Environmental conditions and beekeeping practices associated with *Nosema ceranae* presence in Argentina

Adriana PACINI^{1,2}, Ana MOLINERI^{1,2}, Karina ANTÚNEZ³, Natalia Bulacio CAGNOLO^{1,2},
Julieta MERKE^{1,2}, Emanuel ORELLANO^{1,2}, Ezequiel BERTOZZI², Luis ZAGO⁴,
Andrea AIGNASSE⁵, Hernán PIETRONAVE², Graciela RODRÍGUEZ⁶,
María Alejandra PALACIO⁷, Marcelo SIGNORINI^{1,2}, Agostina GIACOBINO^{1,2}

¹Instituto de Investigación de la Cadena Láctea (INTA-CONICET), Estación Experimental Agropecuaria Rafaela, Ruta 34 Km 227, Rafaela, Santa Fe, Argentina

²Instituto Nacional de Tecnología Agropecuaria EEA Rafaela, Ruta 34 Km 227, 2300, Rafaela, Santa Fe Province, Argentina

³Departamento de Microbiología, Instituto de Investigaciones Biológicas Clemente Estable, Av. Italia 3318, CP 11.600, Montevideo, Uruguay

⁴Instituto Nacional de Tecnología Agropecuaria EEA Resistencia, Av. Wilde 5, 3500, Resistencia, Chaco Province, Argentina

⁵Programa para el Desarrollo Apícola, Ministerio de la Producción de la Provincia de Formosa, Belgrano 878 - 6to. Piso, 3600, Formosa, Formosa Province, Argentina

⁶Instituto Nacional de Tecnología Agropecuaria, EEA Hilario Ascasubi, Ruta 3 Km 794, CP 8142, Hilario Ascasubi, Partido de Villarino Buenos Aires, Argentina

⁷Instituto Nacional de Tecnología Agropecuaria, EEA Balcarce, Ruta 226 Km 73,5, CP 7620, Balcarce, Buenos Aires, Argentina

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Abstract— A longitudinal study was carried out in order to evaluate *Nosema* spp. spore counts in two key moments of the year (late autumn and spring) in honeybee colonies from five eco-regions of Argentina. Three hundred and sixty colonies were randomly selected and the infestation level of *Nosema* spp. and *Varroa destructor* was assessed in autumn and spring of 2015. In addition, a questionnaire concerning all management practices applied in the colonies was answered by the beekeepers. Generalized linear mixed effect models were performed to determine the relationship between risk factors associated with *Nosema* spp. spore counts in each eco-region. Data were scanned to identify colonies cluster with a higher probability of achieving high values of *Nosema* spp. abundance for each sampling period. Spatial distribution of colonies with a higher risk of achieving high *Nosema* spp. abundance seems to be explained by geographical conditions associated to climatic conditions and some management practices implemented in each eco-region.

environment / management practices / *Nosema ceranae* / risk factor / *Varroa destructor*

1. INTRODUCTION

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Corresponding author: A. Pacini,
pacini.adriana@inta.gob.ar

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Apis mellifera colony losses episodes have been registered around the world, especially in Europe and the USA (van Engelsdorp et al., 2009; Higes et al., 2010; Le Conte et al., 2010; Lee et al., 2015; Van Der Zee et al. 2014; Jacques et al., 2017; Meana et al., 2017). More recently, honeybee colony losses

are being focus of research in South America, with similar results to those achieved in Europe and the USA (Giacobino et al. 2016b; Maggi et al. 2016; Antúnez et al. 2016; Requier et al. 2018; Molineri et al. 2018). Different pathogens (e.g., *Varroa destructor*, *Nosema* spp., viruses) and other stressors (starvation and pesticide exposure) have been identified as potential factors responsible for the losses of honeybee colonies (Potts et al., 2010; van Engelsdorp and Meixner 2010; Williams et al., 2010; Pettis et al., 2012; Maggi et al., 2016; Steinhauer et al., 2014; Molineri et al., 2018).

Nosemosis is a disease caused by two species of Microsporidia (Phylum Microspora): *Nosema apis* and *Nosema ceranae* (Higes et al., 2013). Both are commonly found in adult honeybees (*Apis mellifera*), and are obligate parasites of the bee's gut, reducing the lifespan of its host (Higes et al., 2008). However, recent studies also found that *Nosema* spp. infection occurs in brood stages under laboratory (Benavau and Nieh 2017) and natural conditions (Urbieta-Magro et al., 2019), and potentially in larval queens (Traver and Fell 2012). Regarding the impact on *A. mellifera* colonies, some studies have found that winter colony losses and reduced honey production were related to Nosemosis disease (mostly attributed to *N. ceranae*) in Europe (Higes et al. 2008, 2009; Soroker et al. 2011; Chauzat et al. 2016) and in South America (Invernizzi et al., 2011; Bravo et al., 2014). However, in other studies associated to colder climates, no link was found between *N. ceranae* and colony losses (Gisder et al. 2010; Stevanovic et al. n.d.-a, n.d.-b; Dainat et al. 2012; Francis et al. 2014). In Argentina, there are some studies that explored the impact of *Nosema* spp. in honeybees and their interaction with environmental conditions (Sarlo 2010; Signorini et al. 2014; Giacobino et al. 2016a; Pacini et al. 2016a). Some authors concluded that Nosemosis due to *N. ceranae* infection produces a high mortality of *A. mellifera* colonies (Sarlo 2010). Other studies could not find a relationship between the counts of *Nosema* spp. spores and the strength parameters of the colony or mortality related to high *Nosema* infections (Giacobino et al. 2016a; Signorini et al. 2014; Molineri et al. 2018).

