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Nosema ceranae (Microsporidia: Nosematidae) Does Not Cause Collapse of Colonies of Africanized *Apis mellifera* (Hymenoptera: Apidae) in Tropical Climate

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Abstract

Nosemosis is an important disease that affects honey bees (*Apis mellifera* Lineu), caused by obligate intracellular parasites, *Nosema apis* and/or *Nosema ceranae*. Since the initial detection of *N. ceranae* in *A. mellifera* coincided with recent large-scale losses of bee colonies worldwide, the impacts of this parasite under field conditions are of great interest. Here we test two hypotheses, the first one, whether the climatic variables (temperature, air humidity and precipitation) influence the intensity of infection of the microsporidium *Nosema* spp. in Africanized honey bees (*Apis mellifera*), and the second, whether the local of hive installation (outdoor or roofed) influences the intensity of infection of these spores in Africanized honey bees. Between August 2013 and August 2016, samples of Africanized bees were collected weekly from 20 colonies, of which ten were located in an open area (outdoor apiary) and ten under a roof on a concrete floor (roofed apiary). *N. ceranae* was the only species present. The type of apiary did not influence ($p > 0.05$) the number of spores of *N. ceranae* in Africanized bees. However, the infection intensities of the roofed apiary colonies were lower in the autumn. Regarding the meteorological parameters, there was a negative correlation between the winter infection intensities and the minimum temperature in the roofed apiary and the humidity in the outdoor apiary. The highest infection intensities occurred in both apiaries in the spring and summer, which may be related to higher pollen production. On average, the infection intensity was $16.19 \pm 15.81 \times 10^5$, ranging from zero to 100.5×10^5, and there were no records of collapse during the three years.

Introduction

Apis mellifera can be affected by two species of microsporidia (*Nosema apis* and *Nosema ceranae*), which cause the disease called nosemosis or nosemose, with different field symptoms and seasonal prevalence (Fries et al., 2006, 2013, Higes et al., 2008). The current diagnostic techniques include light microscopy to confirm the presence and intensity of infection and molecular tools to distinguish *Nosema* species (Paxton et al., 2007; Fries et al., 2013) or to quantify nucleic acids of the species that are present (Cilia et al., 2018).

Initially, the only etiological agent of this pathology in *Apis mellifera* bees was *N. apis*. However, the presence of *N. ceranae* (Higes et al., 2006; Huang et al., 2007) was also observed in the mid-2000s, initially in *Apis cerana* (Fries et al., 1996). It is believed, however, that *N. ceranae* began infecting *A. mellifera* bees a few decades ago, because analyses of historical samples have detected its presence in several places, such as Uruguay (Invernizzi et al., 2009), Brazil (Teixeira et al., 2013), Italy (Ferroglio et al., 2013), USA (Fell et al., 2015) and México (Guzman-Novoa et al., 2011; Guerreiro-Molina et al., 2016) in decades prior to its first

detection in bees of the genus *Apis* in 1996 (Fries et al., 1996).

Currently, the species *N. ceranae* is considered to be among the most prevalent infecting bees globally (Fries, 2010; Higes et al., 2010a; Gisder et al., 2010; Lecoq et al., 2016; Paxton et al., 2007; Higes et al., 2008; Emsen et al., 2016). *N. ceranae* can infect all members of the colony, be they workers, drones or queens (Alaux et al., 2011), including larvae (Eiri et al., 2015). This microsporidium infects the bees through the ingestion of spores from contaminated sources (pollen and water) and is eliminated in the feces (Fries et al., 1996).

