Detection and characterization of Deformed wing virus (DWV) in apiaries with stationary and migratory management in the province of Entre Ríos, Argentina

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Abstract

The deformed wing virus (DWV) is a highly prevalent pathogen that affects honeybees and is associated to colony losses. In Argentina, the Entre Rios province possesses a floral diversity that allows beekeepers to perform migratory or sedentary management. The aim of this work was to investigate the effect of both types of management on the prevalence and abundance of DWV and to characterize the DWV variant present in the study area.

In migratory apiaries, 86.2% of the colonies gave a detectable result to DWV at the beginning of the season (September 2018), and 62% at the end of the season (April 2019). On the other hand, DWV was detected in 44.12% and 62% of sedentary samples, at the beginning and at the end of the season, respectively. The highest viral loads were obtained from migratory samples collected in September. DWV presence and abundance were associated with migratory management and the time of sampling. The virus was also detected in the brood, mites and pollen from the brood frame. Sequence analysis from migratory and sedentary samples revealed a unique DWV variant (DWV-A).

Migratory activity has become a common approach to maximize honey production. However, this practice has negative consequences for colony health and susceptibility to DWV.

Introduction

Argentina is one of the most important honey producers and exporters worldwide. It is well reported that honeybee (*Apis mellifera L.*) productivity is threatened by numerous parasites and pathogens such as viruses, bacteria, protozoa, and mites [1]. Among the pathogens that affect honeybee health, viruses are a dynamic group with an growing number of members. Bee viruses cause significant losses in honey production and are associated with high morbidity and mortality in both naïve and wild bees [2–4]. Although viruses usually cause covert infections, under stressing conditions, immunosuppression or nutritional deficiencies, viral infections can produce clinical signs associated with the disease and weaken the colony.

The deformed wing virus (DWV) is one of the most important viruses that affects honeybees due to its worldwide prevalence and association with colony collapse disorder. DWV presents different transmission routes; horizontal transmission is mediated by trophallaxis, cannibalism, cleaning, and saliva, while vertical transmission can be conducted by either drones or the queen. In addition, the mite *V. destructor* acts as an efficient vector when it is parasitizing the honeybee. This virus infects all castes and has been detected in different stages of *A. mellifera* (egg, larvae, pupae and adult). DWV´s overt infections are associated with the appearance of bees with deformities in their wings and a short life expectancy, which is manifested at the colony level by a progressive decrease in the population size [5,6]. DWV belongs to the genus *Iflavirus*, is a ssRNA virus, and three variants have been described, including DWV-A, DWV-B (also known as Varroa destructor-1 virus), and DWV-C. Variant C groups all the sequences resulting from recombination between DWV-A and DWV-B [7–10].

Worldwide, the importance of the honeybees is related to their role in the pollination of agricultural crops [11,12] and wild plant species that enhance landscape biodiversity as well as the production of honey. Pollination activity is particularly important in the case of migratory beekeeping, as crops and wild plants from different regions and flowering seasons benefit from honey bee foraging behavior [13].

A growing body of evidence indicates that the health of commercially honey bee colonies is influenced by multiple biotic and abiotic factors that can act in synergistically [14–16]. These factors include parasites, bacterial and fungal brood pathogens [17–19], viruses[20], the mite *V. destructor* [21] chemicals, poor nutrition, climate, reduced genetic diversity [22], queen failure [23], and management practices such as migratory beekeeping [2,24–31].

The damaging effects of these stressors may be exacerbated by apicultural practices. Migratory colonies have a greater risk of parasitism and infectious diseases than stationary ones and although transhumance is a common practice in many regions, few studies have focused on the potentially harmful effects of this management [32–35]. Particularly, there is a lack of research on the consequences of migratory conditions on bee disease incidence. [28,36–38].

The movement to new pollination locations forces the colonies to adapt to new environmental conditions, including daily oscillations in temperature, humidity, and wind patterns. Additionally, their exposure to pathogen might increase, and infections by new pathogens may occur [39,40]. In fact, transportation and pollination services have recently been proposed to increase the infestation rate and abundance of *Nosema ceranae* and some viruses [27–31,37] in *A. mellifera* worker bees.

Stress experienced during transportation also impairs immunity [27] and increases susceptibility to disease, as it reduces forage diversity by pollinating large monocultures [35,41].

In studies carried out in Western US with bee colonies involved in almond pollination, the association between multiple factors, including pathogen seasonality, pathogen abundance, beekeeping operation, colony population size, and level of mite infestation on honey bee colony health and colony losses was investigating. It was observed that sampling date correlated with pathogen prevalence and abundance [30,31]. Pathogen prevalence was higher in samples obtained after almond pollination; additionally, weak colonies showed higher pathogen prevalence than strong ones [31]. Two other controlled experiments were conducted to test whether viral pathogen and parasite loads increased because of migratory practice for almond pollination. Migratory colonies returned with fewer bees and higher BQCV loads than stationary colonies; however, viral loads became similar one month later. On the other hand, DWV prevalence and loads were higher in the migratory colonies upon their return and remained so until the end of the study. Levels of varroa, however, were similar in migratory and stationary colonies, but the former experienced a decrease in mite loads, possible due to lower number of host bees [29].

In recent reports, Jara et al. (2021)[28] studied *V. destructor, Nosema spp*. and DWV infestation and infection rates before, during and after the migratory operation in Spain. They found an increased

incidence of *V. destructor and Nosema ceranae* and a lower DWV viral load in migratory colonies. In addition, long- and short-term effects of managed migration on pathogen loads and immunity were evaluated in experimental honeybee colonies that were maintained with or without migratory movement. Bees exposed to migratory management during adulthood showed increased levels of the AKI virus complex (Acute bee paralysis, Kashmir bee, and Israeli acute bee paralysis viruses) and decreased levels of antiviral gene expression (dicer-like). Moreover, the seasonal increase in DVW was higher in juvenile bees from migratory colonies than in those from stationary ones [27].

In Argentina, migratory beekeeping is a management practice conducted in search of specific flowering during the season. In the province of Entre Rios, colonies from the south remain in a single location the whole year. In the north, however, most beekeepers start the season with monoculture flowering, move their colonies in the middle of the season to native forest looking for forage diversity, and they return to another monoculture crop at the end of the season. All beekeepers in that region manage commercial honeybee colonies solely for honey production.

The hypothesis tested in our study was that stressors associated with migratory management affect colony health and disease transmission, resulting in a higher infection rate of viruses in the migratory colonies. To test this hypothesis, we evaluate DWV prevalence and abundance in commercially managed migratory and stationary honeybee colonies from the Entre Rios province. Furthermore, we investigated the relationship between DWV abundance and colony population as a proxy for colony health.

Results

Honey bee colony monitoring

Commercially managed colonies from Entre Rios province were monitored at the beginning and at the end of the season (i.e., September 2018 to March 2019). Throughout our study, apiaries with both stationary or migratory management practices experienced neither colony losses nor a decrease in honey production.

Stationary apiaries were situated in Gobernador Macia village, where 6 apiaries were sampled. On the other hand, we selected 9 migratory apiaries located in Villa del Rosario village. These migratory colonies were placed in an area with monoculture crops (citrus) at the beginning of the study. Colonies remained in Villa del Rosario from September to November, throughout the citrus flowering season. Subsequently, they moved in search of wild flowering. Four apiaries transported their colonies 106–189 km to the north (Feliciano villages and Bompland- Corrientes province), while the other 5 apiaries moved 320 km to the south, to Nogoya village. This latter location was situated 60 km from Gobernador Macia, where stationary management was conducted. At the end of the season, in early February, all migratory colonies returned to their starting point in Villa del Rosario, where wintering took place (Fig. 1).