Nosemosis is a widely documented disease in temperate, tropical, and subtropical climates

(Martín-Hernández et al., 2018; Grupe and Quandt 2020). Different reports proposed a climatic influence on the dynamics of *N. ceranae* and *N. apis* infections (Klee et al., 2007; Fries 2010) and virulence (Gisder et al. 2010). One of the most supported evidence is that in colder temperatures, *N. ceranae* infectivity is lower than *N. apis* given spores lose their ability to germinate (Fries 2010; Gisder et al. 2010). On the other hand, the number of spores was higher in *N. ceranae* than in *N. apis*-infected cells (up to 96 h post-infection) incubated at 33 °C (Gisder et al. 2017). Therefore, *N. apis* proliferation is favored in colder climate as *N. ceranae* spores are more sensitive to freezing temperatures while warmer climates favored its proliferation. At the same time, beekeeping practices can promote the most virulent forms of pathogens through horizontal transmission (Fries and Camazine 2001). In relation to this point, it has been documented that the replacement of old combs can reduce the damage caused by *N. apis* (Fries 1988). Similarly, re-queening practice was associated to spore counts of *Nosema* spp. as colonies with old queens developed higher infection intensity than colonies with younger queens (Muñoz et al., 2014).

On the other hand, nutritional problems have also been identified as potential factors associated to honeybee colony losses (Goulson et al. 2015). The quality of food for honeybees has markedly declined in the world in the last years (van Engelsdorp and Meixner 2010). Changes in land use are generating a reduction of flower diversity (Kremen et al., 2007), promoted by technological progress and market prices. These reductions in nutritional resources can have a direct impact on honeybee health and particularly is a possible factor associated with Nosemosis (Alaux et al., 2010; Branchiccela et al., 2019).

Seemingly, interspecies competition linked to environmental variables and beekeeping practices are important elements explaining *Nosema* spp. infection (Martín-Hernández et al. 2018). Thus, the aim of the present work is to assess the influence of environmental conditions combined with beekeeping management promoting development of *Nosema* spp. under temperate and subtropical regions in order to provide new information about the epidemiology of the infection.

Table 1. Eco-region characterization based on season average maximum and minimum registered temperature and annual precipitation

	SSF	CSF	CH	CT	CSA
	Late autumn				
T max(°C)	23.5	26.11	28.53	27.7	29.15
T min (°C)	12.55	14.88	14.07	16.72	19.07
H (%)	81.33	80.00	77.00	77.0	77.0
<i>V. destructor</i> infestation level (mean ± standard deviation)	1.85 ± 3.63	0.81 ± 2.69	3.10 ± 5.20	1.00 ± 1.86	2.75 ± 3.41
<i>Nosema</i> spp. prevalence (colonies positive to <i>Nosema</i>)	82%	66%	44%	32%	54%
	Spring				
T max(°C)	20.47	23.56	24.8	32.81	32.74
T min (°C)	14.82	9.26	12.42	13.00	17.14
H (%)	68.00	37.15	75.5	59.76	52.5
<i>V. destructor</i> infestation level (mean ± standard deviation)	1.73 ± 2.54	0.53 ± 1.35	2.26 ± 2.73	1.56 ± 1.90	2.12 ± 0.49
<i>Nosema</i> prevalence (colonies positive to <i>Nosema</i>)	88%	78%	70%	41%	39%
Annual colony losses* (mean, confidence interval 95%)	14% (6–31)	18% (10–31)	14% (8–25)	1% (0.4–2)	5% (2–12)
Annual precipitations (mm)	600–1100	800–900	> 1200	< 1000	550–800

T max , maximum temperature; *T* min , minimum temperature; *H* , humidity; *SSF* , South Santa Fe region; *CSF* , Center Santa Fe region; *CH* , Humid Chaco region; *CT* , Transition Chaco region; *CSA* , Semi-arid Chaco region

*Data extracted from Molineri A, Giacobino A, Pacini A, Bulacio-Cagnolo N, Merke J, Orellano E, Bertozzi E, Masciangelo G, Zago Luis, Aignasse Andrea, Pietronave Hernán, Rodríguez Graciela, Crisanti Paola, Palacio Maria Alejandra, Signorini M. (2018). Environment and *Varroa destructor* management as determinant of colony losses in apiaries under temperate and subtropical climate. Journal of Apicultural Research. DOI: <https://doi.org/10.1080/00218839.2018.1475697>

2. MATERIALS AND METHODS

2.1. Study design and sample size

The study was carried out between autumn and spring of 2015, considering the seasons when *Nosema* spp. spore counts were higher in similar regions from Argentina (Giacobino et al. 2016a). Colonies located in three provinces of north and central Argentina (Santa Fe, Chaco and Formosa) were sampled. Based on nectar flow period, the beekeeping management schedule, the eco-region categorization (Arzamendia and Giraudo 2004; Burkart et al. 1999; Riveros 2009), and agricultural practices (Giorgi et al., 2008; RIAN 2010) five eco-regions were defined: South Santa Fe, Central Santa Fe, Humid Chaco, Transition Chaco, and Semi-arid Chaco (Table 1).

