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Presence of Known and Emerging Honey Bee Pathogens in Apiaries of Veneto Region (Northeast of Italy) during Spring 2020 and 2021

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Abstract: A progressive honey bee population decline has been reported worldwide during the last decades, and it could be attributed to several causes, in particular to the presence of pathogens and parasites that can act individually or in synergy. The health status of nine apiaries located in different areas of the Veneto region (northeast of Italy) was assessed for two consecutive years (2020 and 2021) in spring, during the resumption of honey bee activity, for determining the presence of known (*Nosema* spp., *Varroa* mite and viruses) and less known or emerging pathogens (*Lotmaria passim* and *Crithidia mellificae*) in honey bees. After honey bees sampling from each of the nine apiaries, *Nosema apis*, *Nosema ceranae*, *L. passim*, *C. mellificae*, ABPV, CBPV, IAPV, KBV, BQCV, SBV, DWV-A, DWV-B and *V. destructor* were investigated either by microscopic observation or PCR protocols. The viruses BQCV, SBV, CBPV followed by *N. ceranae* and *L. passim* were the most prevalent pathogens, and many of the investigated hives, despite asymptomatic, had different degrees of co-infection. This study aimed to highlight, during the resumption of honey bee activity in spring, the prevalence and spreading in the regional territory of different honey bee pathogens, which could alone or synergistically alter the homeostasis of bees colonies. The information gathered would increase our knowledge about the presence of these microorganisms and parasites in the territory and could contribute to improve beekeepers practice.

Keywords: honey bee; Italy; microscopy; *Nosema* spp.; pathogen; PCR; real time RT-PCR; RT-PCR; trypanosomatids; virus; *Varroa*

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1. Introduction

Honey bees (*Apis mellifera*) health status has been an important scientific topic in the last years, and the global decline in populations of both managed and wild honey bees has been reported as one of the most severe ecological and economic problems worldwide [1]. To date, honey bee colony losses recognize no single cause, but the interactions between multiple biotic (e.g., pathogens, bee genetics, and queen longevity) and abiotic (e.g., agrochemical exposure, forage quality, management practices and climate change) factors are contributing to reduce populations of these pollinators [2,3]. While studies on the quality of environmental conditions are giving well-established information about their impact on bee health, the role of pathogens such as the microsporidia *Nosema* spp., trypanosomatids, viruses and the ectoparasitic mite *Varroa destructor* in colony mortality and the relationships between their prevalence and abundance have not been fully elucidated and require further investigation. The beekeeping sector is receiving support from the European Union [4], also co-financed by the member states, with the aim to promote the production and marketing

of its products, given also the growing impact of certain pathogens on the health status of honey bees, for the qualitative and quantitative improvement of agricultural production and natural ecosystems.

The resumption of activity in spring is an important moment for the development of the bee colony and it is influenced by both the interventions carried out by beekeepers in the previous autumn and winter seasons and the pathogens occurrence in the hives. To date most pathogens and parasites affecting honey bees have an almost worldwide distribution [5,6], and they include viruses, fungi, bacteria, protozoa and parasitic mites which, alone or in association [7,8], or as a result of particular environmental or hive conditions, could affect the health of the apiaries.

It is well known that both *Nosema* spp. (*Nosema apis* and *Nosema ceranae*) and several viruses are a major cause of honey bee diseases. *Nosema* spp. can affect the productivity and survival of honey bees altering their longevity and behaviour, brood rearing and pollen collection [9,10]. It was also reported that co-infection by virus and *N. ceranae* in honey bees might be associated with colony collapse disorder (CCD) [11]. Currently, more than 30 viruses have been identified in honey bee populations [6] and multiple viral infections have been reported in bees [12–17]. Six of these viruses can be responsible for severe diseases in honey bees: acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), and sac-brood virus (SBV). These pathogens can attack different developmental stages of the honey bees including eggs, larvae, pupae, adult worker, drones and queens. Their transmission can occur both horizontally between bees and vertically from queen to offspring.

ABPV, KBV and IAPV are closely related dicistroviruses [18,19] that normally persist at a low level of infection within the colony, with no obvious symptoms at the individual or colony level. However, they are extremely virulent when injected into pupae or adults [20]. Considering these features upon injection into the bee haemolymph, it is not surprising that they are usually related to *V. destructor*, which, when feeding on the bee's haemolymph, can transfer ABPV among the colony, causing paralysis of adult bees and death in a short time. While ABPV and IAPV have been reported to result in rapidly progressing paralysis and death of honey bees [21,22], KBV affects both brood and adult bees with no visually detectable clinical symptoms and can be spread between bees and from the queen to her offspring [23].

BQCV is one of the most prevalent and widespread viruses in honey bee colonies, with a constant annual incidence in the adult population. Although BQCV does not cause visible symptoms in infected adult bees, it can kill queen larvae and pupae by turning their cells black [24]. BQCV has been related with *Nosema* spp. infestation and other viruses [25], but it is still unclear how they are linked.

CBPV is an unclassified bipartite RNA virus [26] with a worldwide distribution, above all in Asia, Europe and North America [27–29], causing a rare but severe chronic paralysis. Infected honey bees show abnormal trembling, making them unable to fly, and darker, hairless abdomens [30]. Symptomatic individuals seems to be too weakened for pollination or honey production and often die in few days [21,31], leading to mounds of dead bees outside affected colonies, which sometimes collapse.

SBV and DWV are single-stranded positive-sense RNA virus members of the Iflavirus genus [30,32,33]. SBV was the first virus identified in honey bees and is transmitted to larvae by SBV-infected nurse bees [34], leading the larvae to fail to pupate [35,36], acquire a sac-like appearance and finally die. SBV can also affect adult bees without clinical signs of disease, even if such bees may have a shorter life span [37,38]. The virus may remain viable in dead larvae, honey, or pollen for weeks, so the infection can be further spread from one hive to another through swarming and drifting of the virus-infected colonies.

