using the One-Step RT-PCR kit (Qiagen, Germany), as previously described (24). A reaction mixture without RNA served as the negative control, and a known positive sample of each of the 4 viruses as the positive control. The size of each PCR product was compared to the 100-bp DNA ladder (Fermentas, Germany), and the results for each RT-PCR reaction mix were interpreted as positive or negative, according to the expected size of the DNA fragment (24).

Results

Over the 14-month period of visits and observations of clinical signs, none of the 18 investigated honey bee colonies from 6 apiaries showed evident signs of the diseases. Generally, the lowest average number of 4 viruses for each month was detected in pupa samples, followed by hive bees while the highest average number of viruses was detected in forager bees (Figure 1). The BQCV was detected in 4 of 131 (3%) and DWV in 2 of 131 (2%) pupa samples, while all pupa samples were negative for ABPV and CBPV (Figure 2, Figure 3). In hive bee samples, BQCV was found with a high percent (varied from 89% to 100%) for all months, while DWV was detected between 6% and 44% of samples during the 14-month period (Figure 4). The ABPV was detected in April, May, June and August 2010 and in May 2011 with 22%, 17%, 17%, 28% and 89% positive samples, respectively. The CBPV was found only in 6% of hive bee samples in May 2011. The detection of positive results among hive bee samples revealed different percent for each of the 4 viruses, with the highest for BQCV (94%), followed by DWV (24%), ABPV (22%) and CBPV (1%) (Figure 3). In forager bees, BQCV was discovered in all samples (100%), but for DWV was lower and varied between 33% and 67% during the study period. The highest seasonal variation in the forager bees was observed for ABPV; 0% in April 2011, 6% in September 2010, between 39% and 50% from April to August 2010, with the highest percent of positive samples in June 2010 (83%) and May 2011 (100%). The detected variation for CBPV was from 0% (April, May and July 2010 and April 2011) to 28% positive samples (6% in June 2010 and May 2011, 11% in September 2010 and 28% in August 2010) (Figure 5). The comparison of all the results for 144 forager bee samples indicates that BQCV was detected in 100%, DWV in 50%, ABPV in 46% and CBPV in 6% of samples (Figure 3). Multiple virus infections were detected, with the highest number in forager bees for all months, followed by hive bees, while multiple infections were not detected in pupas (Figure 1). The peaks of the highest average number of multiple virus infections were detected in June 2010, August 2010 and May 2011 (Figure 1).

In 75 out of 108 collected samples (69,4%) of accumulated debris at least 1 Varroa mite was found (Table 1). The number of counted Varroa mites in 3 selected colonies within individual apiaries in the same month was similar. The highest numbers of Varroa mites were detected for all months in apiaries 1 and 3, followed by apiaries 2 and 5, with the highest numbers of Varroa mites counted in August and September 2010. In apiary 6, the highest numbers were counted in May and August 2010, while in apiary 4, the infestation was the lowest through the season, with 0 to 5 detected Varroa mites/per month (Table 1). The peak of Varroa mite infestation was detected in August 2010, with an average of 62 mites per colony. August 2010 was also the only month when all colonies were found infested with the Varroa mite (Table 1). The highest numbers of viruses and Varroa mites were detected in apiaries 1 and 3, while the lowest numbers were detected in apiaries 4 and 6, indicating strong correlation between Varroa mite infestation and number of detected viruses (data not shown).

Pupa samples were free of *Nosema sp.*, except 2 samples in May 2010 (0,2 and 0,4 million spores/bee) and 1 sample collected in May 2011 (1,8 million spores/bee). The comparison of average number of counted spores of *Nosema sp.* for 8 months showed the highest number detected in forager bees and a much lower number among hive bees (Figure 6). Both curves showed a similar dynamic; two peaks were recognised, first in April 2010 and second in September 2010. The average number of spores varied from 1.9 million spores per bee in June 2010 and May 2011 to 20.8 million spores per bee in April 2010 in the forager bee samples and from 0 spores per bee in June 2010 to 3.9 million spores per bee in September 2010 in the hive bee samples (Figure 6).