The number of apiaries sampled from each region was determined according to the number of apiaries registered. A total of 360 colonies from 65 apiaries owned by different beekeepers were sampled. The sample size was calculated based on an estimated number of 5300 apiaries in the study area and an expected prevalence of *Nosema* spp. of 86.1% (Giacobino et al. 2016a), with a 95% confidence level and a precision < 10%. Apiaries were randomly chosen following stratified randomization procedures (computerized random numbers) (Moher et al., 2010). Within each apiary, a minimum of five colonies or 10% of the total colonies (Lee et al., 2010) was randomly selected to evaluate *Nosema* spp. spore counts and *V. destructor* infestation level.

2.2. Data collection

Data collection was assessed three times per colony: early autumn (to account for the initial colony size and health status and to agree a visit schedule with the beekeepers), late autumn (approximately 50 days after first visit), and at the beginning of spring (within 15 days). Due to the long distances, the loss of some participating apiaries could not be avoided so 360 colonies in 65 apiaries, 335 colonies in 59 apiaries, and 289 colonies in 55 apiaries were monitored during early autumn, late autumn, and spring respectively.

2.2.1. Honeybee colony strength measures

During each visit to the apiaries, the number of worker bees and the number of cells with sealed brood were estimated visually, according to the Liebefeld method, a suitable method for large amount of high-quality field data (Dainat et al., 2020).

2.2.2. *Nosema* spp. spore counts

During each visit, honeybee foragers were collected at the sealed entrance of the hive and placed in labeled plastic flasks containing 50 mL of 96% ethanol. For each sample, 60 bees from the same hive were used. Abdomens were removed with curved tweezers, and spore suspensions were prepared by adding 60 ml of distilled water to crushed abdomens in a mortar with a pestle (1 ml of water per bee in the pooled sample according to Fries et al., 2013). A 1-ml aliquot was added to a Neubauer hemocytometer, and *Nosema* spp. spores/bee was determined using light microscopy 400X (Cantwell 1970; Fries et al., 2013). Results were expressed as mean numbers of *Nosema* spp. spores/bee. Several studies suggested that proportion of infected bees is a better measurement than the average spore count per bee in a pooled sample (reviewed in Fries et al., 2013). However, the same author stated, “there is a highly significant and positive correlation” between both parameters and that under certain circumstances (e.g., a high number of samples as it is our case) pooled samples can be used.

2.2.3. *V. destructor* infestation level

Adult bees were examined to diagnose the presence of varroa mites during each visit in all selected colonies. In each colony, approximately 250 bees were collected from both sides of three unsealed brood combs in a jar containing 50% ethanol and soap. The mites were separated from the bees by shaking jars and pouring the jar content into a sieve with a mesh size of 2 mm. The intensity of mite infestation on adult bees was expressed in percentage (Dietemann et al., 2013).

Figure 1. Apiaries location and distribution according to eco-regions from Argentina.

2.2.4. Questionnaire

In each visit to the apiaries, management practices performed were obtained from a checklist questionnaire answered by the beekeepers (Supplementary Table S1). The questionnaire gathered information about geographic location, number of colonies, and commonly performed management practices (carbohydrate and protein diets, re-queening, colony migration, apiary location, and the use of plastic bags in winter to protect the brood nest) (Figure 1).

2.2.5. Determination of *Nosema* species

In order to determine the *Nosema* species present in the apiaries, pools of 50 randomly selected bees per apiary (aprox. 8 bees/colony) from the 59

apiaries sampled in late autumn were analyzed. Genomic DNA was isolated from each pool using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Species-specific PCR was carried out as described by Chen et al., (2009). Briefly, DNA extracted from each pool was amplified with two sets of *Nosema* primers individually. For each run of PCR amplification, a negative control and two positive controls were included, one for each specie (*N. apis* and *N. ceranae*).

To confirm those results, a second pool per apiary was analyzed at the Department of Microbiology, IIBCE, Uruguay. DNA was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen), according to the manufacturer's instructions. The presence of *N. ceranae* and/or *Nosema apis* was analyzed by multiplex PCR,

Table II. Definition of explanatory variables selected ($P < 0.15$) by univariate analysis for potential association with *Nosema* spp. abundance in autumn and spring in apiaries located in temperate and subtropical climate from Argentina

