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ORIGINAL RESEARCH ARTICLE

Risk factors associated with the abundance of *Nosema* spp. in apiaries located in temperate and subtropical conditions after honey harvest

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Nosema apis and *Nosema ceranae* are obligate parasites that develop within the honey bee gut reducing the life of their host. The role that they have in colony losses is not clear, and it remains to be elucidated how the geographical and seasonal variations influence pathogenicity of nosema. The aim of this study was to identify risk factors associated with the abundance of *Nosema* spp. spores in apiaries located in temperate and subtropical regions after honey harvest. A total of 361 colonies distributed in five eco-regions of Argentina were examined to evaluate the abundance of *Nosema* spp. spores. Regions differed with regard to temperature, precipitation, and vegetation landscape. The abundance of *Nosema* spp. spores was significantly higher in temperate than in subtropical regions. A south-north gradient of *Nosema* spp. spore abundance was found, with the highest spore counts observed in South Santa Fe, continuously descending towards the northern regions of higher temperature. The observed gradient may be related to weather conditions and/or availability of floral resources in each eco-region. Also, colonies with >3% of *Varroa destructor* infestation showed the highest abundance of *Nosema* spp. spores. *N. ceranae*, *N. apis*, and co-infection were identified in 37.77, 26.66, and 35.55% of the studied colonies, respectively.

Factores de riesgo asociados con la abundancia de *Nosema* spp. en apiarios en condiciones templadas y subtropicales después de la cosecha de miel

Nosema apis y *Nosema ceranae* son parásitos obligados que se desarrollan en el intestino de la abeja de la miel reduciendo la vida del hospedador. El papel que tienen en la pérdida de colonias no está claro, y queda por esclarecer cómo las variaciones geográficas y estacionales influyen en la patogenicidad de *Nosema*. El objetivo de este estudio fue identificar los factores de riesgo asociados con la abundancia de esporas de *Nosema* spp. en colmenares situados en las regiones templadas y subtropicales después de la cosecha de miel. Un total de 361 colonias distribuidas en cinco ecorregiones de Argentina fueron examinadas para evaluar la abundancia de esporas de *Nosema* spp. Estas regiones difieren con respecto a la temperatura, las precipitaciones y la vegetación del paisaje. La abundancia de esporas de *Nosema* spp. fue significativamente mayor en las zonas templadas que en las regiones subtropicales. Se encontró un gradiente sur-norte de abundancia de esporas de *Nosema* spp. con recuentos más altos de esporas observados en el sur de Santa Fe, descendiendo de forma continua hacia las regiones del norte con temperatura más alta. El gradiente observado puede estar relacionada con las condiciones climáticas y / o la disponibilidad de los recursos florales en cada ecorregión. Además, las colonias con> 3% de infestación por *Varroa destructor* presentaron la mayor abundancia de esporas de *Nosema* spp. Se identificó *N. ceranae*, *N. apis* y co-infecciones en 37.77, 26.66 y 35.55% de las colonias estudiadas, respectivamente.

Keywords: *Nosema apis*; *Nosema ceranae*; *Apis mellifera*; environment; *Varroa destructor*; management practices

Introduction

Many stressors are acting to undermine honey bee health. One such is Nosemosis, a disease caused by two species of microsporidia (Phylum Microspora), *Nosema apis* and *Nosema ceranae* (Williams, Sampson, Shutler, & Rogers, 2008). Both are commonly found in adult honey bees (*Apis mellifera*) around the world and represent obligate parasites of the bee gut, reducing the lifespan of their host (Higes et al., 2008).

The pathology caused by *N. apis* has been named Nosemosis type A. The parasite can cause significant tissue damage in the gut, reducing winter survival in temperate climates, honey production and pollination effectiveness (Malone, Gatehouse, & Tregidga, 2001). *N. ceranae* can cause Nosemosis type C which can lead to nutritional and energetic stress (Mayack & Naug, 2009), suppression of the host immune response (Antúnez et al., 2009; Holt, Aronstein, & Grozinger, 2013), and

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decreased host survival at the individual level (Higes, García-Palencia, Martín-Hernández, & Meana, 2007; Dussaubat et al., 2012).

The role that these agents have in colony losses has generated much controversy (Paxton, 2010). In several European countries, the rapid dissemination of *N. ceranae* has been thought to be related with an increasing number of honey bee colony deaths and low production (Higes et al., 2008; 2009; Invernizzi et al., 2011).

Different reports have proposed that climatic factors play a role in the outcome of *N. ceranae* infections (Fries, 2010). Low temperatures inhibited *N. ceranae* germination more than *N. apis* germination (Gisder et al., 2010). Several studies conducted in regions with colder climates (Sweden, Germany) showed a predominance of *N. apis*, but in European countries (Italy, Spain) with hot summers and moderate winters *N. ceranae* was the predominant species (Klee et al., 2007). However, a recent Canadian study suggests that *N. ceranae* may be better adapted than *N. apis* at colder climates (Emsen et al., 2016).

It still remains to be elucidated how geographical and seasonal variations can influence the pathogenicity of *Nosema* spp. In Argentina there are few studies that have analyzed the impact of infections by *Nosema* spp. on bees and their interaction with environmental conditions. Based on different observational studies in the southeastern part of Argentina, Sarlo (2010) concluded that Nosemosis due to *N. ceranae* infection results in a high mortality and a delayed development of *A. mellifera* colonies. That study established that *N. ceranae* spores have a seasonal dynamic in colonies that is negatively correlated with temperature. Other studies, also carried out in Argentina, were unable to find a relationship between the sporulation level and colony strength parameters (Giacobino et al., 2016; Signorini et al., 2010). *Nosema* spp. epidemiology seems to be complex, and multiple factors may be involved simultaneously. The aim of this study was to identify the risk factors associated with the abundance of *Nosema* spp. spores in apiaries located in temperate and subtropical conditions in autumn between the end of honey harvest and the beginning of acaricide treatment.