The transport of honey bees was carried out following the requirements of the Argentine Animal Health Authority, which establishes that the colonies must not present any clinical sign associated with *American foulbrood, Aethina tumiday* and *Tropilaelaps sppand.*

Colony population size was used as an indicator of colony health and was monitored at each sampling event. Consequently, the colonies were classified into 3 categories (C1, C2 and C3). A total of 66 hives from stationary apiaries and 79 hives from migratory apiaries were analyzed.

At the beginning of the season, 34 stationary hives were sampled, with 91.2% (31/34) of the colonies classified in category C1 and 8.8% (3/34) in C2. On the other hand, out of the 32 stationary hives analyzed at the end of the season, 65.6% (21/32) belonged to category C1, 9.4% (3/32) to category C2, and 25% (8/32) to category C3. In the first sampling of migratory apiaries, 29 hives were analyzed, with 62.1% (18/29) categorized in C1 and 37.9% (11/29) in C3. At the end of the season, the hives were classified as follows: 50% (25/50) in category C1, 24% (12/50) in category C2, and 26% (13/50) in category C3 (Fig. 2).

Throughout this study, the monitored colonies did not exhibit clinical signs associated with known pathogens. However, in 2 hives under migratory management, adult bees with deformed wings were detected in the brood frames at the beginning of the season.

Varroa and nosema diagnostic:

At each sampling event, live honey bees were obtained to assess the levels of infestation of the Varroa destructor mite and the presence of the microsporium parasite *Nosema spp.*

In all analyzed apiaries, levels of Nosema were below the recommended treatment threshold (1.000.000 spores/ml); nevertheless, sedentary colonies exhibited lower levels of Nosema than migratory ones, both at the beginning and the end of the season (Supplementary material 1).

In the analysis of *V. destructor*, infestation levels were also found to be below the recommended treatment threshold (3%) application in nearly all samples. Only 2 sedentary and 2 migratory colonies showed infestation levels slightly above 3% at the end of the season. The low levels of mite infestation observed can be attributed to the rigorous and synchronized treatment against varroa that beekeepers applied to their colonies in the study area. This treatment involved the use of Almitraz at the beginning of the season and Oxalic acid at the end (Table 1 and Supplementary material 1).

Table 1
Management description in stationary and migratory apiaries throughout the season (September 2018-March 2019).

Sampling	Variables	Stationary apiary	Migratory apiary
Beginning	N° Hives/ N° Apiary	29/5	34/6
	Hive Category	C1: 31 hive; C2: 3 hive; C3:0 hive	C1: 18 hives; C2: 0 hive; C3: 11 hives
	Varroa treatments*	1	1 in each migratory movement*
	Nosema treatments	no	no
	Replacement of queen	yes	yes
	Dietary supplement	pollen pellets and sucrose syrup.	Pollen pellets and sucrose syrup.
	Type of flowering	Native forest	Citrus
End	N° Hives/ N° Apiary	32/6	50/9
	Hive Category	C1: 21 hive; C2: 3 hive; C3:8 hive	C1: 25hive; C2: 12 hive; C3:13 hive
	Varroa treatments	1	1 in each migratory movement*
	Nosema treatments	No	No
	Replacement of queen	No	No
	Dietary supplement	pollen pellets and sucrose syrup.	Pollen pellets and sucrose syrup.
	Type of flowering	Native forest	Eucalyptus grandis

Varroa treatments*: The migratory apiaries during this work carried out 3 treatments, the apiaries applied the treatments at each migratory operation (September, December and March).

The presence of varroa in its reproductive status was also evaluated by sampling the brood whenever possible. In apiaries with both types of management, mites were found either parasitizing the brood or present in the cell but not associated to the brood. Individuals were pooled for further analysis (Table 3 and Supplementary material 2).

Table 2
DWV positive hives assayed by RT-qPCR, according to the type of management and sampling time.

Sample	Management	Season	Positive	%	Total Hive	Median VL (log_{10}RNA copies/µl)
BEES	Stationary	Beginning	15	44.1	34	3,59
		End	20	53.1	32	3,71
	Migratory	Beginning	25	86.2	29	4,46
		End	31	66.0	50	3,82

Table 3
DWV positive pools from brood frames collected during the beginning and end of season and in apiaries with different types of management.

Sample	Management	Season	N° Positive	%	Total Pools
Parasitized Brood	Stationary	Beginning	6	85.7	7
		End	5	55.6	9
	Migratory	Beginning	27	87.1	31
		End	21	91.3	23
Non Parasitized Brood	Stationary	Beginning	7	87.5	7
		End	6	60.0	10
	Migratory	Beginning	18	78.3	23
		End	19	86.4	22
Varroa	Stationary	Beginning	6	85.7	7
		End	6	66.7	9
	Migratory	Beginning	25	86.2	29
		End	20	90.9	22
Pollen	Stationary	Beginning	0	0.0	2
		End	2	28.6	7
		Wintering	1	25.0	4
	Migratory	Beginning	0	0.0	4
		End	0	0.0	5
		Wintering	7	70.0	10

DWV prevalence and abundance:

The presence and abundance of DWV was assessed using RT-qPCR. In this study, DWV point prevalence was defined as the proportion of colonies that tested positive at each sampling event, while DWV abundance referred to viral RNA abundance quantified by RT-qPCR. DWV was detected in all study areas and in all types of samples, including adult bees, brood, mites and pollen. However, DWV point prevalence and abundance in honey bee colonies varied depending on the sampling date and beekeeper operations. The highest number of positive colonies (86.2%; 25/29) was obtained from honey bee samples with migratory management, collected in September when colonies were located in Villa del Rosario. The viral loads (VL) in those positive colonies had a median of 4.46 log10 RNA copies/µl and reached a maximum value of 10,37 log10 RNA copies/µl. Meanwhile, in stationary apiaries 44.12% (15/34) of colonies tested positive for DWV detection and had a VL median of 3.59 log10 RNA copies/µl.

Immediately after returning from native flowering, migratory colonies showed a decrease in the number of positive colonies. At that time, colonies under both types of management had similar DWV point prevalence and VLs (Table 2, Fig. 3)

The presence of DWV was also evaluated in brood. For this purpose, samples were pooled and categorized as parasitized brood, non-parasitized brood and brood-associated-varroa. Polen samples from the brood frames were also pooled and examined.

At the beginning of the season, a high percentage of positive samples were detected in parasitized brood, non-parasitized broods and varroas, with similar values in both migratory (87.1, 78.3 and 86.2%, respectively) and stationary colonies (85.7, 87.5 and 85.7%, respectively). When comparing the number of positive samples over the course of the season, a decrease in the percentage of positive pools was observed in sedentary colonies from the beginning to the end. In contrast, the values increased in brood and varroa samples when migratory management was carried out (Table 3).

Pollen samples obtained from the brood frames were also tested for the presence of DWV and 10 out of 32 samples resulted DWV positive. Notably, DWV was detected in samples collected in July, suggesting that the virus remained and/or circulated in the colonies during wintering (Table 3 and Supplementary material 2).