Variable	Level	<i>N. ceranae</i> abundance in autumn		<i>N. ceranae</i> abundance in spring	
		Mean $\pm$ SD	<i>P</i> value	Mean	<i>P</i> value
Eco-region	South Santa Fe	316081 $\pm$ 122959	< 0.001	186876 $\pm$ 62237	< 0.001
	Center Santa Fe	184486 $\pm$ 44818		252406 $\pm$ 56117	
	Humid Chaco	9434 $\pm$ 2595		259628 $\pm$ 69206	
Size of the apiary	Transition Chaco	9893 $\pm$ 2958	0.203	8336 $\pm$ 2621	< 0.001
	Semiárid Chaco	47469 $\pm$ 15992		17235 $\pm$ 5419	
	Less than 50 colonies	44909 $\pm$ 6684		74830 $\pm$ 9649	
	Equal or more than 50 colonies	64872 $\pm$ 16028		349360 $\pm$ 83225	
	No	35146 $\pm$ 5275		63509 $\pm$ 9645	
Pollen supply	Pollen cake	104478 $\pm$ 20971	< 0.001	268989 $\pm$ 53188	< 0.001
	Natural pollen	105772 $\pm$ 94156		13385 $\pm$ 11072	
Re-queening	No	44243 $\pm$ 6910	0.045	113672 $\pm$ 17151	0.889
	Yes	78375 $\pm$ 18632		117822 $\pm$ 24628	
Migratory beekeeping	No	57840 $\pm$ 7184	< 0.001	117510 $\pm$ 13549	< 0.001
	Migration to woods	1000 $\pm$ 902		18008 $\pm$ 14134	
	Migration to crops	23924 $\pm$ 15272		2649 $\pm$ 2277	
Winter cover for the colony (protect the colony from cold)	No	40308 $\pm$ 7828	0.032	122959 $\pm$ 21638	0.864
	Yes	73575 $\pm$ 14855		117759 $\pm$ 21231	
	Perennial trees	20661 $\pm$ 4643		37727 $\pm$ 8198	
Apiary location	No perennial trees	57567 $\pm$ 14340	< 0.001	181365 $\pm$ 43206	< 0.001
	Direct sun	410256 $\pm$ 159713		329792 $\pm$ 114329	
	Woods	17547 $\pm$ 3313		23027 $\pm$ 4034	
Surrounding vegetation	Woods and crops	39337 $\pm$ 8425	< 0.001	239312 $\pm$ 51620	< 0.001
	Crops	900554 $\pm$ 321189		355293 $\pm$ 108969	
	No	51336 $\pm$ 11283		48465 $\pm$ 10217	
Neighbors apiaries	Yes	56659 $\pm$ 8726	0.711	180842 $\pm$ 27316	< 0.001
Varroa infestation level early autumn			0.197		0.323
Varroa infestation level late autumn			0.854		0.059

Table II (continued)

Variable	Level	<i>N. ceranae</i> abundance in autumn		<i>N. ceranae</i> abundance in spring	
		Mean $\pm$ SD	<i>P</i> value	Mean	<i>P</i> value
<i>Nosema</i> spp. early autumn	-		< 0.001		0.006
<i>Nosema ceranae</i> late autumn	-		-		0.002
Varroa infestation level in spring			-		0.828

P values in italic showed an association between variables and *N. ceranae* abundance in each moment

as described by Martin-Hernández et al., (2007). Five microliters of obtained DNA or 1/0 dilution was used for PCR. Positive (*N. apis* and *N. ceranae*) and negative controls were included in each run.

2.3. Statistical analysis

Data was analyzed using a two-step approach. First, a univariate analysis using a generalized linear mixed effect model (GLMM) was conducted for selecting explanatory variables potentially associated with *Nosema* spp. spore counts in late autumn and spring (before and after winter). For each univariate model (*Nosema* spp. spore count ~ potential predictor), we assumed a gamma distribution for the outcome variable with a log link function and colony nested in the apiary term as a random effect (as some zeros were measured, we added a constant value of 1000 to all counts). Variables with a *P* value $\leq$ 0.15 were selected for a subsequent multivariable analysis. When two of the explanatory variable explained similar results and were statistically associated (collinearity evaluation), the variable with the lower *P* value was selected for the multivariate model (Dohoo et al. 1996).

Following, a multivariable analysis was performed using a GLMM to evaluate the effect of the selected explanatory variables on *Nosema* spp. spore count at each study moment. As mentioned before, we assumed a gamma distribution for the outcome variable with a log link function and colony nested in the apiary term as a random effect. We included the *Nosema* spp. spore count registered at early autumn as a co-variable in the autumn model and the late autumn spore counts as a co-variable in the spring model. A manually conducted backward elimination strategy was followed by removing one variable with the highest *P* value at a time, and model selection method based on AIC information was performed (Thrusfield et al. 2018). We compared the population size (number of worker bees) and number of sealed brood between *Nosema* spp infected and no-infected colonies during early and late autumn and during spring. In addition, the Spearman rank correlation was determined to analyze the relationship between *Nosema* spp. spore counts and

the number of adult worker bees and of cells with sealed brood at all three sampling times. All statistical analyzes were carried out using InfoStat software (Universidad Nacional de Córdoba, Argentina).