Materials and methods

Study design and sample size

A cross sectional study was carried out from February to June 2015 in north-central Argentina. The sampling time was defined between the ending of honey harvest and the beginning of autumn acaricide treatment. A total of 361 colonies from 64 apiaries (owned by different beekeepers) were sampled. The sample size was estimated based on the fact that there are 5300 apiaries in the study area and 86.1% of expected prevalence of *Nosema* spp. (Giacobino et al., 2015), with 95% confidence level and a precision <10%. Based on the nectar flow period, the beekeeping management schedule, the eco-region categorization (Arzamendia & Giraudo, 2004; Burkart, Barbaro, Sanchez, & Gomez, 1999; Riveros, 2009), and agricultural practices (Giorgi et al., 2008; Red de Información Agropecuaria Nacional [RIAN], [RIAN], [RIAN], 2010) five regions were defined: South Santa Fe; Center Santa Fe; Warm Chaco; Transition Chaco; and Semi-arid Chaco. The number of apiaries from each region was determined according to the number of apiaries registered in each region. Apiaries were randomly chosen following stratified randomization procedures (computerized random numbers) (Moher et al., 2010). Within each apiary, a minimum of six colonies or 10% of the total colonies (Lee, Moon, Burkness, Hutchison, & Spivak, 2010) have been randomly selected to evaluate *Nosema* spp. sporulation level (abundance of *Nosema* spp.) and *V. destructor* infestation level.

South Santa Fe has a temperate climate with an annual media temperature of 18 °C and precipitations of 600 mm in its southwest and 1100 mm in its northeast region. The land use is primarily based on soybean crops, followed by corn and wheat crops. Also alfalfa pastures are cultivated as secondary production. On the other hand, Center Santa Fe (also temperate climate) has an annual media temperature of 17–18 °C, with average precipitations of 800–900 mm. The most relevant production is intensive livestock (dairy farms and wintering animals on alfalfa based pastures). Also agriculture of soy, wheat, corn, and sunflower crops located in high-quality soil is found (Giorgi et al., 2008).

The Chaco eco-region was divided into three regions according to temperature and humidity annual gradients: Warm, Transition and Semi-arid Chaco (Riveros, 2009). Warm Chaco has a subtropical climate with more than 1100 mm of annual precipitation. The annual mean temperature is 23 °C. This is a low lying area with lots of rivers. On the highlands by the coast, agriculture is developed as small farmsteads. Other places are used for livestock, for forest production or for cultivation of special crops as rice (Red de Información Agropecuaria Nacional [RIAN], 2010).

Transition Chaco has a humid to sub-humid subtropical climate. Annual mean temperatures are from 23 to 24 °C (43 °C maximum; −7 °C minimum) with an annual mean precipitation of 1000 mm. Most of the region is used for cereal, oil and cottonseed crops. Farms are generally run as in mixed production systems with livestock production as an alternative (Red de Información Agropecuaria Nacional [RIAN], 2010). Semi-arid Chaco has a semi-arid subtropical climate of with annual mean temperature of 23 °C and 550–900 mm precipitation. Wood harvest without reforestation is the most important land use. Agriculture is limited due to water shortage. Livestock production on natural pastures inside the native vegetation constitutes a low efficiency production (Red de Información Agropecuaria Nacional [RIAN], 2010).

Honey bee colony strength measures

During monitoring the following parameters were measured in colonies: (a) adult bee population (estimated as number of combs covered with adult bees); (b) brood area (estimated as number of combs covered with brood cells); and (c) number of combs covered with honey and pollen cells (vanEngelsdorp, Evans, Saegerman, Mullin, & Haubruge, 2009).

Nosema *spp. abundance and species identification*

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

Determination of Nosema *species*

Genomic DNA was isolated from spores of 50 bees randomly-sampled from each of 59 apiaries (5 apiaries were lost because the sample had not enough bees) using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Species-specific PCR was carried out as described in Chen et al. (2009).

V. destructor *infestation*

Adult bees were examined to diagnose the presence of varroa mites 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. The mites were separated from the bees by pouring the jar content into a sieve with a mesh size of 2 mm (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 percentage of infestation per colony (Dietemann et al., 2013). In previous studies, we determined a critical threshold of 3% as the 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., 2015). 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%).

Questionnaire

Potential explanatory variables were obtained from a checklist questionnaire answered by the beekeepers in order to gather information about: geographic location, number of colonies, and commonly performed management practices (carbohydrate and vitamin diets, queen replacement, making nuclei, colony migration, apiary location, use of “poncho” plastic bags used in winter to protect the brood nest). Additional information concerning apiary management practices could be obtained from the complete questionnaire which is available upon request.

Statistical analysis

The variable abundance of *Nosema* spp. was transformed to the log of nosema spores/bee. Since is not possible to log transform zero values, the response variable was transformed into $\log_{10}$ of the number of nosema spores +1 in order to include negative and positive sample values.

An single variable analysis with apiary as random effect was conducted for selecting explanatory variables potentially associated with the log of nosema spores/bee. Variables with a p-value ≤ 0.15 were selected for a subsequent multivariable analysis. Exclusively the explanatory variable with the higher p-value was selected for the multivariate model when two of them may have explained similar results and were statistically associated (collinearity evaluation).

Multivariable logistic regression analyzes with apiary as random effect were performed using a generalized linear mixed model (GLMM) to evaluate the effect of the selected explanatory variables. The outcome variable ($\log_{10}$ nosema spores/bee) was assumed to follow a Gamma distribution with logarithm link function. A manually conducted backward elimination strategy was followed by removing one variable at a time with the highest p-value. 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, Sandberg, Themudo, & Ersboll, 2012). All statistical analyzes were carried out using InfoStat software (Universidad Nacional de Córdoba, Argentina).