Association of DWV presence and monitored factors.

The relationship between the type of management and various factors, including, DWV point prevalence, the presence of other pathogens, colony health, nutritional state, dietary supplementation, and time of sampling, was further analyzed.

At the beginning of the season, the number of DWV-positive samples was significantly higher in migratory colonies than in sedentary ones ($p = 0.0005$). Significant differences between both types of

management were also observed at that time when analyzing hive category ($p = 0.0002$), nosema infestation ($p = 0.0252$) and nutrition ($p = 0.0551$).

On the other hand, at the end of the season, nosema infestation, nutrition and dietary supplement were the variables that showed significant differences between migratory and sedentary colonies (Table 4).

Table 4
Summary statistics of each variable on beginning and end seasons for Migratory versus Stationary management.

Season	Variable	Level	Stationary apiary	Migratory apiary	χ^2	p-value
Beginning	DWV detection	Positive	0,44	0,86	11,96	**0,0005***
		ND	0,56	0,14		
	Hive Category	1	0,91	0,62	17,16	**0,0002***
		2	0,09	0		
		3	0	0,38		
	Varroa Infestation	ND	0,71	0,79	3,98	0,2633
		Low	0,09	0		
		Medium	0,20	0,17		
		High	0	0,04		
	Nosema Infestation	ND	0,91	0,69	5,01	**0,0252***
		Weak	0,09	0,31		
				0,62		
	Nutrition	R	0,47	0,35	5,80	**0,0551***
		G	0,35	0,03		
		VG	0,18			
	Dietary supplement	Yes	1	1	0,40	0,5287
		No	0	0		
End	DWV detection	Positive	0,53	0,66	1,36	0,2437
		ND	0,47	0,34		
	Hive Category	1	0,66	0,5	3,14	0,2082
		2	0,09	0,24		
		3	0,25	0,26		
	Varroa Infestation	ND	0,66	0,72	1,77	0,6221
		Low	0,06	0,1		
		Medium	0,22	0,16		
		High	0,06	0,02		

Season	Variable	Level	Stationary apiary	Migratory apiary	χ^2	p-value
	Nosema Infestation	ND	0,88	1	4,14	**0,0418***
		Weak	0,12	0		
	Nutrition	R	0,19	0	21,72	**< 0,0001***
		G	0	0,36		
		VG	0,81	0,64		
	Dietary supplement	Yes	0,34	1	44,11	**< 0,0001***
		No	0,66	0		

Results of Pearson's Chi-squared test for each variable among Migratory and Stationary management on each season (beginning and end). Significant differences were denoted by "*" at alpha < 0.05. Proportions for each level are shown on the different variables.

Variables with significant differences between migratory and sedentary colonies (DWV point prevalence and Nosema infestation) were evaluated in a generalized linear mixed effect model (GLMM). The migratory management at the beginning of the season significantly increased (p = 0.0274) the probability of DWV presence with an increase factor of 2% (OR = 2.038); in the case of nosema, the probability of infestation was not found to be related with the type of management in the final model (p = 0.9).

A PCA analysis, considering variables such as the number of frames with bees, varroa and nosema infestation rate, and DWV viral load at the beginning and end of the season for both management types, allowed us to assess the relative weight of each variable in the clustering of the samples throughout the study. The eigenvalues of the two principal components explained 64,1% and 67% of the overall variability, as shown in Fig. 4A and 4B, respectively. The DWV VL at the beginning of the season in migratory management was the variable with the strongest influence on the dispersion of the colonies in the PCA (Fig. 4A), whereas the dispersion of the stationary colonies was not significantly affected by these variables (Fig. 4B).

Given these results, a generalized linear mixed effect model (GLMM) was conducted to analyze the influence of each variable (number of frames with bees, varroa and nosema infestation rate, and DWV viral load) on the management at each time of sampling. A positive association between VLs and migratory management was found at the beginning of the season (p = 0,0588) with an increase factor of 1% (OR = 1,13) (Table 5).

Table 5
Explanatory factors for Migratory management at the beginning and the end of the season.

Season	Variable	Estimate (SE)	Odds	Z-value	p-value
Beginning	Intercept	-5,02 (0,98)	0,00	-5,10	**< 0,0001**
	DWV	0,13 (0,07)	1,13	1,89	**0,0588**
	Bees frames	-0,15 (0,10)	0,86	-1,60	0,1097
	Nosema Infestation	4,1E-06 (5,1E-06)	1	0,80	0,4209
End	Intercept	-5,36 (0,60)	0,00	-8,85	**< 0,0001**
	DWV	0,01 (0,09)	1,01	0,13	0,8935
	Bees frames	-0,02 (0,07)	0,98	-0,34	0,7354
	Nosema Infestation	2,5E-06(3,3E-06)	1	0,75	0,4528

GLMM with binomial distribution for migratory management, random factor 'apiary'. n = 145 colonies, SE = standard error.

Pollen identification:

Pollen samples were useful in identifying the floral resources from which the colonies benefited, providing evidence of clear differences between the two types of management. In sedentary apiaries, the most abundant species found in bee bread were native ones. At the beginning of the season, available resources came from native (e.g.: *Schinus, Celtis*) or exotic (e.g.: *Gleditsia*, *Melia azedarach*) trees or shrubs, or adventitious (i.e.: *Trifolium repens*, *Brassicaceae*) and cultivated (e.g,: Vicia) herbaceous plants. By the end of the season, the pollen found primarily belonged to native species such as the *Trithrinax campestris* palm, shrubs (*Baccharis*), or herbs (*Senecio, Grindelia, Bidens*) with a lesser extent contributions from cultivated forage such as alfalfa (*Medicago sativa*).

For migratory apiaries, at the beginning of the season, the most abundant resource came from Citrus, with a smaller contribution from native plants, both herbaceous (e.g.: Senecio and *Cyperus)*, and arboreal (e.g.: T. *Myrcianthes cisplatensis*). Due to the limited floral diversity offered by monoculture environments, beekeepers needed to move their apiaries to landscapes with more diversity, provided by native species. However, by the end of the season, Eucalyptus plantations became the primary floral resource for colonies in those areas, with additional contributions from native species like *Baccharis and Trithrinax campestris* (Supplementary material 3).

Phylogenetic analysis

To identify the DWV variant circulating in the study areas, we selected 60 samples from sedentary and migratory apiaries, which included adult bees (n = 28), brood (n = 14), varroa (n = 11) and pollen (n = 7) (Supplementary material 4). Additionally, to enhance the sequence dataset from Argentina, DWV-positive

samples from apiaries located in the province of Buenos Aires (a region with the highest number of bee-producing colonies) were included.

The phylogenetic analysis revealed a strong geographic association, where all Argentinean strains belonged to DWV type A and clustered together in a monophyletic and highly supported branch (bootstrap = 100) (Fig. 5A).