2.4. Spatial analysis

The spatial scan statistic cluster-detection method was used to identify and test the significance of specific clusters for a heterogeneous population distribution (Kulldorff and Nagarwalla 1995). The dataset was scanned to detect areas where *Nosema* spp. spore counts were significantly lower or higher than expected by chance. A likelihood-ratio test was calculated for each possible “window” and the scanning upper limit was set at 50% of the population at risk. *Nosema* spp. spore counts were assumed to follow a normal distribution (uses a likelihood function based on the normal distribution, even when the real distribution of the continuous variable may not be normal) , and the most likely cluster along with secondary clusters based on the Gini index criteria were reported (Kulldorff 2014). Finally, risk factors associated with the detected clusters were analyzed using Student’s *t* or Mann Whitney, based on variables distribution. Spatial analysis was performed using SaTScan sof

tware version 9.2 (www.satscan.org).

3. RESULTS

Nosema spp. spores were detected in 181 out of 360 colonies in early autumn (50%), in 181 out of 335 examined colonies in late autumn (54%), and in 190 out of 289 in spring (66%). Table I shows the *Nosema* spp. prevalence and *V. destructor* infestation level per eco-region and sampling moment. Mean *Nosema* spp. spore counts of the infected colonies were $1.21 \times 10^6 \pm 2.94 \times 10^6$ spores/ bee during autumn and $1.07 \times 10^6 \pm 2.85 \times 10^6$ spores/bee during spring. Weak correlations were found between bee population (number of worker bees) and *Nosema* spp. spore counts during early autumn ($R = 0.17$; $P < 0.001$) and during spring ($R = -0.30$; $P < 0.001$). In addition, a significant but weak correlation was found between capped brood cells and *Nosema spp.* spore counts during spring ($R = -0.16$; $P = 0.02$).

In autumn, South Santa Fe showed the maximum *N. ceranae* spores counts (316081 ± 122959 spores/bee) and Humid Chaco the minimum spore counts (9434 ± 2595 spores/bee). As Table I shows, South Santa Fe has a relative higher percentage of humidity and relative lower temperature than Humid Chaco. In spring, Humid Chaco showed the maximum *N. ceranae* spore counts (259628 ± 69206 spores/bee) and a higher percentage of humidity and

Table 3. Final multivariable logistic regression model (backward selection) for *Nosema* spp. abundance in 335 honeybee colonies in autumn and spring in apiaries located in temperate and subtropical climate from Argentina

Random effect	<i>N. ceranae</i> spore counts autumn model			<i>N. ceranae</i> spore counts spring model		
	Estimate	95% CI	<i>P</i> value	Estimate	95% CI	<i>P</i> value
Colony (apiary)	3.51	2.23–3.40	< 0.001	2.19	1.09–4.39	0.005
Fixed effect	F	df	<i>P</i> value	F	df	<i>P</i> value
Eco-region	11.27	4	< 0.001	13.41	4	< 0.001
Surrounding vegetation	13.51	2	< 0.001	9.74	2	< 0.001
Re-queening	6.32	1	0.012	–	–	–
Migratory beekeeping	11.47	1	< 0.001	17.88	2	< 0.001
<i>Nosema</i> spp. early autumn	14.45	1	< 0.001	–	–	–
<i>Nosema</i> spp. late autumn	–	–	–	6.21	1	0.001
Eco-region x Varroa late autumn	–	–	–	4.80	4	0.001

Mean in italic showed the maximun value of *N. ceranae* abundance in each variable associated

lower temperature than Transition Chaco that showed the minimum *N. ceranae* spore counts (8336 ± 2621 spores/bee) (Table II).

A total of 25 apiaries (46%) were positives during late autumn for *N. ceranae* when using primers reported by Chen et al., (2009), being this species the only one detected. This was confirmed using the primers and conditions reported by Martín-Hernández et al., (2007). *Nosema apis* was not detected in any sample although positive controls gave the expected bands.

3.1. Factors associated with *N. ceranae* spore counts in autumn

During late autumn, *N. ceranae*-infected or not infected colonies showed similar bee population and brood cells (*U* Mann Whitney 6838.5; $P = 0.96$; *U* Mann Whitney 8892; $P = 0.31$, respectively). Previous spore counts registered in the colonies, (e.g., *N. ceranae* spore counts at early autumn for autumn model and spore counts in late autumn for spring model) were significantly associated and therefore kept as covariates in both models (Table II).

Eight potential explanatory variables out of the initial fourteen were selected to be included in the multivariable GLMM for *N. ceranae* spore counts in autumn (Table II). The potential explanatory variables were eco-region ($P < 0.001$), migratory beekeeping ($P < 0.001$), pollen supply ($P < 0.001$), re-queening ($P = 0.045$), apiary location ($P < 0.001$), winter cover for the colony ($P = 0.032$), surrounding vegetation ($P < 0.001$), and *Nosema* spp. spore counts before *V. destructor* treatment ($P < 0.001$).

