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Risk factors for the presence of Deformed wing virus and Acute bee paralysis virus under temperate and subtropical climate in Argentinian bee colonies

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ABSTRACT

Beekeepers all across the world are suffering important losses of their colonies, and the parasitic mites *Varroa destructor* and *Nosema* sp, as well as several bee viruses, are being pointed out as the possible causes of these losses, generally associated with environmental and management factors. The objective of the present study was to evaluate the presence of seven virus species (Deformed wing virus –DWV-, Acute bee paralysis virus –ABPV-, Chronic bee paralysis virus –CBPV-, Black queen cell virus –BQCV-, Kashmir bee virus –KBV-, Israeli acute bee paralysis virus –IAPV-, and Sacbrood bee virus –SBV), as well as the prevalence of *Nosema* sp. and *Varroa destructor*, and their possible associated factors, under temperate and subtropical climate conditions in Argentinean colonies. A total of 385 colonies distributed in five Argentinean eco-regions were examined after honey harvest. The final multivariable model revealed only one variable associated with the presence of DWV and two with the presence of ABPV. The apiary random effect was significant in both cases ($P = 0.018$; $P = 0.006$, respectively). Colonies with a Varroa infestation rate >3% showed higher presence of DWV than colonies with <3% of Varroa infestation level (OR = 1.91; 95% CI: 1.02–3.57; $P < 0.044$). The same pattern was observed for the presence of ABPV (OR = 2.23; 95% CI: 1.04–4.77; $P < 0.039$). Also, colonies where replacement of old combs was not a common practice had higher presence of ABPV (OR = 6.02; 95% CI: 1.16–31.25; $P < 0.033$). Regardless of the location of the colonies, virus presence was strongly associated with *V. destructor* level. Therefore, all the factors that directly or indirectly influence the levels of mites will be also influencing the presence of the viruses.

1. Introduction

Honeybees have been suffering high mortality rates in many countries and important losses have been reported in Europe, Middle East, Japan, and the U.S. (reviewed in Neumann and Carreck, 2010). In Latin America, no massive colony losses, or at least no symptoms of colony collapse disorder, have been reported (Vandame and Palacio, 2010).

The most probable explanation for increased over-wintering mortality is the interaction among multiple drivers, including honeybee pathogens associated with environmental and management factors (Van Engelsdorp et al., 2009; Genersch and Aubert, 2010; Meixner et al., 2014; Pirk et al., 2014; Döke et al., 2015; Barron, 2015).

One of the main threats to apiculture all over the world is the parasitic mite *Varroa destructor* (Oldroyd, 1999; Rosenkranz et al., 2010). Nowadays, *V. destructor* is considered the most relevant pathogen causing productive and economic damage in Argentinean honeybee colonies (SENASA, 2007; Giacobino et al., 2014).

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According to Maggi et al. (2012), Korean haplotype of *V. destructor* is cosmopolite in Argentina. Usually, *V. destructor* infestation is associated with virus infections (reviewed in Francis et al., 2013; Emsen et al., 2015), and even several honeybee viruses, including Deformed wing virus (DWV) (Bowen-Walker et al., 1999), Acute bee paralysis virus (ABPV) (Ball, 1983), Kashmir bee virus (KBV) (Chen et al., 2004; Shen et al., 2005a), Israeli acute bee paralysis virus (IAPV) (Di Prisco et al., 2011), and Sacbrood bee virus (SBV) (Shen et al., 2005b), are transmitted by *V. destructor*. On the other hand, the bee parasite *Nosema* sp. is positively associated with Black queen cell virus (BQCV), increasing the susceptibility to BQCV infection (Chen and Siede, 2007).

In Latin America, several viruses have been detected in honeybees of Brazil (Weinstein Teixeira et al., 2008), Uruguay (Antúnez et al., 2005, 2006), and Chile (Barriga et al., 2012). In Argentina, studies on honeybee viruses are still incipient. To date, DWV, ABPV, SBV, IAPV, BQCV, and Chronic bee paralysis virus (CBPV) have been detected in colonies located in temperate climate (Reynaldi et al., 2010, 2011; Castilla et al., 2015; Giacobino et al., 2016). Nevertheless, there are no studies about bee viruses and the environmental and management factors that could be influencing their presence in the colonies. Thus, the objective of the present study was to evaluate the presence of seven virus species (DWV, ABPV, CBPV, BQCV, KBV, IAPV, and SBV), as well as the prevalence of *Nosema* sp. and *Varroa destructor*, and their possible associated factors, under temperate and subtropical climate conditions in Argentinean colonies.