Spatial analysis

The spatial scan statistic (Kulldorff & Nagarwalla, 1995) cluster-detection method was used to identify and test the significance of specific clusters for a heterogeneous population distribution. The data-set was scanned to detect areas where *Nosema* spp. spore abundance was significantly lower o 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 abundance was assumed to follow a normal distribution, and the most likely cluster along with secondary clusters based on the *Gini* index criteria were reported (Kulldorff, 2014). The normal model uses a likelihood function based on the normal distribution, the true distribution of the continuous attribute must not be normal. The statistical inference (*p*-value) is valid for any continuous distribution. The reason for this is that the randomization is not done by generating simulated data from the normal distribution, but rather, by permuting the space-time locations and the continuous attribute (e.g., birth weight) of the observations. All analysis was performed using SaTScan software version 9.2 (www.satscan.org).

Results

Descriptive data

A significant correlation between the $\log_{10}$abundance *Nosema* spp. and colony size ($n = 324$; $r = 0.173$; $p = 0.002$), pollen ($n = 319$; $r = -0.239$; $p < 0.001$) and honey ($n = 319$; $r = -0.250$; $p < 0.001$) was found but the correlation coefficients were low, being the *p*-value influenced by the sample size.

The prevalence of *Nosema* spp. in the colonies was 50.13% (181/361) (Table 1). The average abundance of *Nosema* spp. spores was 2.37 ± 2.75 $\log_{10}$ nosema spores/bee. South and Center Santa Fe showed the highest *Nosema* spp. abundance with 5.03 ± 1.72 and 3.64 ± 2.45 $\log_{10}$ nosema spores/bee, respectively. On the other hand, the subtropical regions showed lower levels than Santa Fe: Warm Chaco 1.47 ± 2.01, Transition Chaco 1.85 ± 2.27, and Semi-arid Chaco1.47 ± 2.08 $\log_{10}$ nosema spores/bee, respectively.

Bivariate analysis

Six potential explanatory variables were selected individually to be included in the mixed-effects logistic regression model with apiary as random effect (selected variables had a significance value $p < 0.15$). Variables selected were: eco-region, colony multiplication, apiary location, carbohydrate diet period, use of a "poncho", and the varroa infestation level (≤3%, >3%) (Table 2).

The variable eco-region was associated with the surrounding vegetation ($p < 0.001$). Almost the half of apiaries located in Santa Fe regions were bounded by forest and crops (62.5% in South Santa Fe and 40% in Center Santa Fe). On the other hand, 66.38% of the apiaries from Chaco eco-regions were delimited by forest and natural grassland, (46.2, 84.6, and 72.7% in Warm Chaco, Transition Chaco, and Semi-arid Chaco, respectively). Therefore, only the eco-region was included in the final model.

Multivariable logistic regression analysis

Complete survey data for the potential risk factors included in the model were available for 340 of the 361 colonies sampled (86%).The apiary as random-effect was significant ($p < 0.001$). The final multivariate model identified two variables associated with the log of abundance of nosema spores: eco-regions ($p = 0.037$) and prevalence of varroa ($p = 0.001$) (Table 3). A particular geographical distribution was observed as the highest abundance was registered in the south (South Santa Fe; 3.50 $\log_{10}$ *Nosema* spores/bees) and these levels were reduced towards the north regions under study. Transition Chaco had the lowest level (1.54 $\log_{10}$ *Nosema* spores/bee). Also, colonies with more than 3% of Varroa infestation showed an increase in levels of Nosema spores/bee (Figure 1).

Those previously selected variables that were non-significant in the final multivariate model were associated with risk factors identified (Figure 2). For instance, the use of "poncho" during winter season ($p < 0.001$), and apiary localization ($p < 0.001$) were associated with eco-regions. Some management practices, as the use of "poncho" (62.5%) were frequently used in South Santa Fe but not in Chaco regions, any apiary in the Chaco regions are located in the direct sun. In addition, prevalence of varroa was associated with carbohydrate diet period ($p < 0.030$), 64.8% of the colonies who applied carbohydrate in other time than autumn had more than 3% of varroa infestation ($p < 0.001$).

Spatial analysis

Two significant clusters were detected across the geographical range. A high value cluster with a total of 124 colonies and a low value cluster with 180 colonies were

Table 1. Descriptive data.

Eco-region (N° of colonies included)	Apiary size (colonies/apiary)	Beekeepers activity (years)	Average production (last 3 years)	Average mortality (last 3 years)	Presence of nosema (%)
South Santa Fe (47)	26.88	10.92	18.65	15.87	91.5
Central Santa Fe (101)	57.07	17.92	25.83	12.86	69.3
Warm Chaco (89)	34.33	7.12	22.73	12.62	30.3
Transition Chaco (63)	34.11	11.41	14.71	16.77	29.5
Semi-arid Chaco (61)	34.10	8.19	17.20	20.08	36.5

Table 2. Definition of explanatory variables selected ($p < 0.15$) by univariate analysis for potential association with abundance of *Nosema* sp. spores.

Definition of variables	Level	Mean[a]	SE[a]	p-value
Carbohydrate diet period	Autumn	1.38	1.97	<0.001
	Other than autumn	3.63	2.47	
Varroa Infestation	Less than 3% (Ref.)	2.21	2.51	<0.001
	3% or more	3.04	2.49	
Colony multiplication	Yes (Ref.)	2.73	2.53	0.025
	No	0.59	1.33	
Eco-regions	South Santa Fe	5.03	1.72	
	Center Santa Fe	3.64	2.45	
	Warm Chaco	1.47	2.01	<0.001
	Transition Chaco	1.85	2.27	
	Semi-arid Chaco	1.47	2.08	
Apiary location	Direct sun	4.07	1.93	
	Perennial trees	1.81	2.19	<0.001
	No perennial trees	2.82	2.63	
"Poncho"	Yes	3.11	2.55	
	No	2.23	2.43	0.124
Surrounding vegetation[b]	Forest and grassland	1.90	2.26	0.022
	Forest and crops	3.11	2.64	
	Crops	3.10	2.52	

Table 3. Final multivariable logistic regression model (backward selection) for abundance of *Nosema* spp. in 361 honey bee colonies distributed in 64 apiaries.