DWV is transmitted vertically and horizontally by the ectoparasite *V. destructor* [32,39], causing deformed wings and shortened abdomens often observed on honey bees with a reduced life span [23,39,40], associated with bee colony weakening and mortality. Three

genetic variants of DWV were discovered and identified as types A, B, and C, but the DWV-A and -B are the most widespread variants [41,42].

As with viruses, *V. destructor* mite is also largely widespread and it weakens the bees, feeding on the fat body [43] both of adult bees and their brood. Many studies shown that it also acts as a vector for many viruses affecting honey bees [44], in particular DWV, leading to the speculation that viruses and mites had a synergistic negative effect on the honey bee colonies [45,46]. However, the mechanisms that underlie *Varroa*-mediated vectoring and transmission of viruses to honey bees remain poorly understood and the correlation between virus detection and *Varroa*-infestation level is still not so clear [47].

The trypanosomatids *Crithidia mellificae* and *Lotmaria passim* are unicellular obligate parasites that infect *A. mellifera* colonizing the digestive system [48,49]. *C. mellificae* was described for the first time in 1967 [50], while Schwarz et al. [48] recently characterized *L. passim*. These pathogens are currently receiving much attention given that their presence has been correlated with colony losses especially when detected in association with *N. ceranae* [51–54], even if their potential pathogenicity and prevalence have not been fully elucidated [55,56]. However, nowadays *L. passim* appears to be the predominant one in honey bees worldwide, in contrast to *C. mellificae* [57–60].

The purpose of this study was to monitor the health status of nine apiaries situated in the Veneto region (northeast of Italy) during spring of 2020 and 2021, evaluating the presence and prevalence in honey bee colonies of 13 pathogens, known and less known or emergent (i.e., *L. passim*, *C. mellificae*, DWV variants). To date, there is a lack of information on the occurrence and spread in the Veneto region, and generally in Italy, so different honey bee colonies across different geographic areas were studied. In this way, we could stimulate and extend epidemiological studies aimed at investigating the potential relationships between colony health and pathogen occurrence and their dynamics on a larger scale, and possibly compare these results with data originating from colonies in the surrounding European countries [61].

2. Materials and Methods

2.1. Sample Collection

According to the Italian National Beekeeping Registry (as of 31 December 2021), the Veneto region recorded 8470 beekeepers, 15,830 apiaries and 128,592 colonies with a mean density of 0.8628 apiary/km^2.

A total of 180 clinically healthy honey bee (*A. mellifera*) colonies from 9 apiaries (10 hives per apiary) distributed over different geographical areas (plain, hills, mountains) of the Veneto region (northeast of Italy) and belonging to different beekeepers were identified. Each apiary has been marked with a letter from A to I (Figure 1), and a specific form has been prepared for the collection data on beekeepers, on geographical information about the territory in which the apiary was located, on the nearby crops and human activities, on disease and *Varroa* mite infestation, on possible treatments against *Varroa* carried out in the previous autumn/winter season and on previous poisoning due to phytochemical treatments.

The apiary A was located in the mountains, within a natural context consisting of meadows for fodder production (0.6297 apiary/km^2). The apiaries E (1.3595 apiary/km^2), G (1.3813 apiary/km^2), H (1.3595 apiary/km^2), and I (0.7765 apiary/km^2) were located in the hills, mostly in an agricultural environment in which fruit trees (Pomacee, Drupacee, grapevine and olive tree), cereal, forage, horticultural and ornamental flower crops were present, with the exception of the apiary F located in an agricultural/urban/industrial context. The apiaries B (1.3595 apiary/km^2), C, D, and F (1.1209 apiary/km^2) were located in the plain, in an agricultural and/or urban context characterized by the presence of poplars and ornamental flower crops or greenhouses and horticultural, cereal and forage crops or cereal and forage crops (Figure 1).