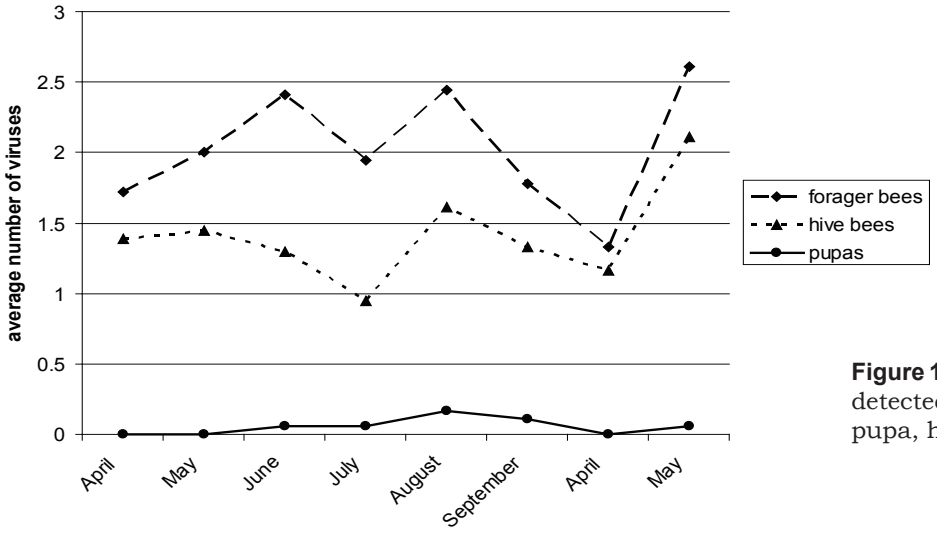

Figure 1: The average number of 4 viruses detected from April 2010 to May 2011 in pupa, hive and forager bee samples

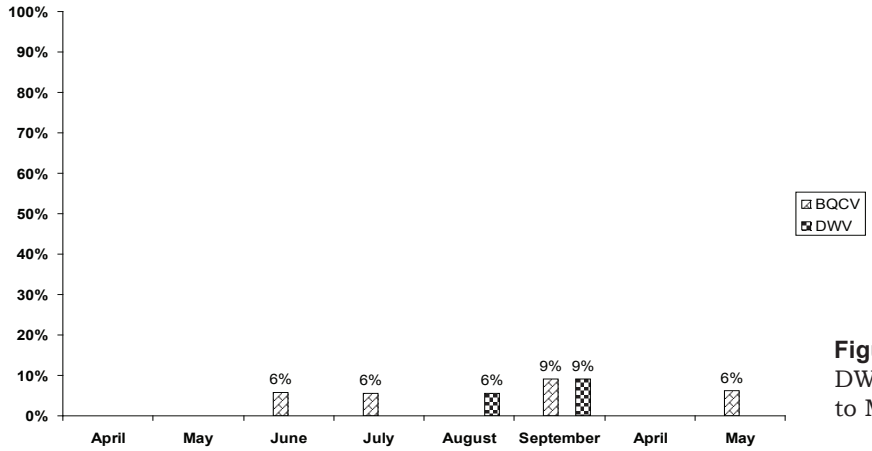

Figure 2: The percentage of BQCV and DWV detected in pupae from April 2010 to May 2011

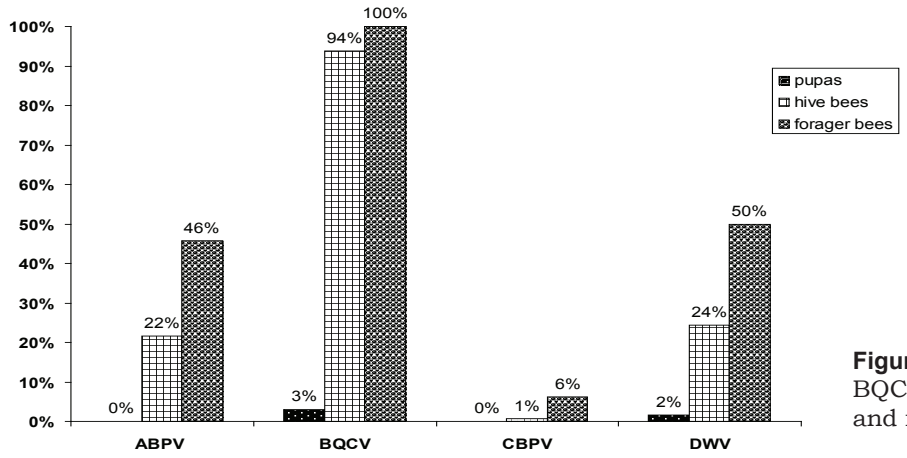

Figure 3: The percentage of ABPV, BQCV, CBPV and DWV in pupa, hive and forager bee samples