Random effect	Estimate	95% CI[a]	Exp B	p-value
Apiary	0.860	0.540; 1.369		<0.001
Fixed effects	Level	95% IC		p-value
Eco-region	South Santa Fe	0.07; 2.207	1.139	0.001
	Central Santa Fe	−0.216; 1.55	0.667	
	Warm Chaco	−0.732; 0.940	0.104	
	Transition Chaco	−0.809; 0.946	0.068	
	Semi-arid Chaco (Ref.)	–		
Varroa Infestation	Less than 3%	−0.737; −0.180	−0.459	0.037
	3% or more than 3%(Ref)	–		

[a]95% confidence interval.

identified (57 colonies were not associated to neither of both). The 77% of the colonies with >3% of varroa infection before autumn treatment were significantly associated to the high value cluster ($p < 0.001$). In addition, apiaries from Center (61.9%) and South Santa Fe (38.1%) were included in the high value cluster, while apiaries from Semi-arid (28%), Transition (31.1%) and Warm Chaco (40.9%) were in the low value cluster (Figure 3).

Nosema *spp. identification*

Of fifty-nine apiaries the infecting nosema species was determined. 76.3% of the samples were found to be positive for *Nosema* spp. In 37.77% samples *N. ceranae* was present and in 26.66% *N. apis*. Co-infection was observed in 35.55% of studied samples.

Discussion

A remarkable regional gradient of nosema spores was observed, with high counts in South Santa Fe, descending towards the northern regions where temperatures are higher. Apparently, high temperatures negatively affect the development of *Nosema* spp., as a higher average temperature was correlated with the observation of a lower spore load in studied colonies (Chen, Chung, Wang, Solter, & Huang, 2012; Mariani et al., 2012). This observation reinforces the idea that climatic factors may play a key role in Nosemosis prevalence (Fries, 2010; Meixner et al., 2014).

However, temperature and/or humidity might not fully explain this pattern. According to our results, apiaries located in different eco-regions also showed differences with regard to the surrounding vegetation. Regions that showed the lowest spore levels were surrounded by forest and grassland, while regions with high levels of *Nosema* spp. sporulation were surrounded by crops (especially soybean and corn). As the use of pesticides and nutritional deficiencies are common in areas with a high proportion of arable crops, Nosemosis as well as other diseases might be promoted under this situation. Furthermore, Alaux, Ducloz,

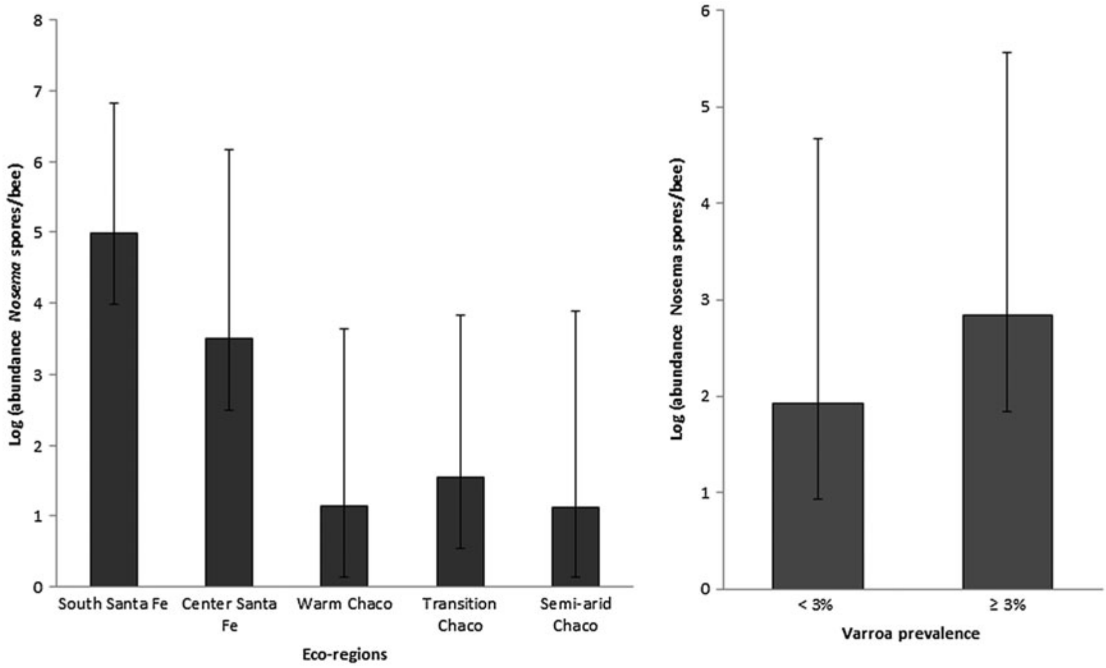

Figure 1. Variables of the final mixed-effects logistic regression model for apiary factors associated with abundance of *Nosema* spp. spores (random effect: apiary; n = 361).

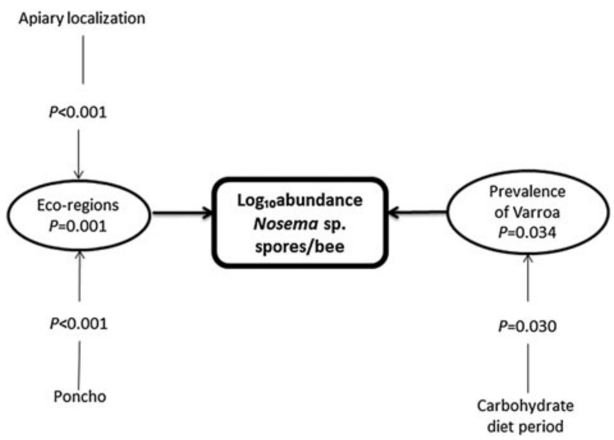

Figure 2. Association network between the significant explanatory variables in the univariate analysis (p < 0.15) and risk factors for abundance of *Nosema* spp. spores identified by the logistic regression mixed model outcome.