2. Materials and methods

2.1. Study design and sample size

A cross-sectional study was carried out from February to June 2015 (autumn), in north-central Argentina. The sampling time was defined between the end of the honey production period and the beginning of the autumn acaricide treatment. A total of 385 colonies from 64 apiaries (owned by different beekeepers) were sampled. This number was consistent with the number of apiaries in the study area (n = 5300; 95% confidence level); precision = 10% and 74% of expected prevalence of colonies with >3% of Varroa adult infestation during autumn (SENASA, 2007). Five eco-regions were defined based on the nectar flow period and their beekeeping management schedule (Burkart et al., 1999; Arzamendia and Giraudo, 2004; Giorgi et al., 2008; Riveros, 2009; RIAN, 2010). The eco-regions were defined as: South Santa Fe, Central Santa Fe, Humid Chaco, Transition Chaco, and Semi-arid Chaco (Table 1; Fig. 1). The number of colonies sampled in each eco-region was defined proportionally considering the total number of colonies in the region. Thus, the number of colonies sampled in each eco-region was: 48 in South Santa Fe, 102 in Central Santa Fe, 91 in Humid Chaco, 78 in Transition Chaco, and 66 in Semi-arid Chaco. Apiaries were randomly chosen following stratified randomization procedures (computerized random numbers) (Moher et al., 2010). Within each apiary, a minimum of 6 or 10% of the total number of colonies (in apiaries larger than 60 colonies) was randomly selected (Lee et al., 2010). The chosen colonies were managed by the beekeeper like the rest of the colonies in the apiary and according to the usual beekeeping practice. The aim was to guarantee that colonies reflected different management techniques in the study region. Samples were collected to evaluate *Varroa destructor* levels, *Nosema* sp. spore counts, and virus titers.

The majority of the beekeepers in Argentina own fewer than 200 colonies, distributed in several apiaries. Honey harvest is performed only during summer, and the treatments against Varroa mites are done during autumn. Most of the beekeepers use commercial acaricide treatments, only a few of them may use homemade formulations (Giacobino et al., 2015). Additionally, early spring treatment is less applied and depends on each year sanitary and economic conditions. Normally, apiaries receive a carbohydrate supply in the course of autumn and spring (often sucrose syrup) and rarely some pollen substitutes (Giacobino et al., 2014). On the other hand, some beekeepers replace their queens (buying new queens from commercial queen producers) and make nuclei from their colonies before nectar flow start.

2.2. Sampling and virus analysis

Approximately 40 nurse bees were collected from brood frames within each colony and maintained alive in plastic containers with breathing holes until they were frozen at −20 °C. Live bees were used to ensure high-quality RNA (Amiri et al., 2015; Francis et al., 2013).

Pools of bees (n = 30) from each hive were macerated in a mortar and homogenized with 7 ml of phosphate buffer (PBS) of pH = 7. The mixture was centrifuged at 4500 rpm at 8 °C for 45 min and the supernatant was collected and stored at −20 °C.

2.3. Viral RNA extraction

Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific/Life Technologies), according to the manufacturer's protocol. RNA samples were dissolved in 10–50 μl ultra-pure water (DNAse-/RNAse-Free Distilled Water; Invitrogen).

2.4. Reverse transcriptase reaction

Copy DNA was synthesized by reverse transcription (RT) reaction from the extracted RNA. The reaction mixture contained 1 μl of RNA (~2 μg), 1 μl of reaction buffer 5 x (Promega), 0.5 μl dNTP 10 mM (Promega), 0.125 μl of RNasin® 40 U/μl (Promega), 0.25 μl of random primers 2 μg/μl, 0.175 μl of reverse transcriptase 200 U/μl (Promega), and completed with volume of 1.95 μl of ultra-pure water (DNAse-/RNAse-Free Distilled Water; Invitrogen) to obtain a total volume of 5 μl of mixture. The reaction was developed in a Biometra Trio-Thermoblock. The thermal cycling profiles were: 42 °C for 45 min, 94 °C for 10 min and 4 °C for 4 min.

2.5. qPCR amplification of DWV, ABPV, CBPV, BQCV, IAPV, SBV, and KBV

To determine the presence of DWV, BQCV, SBV, CBPV, ABPV, KBV, and IAPV, qPCR was carried out, using the method described by Locke et al. (2012a). Negative (H_2O) and positive controls (recombinant plasmid DNA with the virus inserted into the pGEM-T Easy vector) were included in each run of the qPCR reaction. After amplification, a melting curve analysis was performed to determine the specificity of the PCR products. The housekeeping gene β-actin was used as an internal control, where the presence and quantification of this reference gene ensure that the entire procedure from extraction to quantification was performed without degradation of RNA (Francis et al., 2013; Locke et al., 2012a).

For the qPCR amplification, the reaction mixture contained the primers described by Locke et al. (2012b) (0.4 μl 1.5 uM of each pair of primers selected), master mix SYBER green PCR kit QuantiTect (cat 204143) 2.5 μl, 1.45 μl ultra-pure water (DNAse-/RNAse-Free Distilled Water; Invitrogen), and 0.5 μl of cDNA. Samples were amplified using the LightCycler 2.0 Roche Thermocycler with the following thermal cycling profiles: 95 °C for 10 min, 45 cycles at 95° for 15 s and 56 °C for 1 min. The fluorescence emission of the samples was monitored at 530 nm. Samples having a geometric increase in fluorescence emission in the two previous successive

Table 1
Region characterization based on annual mean temperature and precipitation, land use and floral resources.