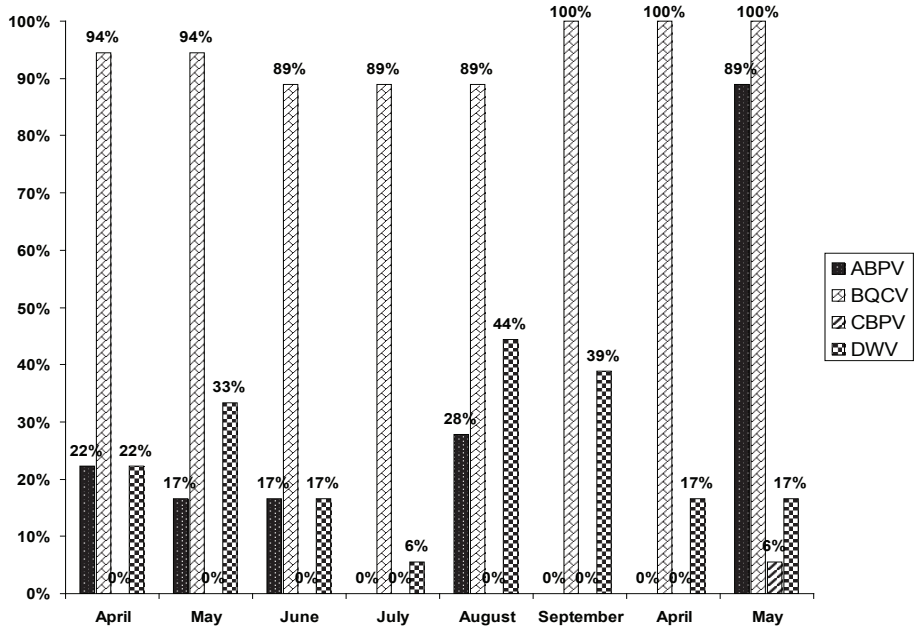

Figure 4: The percentage of ABPV, BQCV, CBPV and DWV detected in hive bee samples from April 2010 to May 2011

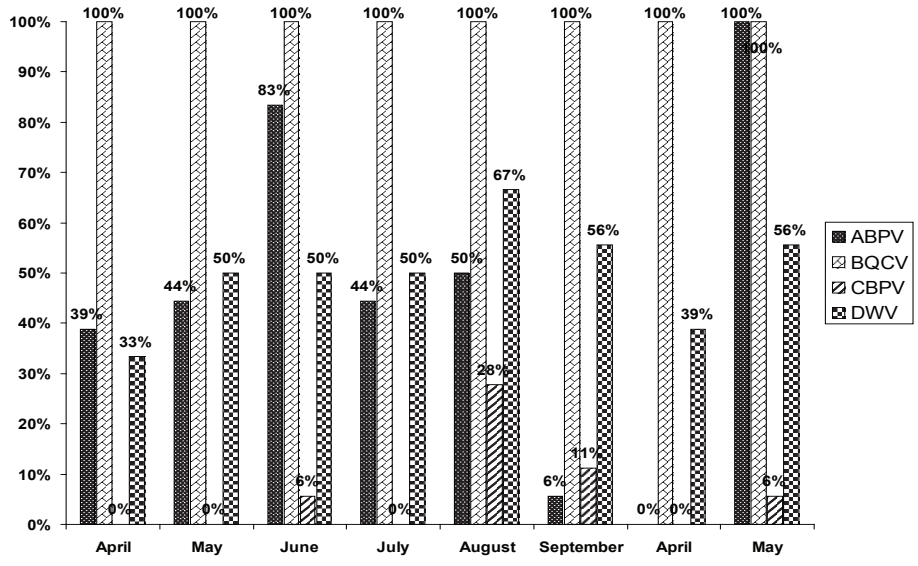

Figure 5: The percentage of ABPV, BQCV, CBPV and DWV detected in forager bee samples from April 2010 to May 2011