The activities of cleaning and feeding in the colony bring new sources of infection (Fries, 2010; Higes et al., 2010b; 2013; Martin Hernandez et al., 2018). The spores germinate in the middle intestine, where the epithelial cells are infected, among other consequences causing the suppression of the immune system (Antúnez et al., 2009), energy stress (Mayack & Naug 2009; Castelli et al., 2020), acceleration of age polyethism (Lecoq et al., 2016), with a consequent decrease in longevity, as well as reduction of honey production and pollination (Botías et al., 2013). Infection with this microsporidium has been associated with colony collapse disorder (CCD) in parts of Europe (Higes et al., 2009; Martín-Hernández et al., 2007), but not in central Europe (Gisder et al., 2010), South America (Invernizzi et al., 2009; Pires et al., 2006), and United States (Cox-Foster et al., 2007; Chen et al., 2008; Guzman-Novoa et al., 2016). According to Fries (2010), the impact of this parasite is different depending on the environment, and research has found conflicting virulence data, either for individual bees and colonies, presenting distinct seasonal patterns between areas.

In Brazil, *N. ceranae* is widely distributed throughout the country, with no pattern of infection intensity observed during the year (Teixeira et al., 2013; Pires et al., 2016). Their presence was first reported by Klee et al. (2007), but later studies have demonstrated their presence since the 1970s (Teixeira et al., 2013), although no studies have so far evaluated the consequences of this infection in the long term. In tropical countries, there is little information about infections and seasonal patterns of *N. ceranae* (Guerrero-Molina et al., 2016; Fleites-Ayil et al., 2018). Since detection of *N. ceranae* in *A. mellifera* coincided with the recent large-scale loss of bee colonies worldwide, data on pathology and management are of significant interest (Fries et al., 2013). Epidemiological assessments and studies conducted in this sense, in Brazil, may help to elucidate the causes of colony decline and sudden losses, since there is possible involvement of pathogens and parasites in this phenomenon (Teixeira et al., 2008a; 2008b; 2012; 2013; Santos et al., 2014; Schwarz et al., 2014), including *N. ceranae*.

Here we test two hypotheses, the first one, whether the climatic variables (temperature, air humidity and precipitation) influence the intensity of infection of the microsporidium *Nosema* spp. in Africanized honey bees (*Apis mellifera*), and the second, whether the local of hive installation (outdoor or roofed) influences the intensity of infection of these spores in Africanized honey bees.

Material and Methods

From August 2013 to August 2016, samples containing about thirty Africanized honey bees were weekly collected from 20 colonies in two apiaries of the Honey Bee Health Laboratory (LASA) of the Biology Institute of the São Paulo State Agribusiness Technology Agency (IB/APTA), in Pindamonhangaba, SP. Langstroth hives were installed at easels (height of 50 cm), ten in the open air (here called "outdoor apiary") and ten under roof (3.2 m high) (here called "roofed apiary").

The spore collections were performed according to Teixeira and Message (2010): the entrance to the hive´s was closed with a strip of common foam to allow the collection of honey bees arriving from the field. These honey bees were swept with a common brush (paint brush) 4 to 5 cm wide into a universal type plastic bottle, containing 70% alcohol. At least 30 bees were collected per hive. The counts of *Nosema* spp. were performed according to Cantwell (1970).

For confirmation of the *Nosema* spp., composite samples were prepared from each beehive containing bees belonging to each weekly collection. Preparation of the samples for DNA extraction was done according to Teixeira et al. (2013) and for identification of the microsporidium species, the samples were submitted to duplex PCR reactions as described by Guimarães-Cestaro et al. (2016).

Data on temperature, air humidity and precipitation were obtained from the Polo do Vale do Paraíba meteorological station in Pindamonhangaba, SP, along with temperature and relative humidity obtained from a thermohygrometer installed in the covered apiary. In addition, pollen production data were obtained from September 2015 to August 2016 with pollen collectors of the intermediate type to evaluate the effect of this variable on the level of infection of the pathogen.

Although meteorological data and pollen quantity were collected daily, a weekly average was performed for the assessments (Table 1 and 2).

Table 1. Sampling number (N value) for each studied variable on outdoor apiary: Spores (honey bee samples), air humidity, precipitation, maximum temperature, and minimum temperature.