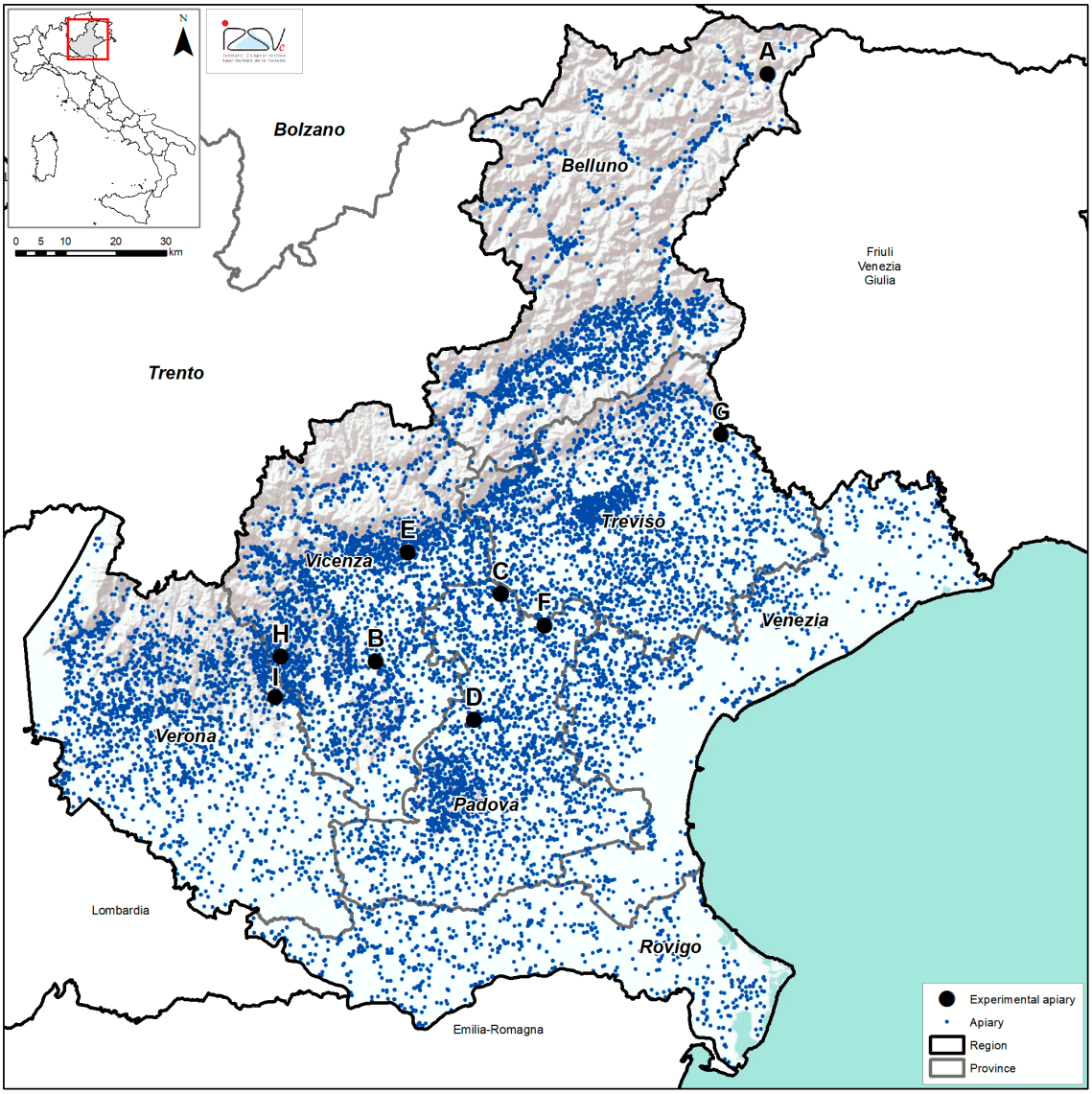

Figure 1. Veneto region (north-east of Italy): distribution of the apiaries involved in the study. Each apiary has been marked with a letter from A to I. Source: Base map from ESRI, USGS, NOAA (ArcGIS Desktop Licence 10.5.1).

Between the end of April and the beginning of May both in 2020 and 2021, about 90 honey bees from each of the 10 hives of each apiary were sampled from the flight board and then stored at −20 °C until analysis. Unfortunately, honey bees were collected only from 171 out of 180 selected hives since in 2020 we received samples from only 1 of the 10 hives of apiary A. *Varroa* infestation was assessed in the field with the powdered sugar test as indicated in the OIE Manual [62]) and detected mites were collected.

Meteorological data were available from the website (www.arpa.veneto.it (accessed on 14 February 2022) of the Environment Protection Agency of Veneto region.

2.2. *Nosema spp.*

For *Nosema* spp. spores detection and quantification, a homogenate of 60 bee abdomens was analysed by light microscopy at 400× according to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals chapter 3.2.4, paragraph B.1.1 [62]. The *Nosema* spp. positive samples were analysed for *Nosema* species (*Nosema apis*/*Nosema ceranae*) identification. DNA extraction was performed from 1 mL homogenate using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with a

lysozyme pre-incubation step. The yield and purity (260/280 and 260/230 nm absorbance ratios) of DNA was determined using the Nanodrop™ One C (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer. The DNA was stored at −20 °C until use. For *N. apis* and *N. ceranae* identification, two different sets of primers described by Martín-Hernández et al. [63] were used. PCR was performed in a final volume of 50 µL using AmpliTaq™ Gold kit (Applied Biosystems™, Waltham, MA, USA), with 200 ng of DNA and containing a final concentration of 0.25 µM for each primers and 0.2 mM of dNTPs. The PCR was carried out using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems™, Waltham, MA, USA), and the thermal cycling profile consisted of an initial denaturation step at 95 °C for 10 min followed by 35 cycles consisting of denaturation at 94 °C for 15 s, annealing at 62 °C for 30 s, extension at 72 °C for 30 s and a final elongation step at 72 °C for 7 min. Negative and positive controls were included in each PCR. Amplification products (218 bp for *N. ceranae* and 312 bp for *N. apis*) were analysed by capillary electrophoresis on LabChip GX Touch HT® (Perkin Elmer, Waltham, MA USA).

2.3. Lotmaria passim and Crithidia mellificae

To detect the presence of *C. mellificae* and *L. passim*, the starting material was the same homogenate of 60 bees' abdomens prepared for *Nosema* spp. spores analysis. DNA extraction and PCR were performed as described in paragraph 2.2. For *C. mellificae* and *L. passim* detection, two different sets of primers described by Bartolomé et al. [64] (Table 1) were used. For *C. mellificae*, a final concentration of 0.25 µM of primers, 0.2 mM of dNTPs and 1.5 mM of $MgCl_2$ were used, while for *L. passim*, the final concentration was 0.3 µM for primers, 0.2 mM for dNTPs and 2 mM for $MgCl_2$. PCR reactions were performed on a Veriti™ 96-Well Thermal Cycler (Applied Biosystems™, Waltham, MA, USA) and the thermal cycling profile consisted of an initial denaturation step at 95 °C for 10 min, followed by 45 cycles consisting of denaturation at 94 °C for 30 s, annealing for 30 s at 58 °C for *C. mellificae* or 68 °C for *L. passim* and extension at 72 °C for 10 s, with a final elongation of 7 min at 72 °C. PCR products of 254 bp and 177 bp of the genes encoding, respectively, for the RNA polymerase II large subunit (RBP1) of *L. passim* and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *C. mellificae* were analysed by capillary electrophoresis on LabChip GX Touch HT® (Perkin Elmer, Waltham, MA, USA).

Table 1. Type of analysis, investigated pathogens and references of primers and probes.

Analysis	Pathogens	References
PCR	*Crithidia mellificae*	[64]
	Lotmaria passim	[64]
	Nosema apis/Nosema ceranae	[63]
RT-PCR	IAPV	[65]
	KBV	[66]
	ABPV	[67]
	BQCV	[68]
Real Time RT-PCR	DWV-A/DWV-B	[69]
	CBPV	[70]
	SBV	[71]

2.4. Honey Bee Viruses

Honey bee samples were analysed for eight honey bee viruses relevant in respect to colony health status: ABPV, CBPV, DWV-A and DWV-B, SBV, BQCV, KBV and IAPV.