Upon conducting a thorough analysis of the Argentinean sequences, it was observed that strains from different management types (stationary and migratory) in two zones with varying floral resources diverged and formed clusters amongst themselves. Specifically, the strains from Villa del Rosario (migratory management) were grouped together in three well-supported clades (clades 1, 2, and 3). Clade 1 primarily comprised of samples from migratory apiaries, including strains collected at the beginning and end of the season derived from adult bees, brood, or varroa mites. Notably, a distinct branch emerged, uniting strains isolated from stationary hives at the end of the season, which had interacted with migratory hives. Clade 2 included samples from both migratory and stationary apiaries, irrespective of the sampling time. This lack of clustering could be attributed to the sharing of feeding sites during a specific period. Clade 3 included strain BA-203-2015 from a stationary apiary located in Buenos Aires, and strains from migratory apiaries collected at the beginning and end of the season. Notably, these migratory apiaries ventured into the border region between the provinces of Buenos Aires and Entre Ríos. Clade 4 grouped strains isolated from stationary apiaries in 2015, along with a migratory strain from 2019 which is related to the strain DWV-Chilensis-A1, from Chile. Clade 5 included exclusively strains from stationary apiaries; strains BA-108-2015 and ER-378-2018 belonged to the beginning of the season, while lineages from the end of the season originate from samples collected in Entre Ríos in 2019. Clade 6 primarily comprised of strains from stationary apiaries from Buenos Aires, with the exception of a pollen-isolated strain, ER-P11-2019 (Fig. 5B).

Discussion

Argentina presents diverse landscapes across its territory, enabling the development of beekeeping based on the available floral diversity in each eco-region [42]. Argentine beekeeping is renowned for its production of clear honeys[43], primarily sourced from apiaries. However, the intensification of agriculture has brought about changes in beekeeping production scenarios, leading to the existence of multifloral or monofloral areas. Consequently, beekeepers employ either migratory or stationary management strategies to enhance honey production, depending on flowering patterns.

Recent studies have emphasized the potential drawbacks of migratory beekeeping, particularly concerning bee colony health. This activity increases the risk of acquiring and spreading pathogens and parasites[29], which can have detrimental effects on colony health. Our findings confirm the harmful effect of migratory activity, as we observed a higher number of migratory colonies testing positive for DWV detection compared to sedentary ones.

Regarding the detection of *Nosema spp*, no significant differences were observed between the type of management or the time of sampling. Only 12% (18/145) of the samples analyzed presented a spore count, which, in all cases, was below the threshold that requires treatment (1,000,000 spores/ml).

Concerning the detection of *Varroa destructor*, low percentages of infestation in the phoretic stage were detected at any time of sampling (beginning vs. end of the season) and irrespective of the type of management (migratory vs. stationary). It is worth noting that samples for varroa detection were collected before the routine treatment against the mite was applied.

When examining the sealed brood, Varroa mites were detected in both migratory and stationary apiaries, with a higher number of mites found in sealed broad from migratory apiaries at both the beginning and end of the season. This indicates that mites were present in both areas, even in colonies with non-detectable phoretic mite, and suggests that a significant portion of the mites present at the time of sampling were in the reproductive stage[44,45]. However, it´s important to note that our study cannot definitively determine whether the type of management influences the presence or spread of these pathogens, as observed in other studies[28,29]. The low level of varroa found can be attributed to the strict management that beekepers applied to their colonies, not only in migratory apiaries but also in stationary ones. It is well reported that migratory management can have a negative impact on mite infestation[28,30], which motivates the rigorous control of varroa. On the other hand, stationary apiaries in the study region have adapted to the presence of migratory colonies in neighboring areas by increasing control measures against these pathogens to prevent significant infestations from non-local apiaries. Therefore, values of Nosema and Varroa were low and similar in both types of management.

The presence of DWV was detected in 62.7% (91/145) of the processed samples. This high percentage of positive samples is consistent with other reports in various regions of the country[46,47]. We found significant associations between the presence of the virus, the sampling time, and the type of management, particularly in migratory apiaries at the beginning of the season (p: 0.0005). This result highlights the negative impact experienced by apiaries located in areas dominated by monoculture, particularly at the beginning of the season. Adequate nutrition is known to improve and maintain colony health, and it´s worth noting that the nutritional content of pollen varies by geographic region[26]. Therefore, pollinators in monofloral crop areas, with reduced floral diversity and nutritional resources, are more susceptible to diseases[26,35,48]. In our study, honeybees from migratory apiaries primarily forage on citrus monocultures at the beginning of the season and eucalyptus spp at the end. Pollen samples collected at each sampling time from inside the colony confirm the dominance of these monocultures. This lack of floral diversity could potentially impact colony strength and, as a result, contribute to the increased circulation of DWV. Our results revealed a higher percentage of positive samples (86.2%) in migratory apiaries that were exposed to monoculture at the beginning of the season. Furthermore, 38% of these hives were categorized as C3, indicating a weakened condition in terms of their population size. Moreover, these colonies exhibited a higher viral load in adult bees. While we assumed that the lack of nutritional diversity could be a factor affecting colony health; however, this assumption must be

considered cautiously, and further analysis of the protein contribution provided by this type of pollen is required. Nevertheless, several studies have demonstrated the impact of nutritional stress, revealing a positive relationship between multifloral areas and the annual survival of hives [49,50].

Recent studies conducted in *Eucalyptus grandis* plantations in Uruguay reported the negative impact of nutritional stress on colony strength. Beehives exposed to monoculture exhibited reduced population sizes, fewer broods, and increased disease susceptibility compared to colonies supplemented with multifloral pollen in their diet. In our study, migratory hives returned to their area of origin (Villa del Rosario) for the flowering of Eucalyptus spp at the end of the season, following a period of transhumance in multifloral areas. Samples collected at that time presented 24% fewer positive colonies and lower VLs than at the beginning of the season. Additionally, the colonies returned with increased strength, with only 24% of the colonies categorized as weak (C3), 26% as average (C2), and 50% as strong (C1).

Despite the period of transhumance in multifloral areas, migratory colonies enter the winter with the storage of monofloral pollen of Eucalyptus spp. This specie has a low lipid content, a low percentage of crude protein and it is deficient in isoleucine [51–53] and, consequently, does not satisfy the minimum requirements for colony maintenance and breeding. As a result, beekeepers must add a dietary supplement to ensure that the colony has sufficient reserves to survive the winter. Migratory apiaries under these conditions may not survive the winter, and those that manage to arrive at the beginning of the following season and recover their population size in spring are the ones that received the greatest floral diversity at the end of the season[52]. Our results align with this dynamic, as weak hives at the end of the season tend to have a lower population in the following spring and exhibit the highest susceptibility to DWV infection.

Besides these harmful effects, the stress induced by long transport of honey bees led to physiological changes including a reduction in hypopharyngeal gland size and downregulation of certain immune and stress resistance genes[34].

For the stationary apiaries located in the central region of the Entre Ríos province, the high floral diversity enables varied pollen intake into the colonies[54], promoting colony health at the beginning of the season with a majority classified as C1 (strong health condition, 91%). However, by the end of the season, after a period of coexistence with migratory hives, there was a reduction in strong colonies (66% classified as C1), an increase in weak ones (25% classified as C3) and a higher number of DWV positive colonies. These results support the negative impact of the migratory movement, as bees from both colony types share floral resources and feeding sites, potentially enhancing the horizontal transmission of pathogens. Notably, despite a slight increase in the number of positive colonies during the end of the season in this area, stationary colonies consistently maintained relatively low DWV loads in adult samples regardless of the sampling time.