	Outdoor Apiary				
	Spores	Air humidity	Precipitation	Max. Temperature	Min. Temperature
Autumn	360	30	33	36	36
Spring	380	36	26	38	38
Summer	390	26	27	39	39
Winter	390	26	39	39	39
Total	**1520**	**118**	**125**	**152**	**152**

Table 2. Sampling number (N value) for each studied variable on roofed apiary: Spores (honey bee samples), air humidity, precipitation, maximum temperature, and minimum temperature.

	Covered Apiary				
	Spores	Air humidity	Precipitation	Max. Temperature	Min. Temperature
Autumn	337	22	33	22	22
Spring	380	22	26	22	22
Summer	382	26	27	26	26
Winter	364	26	39	26	26
Total	**1463**	**96**	**125**	**96**	**96**

A completely randomized factorial experimental design was used (2 apiary types x 4 seasons), with 10 replicates (represented by hives). The data were analyzed through the MIXED procedure of the SAS (Statistical Analysis System) program (SAS Institute, 2001), to determine the matrix structure of variance and covariance. The significance level adopted for the analysis of variance was 5%. The apiary averages were compared by the F-test and the seasonal differences by the Tukey-Kramer test. Pearson and Spearman correlations between spore fluctuation and climatic variables (maximum and minimum temperature, humidity, and precipitation) and pollen production in each season of the year were calculated.

Results

All the analyzed samples presented positive results for the species *Nosema ceranae*, while *Nosema apis* was not detected (Fig 1).

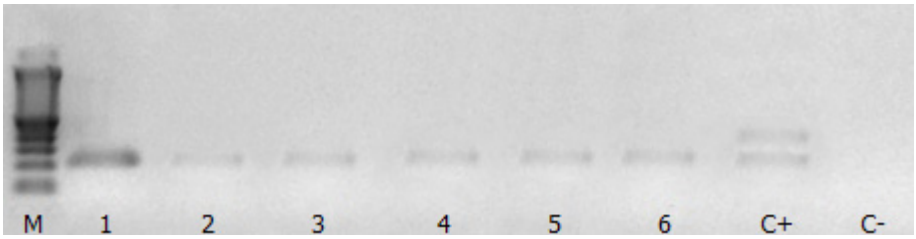

Fig 1. Agarose gel (2%) showing duplex PCR products (*Nosema apis*/*Nosema ceranae*). M: Marker. 1-6: Samples infected with *Nosema ceranae*. C +: Positive control of *Nosema apis* (321bp) and *Nosema ceranae* (218bp). C-: Negative control.

Although outdoor hives had a lower mean number of spores than covered hives, *N. ceranae* infection intensities did not differ (p=0.0706) between the place of installation of the apiaries and the seasons, except for autumn in comparison with the other seasons in the colonies of the covered apiary (p<0.0001) (Table 3).

The meteorological data were correlated with the fluctuation of the number of spores per bee (p<0.0001) only when considering season. In winter, the minimum temperature had a negative correlation with the number of spores in the outdoor apiary (Fig 2) and the humidity had a negative correlation with the number of spores in the covered apiary (Fig 3). Precipitation data had no influence on the intensity of infection (Fig 4).

The highest pollen production occurred in the summer and spring, when spore prevalence in bees was also high (Fig 5), without, however, presenting a significant difference (Table 4).

Table 3. Intensity of natural infection of *Nosema ceranae* (average number of spores/bee x10^5) in hives located in open air and covered apiaries from August 2013 to August 2016 in relation to the seasons of the year.

Season	Outdoor Apiary	Covered Apiary	Mean
Spring	16.86±17.40aA	21.51±16.39aA	19.19±3.29
Summer	14.91±15.79aA	19.02±17.03aA	16.96±2.91
Autumn	14.01±14.18aA	11.09±11.70aB	12.55±2.06
Winter	14.34±15.99aA	18.21±15.03aA	16.38±2.74
Mean	15.03±1.28	17.46±4.47	

†Means followed by different lowercase letters in the rows and upper case in the columns differ from each other by the Tukey-Kramer test ($p \leq 0.05$).