Region	Annual temperature (°C)	Annual precipitation (mm)	Main Land use	Nectar/Pollen Flow
South Santa Fe	18	600–1100	Soy, corn, and wheat	Short (less than three months)
Central Santa Fe	17–18	800–900	Dairy farms and wintering animals on alfalfa pastures	Intermediate (three –four months)
Humid Chaco	23	1200–1500	Small farmstead, livestock or forest and rice production	Long (between 9 and 10 months)
Transition Chaco	23 to 24	900–1000	Cereals, oleaginous, and cottonseed crops mixed with livestock production	Long (between 9 and 10 months)
Semi-arid Chaco	23	550–800	Forest production	Long (between 9 and 10 months)

cycles of cycling number 45 were considered positive. The first of these emissions lifting cycles was considered as the first cycle of positivity (CP). Negative (H_2O) and positive controls (recombinant plasmid DNA with the virus inserted into the pGEM-T Easy vector) were included in each run of the RT-PCR reaction.

The viral loads of positive samples were estimated using standard curves prepared with cycle threshold (C_t) data obtained from known concentrations of cDNA fragment copies of each virus studied. To convert the C_t values generated by qPCR from experimental samples to RNA genome copies per μl, serial 10-fold dilutions of *in vitro* RNA synthesized from the plasmids of known concentration were analyzed by the qPCR protocol described above. The equation of the curve of RNA copy versus the normalized C_t value was used for subsequent conversions (Pfaffl 2001).

2.6. Sampling and Varroa analysis

Adult nurse bees were examined to diagnose the presence of Varroa mites in all the colonies evaluated. Approximately 250 bees per colony were collected from both sides of three unsealed brood combs in a jar containing 50% ethanol. The mites were separated from the bees by pouring the jar content into a sieve with a 2-mm mesh size (Dietemann et al., 2013). The intensity of mite infestation on adult bees was calculated dividing the number of mites counted by the number of bees in the sample to determine the proportion of infested individuals and multiplying by 100 to obtain the infestation rate per colony (Dietemann et al., 2013).

2.7. Sampling and Nosema analysis

Forager honeybee samples were collected from the hive entrance (Fries et al., 2013). A minimum of 60 bees were gathered and placed in labeled plastic flasks containing 60 ml of 96° alcohol. Spore suspensions were prepared for each colony by adding 60 ml of distilled water to crushed abdomens of 60 randomly selected individuals. The number of *Nosema* spp. spores/bee (transformed to $\log_{10}$) was determined using light microscopy 40 X and a hemocytometer. For each sample, we counted the number of spores in 80 hemocytometer squares (5 groups of 16 squares) (Cantwell, 1970; Human et al., 2013). This sampling method is the most frequently used and can detect 5% of sick bees with 95% of confidence (Fries, 1988).

2.8. Questionnaire

Potential explanatory variables were obtained from a checklist questionnaire concerning management practices answered by the beekeepers (available as supplemental material). The questionnaire included questions with reference to the geographic location, number of colonies, and frequency of management practices such as supplementation of diets with carbohydrates and proteins, monitoring of mite levels in the colonies, queen replacement, division of the colony into nuclei, colony migration and treatment against Varroa mites. Table 2 show all the explanatory variables obtained from the survey.

2.9. Prevalence estimation

A colony was considered to be positive to a virus when a pooled sample of 30 bees was positive to the specific PCR. The prevalence of each virus was calculated as positive colonies over all the colonies under study.

For Nosema, a colony with spores presence was considered positive and the prevalence was calculated as positive colonies over all the colonies under study.

In previous studies, we determined a critical threshold of 3% (mite load above which it is recommended to treat colonies during autumn to avoid severe winter losses). Our results suggested that colonies that go through winter with more than 3% of mite load hardly survive until the following spring (Bulacio Cagnolo, 2011; Giacobino et al., 2014). To establish a relative sanitary condition, previous results were used to subcategorize the colonies into two levels: high and low, according to their autumn infestation with Varroa mites (high: >3%; low: ≤3%). The prevalence of colonies with >3% of Varroa infestation level and the prevalence of colonies with <3% of Varroa infestation level within each region were calculated.

2.10. Statistical analysis

At first a univariate analysis (a generalized linear mixed model with apiary as random effect) was conducted between the outcome *Nosema* sp. (quantitative variable: spores/bee and binary variable: presence/absence of spores) and region. The same analysis was performed with the outcome Varroa infestation level (binary variable: >3% and ≤3% of Varroa infestation level) and region. The objective of this first approach was to describe the geographical distribution of the diseases (Table 3, step 1).

Univariate analysis (a generalized linear mixed model with apiary as random effect) was conducted to select explanatory variables potentially associated with the presence of DWV, ABPV, and BQCV and those having a P-value ≤ 0.15 were selected for multivariable analysis (Table 3, step 2). Risk factors were looked only for DWV, ABPV and BQCV because they were the top-three prevalent viruses in the study.

We evaluated the management practices variables using a generalized linear mixed model (GLMM) with apiary as random effect as all colonies from the same apiary are uniformly managed (Table 3, step 2). All variables with a P-value <0.15 were selected to be included in the final multivariable model after evaluating the potential confounding effects (Table 3, step 3).