From each hive a pool of five honey bees were analysed after homogenization by Tissue Lyser II (Qiagen, Hilden, Germany) (2 cycles, 1 min each, at 30 Hz) in the presence of a 5 mm stainless steel bead. Total RNA extraction was performed on KingFisher Flex Purification System (Thermo Fisher Scientific, Waltham, MA, USA) for automated nucleic acid purification using the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems™,

Waltham, MA, USA), according to the manufacturer's instructions. To detect viral RNA both real time RT-PCR and end point RT-PCR were performed.

For ABPV, CBPV, DWV-A, DWV-B, SBV and BQCV the amplification reactions were carried out on CFX96 Real-Time detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Real-Time One-Step RT-PCR reactions were performed using 250 ng of RNA and the QuantiTect Probe RT-PCR kit (Qiagen, Hilden, Germany) in a final volume of 25 µL with primers and fluorescent probe concentrations of 0.8 µM and 0.2 µM for ABPV, CBPV, SBV and BQCV, respectively, and 0.35 µM and 0.1 µM for DWV-A and 1.2 µM and 0.4 µM for DWV-B. After the initial reverse transcription of RNA at 50 °C for 30 min, the amplification profile consisted of a denaturation step at 95 °C for 10 min following by 40 cycles consisting of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min.

For IAPV and KBV detection, the RT-PCR was performed using the One-step RT-PCR kit (Qiagen, Hilden, Germany) on Veriti™ 96-Well Thermal Cycler (Applied Biosystems™, Waltham, MA, USA) in a final volume of 50 µL containing 250 ng of RNA and 0.4 µM of each primers. PCR products (767 bp for IAPV and 659 for KBV) were analysed by capillary electrophoresis on LabChip GX Touch HT® (Perkin Elmer, Waltham, MA, USA).

Negative and positive controls were included in each PCR. The references of all primers and probes used are reported in Table 1.

2.5. Varroa Mite

For the detection of DWV-A and DWV-B in the *Varroa* samples, a pool of at least 2 specimens per hive was homogenized by Tissue Lyser II (Qiagen, Hilden, Germany) in the presence of a 5 mm stainless steel beads. Total RNA was extracted using the RNA Isolation-NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Samples were analysed for DWV-A and DWV-B using primers, probes and amplification conditions used for the detection of the same viruses in bees (Table 1).

2.6. Statistical Analysis

All statistical analyses were performed using the STATA 17.0 (Stata Corp LLC. College Station, TX, USA) software, and Fisher Exact Test was carried out to determine if there was any association between the pathogens found. It was considered statistically significant if p-value $< \alpha$ adjusted with the number of possible comparisons for each pathogen with other pathogens with which it is possible to calculate the test (pathogens with all positive or negative samples excluded). A 5% level of significance was considered. Main statistical measures, such as median, have been calculated for the quantitative variable number of co-infection, and stratified for the landscape (plain, hills, and mountains).

3. Results

The prevalence of the honey bee pathogens is shown in Figure 2.

3.1. Nosema spp.

All the samples analysed in 2020 and 2021 were negative for *N. apis*. In the first year, of sampling the overall prevalence in all the hives tested for *N. ceranae* was 71.3%, while in the second year, a slight decrease of *N. ceranae* positive hives was observed with an overall value of 64.4%. In 2020, only one apiary (A) had all the hives negative for *Nosema* spp., while in four apiaries (B, E, H, I) all the hives resulted positive for this pathogen. In 2021, *N. ceranae* positive samples were found in all the apiaries with an occurrence ranging between 10% (apiary A) and 100% (apiaries C, D, I). In regards to the *N. ceranae* infection rate, expressed as the number of *N. ceranae* spores per bee, the mean value per apiary was between 3.43×10^5 (apiary F) and 1.32×10^7 (apiary I) in 2020, while in 2021, it lowered to between 2.97×10^5 (apiary B) and 3.42×10^6 (apiary D). The data are shown in Figure 2.