Table 4. Pearson's correlation coefficient between pollen production and spore fluctuation in each season evaluated.

Season	Outdoor Apiary R value (P value)	Covered Apiary R value (P value)
Spring	-0,4301 (0,1726)	-0,4348 (0,1376)
Summer	-0,4447 (0,1705)	-0,2662 (0,4289)
Autumn	0,1043 (0,7345)	-0,1505 (0,6235)
Winter	-0,4215 (0,1724)	-0,3537 (0,2593)

Discussion

The presence of *Nosema ceranae* and absence of *Nosema apis* corroborates the results obtained by Teixeira et al. (2013), Santos et al. (2014) and Guimarães-Cestaro et al. (2017a, 2017b), indicating possible supression of *N. ceranae*

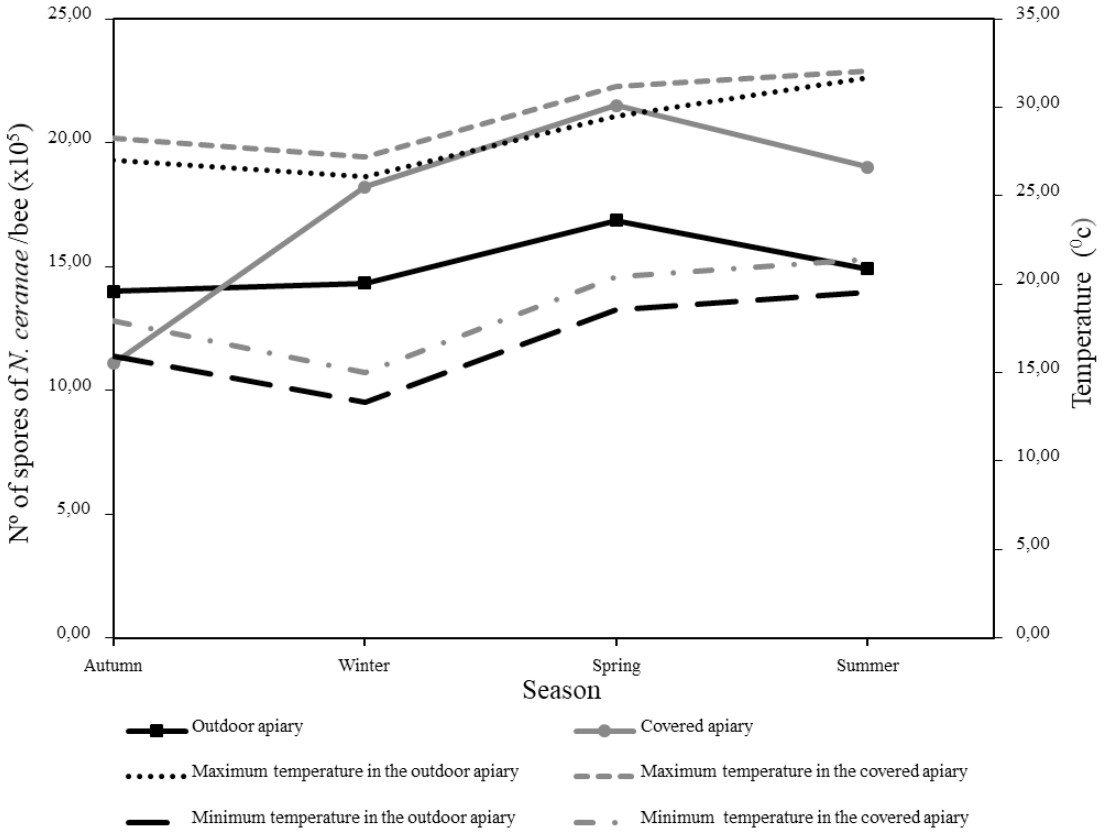

Fig 2. Intensity of natural infection of *Nosema ceranae* (mean number of spores/bee x10^5) and maximum and minimum temperatures in hives located in outdoor apiary and covered apiary from August 2013 to August 2016 in relation to seasons.