While the presence of viruses affecting honey bees has been reported in various provinces of Argentina[46], the identification of DWV A and B circulating variant was recently reported in apiaries located in Buenos Aires and Santa Fe provinces. Given the limited information available regarding DWV in our study area, we aimed to determine the DWV variant present in the collected samples. We successfully identified the DWV-A variant in 60 samples obtained from adult bees, parasitized brood, non-parasitized brood, and varroa mites. The sequences analyzed from both migratory and stationary apiaries in Entre Ríos province revealed the presence of the same strain of DWV (DWV-A). All the Argentine strains exhibited a common geographic structure, as they do not cluster with any other strains worldwide, except for one strain from Chile.

Additionally, in one of the apiaries, bees displaying wing deformities were observed (Supplementary material 1, COD 333). Interestingly, the sequences obtained from both symptomatic and asymptomatic colonies exhibited a 99% amino acid similarity, indicating the presence of the same circulating variant.

In conclusion, the intensification of agriculture in Argentina has brought about changes in beekeeping practices, with migratory activity becoming a common approach to maximize honey production. However, this practice has been shown to have negative consequences for colony health and increased susceptibility to DWV. Further research is necessary to better understand and mitigate the impacts of migratory activity on beekeeping in Argentina.

Materials and Mathods

Sampling strategy:

During the period from September 2018 (early spring in the southern hemisphere) to March 2019 (early autumn in the southern hemisphere), two samplings were carried out in the province of Entre Ríos, Argentina. The apiaries were located in two different zones based on their management practices. Zone 1 is characterized by stationary operations (stationary apiaries), meaning that the colony remains in the same location throughout the season (from September to March). In Zone 2, migratory operations (migratory apiaries) are performed from December to February.

The beekeepers manage more than 1000 hives; with each apiary containing between 60 and 100 hives. All the apiaries were dedicated to honey production. In the migratory apiaries, honey was harvested at the end of each monoculture flowering season (October, January, March). However, for the stationary apiaries, the harvest took place between the end of spring and the beginning of autumn (October, March).

The beekeepers rigorously applied the varroa treatments with Amitraz or Oxalic. The stationary apiaries conducted two treatments at the beginning and end of the season, while the migratory apiaries applied the treatments at each migratory operation. In addition, queen replacements were conducted annually in all hives, typically in October.

Sample collection:

Sampling was conducted at two distinct time points: at the outset of the season in September 2018 and again at its conclusion in March 2019. This sampling occurred prior to the application of acaricide treatment. A total of 145 hives, distributed across 15 different apiaries within zones 1 and 2, were included in the study (as detailed in Table 1). Within each apiary, a minimum of six colonies were randomly selected for sampling. In cases where an apiary had more than 60 hives, 10% of the total number of colonies were chosen for sampling. All collected samples were promptly shipped to the laboratory on dry ice and subsequently stored at -80°C until further analysis.

Approximately, 300 nurse bees were collected from the brood frame to detect and quantify virus and to calculate the percentage of phoretic mite infestation. The samples consisted of female bees representing various age groups, encompassing nurse bees, worker bees, and forager bees. For the detection of Nosema ssp., foraging bees were specifically collected at the hive entrance.

During each sampling event, a minimum of one hive per apiary was chosen for brood analysis. A portion of the sealed brood was carefully extracted to assess the presence of *V. destructor* and DWV. Additionally, pollen samples were collected. The presence of DWV was subsequently examined in brood, varroa, and pollen samples.

Quantification of beehive health

Beehive health was evaluated using the classification described by Cayley Faurot-Daniels et al. (2020)[55]. Briefly, beehives were categorized as either strong (C1: category 1), average (C2: category 2), or weak (C3: category 3), primarily based on their population size. This assessment was determined by the number of frames occupied by bees. To account for variations in flower availability between the beginning and end of the season, the criteria for each category were adjusted according to the specific sampling time. At the beginning of the season in September 2018, hive categories were established as follows: weak (C3) represented hives with fewer than 5 frames covered by bees, average (C2) included hives with 5 to 7 frames covered by bees, and strong (C1) encompassed hives with 8 to 10 frames covered by bees. By the end of the season in March 2019, the criteria for each category were adjusted to reflect the number of frames covered by bees as follows: weak: fewer than 5 frames, average: 6 frames, and strong: 8 frames.

Furthermore, a visual inspection of the adult bee population and hatchlings was conducted. During this inspection, the presence of disease symptoms such as bees with deformed wings, adult and larvae mortality, and significant reductions in the adult population were recorded. Additionally, the presence of *V. destructor* and the mortality rate per colony were assessed.

Similarly, hives were categorized based on their nutritional status, determined by the availability of food within the brood chamber. The classifications included “very good” (VG), denoting hives with four frames containing pollen and honey; 'good' (G), signifying hives with two frames; and 'regular' (R), indicating hives with only one frame of stored food. At the beginning of the season, all hives located in Zone 1 and Zone 2 received an identical dietary supplement, which consisted of pollen granules and sucrose syrup.

By the end of the season, all the hives in zone 1 and zone were maintained at a level of 2 to 4 frames containing pollen and honey. This was achieved by providing additional dietary supplements to ensure the hives´ survival through the winter.

Quantification of V.destructor:

V. destructor infestation was monitored in each colony by analyzing 300 nurse bees collected from the brood frame. This analysis followed the methodology outlined in Dietemann et al. (2013)[56]. The infestation level for each apiary was expressed as the percentage of mites found in the total sample analyzed.

Detection of Nosema spp spores:

The level of *Nosema ssp* infection within each colony was determined by examining a pool of 60 bees collected at the hive entrance. The spore count was conducted according to the method described by Catwell (1970)[57].

Detection and quantification of DWV:

For RNA extraction, a group of 30 nurse bees collected from the central brood frames was processed. To analyzed both parasitized and non-parasitized brood, a pooled sample of 10 brood from each respective category was examined. Varroa mites obtained from the parasitized brood were analyzed based on the level of infestation: if the brood presented fewer than 5 varroa mites, they were combined into a single 1 pool; otherwise, when more than 5 varroa mites were found per brood, pools consisting of 5–7 mites were created.

All bee and brood samples were processed by macerating them in a mortar with an appropriate volume of PBS pH 7 (7 ml for adult bees and 3 ml for brood). The varroas were first frozen with liquid nitrogen and subsequently macerated in a mortar with 0.5 ml of PBS at pH 7. Bee bread was collected in those frames where brood samples were taken. The material was extracted from 50 cells, resulting in one pool per hive. Each pool consisted of 100 mg of material, which was resuspended in 0.5 ml of PBS at pH 7.

The complete mixtures were centrifuged at 4500 rpm at 4°C for 45 minutes. From the resulting supernatant, 200 µl were utilized for RNA extraction using the High Purity Viral RNA Kit (Roche) and following the manufacturer's recommendations. The remaining supernatant was collected and stored at -80°C.

cDNA synthesis was performed using M-MLV Reverse transcriptase (Promega). The reaction mixture contained 5 µl of RNA, 5 µl of reaction buffer 5x (Promega), 0.5 µl of dNTP 10 mM (Promega), 0.5 µl of random primers 2 µg/µl, 0.25 µl of reverse transcriptase 200 U/µl, and 13.75 µl of ultra-pure water (Distilled Water DNAse, RNAse Free; Invitrogen/Sigma) to obtain a total volume of 25 µl. Reverse transcription was performed at 42ºC for 45 min followed by a denaturation step at 94 ºC for 10 min to inactivate the reverse transcriptase.

The viral load of Deformed Wing Virus (DWV) was assessed by quantitative PCR using primers that were previously descripted by Bradford et al. 2017 (Pan-DWV). This set of primers was designed to amplify a region of 179 bp within the helicase protein, enabling the detection of all DWV variants.