Multivariable logistic regression analyses with apiary as random effect (using a GLMM) were performed to evaluate the effect of the selected explanatory variables on the binary outcome variables presence/absence of DWV, presence/absence of ABPV and

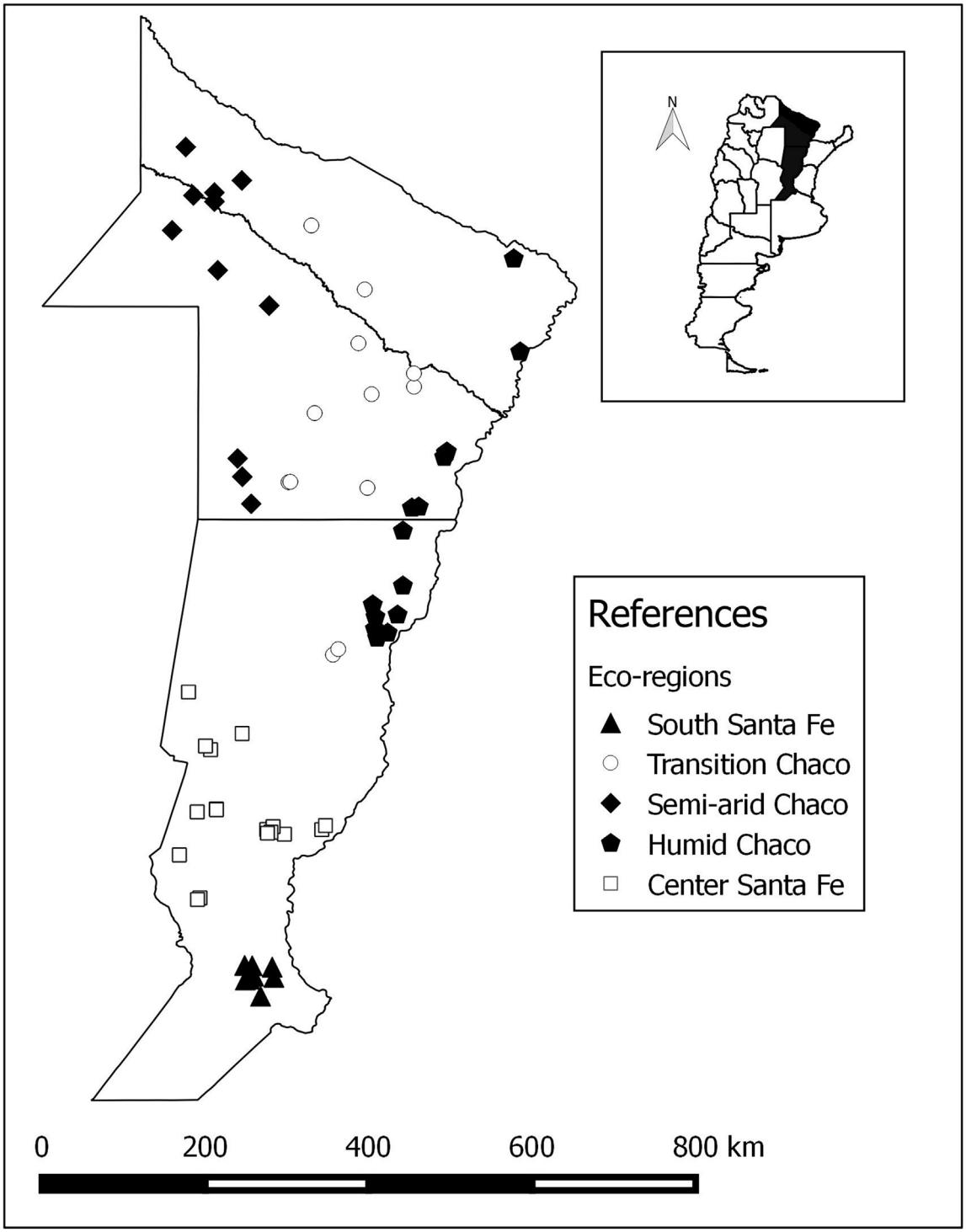

Fig. 1. Apiaries location and distribution according to eco-regions of Argentina.

presence/absence of BQCV. A manually conducted backward elimination strategy was followed by removing one variable at a time with the highest *P*-value (Table 3, step 4). We tested the potential confounders by evaluating change in odds ratio after including them in the multivariable model. With each variable removed from the model, the coefficient of significant variables was checked and if it resulted in more than 20% change in estimates, the variable was retained in the model to account for its confounding effect (Chowdhury et al., 2012). We evaluated for potential collinearity *a priori* of the multivariable analysis by testing that a variable is not in the causal pathway using causal diagrams: we tested correlation between all explanatory variables before including them in the multivariable model (Table 3, step 3). A univariate analysis was performed with the aim to establish the relation between the variables included in the final model and each of the variable not included in the final model (Table 3, step 5). All statistical analyses were carried out using InfoStat software (Universidad Nacional de Córdoba, Argentina).

Table 2
Summary of explanatory variables derived from the questionnaire and assessed as potential risk factors for DWV and ABPV presence.