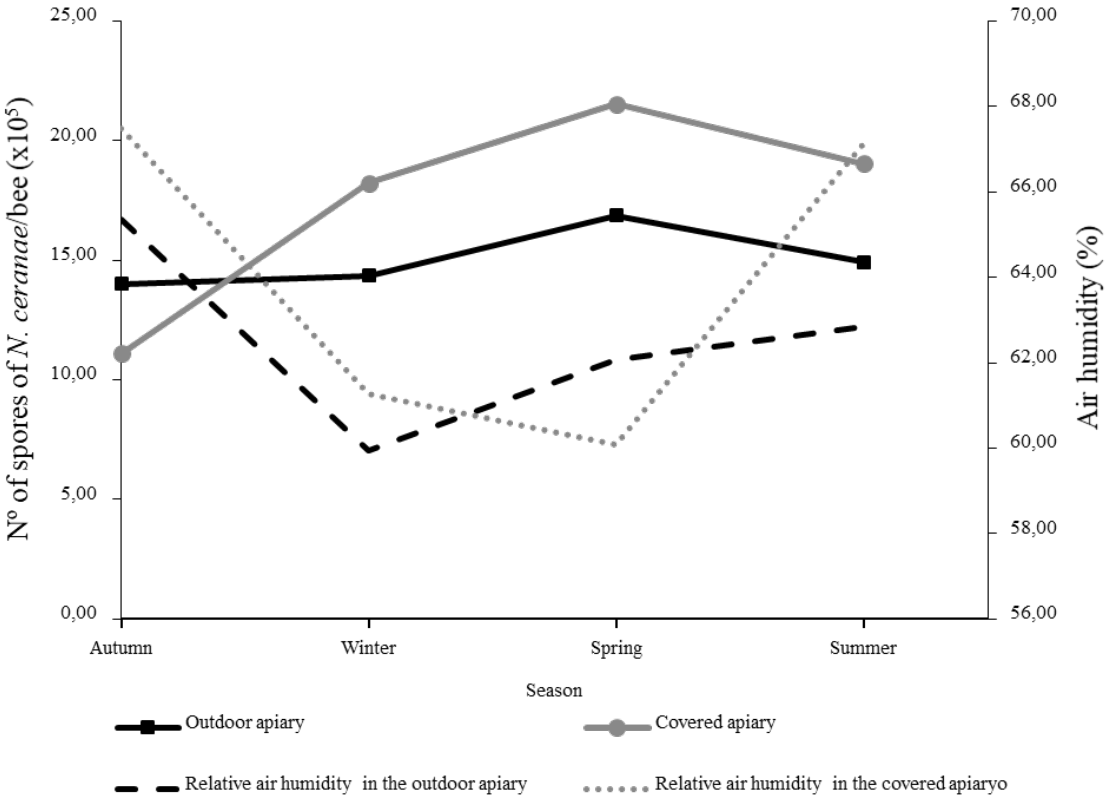

Fig 3. Intensity of natural infection of *Nosema ceranae* (mean number of spores/bee x10^5) and relative air humidity in hives located in open air apiary and covered apiary from August 2013 to August 2016 in relation to seasons.