All qPCRs were performed in a final volume of 12,5 µl containing 6,25 µl iTaq Universal SYBR Green Supermix (Bio-Rad), 0.5 µl of each primer (10 µM), 2.75 µl H2O and 2.5 µl of cDNA template. The thermal qPCR profile included an initial denaturation step of 3 minutes at 95°C, followed by 40 cycles of amplification, each consisting of 15 seconds 95°C and 1 minute of 60°C. A melting curve analysis was performed, ranging from 65°C to 95°C, with the temperature increasing 0,5°C every 5 seconds. A five-points standard curve was prepared with Ct data derived from known concentrations of a plasmid containing the target sequence. These concentrations encompassed 10-fold dilutions, ranging from 7,3 x 106 to 10 2 genetic copies/µl). Each point on the standard curve was assayed in triplicate. Viral loads were expressed as genome copies/µl per reaction. The housekeeping gene β-actin was amplified as an internal control using the primers described by Chen et al. (2006)[58].

Statistical analysis:

Initially, a descriptive analyze was conducted examining various categorical variables, including DWV point prevalence, nosema and varroa presence, nutrition, dietary supplement, and hive category (according to population size). The chi-square test was employed to assess the relationships between these variables.

Variables that exhibited significant differences between migratory and sedentary colonies were then included in the construction of a generalized linear mixed effect model (GLMM). This model was utilized to estimate the individual effect of each variable. Models were compared by ANOVA's, and the Akaike Information Criterion (AIC) was considered as an additional criterion for selection.

Subsequently, a Principal Component Analysis (PCA) was also performed to evaluate the relative weight of various quantitative variables (number of bees frames, varroa and nosema infestation rate, and DWV viral load). The PCA aimed to assess how these variables contributed to the dispersion of colonies, particularly concerning the type of beekeeping management at the beginning and end of the season. Additionally, the explanatory power of these variables was analyzed in this context.

Finally, another generalized linear mixed effect model (GLMM) was employed to investigate the impact of each variable, including the number of frames with bees, varroa and nosema infestation rate, and DWV viral load), specifically in the context of migratory management at each time of sampling (ie beginning and end of season).

All statistical analyses were performed using InfoStat software (National University of Córdoba, Argentina)

Pollen identification:

The examination of pollen collected from both transhumant and sedentary apiaries was conducted through the analysis of bee bread. To process the bee bread, the protocol described by Fagúndez et al. (2011)[59], were followed. For observation and analysis under an optical microscope, the pollen residue was subjected to acetolization, following the methodology originally described by Erdtman et al. (1960)[60]. The contribution of each type of pollen in the diet of bees was calculated using the method outlined by O'Rourke & Buchmann et al. (1991)[61]

Sequencing:

From the sampling conducted in Entre Rios, samples that tested positive for Deformed Wing Virus (DWV) were selected for sequencing. These samples included positive specimens of bees, brood, varroas, and pollen. Additionally, DWV-positive samples from sedentary apiaries located in the province of Buenos Aires, which had been previously analyzed in our laboratory, were also included in the study. The selected samples were amplified by RT-nPCR using the primers described by Ryabov et al., (2014, 2017)[10,62] that targeted a 1600 bp fragment within the helicase coding region. Briefly, cDNA synthesis was performed as previously mentioned. All nested PCRs were carried out using the Gotaq polymerase (Promega). The reaction mix contained 5 µl cDNA, 5 µl 5x reaction buffer (Promega-1.5 mM magnesium chloride in final reaction volume), 0.5 µl 10 mM dNTPs (Promega), 0.5 µl primers (10 µM), 0.25 µl 5U/µl polymerase, and 13.75 µl ultrapure water (DNase distilled water, RNase-free; Invitrogen/Sigma), in a total volume of 25 µl. Positive and negative controls were incorporated in all assays. The thermal nPCR profile was as follows: an initial denaturation step of 5 minutes at 95ºC, followed by 35 cycles, each consisting of 30 seconds at 95ºC, 45 seconds at 52ºC, and 1 minute at 72ºC, concluding with a final elongation step of 7 minutes. Purification of the PCR products and sequencing by the dideoxynucleotide chain termination method were performed using the services of Macrogen Inc (City, Korea).

Sequences generated in this study were deposited into GenBank (Supplementary material S4).

Phylogenetic analysis:

Datasets were constructed with the most related strains identified through Basic Local Alignment Search Tool (BLAST) analysis on the NCBI website (https://blast.ncbi.nlm.nih.gov/). A total of 95 strain sequences were compilated to create the dataset for DWV. Subsequently, multiple sequence alignments based on 1500 nucleotides (spanning positions 5083 to 6583) were performed and edited using Muscle available in AliView v1.16 (Larsson, 2014)[63]. Before proceeding with the phylogenetic analysis, a quality assessment of the dataset was performed. Briefly, the phylogenetic information was estimated using IQ-Tree (REF) and the PHI test for recombination was carried out in Split-Tree4 (REF). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version X (Kumar et al., 2018)[64]. The Neighbor-Joining (NJ) method (Saitou and Nei, 1987)[65] was performed. The evolutionary model selected based on the Bayesian Information Criterion (BIC) was Tamura-Nei (TN93); this model included a discrete Gamma distribution (+ G) with 5 rate categories, and it assumed that a certain fraction of sites are evolutionarily invariable (+ I), which is referred to as the TN93 + G + I. The branch supports were estimated using the bootstrap method including 1000 pseudo-replicates. All positions with less than 95% site

coverage were eliminated, meaning that fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option = 95%).

Declarations

Acknowledgements (optional)

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Author contributions (names must be given as initials)

FG and MJDS conceived and designed experiments and wrote the main manuscript text. FG carried out the experiments. GR provided financial resources. CF performed the statistical analysis. SM conducted the phylogenetic analysis. GF conducted the pollen identification and FR performed the analysis of varroa and nosema.

All authors reviewed the manuscript.

Data availability statement (mandatory)

Sequences generated in this study were deposited into GenBank and are specified in Supplementary material S4.

Additional Information (including a Competing Interests Statement)

The authors declare that they have no competing interests.