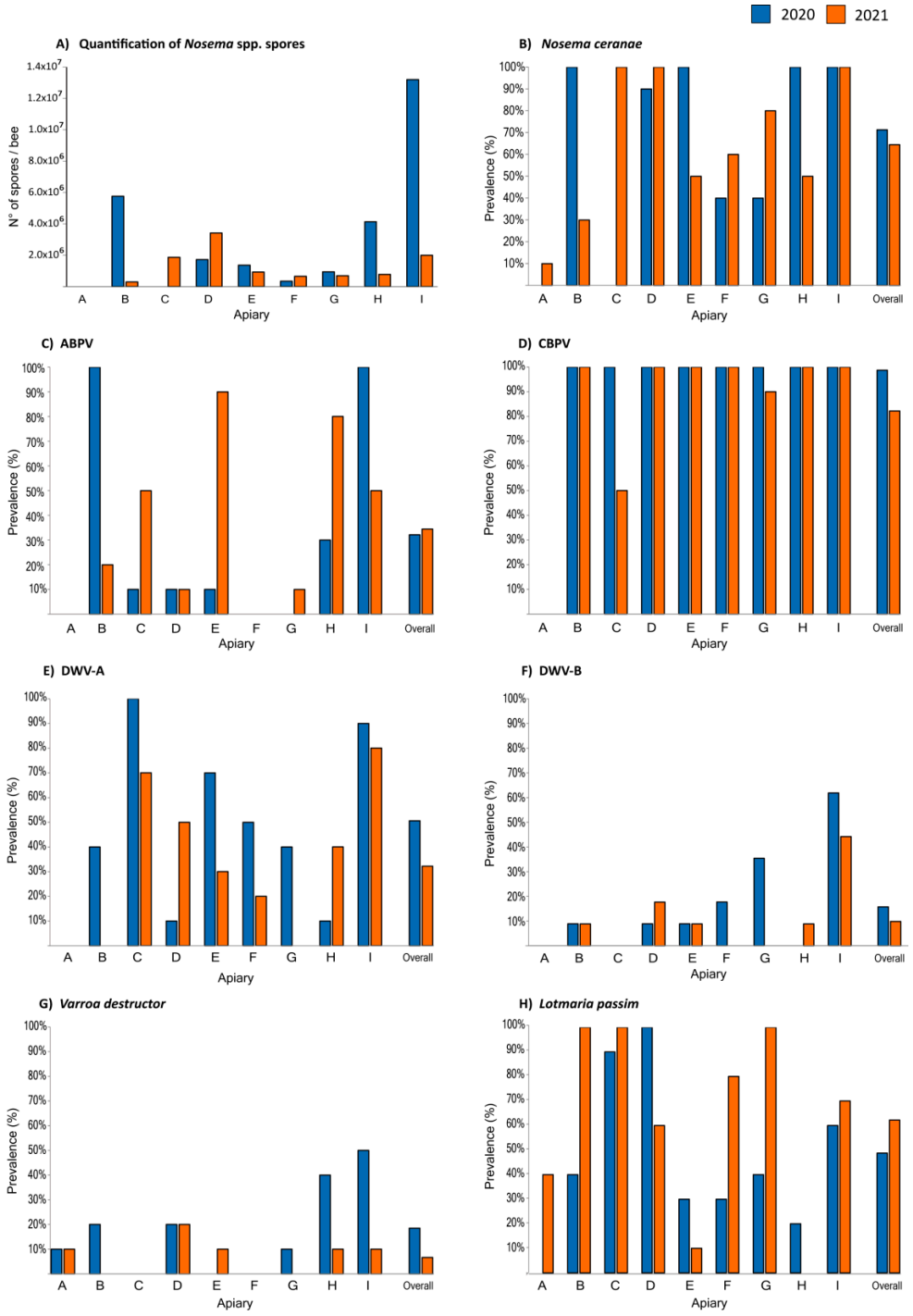

Figure 2. Number of *Nosema* spp. spores and overall pathogens' prevalence in sampled apiaries (from A to I). Panel (**A**): mean number of *Nosema* spp. spores per bee, detected in the sampled apiaries both in 2020 (blue bars) and 2021 (orange bars), is shown. Panels (**B**–**H**): frequencies (in percentage) of *N. ceranae* (**B**), ABPV (**C**), CBPV (**D**), DWV-A (**E**), DWV-B (**F**), *V. destructor* (**G**) and *L. passim* (**H**), detected in the sampled apiaries and in the overall population both in 2020 (blue bars) and 2021 (orange bars), are presented. *N. apis*, IAPV and KBV prevalence are not shown because all the analysed samples were negative. *C. mellificae* prevalence is not shown because positive honey bees were found only in one hive of apiary B in 2020. SBV prevalence is not shown because all the analysed samples resulted positive. BQCV prevalence is not shown because all the analysed samples resulted positive in 2020, and 97.8% of all hives (only two hives in apiary A were negative) resulted positive in 2021.

3.2. Lotmaria passim and Crithidia mellificae

In both years of the study, *L. passim* was detected in almost all of the apiaries analysed with an overall positivity rate of 48.8% in 2020, while an increase was observed in 2021 with an overall value of 62.2%. In 2020, only one apiary (D) had 100% *L. passim* positive hives, while in 2021, the number of apiaries with all the hives positive has increased to three (B, C, G). Concerning *C. mellificae*, it was detected only in one hive of the apiary B in 2020, while in 2021 no positive hives were found.

3.3. Viruses

In general, virus occurrence was higher in 2020 than in 2021. Of the eight viral pathogens tested, KBV and IAPV were not detected in any of the apiaries in both years. SBV and BQCV were the most prevalent viruses detected in honey bees (100% of the apiaries) in 2020, with a very slight overall decrease in 2021 as regards BQCV (97.8%). The other viruses (ABPV, CBPV, DWV-A and DWV-B) affected in different percentages the analysed apiaries (Figure 2). In 2020, the overall prevalence was 32.1% for ABPV, 98.8% for CBPV, 50.6% and 17.8% for DWV-A and DWV-B, respectively. In 2021, viral occurrences observed more or less reflected the trend of the previous year: 34.4% for ABPV, 82.2% for CBPV, 32.2% for DWV-A and 12.2% for DWV-B.

3.4. Varroa Destructor Mite

From the information provided by beekeepers, all the colonies were treated against *Varroa* mite infestation in the previous autumn/winter season (2019 and 2020) by means of oxalic acid-based veterinary medicines. In 2020, *V. destructor* was detected in six out of nine apiaries (A, B, D, G, H, I) with an overall prevalence of 18.5%. Few specimens were found in four apiaries (A, B, D, G), while in the other ones (H and I, located in the hills) the number of specimens varied between 10 and 28. During 2021, *Varroa* was found in five out of nine apiaries (A, D, E, H, I), with an overall prevalence of 6.7%, and only in the apiary A (located in the mountains) the number of specimens was more than three (N = 18). The correlation between the presence of *V. destructor* and the DWV variants was investigated in the apiaries where the number of specimens was equal or more than three: in 2020, in apiaries D, H, I; in 2021, in apiaries A and D. None of the analysed *Varroa* specimens tested positive for DWV-A and DWV-B in the two years of sampling.

3.5. Associations between Pathogen Occurrences

Simultaneous multiple infections with several combinations of different pathogens have been detected in several hives from the nine apiaries (Figure 3).