Crauser, and Le Conte (2010) studied the relationship between nutrition and immunity in honey bees, noting that exposure to a polyfloral diet improved some immune functions compared with monofloral diets. The same authors demonstrated the interactive/synergistic detrimental effects between microsporidia and pesticides, both factors that weaken the honey bee health (Alaux et al., 2010). Invernizzi et al. (2011) carried out a sanitary and nutritional characterization of honey bees in *Eucalyptus grandis* plantations and concluded that availability of pollen from diverse botanical origins with good nutritional value (and eventually a substitute) could attenuate infection with *N. ceranae*.

Moreover, in our study some management practices were also related to the eco-region. One of these management practices, the use of a "poncho", is commonly used in cold areas and reduces honey consumption by insulating the winter cluster. The use of a "poncho" in these areas generates optimal temperature conditions for the development of the parasites (Sarlo, 2010).

A second risk factor associated with the abundance of *Nosema* spp. was the varroa infestation level. Colonies with more than 3% of varroa on adult bees showed a higher abundance of nosema spores. That mite infestation may contribute to nosema development due to parasite interactions between *V. destructor* and nosema has been proposed by Mariani et al. (2012). Accordingly, Botías et al. (2012) reported that infection with *Nosema* spp. may negatively affect the behavior of worker bees and consequently, varroa hygiene may be lower in colonies affected by Nosemosis. The behavioral alterations induced by nosema infection may disturb colony homeostasis and organization, also affecting the foraging behavior (Campbell, Kessler, Mayack, & Naug, 2010; Dussaubat et al., 2010; Krajl & Fuchs, 2010; Woyciechowski & Moroń, 2009).

Risk factors associated with the abundance of nosema were connected with high and low value clusters identified in the spatial analysis. We found that most colonies in the high value cluster came from Center (61.9%) and South Santa Fe (38.1%) and the colonies in the low value cluster were mostly from Semi-arid Chaco (28%), Transition Chaco (31.1%), and Warm Chaco (40.9%).

Furthermore, varroa infestation level was also associated with clusters distribution. Mainly colonies from the high value cluster had >3% of Varroa infestation. Hetero-

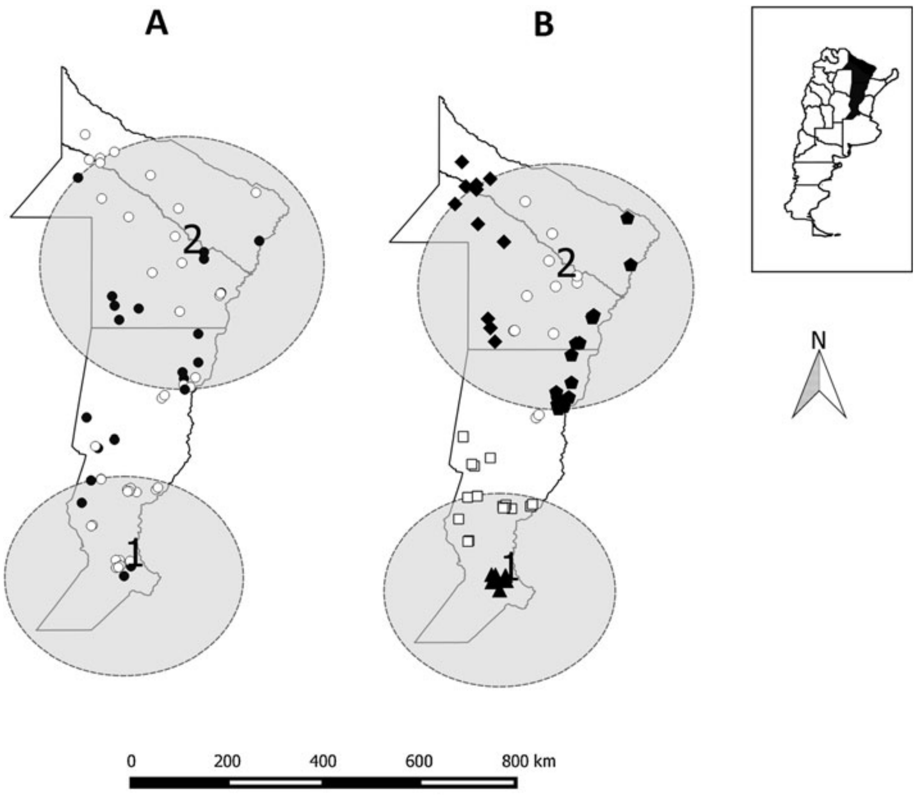

Figure 3. Monitored apiaries distribution from eco-regions (n = 64).
Note: **A**. Spatial association between cluster distribution of abundance of *Nosema* spp. spores and percentage of infestation with *V. destructor* (Black circle: >3% of infestation per colony; White circle: ≤3% of infestation per colony). **B.** Spatial association between cluster distribution of abundance of *Nosema* spp. spores and eco-regions. ○ = Transition Chaco; ◆ = semi-arid Chaco; ▲ = warm Chaco; □ = Center Santa Fe; ▲ = South Santa Fe.

geneity in environmental conditions, availability of nutritional resources, and management practices might explain to some extent the results found. This study was conducted at a time of the year when varroa infestation is high and could have affected the development of Nosemosis. However, further studies are needed in order to clarify the effect of varroa on the presence of *Nosema* spp.

Both *Nosema* species (*N. apis* and *N. ceranae*) were observed in this study alone or as co-infections. Lately, *N. ceranae* has become more common in some regions (Klee et al., 2007; Paxton, Klee, Korpela, & Fries, 2007); the niche of *N. apis* may become diminished by the presence of *N. ceranae* (Martín-Hernández et al., 2012). In Argentina, there were studies that support this idea; Medici, Sarlo, Porrini, Braunstein, and Eguaras (2012) proposed *N. ceranae* as the main causal agent of Nosemosis in Argentina since *N. apis* was found only in 2.6% of the positives samples. Likewise, Invernizzi et al. (2009) and Plischuk, Martín-Hernández, Lange, Higes, and Pascual (2008) did not find *N. apis* in analogous regions from Argentina and Uruguay.