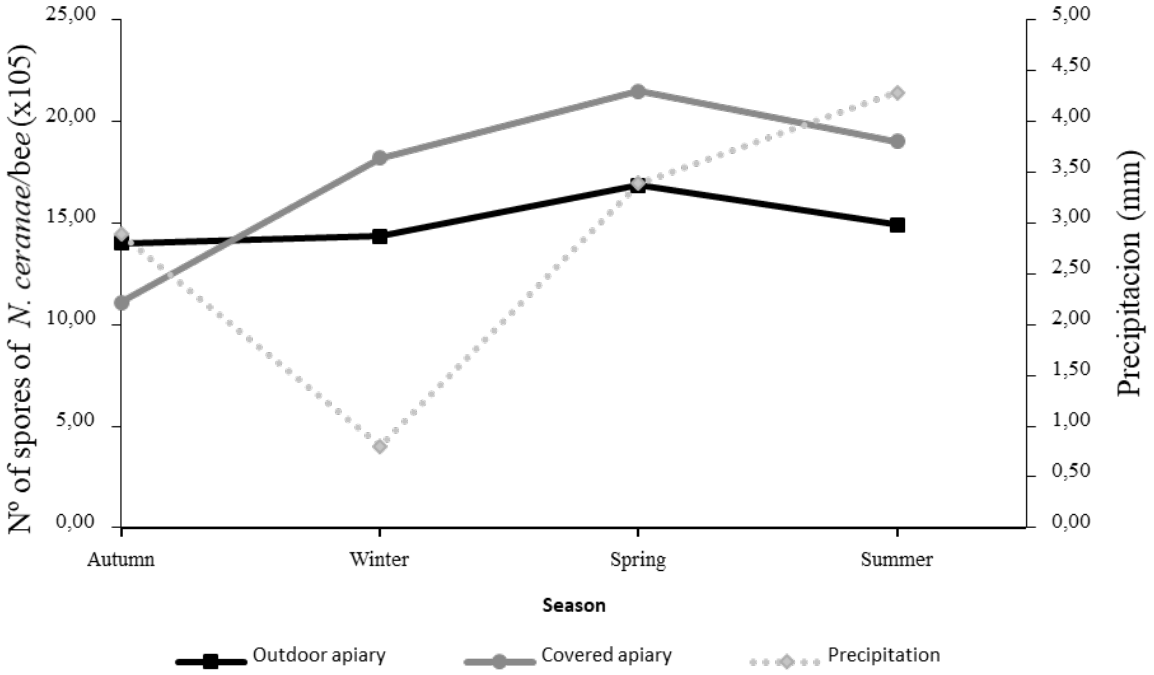

Fig 5. Intensity of natural infection of *Nosema ceranae* (average number of spores/bee x10^5) and pollen production in hives located in open air apiary and covered apiary from August 2015 to August 2016 in relation to the seasons of the year.

in the *N. apis* species in Africanized *Apis mellifera* bees in Brazil, as observed in honey bees in Europe (Klee et al., 2007; Paxton et al., 2007). However, it should be considered that reports of interactions between the two species of *Nosema* in bees are contradictory. Several authors have observed that in countries with hot summers and mild winters, there is predominance of *N. ceranae* (Higes et al., 2006; Klee et al., 2007; Martín-Hernández et al., 2007), but in countries with cooler and longer winters, *N. apis* is predominant (Gisder et al., 2010; 2017). This can also be related to the presence of *N. ceranae* and the absence of *N. apis* in the samples analyzed in the present study and others performed in Brazil (Teixeira et al., 2013; Santos et al., 2014; Guimarães-Cestaro et al., 2017a; 2017b).