In 2020, honey bees infected by three different combinations of pathogens were found in 3.7% of the hives, while co-infections with several combinations of four or five or more than five different pathogens were found in 21%, 37% and 38.3% of the honey bee samples, respectively. The most detected co-infections in 2020 were due to the simultaneous presence of: *L. passim* + CBPV + DWV-A + SBV + BQCV in the 12.3% of the hives; *N. ceranae* + *L. passim* + CBPV + SBV + BQCV in the 11.1% of the hives; *N. ceranae* + CBPV + SBV + BQCV in the 9.9% of the hives.

In 2021, only the 5.6% of the hives were co-infected with only two pathogens (SBV and BQCV), while three or four or five different pathogens in several combinations were simultaneously found in 6.7%, 21.1% and 34.4% of colonies, respectively. Finally, more than five pathogens were present in 32.2% of honey bee samples. The most detected co-infections in 2021 were due to the simultaneous presence of *N. ceranae* + *L. passim* + CBPV + SBV + BQCV in the 18.9% of the hives and *L. passim* + CBPV + SBV + BQCV in the 8.9% of the hives. Statistical analyses showed that in both years of sampling the combination *N. ceranae* + *L. passim* + CBPV + SBV + BQCV is present in more than 10% of the hives analysed, with an increase in 2021 compared to 2020. However, in 2020 a significant association only between the presence of ABPV and *N. ceranae* ($p = 0.000$) was found; in 2021, the significant pathogen associations were three and precisely between APV and *L. passim* ($p = 0.004$),

DWV-A and DWV-B (p = 0.004) and DW-A and *N. ceranae* (p = 0.010 significant at a 10% level). No other significant associations were observed in both years.

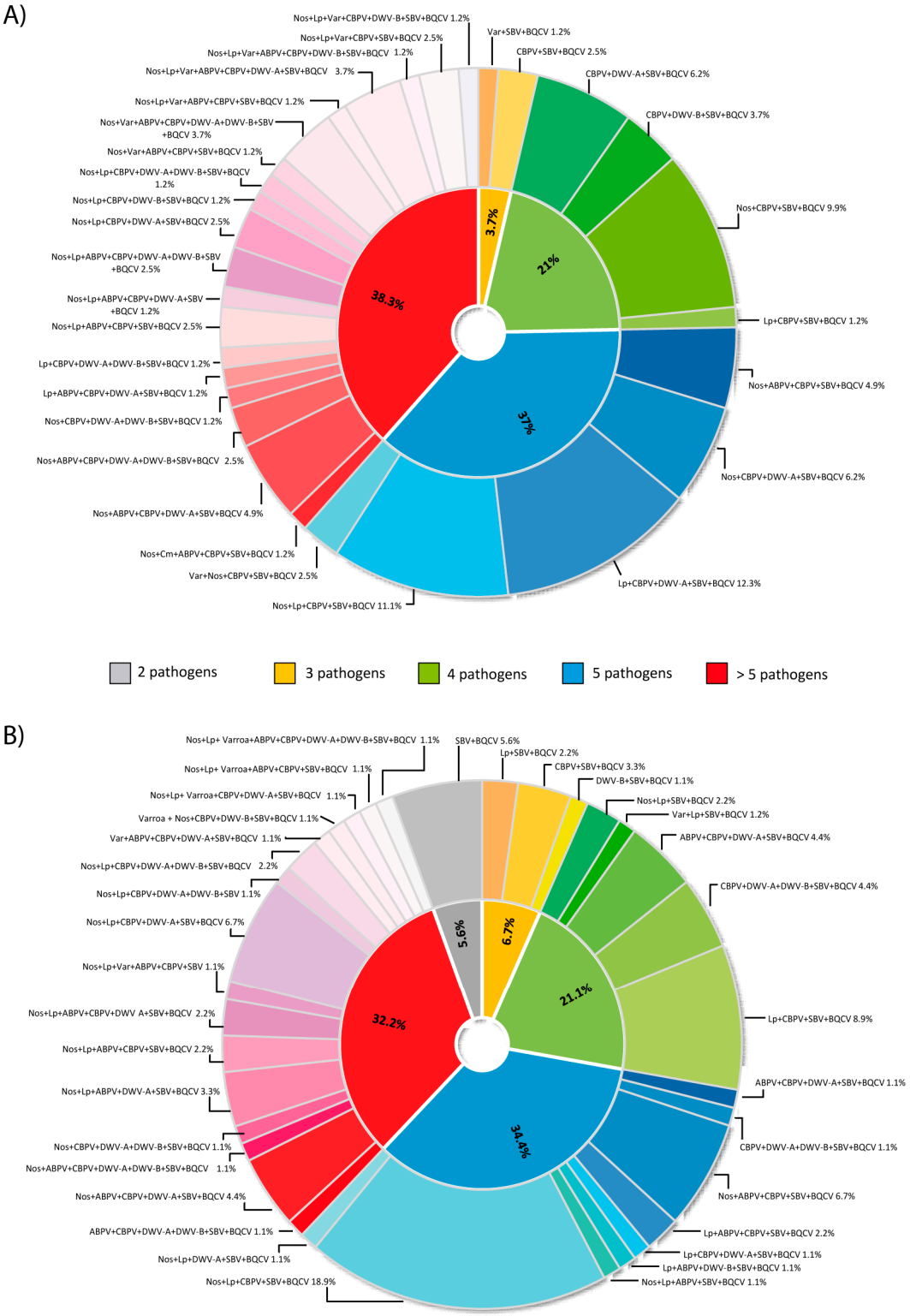

Figure 3. Percentage and type of multiple infections found in all nine apiaries in 2020 (**A**) and 2021 (**B**). Co-infections with 2 pathogens are shown in the shades of grey, with 3 pathogens in the shades of yellow, with 4 pathogens in the shades of green, with 5 pathogens in the shades of blue, and with 6 pathogens or more in the shades of red.

Median values of the number of co-infections have been calculated and stratified for the landscape (plain, hills, and mountains). From the data obtained in 2020, a median decrease from the plain (5.3) and hills (5.7) to the mountains (3.0) could be observed. Similarly, in 2021, co-infections detected in the mountain environment had a lower median value (2.7) than those of the plain (5.1) and hills (5.3).