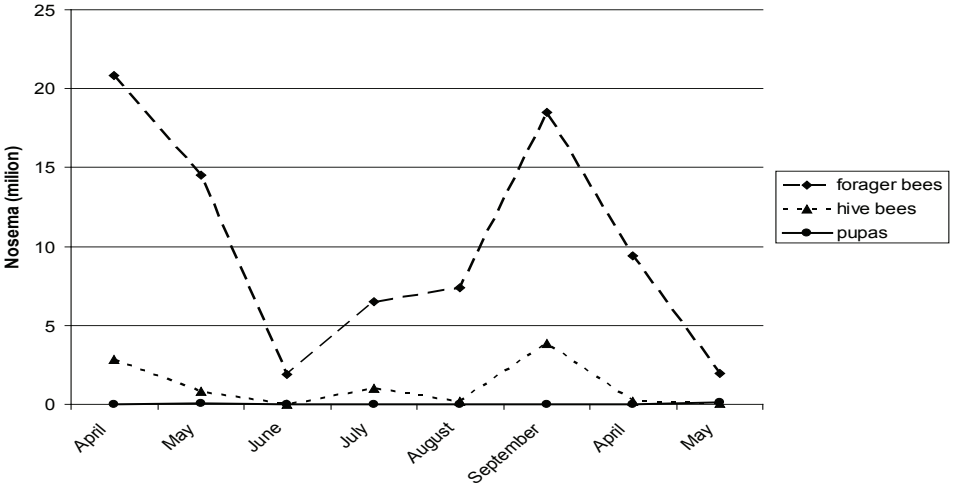

Figure 6: The average number of *Nosema sp.* (million/bee) from April 2010 to May 2011 in pupa, hive and forager bee samples

Table 1: Number of fallen Varroa mite in 18 investigated colonies detected from May 2010 to May 2011

		April	May	June	July	August	September	April	May
apiary 1	**colony 1**		4	11	42	73	21		5
	colony 2		3	49	15	45	35		3
	colony 3		17	23	200	124	30		7
apiary 2	**colony 1**		0	0	0	60	13		0
	colony 2		0	2	7	60	8		0
	colony 3		0	0	0	30	17		0
apiary 3	**colony 1**		20	52	140	200	100		3
	colony 2		21	15	33	100	100		2
	colony 3		18	29	45	300	25		5
apiary 4	**colony 1**		0	0	3	5	4		0
	colony 2		0	1	2	1	2		0
	colony 3		0	0	5	2	0		0
apiary 5	**colony 1**		0	1	1	28	15		0
	colony 2		0	0	0	14	15		0
	colony 3		0	0	4	33	0		0
apiary 6	**colony 1**		4	0	1	2	3		0
	colony 2		40	0	2	20	4		1
	colony 3		35	0	1	18	8		5

Discussion

In the last decade, several studies on the viruses in *Apis mellifera* from different countries have been reported, but there are only a few reports for seasonal variations of virus infections within colonies and apiaries. Viruses can infect all bee casts and developmental stages of the bees in the colony, including eggs, brood and adults. Most of the reports describe the virus prevalence for adult bees, but only limited or partial data are known about the seasonal virus prevalence for different age categories of the bees (2, 5, 15).

This study presents the prevalence of 4 honey bee viruses in pupae, hive bees and forager bees collected in 18 colonies from 6 apiaries over a 14-month period. To our best knowledge, this is the first study on the seasonal variation of 4 viruses in 3 different age categories of the bees, sampled monthly through the whole beekeeping season. In addition, the Varroa mite infection and *Nosema sp.* spores infestation in each investigated colony were screened monthly. The general prevalence of virus infections from the lowest to the highest was pupae < hive bees < forager bees. In our study, pupa samples were almost free of viruses; only in a very low number of samples BQCV and DWV was detected. The observed percent of positive samples detected in pupae was much lower, in comparison to the results of