However, studies from European countries, found both species in an equivalent proportion (Genersch et al., 2010; Gisder et al., 2010). Martín-Hernández et al. (2012) studied the occurrence of the two species in Spain and concluded that they co-exist, however, they observed the presence of *N. ceranae* in all seasons but *N apis* was found only in autumn and spring. If this observation applies also to our study region, this needs to be investigated in a long term investigation. It is possible that the occurrence of both species is explained partially by regional differences as our study included a more extensive area and considered subtropical and temperate environmental conditions with different beekeeping management. Further studies should include a species-specific analysis for region to understand the difference in their occurrence and distribution.

In our study, Eco-regions and prevalence of varroa were the main factors found to be associated with a high number of nosema spores. The information provided by this study shows that environmental conditions and availability of nutritional resources play a significant role in the development of this parasitosis. The high prevalence of nosema in apiaries with a varroa infestation higher than 3% might be related to immune suppression of these colonies with respect to both pathogens. Management practices that reduce varroa loads may also decrease the nosema spore load. In relation to the presence of *Nosema* species, both *N. apis* as *N. ceranae* were found in apiaries of all eco-regions analyzed.

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References

Alaux, C., Ducloz, F., Crauser, D., & Le Conte, Y. (2010). Diet effects on honey bee immune competence. *Biology Letters, 6*, 562–565. doi:10.1098/rsbl.2009.0986

Antúnez, K., Martín-Hernández, R., Prieto, L., Meana, A., Zunino, P., & Higes, M. (2009). Immune-suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae*. *Environmental Microbiology, 11*, 2284–2290. doi:10.1111/j.1462-2920.2009.01953.x

Arzamendia, V., & Giraudo, A. R. (2004). Usando patrones de biodiversidad para la evaluación y diseño de áreas protegidas: Las serpientes de la provincia de Santa Fe (Argentina) como ejemplo [Using biodiversity patterns for the evaluation and design of protected areas: As an example, snakes in the Santa Fe province (Argentina)]. *Revista Chilena de Historia Natural, 77*, 335–348. doi:10.4067/S0716-078X2004000200011

Botías, C., Martín-Hernández, R., Barrios, L., Garrido-Bailón, E., Nanetti, A., & Meana, A. (2012). *Nosema* spp. parasitization decreases the effectiveness of acaricide strips (Apivar) in treating varroosis of honey bee (*Apis mellifera iberiensis*) colonies. *Environmental Microbiology Reports, 4*, 57–65. doi:10.1111/j.1462-2920.2011.02647.x

Bulacio Cagnolo, N. (2011). *Manejo Integrado de* Varroa destructor *(Acari: Varroidae) en colonias de* Apis mellifera *(Hymenoptera: Apidae) en el centro oeste de la provincia de Santa Fe [Integrated Management of* Varroa destructor *(Acari Varroidae) in colonies of* Apis mellifera *(Hymenoptera: Apidae) in west central Santa Fe province]* (Unpublished doctoral thesis). Facultad de Ciencias Naturales, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina.

Burkart, R., Barbaro, N. O., Sanchez, R. O., & Gomez, D. A. (1999). *Ecoregiones de la Argentina. [Ecoregions from Argentina]*. Administración de parques nacionales, Buenos Aires. [Adobe Digital Editions version]. Retrieved from http://www.sib.gov.ar/archivos/Eco-Regiones_de_la_Argentina.pdf

Campbell, J., Kessler, B., Mayack, C., & Naug, D. (2010). Behavioural fever in infected honey bees: Parasitic manipulation or coincidental benefit? *Parasitology, 137*, 1487–1491. doi:10.1017/S0031182010000235

Cantwell, G. (1970). Standard methods for counting nosema spores. *American Bee Journal, 110*, 222–223.

Chen, Y., Evans, J. D., Zhou, L., Boncristiani, H., Kimura, K., Xiao, T., … , Pettis, J. (2009). Asymmetrical coexistence of *Nosema ceranae* and *Nosema apis* in honey bees. *Journal of Invertebrate Pathology, 101*, 204–209. doi:10.1016/j.jip.2009.05.012

Chen, Y. W., Chung, W. P., Wang, C. H., Solter, L. F., & Huang, W. F. (2012). *Nosema ceranae* infection intensity highly correlates with temperature. *Journal of Invertebrate Pathology, 111*, 264–267. doi:10.1016/j.jip.2012.08.014

Chowdhury, S., Sandberg, M., Themudo, G. E., & Ersboll, A. K. (2012). Risk factors for Campylobacter infection in Danish broiler chickens. *Poultry Science, 91*, 2701–2709. doi:10.3382/ps.2012-02412

Dietemann, V., Nazzi, F., Martin, S. J., Anderson, D., Locke, B., Delaplane, K. S., … Ellis, J. D. (2013). Standard methods for varroa research. In V. Dietemann, J. D. Ellis, & P. Neumann (Eds.), *The COLOSS BEEBOOK, Volume II: Standard methods for Apis mellifera pest and pathogen research. Journal of Apicultural Research, 52*(1). doi:10.3896/IBRA.1.52.1.09

Dussaubat, C., Maisonnasse, A., Alaux, C., Tchamitchan, S., Brunet, J., Plettner, E., …, Le Conte, Y. (2010). *Nosema* spp. infection alters pheromone production in honey bees (*Apis mellifera*). *Journal of Chemical Ecology, 36*, 522–525. doi:10.1007/s10886-010-9786-2

Dussaubat, C., Sagastume, S., Gómez-Moracho, T., Botías, C., García-Palencia, P., Martín-Hernández, R., …, Higes, M. (2012). Comparative study of *Nosema ceranae* (Microsporidia) isolates from two diferente geographic origins. *Veterinary Microbiology, 162*, 670–678. doi:10.1016/j.vetmic.2012.09.012