General Management on the apiary	Variable description
Geographical Region	Geographic location: South and Center Santa Fe, Humid, Semi-arid and Transition Chaco.
Number of colonies within each apiary	To know the size of the apiary
Multivitamin supplements	If they supplement with vitamins (no/yes)
Kind of protein diet	Feed colonies during autumn or spring with natural pollen, supplements or substitutes (homemade or commercial patties)
Carbohydrate supply	Feed colonies during autumn or spring with sucrose syrup or high fructose corn syrup and frequency of the supplementation.
Colonies migration	If normally colonies are moved during winter (no/yes)
Splitting colonies	Percentage of colonies within the apiary that has been used for nuclei production during last spring
Annual comb replacement	How many combs per colony are replaced by new ones per year
Queen replacement	If the queen is replaced frequently in the colony (no/yes)
Frequency of requeening	How frequently (in years) a queen is replaced in each colony by the beekeepers
Wooden ware disinfection	Do you normally disinfect the wooden ware before storage it after harvest season. If disinfection takes place, how?
Autumn treatment against Varroa mites	If they normally applies it and what they use
Monitoring Varroa infestation level	Checking for the% of varroa infestation level in adult bees, prior to and after treatment
Apiaries closer	If they have any apiary close to theirs (near than 1500 mts)
Surrounding vegetation	What do they have around the apiary: crops, grassland, forest.
Varroa infestation level	We measure this by sampling the colony and doing the analysis as described in M&M section
Nosema Sp. presence	We measure this by sampling the colony and doing the analysis as described in M&M section.

Table 3
Overview of the statistic tests applied to analyzed the data resulting from colonies at the end of the honey harvest season and prior to acaricide treatment under tropical and subtropical climate (Argentine, 2015).

Statistical analysis	Outcome variable	Independent variable	Objective
Step 1: Generalized Linear Mixed Model (GLMM) Apiary as random effect	*Nosema* sp. Presence/absence (binary variable)	Region	to describe the regional distribution of the main pathogens
	Nosema sp. spore count (quantitative variable) Varroa infestation level >3% and ≤3% (binary variable)		
Step 2: Univariate GLMM Apiary as random effect	DWV presence/absence	Management practices, environmental conditions, region, presence of other diseases	to identify explanatory variables potentially associated with the presence of each virus
	ABPV presence/absence BQCV presence/absence		
Step 3: Causal diagrams/Spearman or Pearson Correlation (*a priori* of the multivariable analysis)	All variables to be included in the multivariable model	All variables to be included in the multivariable model	to evaluate for potential collinearity by testing that a variable is not in the causal pathway and correlation between the variables to be include in the multivariable model
Step 4: Multivariable GLMM (apiary as random effect) with backward elimination strategy	DWV presence/absence	Variables from the univariate analysis with $P < 0.15$	to evaluate the effect of the selected explanatory variables on the viral status
	ABPV presence/absence BQCV presence/absence		
Step 5: Univariate GLMM (apiary as random effect)	Variables in the final model	Variables out of the final model	to define the relation between the variables

3. Results

3.1. Descriptive data

The mean size of each apiary was 40 ± 27 colonies (mean ± S.D.) and most beekeepers had been in the activity for more than 10 years (12 ± 8 years). All apiaries received some carbohydrate supply at least once a year, independently of the kind of syrup (sucrose or high fructose corn). Pollen substitute and synthetic vitamin supplementation were used only by 36.1% and 23.4% of the beekeepers respectively.

3.2. Regional distribution of the main pathogen

The number of virus samples (n = 363) was lower than the estimated sample size (94.3%). This was because bad climate conditions hampered the access to the apiaries and because some samples were lost. For the same reason, 24 of the samples of Phoretic *Varroa* were missing (total Varroa samples = 361, 93.8%).

The mean Varroa infestation per colony prior to the autumn treatment was 7.13 ± 8.7%. Varroa was not detected in 25 of the analyzed colonies (6.9%). The prevalence of colonies with high infestation (>3%) with *V. destructor* was 57.1% (220 of the 361

colonies examined). Semi-arid Chaco had the lower prevalence of colonies with high infestation (41.3%) ($P = 0.021$). The other regions had similar prevalence (70.8% South Santa Fe, 69.6% Central Santa Fe, 57.7% Humid Chaco and, 62.9% Transition Chaco) (Table 3, step 1).

Neither KBV nor IAPV were detected in the colonies analyzed. The other five viruses were found in different prevalence: DWV (35%), ABPV (21.5%), BQCV (8.0%), CBPV (2.2%), and SBV (1.1%). The prevalence of DWV and ABPV per region are shown in Tables 4 and 5, respectively. CBPV was only present in South Santa Fe, Center Santa Fe and Humid Chaco (6.3%, 2.9%, and 2.2%, respectively). SBV was found in South Santa Fe (4.2%), Center Santa Fe (1%) and Semi-arid Chaco (2.1%). BQCV was found in all the regions (South Santa Fe 8.3%, Center Santa Fe 10.8%, Humid Chaco and Transition Chaco 6.7% in each one and 6.3% in Semi-arid Chaco). Mean titers (taking into account all samples) were 1.63 $\log_{10}$ virus/bee for DWV (SE = 0.10$\log_{10}$ virus/bee), 0.42 $\log_{10}$ virus/bee for ABPV (SE = 0.06 $\log_{10}$ virus/bee), 0.37 $\log_{10}$ virus/bee for BQCV (SE = 0.37 $\log_{10}$ virus/bee), 0.023 $\log_{10}$ virus/bee for CBPV, and 0.002 $\log_{10}$ virus/bee for SBV (SE = 0.01 $\log_{10}$ virus/bee; SE = 0.002 $\log_{10}$ virus/bee).