a similar study published in 2002 in France (2). Although the prevalence of BQCV in hive bees and forager bees was almost 100% in all colonies, pupae were free of BQCV. A pattern similar to BQCV was observed for ABPV and DWV (Figure 3). A very possible interpretation of our results is that the majority of infections with these three viruses are the result of horizontal transmission, through contacts between social groups in the direction from forager to hive bees. The results from this study also suggest that the viral infections are less frequently transmitted vertically and few positive pupae samples are probably the result of the horizontal transmission from the infected hive bees, which were found positive in the same apiary, similar as described previously (4). Our hypothesis for possible seasonal variations of virus infections was proven, but not on the same level for different viruses. The percent of BQCV positive samples was stable through the months confirming that BQCV is present as a persistent infection without any clinical signs of infection in adult bees. Contrary, when comparing the results of ABPV in April and May 2010 to those in April and May 2011, some huge differences were observed. It is not clear why all the colonies were negative in April 2011, while this virus was frequently detected in season 2010 among forager bees in almost 50% of the colonies (Figure 5), but in May 2011, all colonies (100%) were detected as ABPV-positive, as completely opposed to one month ago (April 2011). This observation was also supported with the results of 89% ABPV-positive samples of hive bees in May 2011, confirming the results with the detection of high percent of positive forager bee samples and apiaries during last sampling, in May 2011. The detected seasonal variation for ABPV (0%-100%) in our study was much higher than observed in France (0%-25%) in 2002 (2) confirming high dynamic of this virus in short time period, which can be missed if period between two samplings is too long. In one of our previous studies (25), when the ABPV positive colonies had no clinical signs, low virulent ABPV strain was detected. This was confirmed already by the sequencing of part of the ORF 1 and ORF 2 regions of ABPV-positive samples with the identification of two genetic clusters of ABPV in Slovenia (25). All ABPV samples, except one which was collected from apiaries without clinical signs, belonged to the same genetic cluster 1, but for other apiaries infected with ABPV from cluster 2, clinical signs were described (25). The observed seasonal variations for DWV in forager bees ranged from 33% to 67%, confirming that this virus is constantly detected in all months of the season in almost half of the examined colonies. In our study low percent of pupae samples was discovered as DWV-positive (0%-9%), in comparison to the 95% DWV-positive pupae samples in DWV positive bee colonies in previous study (18). This observation confirmed that the comparison of the results between different studies is not easy and may be misleading if the purpose for both studies was different. In our study, CBPV was found mainly in forager bees, with a prevalence of 6% in June, 28% in August, 11% in September 2010 and 6% in May 2011 in hive and forager bees, while in other months, the virus was not detected. The direct comparison of the percent detected for ABPV, BQCV, CBPV and DWV in healthy forager bees from this study to the two previous publications of virus infections in clinically affected honey bees in Slovenia revealed a similar pattern for all 4 viruses (8, 24), but monthly variations for four viruses can be observed here for the first time for Carniolan gray bee. Our study also confirmed that multiple viral infections were detected through the whole season, with the highest frequency in forager bees, followed by hive bees, with some variations in the number of the detected viruses in different months, while only BQCV and DWV were found in a few pupae samples of the same colonies (Figures 1, 2, 4, 5). It is well known that a high number of the Varroa mite and *Nosema sp.* can weaken the bee's immune system, suppressing the expression of immune-related genes and increasing viral titers, both of which reduce honey bee life span and colony strength (26, 27). The numbers of viruses have been shown to be related to the degree of the Varroa mite infestation (2, 28, 29, 30, 31, 32). Worldwide and also in Slovenia, there are Varroa mite control strategies and programs to keep down the infestation at a low level. All bee colonies in our study were positive for the presence of Varroa mites at various levels. The monthly collected data for the fall of Varroa mites in 18 colonies revealed the major differences among the apiaries, while among the colonies of the same apiary, these differences were negligible (Table 1). The Varroa mite infestation was the highest in apiaries 1 and 3, but still far below the economic threshold and deviated from the results of the other four apiaries, where lower Varroa mite

infestation was detected (Table 1). The number of Varroa mites during each month showed the dynamic and classical relationship to the bee brood development, with increasing numbers of Varroa mites in spring and summer months (from May to July, peaking in August) and a drop in September (Table 1). When comparing the data to present the direct relationship between the number of Varroa mites and the number of viruses detected in each apiary, tendencies were confirmed; apiaries 1 and 3 (with the highest number of Varroa mites) also had the highest numbers of detected viruses, in comparison to those in apiaries 4 and 5 (data not shown). This is consistent with the previous observations that the Varroa mite is an important factor for spreading viruses (ABPV, DWV), but does not necessarily indicates if a colony will survive or collapse the following winter (15).