After the backward analysis, the final model showed five variables significantly associated with autumn *N. ceranae* spore counts (Table III). The colony (apiary) random-effect was significant ($P < 0.001$). Eco-region was also associated, as Santa Fe's eco-regions had higher spore counts than Chaco's eco-regions ($P < 0.001$). Similarly, migratory beekeeping ($P < 0.001$) and surrounding vegetation ($P < 0.001$) impact colonies health as fixed apiaries had higher spore counts than migratory apiaries and apiaries surrounded by crops showed the highest spore counts. In the survey, we asked to the respondents to identify "type of vegetation" (woods or/and crops) and if possible to indicate crop

diversity. Regarding the latter, beekeepers pointed out that apiaries located solely near crops where surrounded mainly by soybean, maize and/or alfalfa. In contrast, apiaries where re-queening is a frequent practice exhibited lower spore counts than those where re-queening was not performed ($P = 0.012$). In addition, *N. ceranae* spore counts at early autumn were positively correlated with *N. ceranae* spore counts during late autumn ($P < 0.001$).

3.2. Factors associated with *N. ceranae* spore counts in spring

During spring, *N. ceranae*-infected colonies showed significantly lower bee population than not infected colonies (15067 ± 4420 and 17362 ± 3918 bees, respectively; *U* Mann Whitney = 2692.5; $P = 0.001$). Those colonies also showed lower brood cells (25663 ± 13324 and 32232 ± 15982 cells for infected or not infected colonies, respectively; Mann Whitney = 3020; $P = 0.01$).

Eleven potential explanatory variables out of the initial fourteen were selected to be included in the multivariable GLMM for *N. ceranae* spore counts in spring (Table II). The potential explanatory variables were eco-region ($P < 0.001$), apiary size ($P < 0.001$), pollen supply ($P < 0.001$), apiary location ($P < 0.001$), neighboring apiaries ($P < 0.001$), migratory beekeeping ($P < 0.001$), surrounding vegetation ($P < 0.001$), *Nosema* spp. spore counts at late autumn ($P = 0.006$), *N. ceranae* spore counts in autumn ($P = 0.002$), and varroa infestation in late autumn ($P = 0.059$).

After the backward analysis, the final model included five variables significantly associated with *Nosema* spp. spore counts in spring (Table III). The colony (apiary) random effect was significant ($P = 0.005$). Eco-region was also associated, as Humid Chaco and Central Santa Fe had higher spore counts than the rest of the eco-regions ($P < 0.001$). Like in autumn, migratory beekeeping ($P < 0.001$) and surrounding vegetation ($P < 0.001$) impact colonies health as fixed apiaries had higher spore counts than migratory apiaries and apiaries surrounded by crops showed the highest spore counts. Furthermore, *N. ceranae* spore counts in spring were positive correlated to spore counts in late autumn ($P = 0.013$) and significantly associated with varroa level x eco-

Figure 2. Cluster distribution in autumn and spring in apiaries located under subtropical and temperate climate in Argentina. Dark grey, high *Nosema* spp. abundance cluster; light grey, low *Nosema* spp. abundance cluster. X, South Santa Fe; triangle, Central Santa Fe; black square, Warm Chaco; gray circle, Transition Chaco; +, Semi-arid Chaco.

region interaction ($P = 0.001$). Humid Chaco had higher varroa infestation in autumn than the rest of the regions and showed the highest *N. ceranae* spore counts during spring.

3.3. Spatial analysis

Three significant clusters were detected in the studied region for *N. ceranae* spore counts in autumn ($P < 0.05$). Two of them were high-count clusters of 1.23×10^6 (47 colonies) and 3.65×10^5 spores/bee (five colonies) respectively. Colonies included in these clusters came from Central Santa Fe (90.4%) and Semi-arid Chaco region (9.6%) and were mainly surrounded by wood/crops (42.5%), and only crops (42.5%). Moreover, all colonies within the high-count clusters were from fixed apiaries. The low-count cluster included 133 colonies and had 8.79×10^4 spores/bee (Figure 2a). In low count-cluster, only 9.5% of the colonies were

surrounded by crops. Most colonies included in this cluster made re-queening (72.5%).

Two significant clusters were detected in the studied region for *N. ceranae* spore counts in spring: one of high-count and another of low-count ($P < 0.05$). The high-count cluster has 5.35×10^6 spores/bee (19 colonies), and all colonies included came from Humid Chaco region and was represented by 78.9% of colonies surrounded by wood/crops. The low-count cluster has 6.20×10^4 spores/bee (91 colonies) (Figure 2b). Only 17.6% of the colonies included in this cluster were surrounded by crops.

4. DISCUSSION

Despite the fact that this study evaluated *Nosema* spp. infection in a large number of honeybee colonies distributed in different regions, the total number of apiaries included in each region is low to account for greater geographical and

biological variations, and therefore, the conclusions are limited.