The prevalence of *Nosema* sp. in the colonies was 50.13% (181/361). However, the prevalence in each eco-region was different ($P < 0.001$): 23.8% in South Santa Fe, 38.7% in Central Santa Fe, 14.9% in Humid Chaco, 12.7% in Transition Chaco, and 9.9% in Semi-arid Chaco. The average abundance of *Nosema* sp. spores was 2.37 ± 2.75 $\log_{10}$ *Nosema* spores/bee. South and Central Santa Fe showed the highest *Nosema* sp. abundance, with 4.99 ± 1.84 and 3.50 ± 2.66 $\log_{10}$ *Nosema* spores/bee, respectively. On the other hand, the subtropical regions showed lower levels than Santa Fe: Humid Chaco 1.14 ± 2.50, Transition Chaco 1.54 ± 2.29, and Semi-arid Chaco1.13 ± 2.75 $\log_{10}$ *Nosema* spores/bee, respectively ($P < 0.001$) (Table 3, step 1).

3.3. Univariate analysis

Risk factors were looked only for DWV, ABPV and BQCV because they were the top-three prevalent viruses in the study. Eight and five out of the 19 potential explanatory variables tested were selected after the DWV (Table 4) and ABPV (Table 5) univariate analysis, respectively, to be included in the multivariable analysis (the selected variables had a significance value $P < 0.15$; Table 3, step 2). For BQCV, no variable was selected and no more analysis was performed.

3.4. Multivariable analysis

The multivariable model (Table 3, step 4) revealed only one variable associated with the presence of DWV and two with the presence of ABPV. The apiary random effect was significant in both cases ($P = 0.018$; $P = 0.006$, respectively). Colonies that had a Varroa infestation rate >3% showed higher presence of DWV (OR = 1.91; 95% CI: 1.02-3.57; $P < 0.044$). The same pattern was observed for the presence of ABPV (OR = 2.23; 95% CI: 1.04-4.77; $P < 0.039$). Also, colonies where the replacement of old combs was not a common practice had higher presence of ABPV (OR = 6.02; 95% CI: 1.16-31.25; $P < 0.033$).

The variables removed from the DWV multivariable model were associated with the variables included in the model (Table 3, step 5). Regular autumn sampling for Varroa ($P = 0.022$), the diet supplementation with multivitamin ($P = 0.074$), the carbohydrate diet period ($P = 0.001$), and colony migration ($P = 0.025$) were associated with the Varroa infestation rate. Colonies where regular autumn sampling for Varroa was not done had more Varroa. The same relationship was observed with colonies supplemented with multivitamins. Diet supplementation with multivitamins was a common practice in South Santa Fe, a region where Varroa was more prevalent. When carbohydrate was added to the diet in moments other than autumn, colonies had higher prevalence of Varroa. Colonies that migrated had less Varroa infestation level.

The variables removed from the ABPV multivariable model were associated with the variables included in the model (Table 3, step 5). The surrounding vegetation was associated with Varroa infestation rate. When there were more crops and less forest and grassland, Varroa was more prevalent ($P = 0.091$). Splitting colonies ($P < 0.001$) and carbohydrate diet frequency ($P < 0.001$) were associated with the replacement of old combs. Most of the beekeepers that replaced old combs also split colonies, thus being associated management practices. In addition, beekeepers that usually replaced more combs, also provided carbohydrates to the colonies every year.

4. Discussion

A substantial number of honeybee colonies are lost around the world (Genersch, 2010; Higes et al., 2010; Le Conte et al., 2010; Nazzi et al., 2012; Francis et al., 2013; Döke et al., 2015). As honeybee diseases are at least partially responsible, identifying and preventing risk factors associated with the appearance of pathogens may help to prevent further colony losses.

4.1. Risk factors associated with the presence of DWV and ABPV

The results of the present study show how the Varroa infestation rate and certain management practices are associated with the presence of some viruses such as DWV and ABPV. The Varroa infestation rate was the only risk factor associated with the presence of DWV in the colonies, while both the Varroa infestation rate and the number of combs replaced per year in the colonies were associated with the presence of ABPV. Varroa mites are efficient vectors for both viruses (Genersch and Aubert, 2010) and could affect the immune system of bees (Yang and Cox-Foster, 2005). Furthermore, the presence of Varroa is a known stress factor capable of turning a virus covert infection into an overt one (De Miranda and Genersch, 2010; Sguazza and Reynaldi, 2015). In fact, in our study, when Varroa prevalence was >3% the presence of DWV and ABPV increased. Martin et al. (2012) found that after the arrival of *V. destructor* in previously varroa-free areas, DWV loads increase.