Monitoring the *Nosema sp.* infestation showed that the highest number was detected among forager bees, while the infestation in hive bees was significantly lower and the pupae were almost free of the pathogen, as expected.

Our study confirmed previous observations that BQCV is associated with *Nosema sp.*, although no symptoms in adult bees were observed if both pathogens were present (33). In the early spring, when infested long-living winter bees were still present in the colony, we observed a clear peak of the *Nosema sp.* infestation in April and a second peak later in September 2010, similar to another authors observation (23). Surprisingly, in April 2011, *Nosema sp.* infestation was much lower than that observed in April 2010, confirming that variations from season to season exist, as previously reported (34).

This study also provides further evidence for the long-lasting co-existence of multiple bee pathogens in apparently healthy Carniolan gray bee colonies, similar as observed before (2). So far neither of published results for viruses, together with the Varroa mite and *Nosema sp.*, were presented. Major differences in the virus prevalence among forager bees, hive bees and pupae collected from the same colonies/apiaries were observed. The results also showed that pupae were almost free of the pathogens, while hive bees were infected with viruses in a lower percentage than forager bees. It is reasonable to believe that these data reflect the horizontal transmission of viruses, as a result of the contacts among bees within the same colony. The Varroa mite and *Nosema sp.* were detected in every month of the sampling period with observed differences in prevalence between apiaries. Neither of these pathogens (the Varroa mite and *Nosema sp.*) was confirmed to be independently responsible for colony losses. Most probably, the results of multiple pathogen infections in different age categories of bees in a colony have pathological and/or synergistic effects on the individual honey bee immune system. Our research is presenting the complexity of the viral infections in Carniolan honey bees, which should be followed by further investigations.

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SEZONSKO POJAVLJANJE ŠTIRIH ČEBELJIH VIRUSOV V BUBAH, TER PANJSKIH IN PAŠNIH ČEBELAH KRANJSKE SIVKE (*APIS MELLIFERA CARNICA*)

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Povzetek: V študiji smo z metodo reverzne transkripcije in verižne reakcije s polimerazo (RT-PCR) ugotavljali prisotnost štirih različnih čebeljih virusov v 18 čebeljih družinah iz šestih izbranih čebelnjakov. V vsaki čebelji družini smo enkrat mesečno v obdobju 14 mesecev vzorčili bube ter panjske in pašne čebele in v odvzetih vzorcih dokazovali prisotnost virusa akutne paralize čebel (ABPV), virusa kronične paralize čebel (CBPV), virusa deformiranih kril (DWV) in virusa črnih matičnikov (BQCV). V vzorcih bub smo BQCV in DWV dokazali v zelo nizkem odstotku, brez pomembnejših razlik v spremljanem 14-mesečnem obdobju. Prisotnost vseh štirih virusov (ABPV, BQCV, CBPV in DWV) smo ugotovili pri panjskih čebelah, vendar v nižjih odstotkih kot pri pašnih čebelah, pri katerih smo ugotovili najvišje število virusov. Prisotnost BQCV smo ugotovili v 100 % vzorcev pašnih čebel in v 94 % vzorcev panjskih čebel. Pri panjskih in pašnih čebelah smo ugotavljali pomembna sezonska nihanja odstotkov pri ostalih treh virusih, najvišja nihanja pa smo ugotovili v prisotnosti ABPV. Ta študija zagotavlja tudi dokaze o možnem horizontalnem prenosu ABPV, BQCV in DWV od pašnih na panjske čebele med neposrednimi stiki med čebelami.

Ključne besede: virusi pri čebelah; RT-PCR; bube; panjske čebele; pašne čebele