Emsen, B., Guzman-Novoa, E., Hamiduzzaman, M., Eccles, L., Lacey, B., Ruiz-Pérez, L., & Nasr, M. (2016). Higher prevalence and levels of *Nosema ceranae* than *Nosema apis* infections in Canadian honey bee colonies. *Parasitology Research, 115*, 175–181. doi:10.1007/s00436-015-4733-3

Fries, I. (1988). Infectivity and multiplication of *Nosema apis* Z. in the ventriculus of the honey bee. *Apidologie, 19*(3), 319–328. doi:10.1051/apido:19880310

Fries, I. (2010). *Nosema ceranae* in European honey bees (*Apis mellifera*). *Journal of Invertebrate Pathology., 103*, S73–S79. doi:10.1016/j.jip.2009.06.017

Fries, I., Chauzat, M.-P., Chen, Y.-P., Doublet, V., Genersch, E., Gisder, S., … Williams, G. R. (2013). Standard methods for nosema research. In V. Dietemann, J. D. Ellis, & P. Neumann (Eds.), *The COLOSS BEEBOOK: Volume II: Standard methods for Apis mellifera pest and pathogen research. Journal of Apicultural Research, 52*(1). doi:10.3896/IBRA.1.52.1.14

Giacobino, A., Molineri, A., Cagnolo, N., Merke, J., Orellano, E., Bertozzi, E., Masciángelo, G., …, Signorini, M. (2015). Risk factors associated with failures of Varroa treatments in honey bee colonies without broodless period. *Apidologie, 46*, 573–582. doi:10.1007/s13592-015-0347-0

Giacobino, A., Rivero, R., Molineri, A., Bulacio-Cagnolo, N., Merke, J., Orellano, E., …, Signorini, M. (2016). Fumagillin control of *Nosema ceranae* (Microsporidia: Nosematidae) infection in honey bee (Hymenoptera: Apidae) colonies in Argentina. *Veterinaria Italiana, 52*, 145–151. doi:10.12834/VetIt.120.337.6

Genersch, E., von der Ohe, W., Kaatz, H., Schroeder, A., Otten, C., Büchler, R., …, Peter, R. (2010). The German bee monitoring project: A long term study to understand periodically high winter losses of honey bee colonies. *Apidologie, 41*, 332–352. doi:10.1051/apido/2010014

Giorgi, R., Tosolini, R., Sapino, V., Villar, J., León, C., & Chiavassa, A. (2008). Zonificación Agroeconómica de la provincia de Santa Fe. [Agroeconomic zonification in Santa Fe province] (pp. 215–224). Argentina: INTA 110, ISSN 0325-9137.

Gisder, S., Hedtke, K., Mocke, I. N., Frielitz, M. C., Linde, A., & Genersch, E. (2010). Five-year cohort study of *Nosema* spp. in Germany: Does climate shape virulence and