As expected from previous studies, *N. ceranae* prevalence was similar between early and late autumn and higher during spring (Gisder et al. 2010, 2017). While our study only include three samples (early and late autumn and spring) during 1 year (2015), results support previous findings of *Nosema* spp. prevalence reaching its peak during late spring and early summer (Gisder et al. 2010; Traver and Fell 2011; Traver et al. 2012). *Nosema* spp. autumn prevalence reported here was around levels found in Switzerland all over 18 months including all seasons' samples (46.7%). However, spring proportion of positive *Nosema* spp. samples was considerably higher than prevalence found in German colonies during the same period (22–35%) (Gisder et al. 2010, 2017) but similar to those reported in Virginia state, USA (Traver and Fell 2011) and French colonies (Chauzat et al., 2007). Instead, *Nosema* crer. intensities quantified here were similar in both seasons. These results are consistent with *N. ceranae* intensity curve described in Chen et al., (2012) for tropical and subtropical conditions. According to the authors, *N. ceranae* found a peak in winter and infection level is lower than the annual average when the temperature exceeds 23.8 °C. Therefore, spore counts might be similar between spring and autumn because infection level is intensifying from summer to winter and descending from winter to summer. Described mean values (1.21×10^6 and 1.07×10^6 spores/bee for autumn and spring samples, respectively) are relative low but comparable to previous reports for pooled samples that might include uninfected bees (Retschnig et al., 2017).

Infection with *N. ceranae* was associated with a significant decrease in bee populations during spring but not during previous autumn, suggesting that winter losses are not fully explained by *Nosema* spp. presence or intensity (Fernández et al., 2012; Stevanovic et al., n.d.-a, n.d.-b; Meixner et al., 2014; Traver et al., 2012). Other studies also failed to associate *N. ceranae* infection intensities and colony strength ratings during spring (Traver and Fell 2011; Traver et al., 2012). Moreover, winter and total colony losses registered

in the same study area were not associated to *Nosema* spp. prevalence (Molineri et al., 2018).

While both *Nosema* species have been detected in honeybee colonies in Argentina in the past (Pacini et al., 2016a), in this study, *N. ceranae* appears to be the most prevalent as all the positive colonies were infected exclusively with *N. ceranae*. Medici et al., (2012) reported the predominance of *N. ceranae* in temperate climate from Argentina; however, it does not explain changes in the proportion of samples that resulted positive for *N. apis* earlier during the same year in the same regions (Pacini et al., 2016a). There is evidence suggesting that *N. ceranae* is displacing *N. apis* (reviewed by Martín-Hernández et al., 2012) but it seems to be a slower process than the time period observed here and it should be well documented in order to prove it (Emsen et al. 2015; Gisder et al. 2017). Emsen et al. (2015) found higher levels of infection in *N. ceranae* compared to *N. apis* parasitized colonies. Additionally, a longitudinal cohort study observed that the autumn prevalence of *N. apis* infections decreased by about 11% per year (Gisder et al. 2017). Also, regarding *N. apis* seasonality, a small peak in the autumn was reported by Fries (1993) which might coincide with early autumn (March–April) detection reported previously in the same region (Pacini et al., 2016a). Results from late autumn samples in this study were evaluated twice independently in order to check *Nosema* species in the colonies. Therefore, the difference in *N. apis* presence could be a consequence of *N. apis* seasonal trend combined with relative higher infections of *N. ceranae*.

4.1. Factors associated to *Nosema ceranae* spore counts

In this study, we identified five variables associated with autumn and spring spore counts in colonies from *Apis mellifera*. Environmental factors like eco-region and surrounding vegetation along with beekeeping practices like re-queening and migratory beekeeping explained (at least partially) the *N. ceranae* spore counts distribution during late autumn season. Similarly, eco-region and surrounding vegetation as well as migratory

beekeeping described spore counts distribution in the apiaries during spring.

Climate is a main trait in the eco-regions' assemblage and a main factor regarding *Microsporidia* life cycle (Fries 2010; Meixner et al., 2014). On the one hand, the potential for exposure to spores within the colony increases when adverse weather prevents the cleaning flight by infected bees (Moeller 1972); consequently, temperature may impact on parasite intensity (Retschnig et al., 2017). Colder climates limited the worker ability to defecate outside (Meixner et al., 2014), so it is expected to find higher spore counts in temperate regions compared to more benign environment like subtropical regions. On the other hand, a significant negative correlation of *N. ceranae* intensities with temperature under subtropical climate was reported (Chen et al., 2012). However, a longitudinal study indicated that temperature did not have a clear impact on *Nosema* spp. infection levels in feral honeybees from the USA (Rangel et al., 2016) suggesting that other factors might be involved. As our results suggested, variations in temperature and humidity alone are not enough to explain *Nosema* spp. infection level in different regions. *Nosema* spp. intensity of the infected colonies during autumn and spring is more linked to a group of interconnected drivers, including climate (temperate/subtropical) than to a single factor.

According to the beekeepers answers, soybean, maize, and alfalfa were predominately when apiaries were uniquely surrounded by crops; however, this data rely on each respondent description. Apiaries surrounded mainly by crops showed higher spore counts than colonies located near the woods which may indicate nutritional deficiencies or increased pesticide exposure. For instance, maize produces poor-quality pollen that provides to bees with an inadequate diet for proper development (Di Pasquale et al., 2016). Nutritional deficiencies are strongly linked to *Nosema* spp. presence (Di Pasquale et al., 2013; Invernizzi et al., 2011). Moreover, Branchiccela et al., (2019) found higher levels of *Nosema* spp. in colonies under nutritional stress associated to the low quality of *Eucalyptus* spp pollen. On the other hand, *Nosema* spp.-infected bees showed elevated spore loads after exposure to some

pesticides that are frequently use in extensive agricultural system (Vidau et al., 2011; Pettis et al., 2012 reviewed in Goblirsch 2018). Moreover, Schaaf (2013) conducted a survey of the types of pesticides used in Santa Fe region that revealed the presence of several pesticides linked to higher *Nosema* spp intensity.