4. Discussion

In recent years, numerous events of honey bee mortality or beehive depopulation have been reported in Europe and other countries around the world. Several causes have been identified as responsible stressors, in particular, the presence of different pathogens and parasites that can act individually or in synergy. During the last years, monitoring plans have, therefore, been set up at national level to know the health status of bee colonies and, in Italy, the ApeNet (2009–2010) and BeeNet (2011–2014) projects and other studies were established to monitor hundreds of apiaries distributed over almost the entire Italian territory in order to assess the health of *A. mellifera* [28,72,73].

The resumption of activity in spring is an important moment for the development of the bee colony and is influenced by both the interventions carried out by beekeepers in the previous autumn and winter seasons and the pathogens presence in the hives. In order to collect information on the health status of nine apiaries situated in the Veneto region, in northeast of Italy, we investigated in spring the presence, distribution and prevalence of eight viruses (ABPV, CBPV, DWV-A, DWV-B, SBV, BQCV, IAPV and KBV), fungi (*Nosema* spp.), trypanosomatids (*L. passim* and *C. mellificae*) and *Varroa* mite.

A total of ten pathogens (*N. ceranae*, *L. passim*, *C. mellificae*, ABPV, CBPV, BQCV, SBV, DWV-A, DWV-B, *V. destructor*) were found in spring 2020 and 2021, even if all the honey bees analysed for each year showed no clinical symptoms of disease.

BQCV and SBV were the most prevalent pathogens over the two years in all the apiaries investigated (Figure 2). This not only confirm what has already been shown in studies previously conducted in many European countries as Italy [28], Austria [74], Belgium [75,76], Germany [1], Spain [58], Denmark [77] and France [78], but the infection levels of these viruses in Veneto was higher than the level reported in the literature. SBV is the most widely distributed of all honey bee viruses [36]. As reported by Morawetz et al. [79], high prevalence of SBV is frequent in European honey bee colonies, particularly in spring and summer, when the colony is growing most rapidly and large numbers of larvae are available [78], and it is generally higher both in symptomatic and asymptomatic honey bees [80]. The BQCV infection mainly affects queen larvae and pupae instead and has the highest incidence in spring and early summer; BQCV infected adult bees normally do not exhibit outward disease symptoms [78]. Moreover, several previous reports linked BQCV disease outbreaks with infection of *N. apis* [74,78,81] and *N. ceranae* [25,82–85], suggesting that these pathogens can act synergistically. Even if many apiaries analysed in this study showed co-infections of BQCV with *N. ceranae*, no statistically significant association was found, since BQCV infection was always present in almost all the hives.

As already mentioned above, *N. ceranae* spores were detected, even in considerable number, in several honey bee colonies while *N. apis* was never found. The overall prevalence values of *N. ceranae*, 71.3% in 2020 and 64.4% in 2021, respectively, confirmed the higher diffusion of *N. ceranae* previously reported in different Italian regions [86–88], in other European countries [53,58,89,90] and worldwide [51,63,91–93]. The high prevalence of *N. ceranae* together with the absence of *N. apis* infection corroborates the findings of other authors that *N. ceranae* is definitely spread in Italy and has basically replaced *N. apis* [86]. The hives of apiary A were the only colonies with the lower levels of *N. ceranae* infection, but while in 2020 only one hive has been analysed, since the beekeeper was unable to sample a sufficient number of bees, in 2021 only 10% of the hives were positive to *N. ceranae*. All the hives of apiary C tested negative to *N. ceranae* in 2020, whereas in 2021 all of them were positive. As regards the others apiaries, B, E and H, they halved the prevalence of *N. ceranae* from 2020 to 2021, while the apiaries D, F and G showed an increase in the

number of positive colonies. Only apiary I kept at 100% *N. ceranae* prevalence in both years. Moreover, the mean value of the number of *N. ceranae* spores per bee per apiary was higher in 2020 than in 2021. Given that *N. ceranae* could negatively affect honey bee health causing weakening and depression of the immune system [94] of insects, favouring viral infections or under certain conditions bring the colony to collapse [95], the presence of a significant association between *N. ceranae* and other pathogens was analysed in this study. The data obtained from statistical analysis of the observed infections highlighted particular significant associations between *N. ceranae* with ABPV and the DWV-A variant. In addition, *N. ceranae* infected colonies tested positive to SBV and/or CBPV and/or BQCV, supporting the fact that *N. ceranae* occurrence could increase honey bees susceptibility to other pathogens [65].

Besides *N. ceranae*, the prevalence of trypanosomatid *L. passim* was very high in both years: indeed more than half of the analysed hives tested positive to *L. passim* (51.3% in 2020 and 62.2% in 2021, respectively) and in 2021 its occurrence increased in all apiaries, except for apiaries D, E and H, confirming its greater diffusion in the world [48]. Although many researches have demonstrated that *L. passim* can be present with other pathogens too and especially a relationship between *N. ceranae* and *L. passim* have been reported [96] as well as between *L. passim* and viruses, making plausible synergistic effects [51], the results here reported did not show a significant association of *L. passim* with *N. ceranae*, but it was significantly associated with ABPV. How *L. passim* could influence the occurrence and virulence of the other pathogens in the host is unknown and requires further studies. The other protozoan *C. mellificae*, recently correlated with colony losses in the USA and Belgium, was often detected in association with *N. ceranae* [51,53,97]. However, in the present study it was found only in one hive of the apiary B in 2020.