assertiveness of *Nosema ceranae? Applied and Environmental Microbiology, 76*, 3032–3038. doi:10.1128/AEM.03097-09
Higes, M., García-Palencia, P., Martín-Hernández, R., & Meana, A. (2007). Experimental infection of *Apis mellifera* honey bees with *Nosema ceranae* (Microsporidia). *Journal of Invertebrate Pathology, 94*, 211–217. doi:10.1016/j.jip.2006.11.001
Higes, M., Martin-Hernandez, R., Garcia-Palencia, P., Marín, P., & Meana, A. (2009). Horizontal transmission of *Nosema ceranae* (Microsporidia) from worker honey bees to queens (*Apis mellifera*). *Environmental Microbiology Reports, 1*, 495–498. doi:10.1111/j1758-2229.00052.x
Higes, M., Martín-Hernández, R., Botías, C., Bailón, E., González, Porto A. V., … Meana, A. (2008). How natural infection by *Nosema ceranae* causes honey bee colony collapse. *Environmental Microbiology, 10*, 2659–2669. doi:10.1111/j.1462-2920.2008.01687.x
Holt, H., Aronstein, K., & Grozinger, C. (2013). Chronic parasitization by Nosema microsporidia causes global expression changes in core nutritional, metabolic and behavioral pathways in honey bee workers (*Apis mellifera*). *BMC Genomics, 14*, 799. doi:10.1186/1471-2164-14-799
Invernizzi, C., Abud, C., Tomasco, I. H., Harriet, J., Ramallo, G., Campá, J., … Mendoza, Y. (2009). Presence of *Nosema ceranae* in honey bees (*Apis mellifera*) in Uruguay. *Journal of Invertebrate Pathology, 101*, 150–153. doi:10.1016/j.jip.2009.03.006
Invernizzi, C., Santos, E., Garcia, E., Daners, G., Di Landro, R., Saadoun, A., & Cabrera, C., (2011). Sanitary and nutritional characterization of honey bees colonies in *Eucalyptus grandis* plantations. *Arch. Zootec, 60*, 1303–1314. Retrieved from https://dialnet.unirioja.es/servlet/articulo?codigo=5136599
Klee, J., Besana, A. M., Genersch, E., Gisder, S., Nanetti, A., Tam, D. Q., … Paxton, R. J. (2007). Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee. *Journal of Invertebrate Pathology, 96*, 1–10. doi:10.1016/j.jip.2007.02.014
Krajl, J., & Fuchs, S. (2010). *Nosema* sp. influence flight behavior of infected honey bees (*Apis mellifera*) foragers. *Apidologie, 41*, 21–28. doi:10.1051/apido/2009046
Kulldorff, M. (2014). *SaTScan User Guide for version 9.3* [Adobe Digital Editions version]. Retrieved from http://www.satscan.org
Kulldorff, M., & Nagarwalla, N. (1995). Spatial disease clusters: Detection and inference. *Statistics in Medicine, 14*, 799–810. doi:10.1002/sim.4780140809
Lee, K. V., Moon, R. D., Burkness, E. C., Hutchison, W. D., & Spivak, M. (2010). Practical sampling plans for *Varroa destructor* (Acari: Varroidae) in *Apis mellifera* (Hymenoptera: Apidae) colonies and apiaries. *Journal of Economic Entomology, 103*, 1039–1050.
Malone, L. A., Gatehouse, H. S., & Tregidga, E. L. (2001). Effects of time, temperature and honey on *Nosema apis* (Microsporidia: Nosematidae), a parasite of the honey bee, *Apis mellifera* (Hymenoptera: Apidae). *Journal of Invertebrate Pathology, 77*, 258–268. doi:10.1006/jipa.2001.5028
Mariani, F., Maggi, M., Porrini, M., Fuselli, S., Caraballo, G., Brasesco, C., …, Eguaras, M. (2012). Parasitic interactions between *Nosema* spp. and *Varroa destructor* in *Apis mellifera* colonies. *Zootecnia Trop, 30*, 81–90. Retrieved from http://www.scielo.org.ve/pdf/zt/v30n1/art09.pdf
Martín-Hernández, R., Botías, C., Bailón, E., Martínez-Salvador, A., Prieto, L., Meana, A., & Higes, M. (2012). Microsporidia infecting *Apis mellifera*: Coexistence or competition. Is *Nosema ceranae* replacing *Nosema apis? Environmental Microbiology, 14*, 2127–2138. doi:10.1111/j.1462-2920.2011.02645.x
Mayack, C., & Naug, D. (2009). Energetic stress in the honey bee *Apis mellifera* from *Nosema ceranae* infection. *Journal of Invertebrate Pathology, 100*, 185–188. doi:10.1016/j.jip.2008.12.001
Medici, S., Sarlo, E. G., Porrini, M., Braunstein, M., & Eguaras, M. J. (2012). Genetic variation and widespread dispersal of *Nosema ceranae* in *Apis mellifera* apiaries from Argentina. *Parasitology Research, 110*, 859–864. doi:10.1007/s00436-011-2566-2
Meixner, M. D., Francis, R. M., Gajda, A., Kryger, P., Andonov, S., Uzunov, A., …, Wilde, J. (2014). Occurrence of parasites and pathogens in honey bee colonies used in a European genotype-environment interactions experiment. *Journal of Apicultural Research, 53*, 215–229. doi:10.3896/IBRA.1.53.2.04
Moher, D., Hopewell, S., Schulz, K. F., Montori, V., Gotzsche, P. C., Devereaux, P.J., …, Elbourne, D. G. (2010). CONSORT 2010 explanation and elaboration: Updated guidelines for reporting parallel group randomized trials. *BMJ, 340*, c869. doi:10.1136/bmj.c869
Paxton, R. J. (2010). Does infection by *Nosema ceranae* cause “Colony Collapse Disorder” in honey bees (*Apis mellifera*)? *Journal of Apicultural Research, 49*, 80–84. doi:10.3896/IBRA.1.49.1.11
Paxton, R. J., Klee, J., Korpela, S., & Fries, I. (2007). *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. *Apidologie, 38*, 1–9. doi:10.1051/apido:2007037
Plischuk, S., Martín-Hernández, R., Lange, C. E., Higes, M., & Pascual. (2008). *Detección de* Nosema ceranae *(Microsporidia) en* Apis mellifera *(Hymenoptera: Apidae) de la región Pampeana [Detection of* Nosema ceranae *(Microsporidia) in* Apis mellifera *(Hymenoptera: Apidae) from Pampeana region]*. VII° Congreso Argentino de Entomología. Huerta Grande, Córdoba, Argentina.
Red de Información Agropecuaria Nacional [RIAN]. (2010). *Zonificación RIAN Chaco y Formosa [RIAN zonification Chaco and Formosa]*. EEA Saenz Peña, Chaco, Argentina: Publicación INTA.
Riveros, F. (2009). *El gran Chaco [The bigest Chaco]*. Retrieved from http://www.fao.org/ag/agp/agpc/doc/counprof/spanishtrad/argentina_sp/granchaco/GranChaco_sp.htm
Sarlo, E. G. (2010). *Aportes al conocimiento de la naturaleza y control de la Microsporidiosis causada por* Nosema ceranae *(Microsporidia, Nosematidae) en las colonias de* Apis mellifera *(Hymenoptera, Apidae) asentadas en la región sudeste de la provincia de Buenos Aires, Argentina. [Contributions to the knowledge of the nature and control of Microsporidiosis caused by* Nosema ceranae *(Microsporidia, Nosematidae) in colonies of* Apis mellifera *(Hymenoptera, Apidae) settled in the southeastern region of the province of Buenos Aires, Argentina]* (Thesis Doctoral). Facultad de Ciencias Exactas y Naturales. Universidad Nacional de Mar del Plata, Mar del Plata, Argentina.
Signorini, M., Molineri, A., Bulacio-Cagnolo, N., Merke, J., Luiselli, S., & Caporgno, J. (2010). Evaluación a campo de la efectividad del tratamiento contra Nosemosis en apiarios de la Provincia de Santa Fe. [Field evaluation of the effectiveness of Nosemosis treatment in apiaries of the Province of Santa Fe]. *Revista FAVE - Ciencias Veterinarias, 9*(1), 7–16.
vanEngelsdorp, D., Evans, J. D., Saegerman, C., Mullin, C., & Haubruge, E. (2009). Colony collapse disorder: A descriptive study. *PLoS ONE, 4*, e6481. doi:10.1371/journal.pone.0006481
Williams, G. R., Sampson, M. A., Shutler, D., & Rogers, R. E. L. (2008). Does fumagillin control the recently detected invasive parasite *Nosema ceranae* in western honey bees (*Apis mellifera*)? *Journal of Invertebrate Pathology, 99*, 342–344. doi:10.1016/j.jip.2008.04.005
Woyciechowski, M., & Moroń, D. (2009). Life expectancy and onset of foraging in the honey bee (*Apis mellifera*). *Insectes Sociaux, 56*, 193–201. doi:10.1007/s00040-009-0012-6