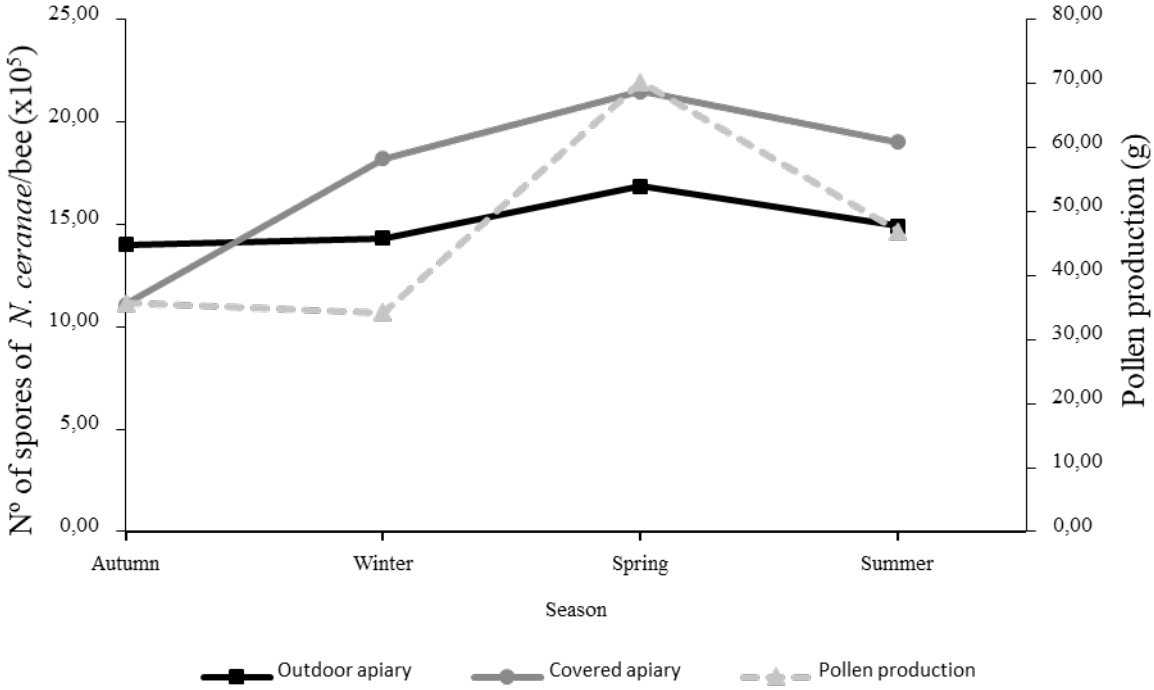

Fig 4. Intensity of natural infection of *Nosema ceranae* (mean number of spores/bee x10^5) and precipitation in hives located in outdoor apiary and covered apiary from August 2013 to August 2016 in relation to the seasons.

It is believed that cold weather is one of the limiting factors of *N. ceranae* distribution (Fries, 2010; Gisder et al., 2010; Ozkirim et al., 2019), although environmental variables and interspecies competition are important elements to explain the differential prevalence of *Nosema* spp. in different climatic regions (Martín-Hernandez et al., 2018).

In our study, the climatic parameters did not influence ($p<0.0001$) the number of spores per bee, but considering the seasons, the largest number of spores was observed in the spring and the smallest in the autumn. These results can be explained by the development of the swarm in two different periods of nectariferous secretion that occur at the study site during the year. The first and most important occurs from February to mid-May and the second from mid-July to September (Silva et al., 1995). During the main flow, the colonies had a higher population of bees, and with the end of the flowering period (which coincides with autumn), the population decreased, with a substantial decline in the queen's posture, but without completely ceasing. With the development of a secondary flow, the colonies resumed their growth, with increased posture and population.

The lowest infection rate was observed when the colonies' activity and population were reduced, due to lower temperatures and less availability of pollen, a product that is an important source of contamination by spores of *Nosema* spp. and other bee pathogens (Higes et al., 2008; Zheng et al., 2014; Guimarães-Cestaro et al., 2016; Teixeira et al., 2018). At the end of winter and early spring, with the increase of comb cleaning for posture and consequent feeding of the larvae, there was higher availability of pollen (Fig 5), higher temperatures, in addition to greater wind intensity, which possibly facilitates the spread of spores through the air (Sulborska et al, 2019), increasing prevalence of this pathogen.