4.2. Management practices associated with the presence of Varroa might indirectly affect virus infection

Some management practices indirectly affect the presence of viruses since they are associated with the risk factors identified, especially with the Varroa infestation rate (Giacobino et al., 2014). The feeding with carbohydrate and multivitamin supplements in the colonies might be related to the autumn mite level since better nourished bees have an improved response to nutritional stresses accumulated in managed colonies (Mattila and Otis, 2006). A healthy colony is not only defined by the absence of diseases but also by the presence of well-nourished and reproductive individuals (Brodschneider and Crailsheim, 2010). Similarly, regular autumn sampling for Varroa is a useful tool to monitor and control Varroa. Probably, beekeepers that have monitored their colonies and found a high Varroa infestation will use a synthetic acaricide, with high effectiveness (Marcangeli et al., 2005). In addition, colony migration was associated with a lower Varroa infestation rate and other variables were associated with this strategy. Traynor et al. (2016) also found that Varroa prevalence and loads were significantly lower in migrating colonies. Beekepers that migrate colonies regularly take samples for Varroa and have to apply acari-

Table 4
Univariate analysis (GLMM with apiary as random effect) for apiary factors associated with Deformed wing virus (DWV) prevalence in colonies at the end of the honey harvest season and prior to acaricide treatment (n = 363; Argentine, 2015).

Variable	Level	DWV prevalence (%)	Odds Ratio (OR)	95% IC[a] (OR)	*P*-Value
Geographical region	South Santa Fe	72.9	0.755	0.128–1.358	0.586
	Central Santa Fe	64.7	1.163	0.277–4.884	
	Humid Chaco	58.9	1.477	0.342–6.378	
	Transition Chaco	65.3	1.007	0.215–4.698	
	Semi-arid Chaco (Ref.)	68.8	–	–	
Number of colonies per apiary	Continuous	–	0.998	0.98–1.016	0.832
Multivitamin supplements	No	61.4	**2.416**	**0.875–6.671**	**0.087**
	Yes (Ref.)	76.7			
Kind of Protein diet	No	65.8	1	0.361–2.770	0.948
	Pollen	66.7	1.115	0.040–31.092	
	Commercial patties	58.3	1.405	0.280–7.056	
	Homemade patties (Ref.)	65.6	–	–	
Carbohydrate diet	Sucrose syrup/Honey	65.2	0.648	0.069–6.071	0.707
	HFCS (Ref.)	58.3			
Carbohydrate diet period	autumn	54.8	**2.631**	**1.168–5.926**	**0.019***
	Other than autumn (Ref.)	73.8	–	–	
Carbohydrate diet frequency	When is necessary	81.5	**3.459**	**1.219–9.815**	**0.020***
	Every year (Ref.)	60.3	–	–	
Queen replacement	No	71.1	0.616	0.259–1.46	0.264
	Yes (Ref.)	61.4	–	–	
Frequency of queen replacement in the apiary	No/more than 2 years	67.1	0.702	0.291–1.698	0.419
	Every year (Ref.)	61.1	–	–	
Splitting colonies	No	71.4	0.706	0.124–4.013	0.253
	Yes (Ref.)	64.6	–	–	
Old combs replacement per colony per year	No	71.8	0.420	0.093–1.895	**0.020**
	<3 combs	79.6	0.257	0.081–0.816	
	3 combs	57.6	0.896	0.311–2.582	
	>3 combs (Ref.)	54.8	–	–	
Material disinfection	No	63.4	1.175	0.502–2.750	0.724
	Yes (Ref.)	66.2	–	–	
Colonies migration	No	66.4	**0.243**	**0.037–1.602**	**0.144**
	Yes (Ref.)	38.9	–	–	
Regular Autumn sampling for Varroa	No	74.4	0.422	0.152–1.171	**0.088**
	Pre-treatment only	87.5	0.184	0.027–1.273	
	Post-treatment only	75	0.404	0.035–4.696	
	Pre-and post-treatment (Ref.)	58.4	–	–	
Regular Autumn treatment	No	81.3	**0.319**	**0.082–1.244**	**0.099**
	Yes (Ref.)	62.5	–	–	
Apiaries closer than 1500 m	No	79.6	**0.312**	**0.126–0.772**	**0.012**
	Yes (Ref.)	57.8	–	–	
Surrounding vegetation	Forest and grasslands	68.9	1.139	0.291–4.463	0.378
	grasslands and crops	58.7	1.868	0.453–7.702	
	Only crops (Ref.)	70.8	–	–	
Varroa infestation level	>3%	75.0	**2.036**	**1.143–3.626**	**0.018**
	≤3% (Ref.)	53.0	–	–	
Nosema sp. presence	No	66.3	0.889	0.483–1.635	0.705
	Yes (Ref.)	63.8	–	–	

*Collinearity between carbohydrate diet period and carbohydrate diet frequency (Table 3, step 3).
Percentages indicates prevalence of DWV for each level of the variable.
[a] HFCS: High fructose corn syrup. 95% confidence interval. Random effect: apiary.

cide treatment before moving to other areas in order to follow good management practices (Giacobino et al., 2015).