Another factor associated with the occurrence of *N. ceranae* spore counts was the migratory beekeeping. Apiaries owned by beekeepers that move the colonies to the woods had reduced spore counts. There are two aspects to consider regarding this practice. On the one hand, as migratory beekeeping in Argentina is carried out principally to benefit from better and more diverse blooms and increase honey production; these colonies are less likely to undergo nutritional stress eventually associated to *Nosema* spp. occurrence (Branchiccela et al., 2019). On the other hand, the general health status of these bees tends to be better because beekeepers are encouraged to control diseases before moving the colonies (SENASA 2013). There is a completely different situation when colonies are moved to large monocultures (e.g., almond growing regions in the USA) increasing exposure to pesticides and limiting the floral resources, among other potential negative effects (Simone-Finstrom et al., 2016). Thus, is probable that the advantage of moving the colonies (in this case a lower spore count than colonies from fixed apiaries), it is more associated with where and how it is done than with the practice itself. Some authors point out that is essential the presence of young productive queens in colonies by regular replacement on an annual or biannual basis to maintain colony health and survival, and to increase strength and productivity (Invernizzi et al., 2009, Giacobino et al. 2015 y 2016b). Here, we found that performing requeening is a good predictor for low *N. ceranae* spore counts in autumn season but not for spring counts, probably because most beekeepers performed this practice during late spring during a good nectar flow (Büchler et al., 2014). Some authors, (Simeunovic et al., 2014; Muñoz et al., 2014; Botías et al. 2012b), showed that the replacement with a younger queen decreases the proportion of adults bees infected with *Nosema* spp. This is associated with the role of a vigorous

young queen in bee population renewal and the replacement of forager bee lost due to *Nosema* spp. infection (Moeller 1978; Harris 2008).

Spring model also showed a significant effect of varroa mites and eco-region interaction on spore counts suggesting a strong influence of other pathogens on *N. ceranae* presence (Mariani et al. 2012; Botías et al. 2012a; Pacini et al. 2016b). This might explain at least partially the higher *N. ceranae* spore counts during spring in the Humid Chaco region (subtropical). While in most of the regions, beekeepers monitored and controlled varroa populations during autumn, colonies from Humid Chaco had the highest varroa mites infestation and variation before winter. Parasitism caused by *V. destructor* is capable of modifying *Nosema* spp. annual cycle (Mariani et al. 2012; Botías et al. 2012a; Pacini et al. 2016b) thus controlling *V. destructor* infestations in colonies may be vital to mitigate *Nosema* spp. infection and managing *Nosema* spp. infections may be beneficial to control the spread of *V. destructor* mites (Little et al., 2016).

4.2. Spatial analyses

The location and traits of the cluster groups provide information about the conditions that are favorable for the development and spread of the disease (Pfeiffer et al., 2008). Three high *Nosema* infestation level clusters were found, two clusters were found in autumn, and one cluster in spring. The clusters analyzed indicated similar associations to those found in relation to risk factors. The high-rate cluster not migrate, almost half of the colonies do not re-queening and were mainly surrounded by crops.

Based on the results from the present study, *N. ceranae* spore counts in autumn and spring are associated with both environmental factors and management practices. Subtropical regions showed reduced spore counts during autumn probably linked to climate and vegetation traits that benefit cleaning behavior, reduced pesticide exposure, and nutritional status. However, migratory beekeeping, re-queening, and *V. destructor* control had a substantial impact on spore counts in both autumn and spring. Consequently, environmental factors alone are not enough to describe

N. ceranae spore counts distribution. This complex interaction between very diverse drivers varying in time and space might explain why a seasonal pattern is difficult to obtain for *N. ceranae* and *N. apis* in different geographic areas.

Scientist and beekeepers have several questions regarding *Nosema* spp prevalence, intensity, and worldwide distribution; therefore, it is still challenging in the apiculture universe to study these parasites. Further studies should be conducted in order to explain *Nosema* species seasonality and co-existence under different climates, considering beekeeping practices impact and agricultural landscape interaction.

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AUTHOR CONTRIBUTIONS

PA, MA, SM and GA designed experiments and interpreted the data. BCN, MJ, OE, PMA and RG participated in the design. PA, MA, OE, ZL, BE, AA, PH and GA performed experiments. AK conducted the analysis of *Nosema*. PA and GA wrote the paper. All authors revised the manuscript.

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Conditions environnementales et pratiques apicoles associées à la présence de *Nosema ceranae* en Argentine.

Environnement / Pratiques de gestion / *Nosema ceranae* / Facteur de risque / *Varroa destructor*.

Einfluss der Umgebungsbedingungen und der imkerlichen Betriebsweisen auf das Vorkommen von *Nosema ceranae* in Argentinien.

Umgebung / Betriebsweisen / *Nosema ceranae* / Risikofaktoren / *Varroa destructor*.

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