Different results ($p<0.05$) were obtained only for colonies of the covered apiary, which presented negative correlation for minimum winter temperature (14.98 ± 1.58 °C) (Fig 2), and for humidity in the open apiary (59.93 ± 10.91) (Fig 3). Similar data were obtained by Chen et al., (2012) in Taiwan, where infection by *Nosema* spp. had negative correlation with temperature, and higher spore counts were observed at mean temperature of 15 ºC. These results were associated with an increase in the bee population in the spring (Winston ,1987), and are also related to the fact that during this period, there is increased risk of the disease due to spore ingestion as the bees clean the combs and feed the larvae, while the presence of spores decreases in periods of low growth (Guerreiro-Molina et al., 2016).

The type of apiary did not influence ($p=0.0706$) the number of spores per bee. Except during the autumn, in all seasons the hives in the roofed apiary tended to have higher infestation of *N. ceranae*, but without statistical difference.

The hives located in the open and roofed apiaries had, on average, $15.03 \pm 1.28 \times 10^5$ and $17.46 \pm 4.47 \times 10^5$ spores per bee, respectively, values close to those found by Santos *et al.* (2014) ($16.5 \pm 114 \times 10^5$ spores per bee) at this same location, and higher than those obtained in other municipalities of the state of São Paulo: $637 \pm 36 \times 10^3$ (Santos *et al* 2014) and 1070×10^3 (Guimarães-Cestaro *et al* 2017b). The highest intensity of infection may be related to the weeklyfrequency and kind of management adopted (equalization of the population, comb exchanges between colonies, supplementation, among others).

In the current study, even considering the high infection rates in comparison to the other municipalities of the state of São Paulo (Santos et al., 2014; Guimarães-Cestaro et al., 2017a; 2017b), no negative effects were observed that could be associated directly to infection by *N. ceranae*, and no collapse of colonies occurred during the three years analyzed. This article presents the first data on the infection intensity of *N. ceranae* during three consecutive years in Africanized bees in a tropical climate, allowing to infer that this pathogen does not cause a collapse in colonies frequently assisted of this honey bee hybrid in tropical areas.

Guerrero-Molina et al. (2016), concluded that Africanized bees infected with the pathogen suffer moderate effects when compared to European bees in temperate areas. Santos et al. (2014) and Guimarães-Cestaro et al. (2017b) verified the highest prevalence of *N. ceranae* in the autumn, but it is important to note that in these studies, the collections occurred only once in the season, unlike the current study, where bees were sampled were performed weekly. According to Teixeira et al. (2013) and Pires et al. (2016), in Brazil there is no pattern regarding the sporulation curve in different colonies and in geographically different places, as also mentioned by Fries et al. (2010) in the Northern Hemisphere.

The absence of colony collapses may be related with the constant and routinely management of apiaries, which made it possible to identify the early needs, for avoinding the substitution of queens, as consequence of absence or poor posture, as well as the needs of artificial suplementatation, timely offer of new wax foundations, adjustments in order to increase or decrease space according to population development, supplementation, among others. Studies carried in cages, with artificial infection by *Nosema* spp. have demonstrated the mortality of bees only a few days after infection (Higes et al., 2006; Paxton et al., 2007; Martín-Hernandez et al., 2011; Dussaubat et al., 2012). The technical management routinely adopted may have been crucial to allow the colonies to survive and to avoid losses reported in studies under artificial or natural conditions but not as often supervised and kept watch over.

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Author Contribution Statement

LGC did the molecular analysis and helped to wrote the manuscript, RM and TMS executed sampling, helped with the

experimental analysis and the initial drafts of the manuscript, IPO performed the statistical analyses, DM helped to plan the work, EWT and MLTMFA were in charge of the assembly and maintenance of the hives and the monthly collection samples, EWT planed and coordinated the work, supervised all the steps of the investigation and wrote the manuscript, all authors collaborated in the revision of the final version of the manuscript.

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