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Figures

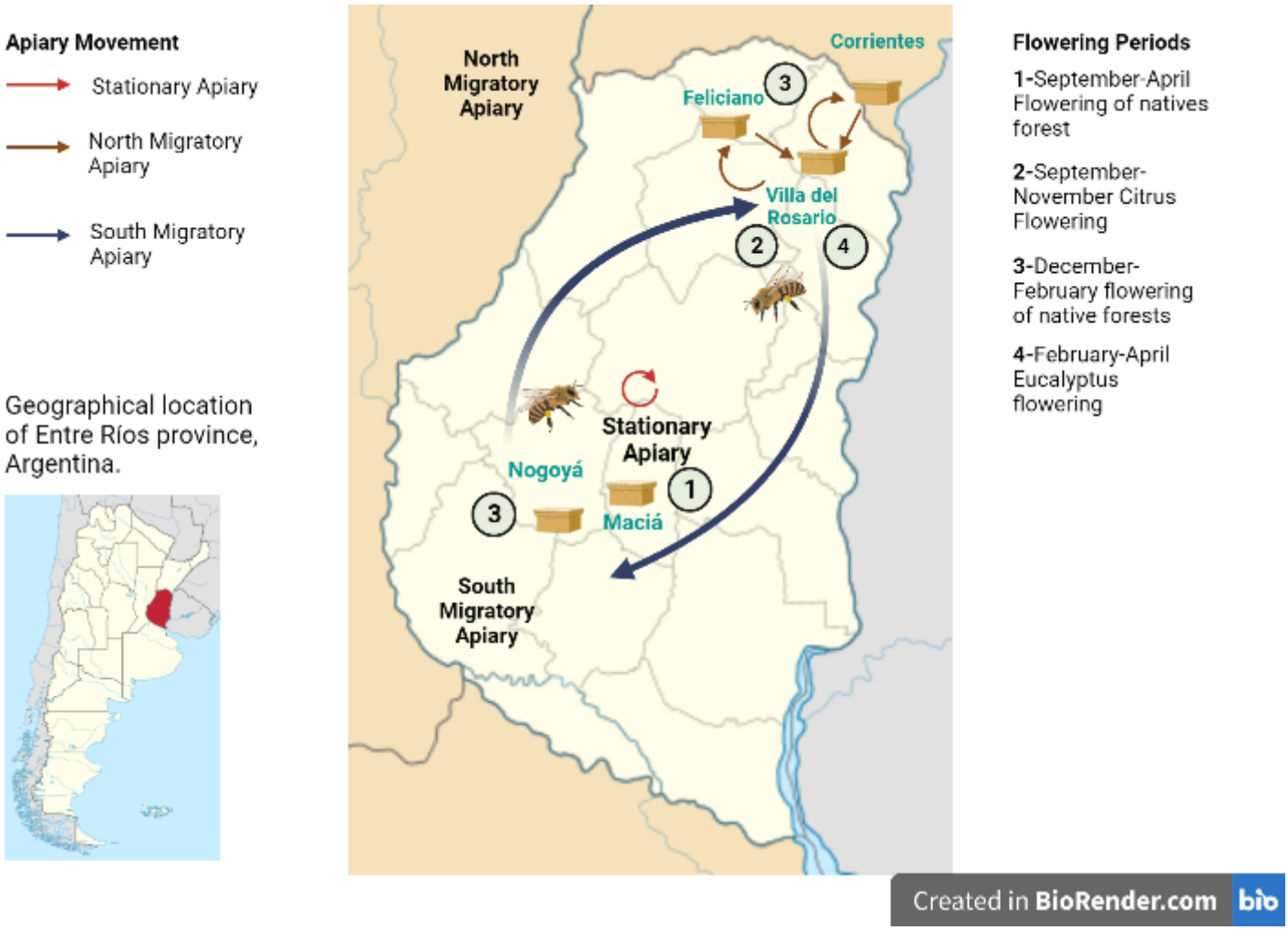

Figure 1

Scheme of the movement of hives and their geographical location.

This diagram represents the movements undertaken by beekeepers engaged in migratory activities, as well as the fixed positions of stationary apiaries throughout the season. For migratory hives, the season begins with the flowering of citrus fruit trees, which lasts for 2 months. After this flowering period, the hives migrate either northward (indicated by brown arrows) or southward (indicated by blue arrows) in search of more diverse floral resources, particularly native forests. At the end of the season, they return to their original location (Villa del Rosario) to take advantage of eucalyptus flowering. In contrast, stationary hives remain in the same central zone of the Entre Ríos province throughout the entire season, taking advantage of the floral diversity available in that area (highlighted by the red arrow).

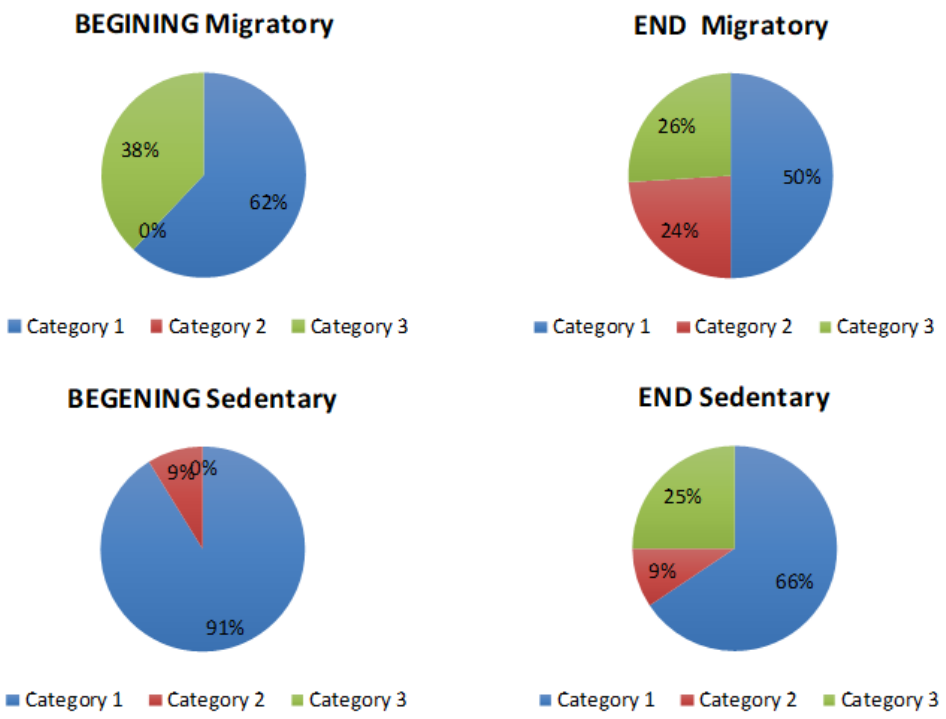

Figure 2

Categorization of hives in apiaries with stationary and migratory management during the 2018-2019 season.

Hives were classified as strong (C1: category 1, blue), medium (C2: category 2, red) and weak (C3: category 3, green) according to their population size, which was determined by the number of frames

covered by the bees. Classification at the beginning of the season: C1: 8-10 frames with bees; C2: 5-7 frames with bees; C3: <5 frames with bees. Classification at the end of the season: C1: up to 8 frames; C2: 6 frames and C3: up to 5 frames. The circular graphs display the percentage of hives in each category at the beginning and end of the season, categorized by management type.

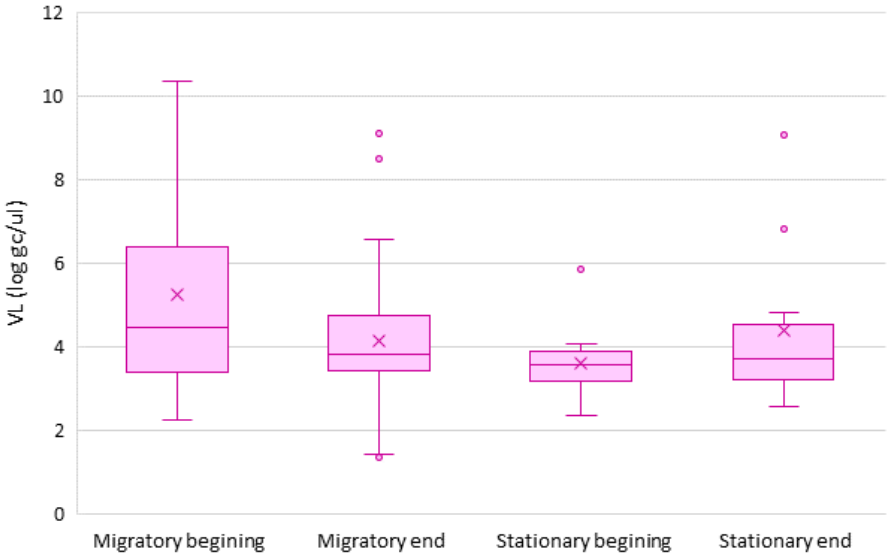

Figure 3

Quantification of DWV loads in migratory and stationary apiaries.