Several studies reported interactions, sometimes controversial, between *N. ceranae* and DWV virus [27,96,98]. Our statistical analysis confirms a significant association between *N. ceranae* and DWV-A variant in both years of sampling. DWV was described as the most prevalent virus in Europe [2] and it has been suggested that its widespread was most probably due to the *Varroa* mites infestation [99], which could be a new viral transmission route, in particular in summer and autumn than in spring [78,79], causing elevated viral loads [45,100,101] and selecting for DWV variants [102]. About these variants, three were mainly reported, each one with a different prevalence [42]. DWV-A was initially the dominant genotype, but over the years, DWV-B prevalence has increased and numerous studies reported now DWV-B as the dominant variant in Europe [103]. DWV-B has been considered a more virulent variant than the DWV-A genotype [41] and mostly associated to *Varroa* mite infestation, whose transmission could exert strong selection on DWV genomes [104], favouring DWV-B. However, little is still known about the prevalence of DWV variants in Italy. The data provided by this study reported overall prevalence values of DWV-A variant ranging between 50.6% (2020) and 32.2% (2021) of hives, while for DWV-B between 17.8% (2020) and 12.2% (2021). In particular, apiary I kept high levels of DWV infection in both years, and this apiary was the one in which most of all investigated pathogens were found. Except for the apiary A, DWV-A variant was found in 2020 in all the apiaries, while for DWV-B variant apiaries A, C and H resulted negative. Interestingly, the prevalence of DWV-A decreased in 2021 in all the apiaries found positive the previous year, except in apiary A, which still remained negative, and apiaries D and H. DWV-B, instead, behaved in a heterogeneous way among all the apiaries (except in apiaries A and C, with no positive samples found in both years) with an overall slight decrease from 2020 to 2021. Although a strong connection between DWV-B and *Varroa* was previously demonstrated [44,105,106], no significant association was found in this survey even if the overall prevalence of *Varroa* in the sampled apiaries declined in 2021 similarly to DWV-B.

Regarding the other viruses investigated in this study, IAPV and KBV were not detected, in accordance with their distribution, which is predominant in Israel, Australia, Asia and USA and less prevalent in Europe [2,22,28,33,65,78,94], while ABPV and CBPV were observed in almost all the nine apiaries. As reported by Berényi et al. [74], since

ABPV, IAPV and KBV share a high level of genetic similarity (the high virulent "AKI complex"), it is supposed that these viruses derive from a common ancestor and then evolved and distributed independently in different geographical areas around the world. This hypothesis is also reinforced by the fact that in Veneto region and in Italy in general, as well as in the near Austria, Slovenia and Croatia [74,107,108], most of the reported cases identified ABPV as the predominant viral species instead of KBV or IAPV. The overall prevalence of ABPV found in Veneto region was between 32.1% (2020) and 34.4% (2021), but not in all the apiaries analysed the value increased from one year to the next: if indeed in apiaries C, E, F and G the ABPV positive hives increased with different percentages, in the apiaries B and I there was a decrease of more than half of infected colonies, and in the apiaries A and F the virus has never been detected. It is known that this virus is quite commonly found in apparently healthy families and it plays a decisive role in the sudden and asymptomatic collapse of the colonies, especially in winter. Moreover, it was supposed that ABPV infection, like DWV, might be spread to honey bees by *V. destructor*, but the presence of very low infested colonies in the apiaries analysed suggested that these viruses could replicate and transmit through other ways.

CBPV occurred in eight out of nine apiaries (the mountain apiary A always tested negative in both years) with an overall prevalence of 98.8% in 2020 and 82.2% in 2021. Although CBPV is widespread in honey bees worldwide and probably due to intensive commercial exchange of insects and hive materials [81], these values were very high compared to what was reported in other European countries, such as France (28%), Slovenia (18.3%), Austria (9%), Czech Republic (8%) and Denmark (4%) [74,78,90,108,109], even if the colonies considered in these studies, unlike ours, showed clinical symptoms of viral infection. The appearance of the clinical form of the disease is linked to episodes of overcrowding in the hive, especially in spring or summer, in the event of persistent adverse weather conditions, and/or the consumption of honeydew. No data about this kind of event resulted from the questionnaires provided by the beekeepers, and the honey bees did not show any symptom of paralysis or malaise.

Finally, different pathogens prevalence and distribution were observed when comparing the different landscapes (plain, hill, and mountain) where the apiaries were located. From the data obtained in 2020, a median decrease in the number of coinfections from the plain (5.3) and hills (5.7) to the mountains (3.0) could be observed. However, the number of co-infections in the honey bees reared in the mountain area derived from the analysis of only one hive of the apiary A. For this reason, the three locations could not be evaluated from a statistical point of view. A similar consideration could also be made for 2021 sampling, where co-infections detected in the mountain environment had a lower median value (2.7) than those of the plain (5.1) and hills (5.3), but since they resulted from a single apiary, they were not very informative and would probably need at least one other apiary in the same geographic area to make the data more robust. However, since the treatments against *Varroa* carried out on the colonies in the previous autumn/winter season (2019 and 2020) were the same, the environmental conditions of the mountain (lower apiary density/km^2) may probably have reduced contacts among honey bees of different apiaries and/or drifting, and hence the spread of infectious agents. On the other hand, with the intensive trade and transport of bee colonies, queens and hives from place to place or in general, the application or not of best beekeeping practices could vary the transmission and spread of these pathogens between apiaries.

5. Conclusions

The global decline in the bee population poses a serious ecological and economic threat: honey bees are critically important to the environment, supporting biodiversity with pollination for a wide range of crops and wild plants, and they also contribute to human wealth and well-being. The impact of anthropogenic activities is increasingly reducing their number and vigour worldwide, in particular by reducing their resistance to parasites (viruses, bacteria, fungi, mites) and consequently their efficiency in pollination and

production of honey. The current descriptive study showed that honey bee hives distributed in several apiaries of the Veneto region were infected with more pathogens. Among these, the most prevalent were BQCV, SBV and CBPV viruses followed by *N. ceranae* and *L. passim*, which are currently distributed worldwide. Studies like this, even if conducted on a regional scale, will help advance our understanding of pathogen's behaviour and focus on the effects of the interactions between them and bee colony. In this way, keeping monitor bee colonies not only locally or nationally but globally, we could promptly intervene on potential problems that could arise and combat honey bee diseases taking in appropriate consideration the environment and the consumer's health.

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