The surrounding vegetation was another factor that indirectly affected virus presence *via* its association with the Varroa infestation rate. Temperature and humidity have a direct effect on mite population growth (Harris et al., 2003). Therefore, the Varroa level might be influenced by a combination of climatic conditions and food availability (Giacobino et al., 2014). When floral resources are available all year, or when winters are extremely short, the Varroa population could grow faster than in regions with long harsh winters and a remarkable broodless period (Calis et al., 1999)

4.3. Beekeeping practices associated with recommended apiary management

The number of old combs replaced per year was a variable associated with the presence of ABPV (the higher the number of combs replaced, the lower the presence of ABPV). Likewise, this practice was associated with splitting the colonies and with the

Table 5
Univariate analysis (GLMM with apiary as random effect) for apiary factors associated with Acute bee paralysis virus (ABPV) prevalence in colonies at the end of the honey harvest season and prior to acaricide treatment (n = 363; Argentine, 2015).

Variable	Level	ABPV prevalence (%)	Odds Ratio (OR)	95% IC[b] (OR)	*P*-Value
Geographical region	South Santa Fe	22.9	2.42	0.43–13.56	0.27
	Central Santa Fe	11.8	5.87	1.26–27.25	
	Humid Chaco	15.6	4.07	0.87–18.94	
	Transition Chaco	29.3	1.78	0.38–8.36	
	Semi-arid Chaco (Ref.)	39.6	–	–	
Number of colonies per apiary	Continuous	–	1.01	0.99–1.03	0.24
Multivitamin supplements	No	19.1	**1.92**	**0.66–5.54**	0.22
	Yes (Ref.)	30.0	–	–	
Kind of Protein diet	No	20.3	0.97	0.30–3.14	0.332
	Pollen	50.0	0.18	0.006–5.97	
	Commercial patties	27.8	0.58	0.09–3.55	
	Homemade patties (Ref.)	20.0	–	–	
Carbohydrate diet	Sucrose syrup/Honey	21.7	0.97	0.07–12.68	0.985
	HFCS (Ref.)	16.7			
Carbohydrate diet period	autumn	18.5	1.41	0.54–3.66	0.463
	Other than autumn (Ref.)	24.1	–	–	
Carbohydrate diet frequency	When is necessary	33.3	**2.73**	**0.94–7.92**	**0.061**
	Every year (Ref.)	18.1	–	–	
Queen replacement	No	23.0	0.85	0.32–2.26	0.729
	Yes (Ref.)	20.6	–	–	
Frequency of queen replacement in the apiary	No/more than 2 years	19.8	1.44	0.53–3.88	0.457
	Every year (Ref.)	24.6	–	–	
Splitting colonies	No	42.9	0.25	0.04–1.52	**0.132**
	Yes (Ref.)	20.2	–	–	
Old combs replacement per colony per year	No	48.7	0.15	0.03–0.76	**0.020**
	<3 combs	17.6	0.90	0.23–3.51	
	3 combs	19.7	0.75	0.21–2.75	
	>3 combs (Ref.)	16.7	–	–	
Material disinfection	No	19.0	1.26	0.48–3.32	0.643
	Yes (Ref.)	23.3	–	–	
Colonies migration	No	22.0	0.54	0.05–5.35	0.601
	Yes (Ref.)	11.1	–	–	
Regular Autumn sampling for Varroa	No	20	0.97	0.31–3.01	0.219
	Pre-treatment only	25	0.69	0.10–4.54	
	Post-treatment only	50	0.17	0.01–2.43	
	Pre-and post-treatment (Ref.)	18.6	–	–	
Regular Autumn treatment	No	31.1	0.47	0.13–1.76	0.260
	Yes	18.8	–	–	
Apiaries closer than 1500 m	No	15.7	1.71	2.4–7.54	0.332
	Yes (Ref.)	23.7	–	–	
Surrounding vegetation	Forest and grasslands	26.6	0.80	0.19–3.33	**0.109**
	grasslands and crops	15.9	1.92	0.41–8.98	
	Only crops (Ref.)	22.9	–	–	
Varroa infestation level	>3%	26.4	**1.92**	**0.93–3.94**	**0.079**
	≤3% (Ref.)	13.6	–	–	
Nosema sp. presence	No	25.3	0.75	0.37–1.54	0.436
	Yes (Ref.)	16.9	–	–	

*Collinearity between carbohydrate diet period and carbohydrate diet frequency (Table 3, step 3).
Percentages indicates prevalence of ABPV for each level of the variable.
[b] HFCS: High fructose corn syrup. 95% confidence interval. Random effect: apiary.

carbohydrate feeding frequency in the colonies. This is expected because, generally, beekeepers that adhere to a management program implement several suggested practices that, combined, are expected to improve the sanitary conditions of the hives (Giacobino et al., 2014, 2015). For instance, most colonies without presence of ABPV are owned by beekeepers reporting to split the colonies and replace the old combs. Another management practice which can reduce the Varroa infestation rate is dividing the colony to make nuclei. Making nucleus splits is not *per se* a control method but divides the present mite population and may thus allow the beekeeper to wait longer before a chemical treatment needs to be used (Wallner and Fries, 2003).

5. Conclusion

Virus presence is strongly correlated to *V. destructor* levels, independently of the climate conditions of where the colonies are located. So, all the factors that directly or indirectly influence mite levels will also be influencing virus presence.

Conflicts of interest statement

There are no conflicts of interest to be declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.prevetmed.2017.02.019.

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