DWV loads detected by RT-qPCR in migratory and stationary colonies during the beginning and end of the season. VLs are expressed as Log10 DWV RNA copies/µl of sample.

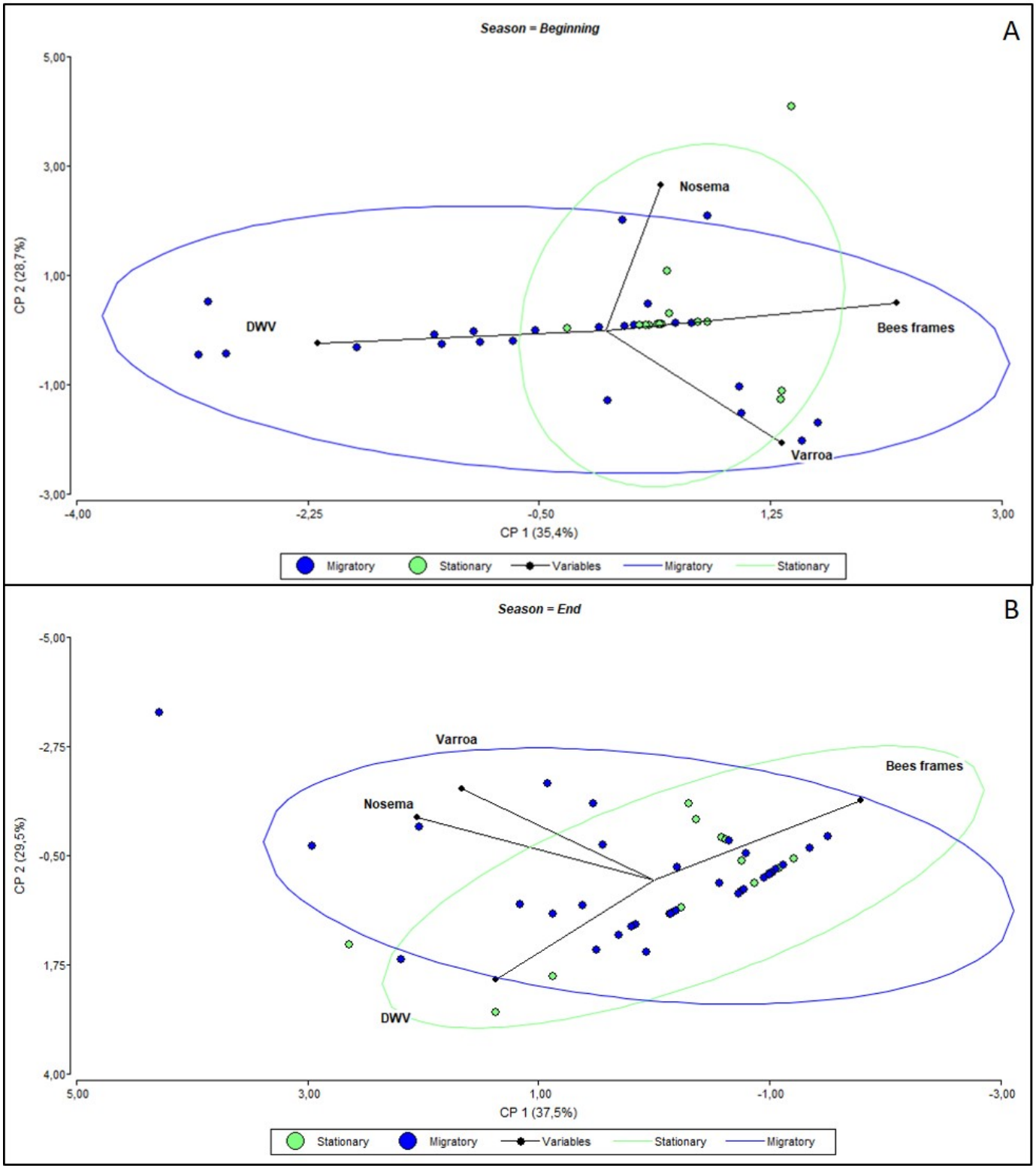

Figure 4

Principal component analysis.

Biplot of the colonies showing dissimilar dispersion of stationary and migratory managements for the beginning and the end of the season, according to each variable: frames with bees, % foretic varroa, Nosema spore counts and DWV loads. Stationary colonies are represented in green, while migratory colonies are in blue.

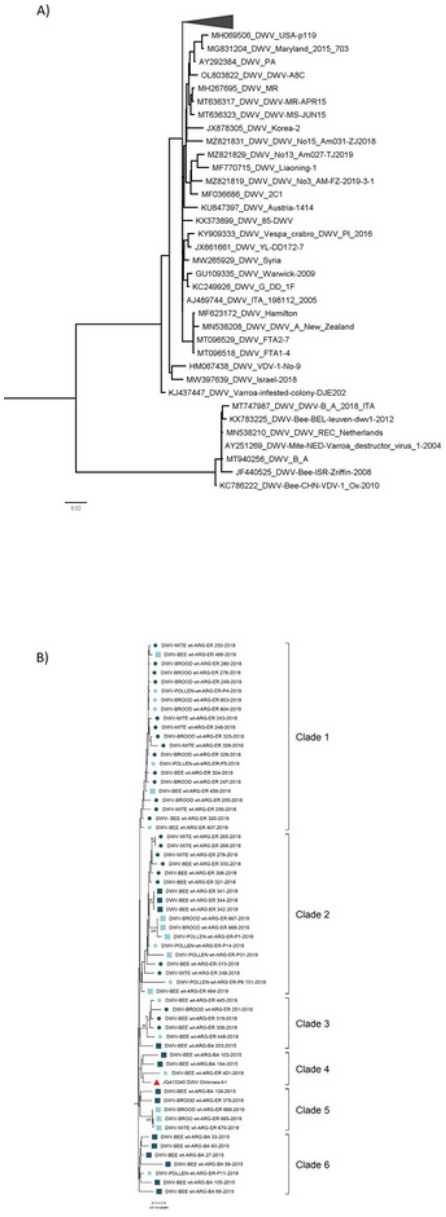

Figure 5. Phylogenetic analysis of DWV

A fragment of 1500 nucleotides (positions 5083-6583) of the DWV helicase gene was amplified and sequenced from 60 samples that belonged to migratory and sedentary apiaries.

(A) Neighbor-Joining phylogenetic tree showing the clustering of Argentinean sequences (◄) related to references strains from different countries. (B) Phylogenetic tree of Argentinean DWV-A sequences from migratory colonies at the beginning (blue circles) and the end (light blue circles) of season, and from sedentary colonies at the beginning (blue squares) and the end (light blue squares) of the season. The red triangle refers to a whole genome sequence from Chile.

Figure 5

See image above for figure legend

Supplementary Files

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- SUPPLEMENTARYMATERIAL.xlsx