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ORIGINAL RESEARCH ARTICLE

Glyphosate-based herbicides and *Nosema sp.* microsporidia reduce honey bee (*Apis mellifera* L.) survivability under laboratory conditions

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Reduction in the population of pollinators can compromise the stability of natural and agricultural ecosystems. One cause of this reduction is contact between pollinators and pesticides. More specifically, pollen and nectar which contain pesticide residues are carried into the colony, in turn, decreasing the resistance of bees to parasites. Therefore, this study aimed to evaluate the mortality and food consumption of *Apis mellifera* workers infected, or not, with *Nosema* microsporidia spores and exposed to a diet containing Roundup® at the field dose recommended by the manufacturer. Each bioassay was composed of four dietary treatments: control, Roundup®, *Nosema* microsporidia spores, and both Roundup® and *Nosema* microsporidia spores. Results of both winter and spring bioassays showed that the interaction between Roundup® and *Nosema* microsporidia significantly reduced survival rate and increased food consumption of the bees. Therefore, it can be concluded that the large amounts of glyphosate-based herbicides employed on extensive monocultures can, under current agroecosystem conditions, compromise the survival of *A. mellifera* colonies.

Keywords: Nosemosis; Roundup®; mortality; interaction pesticides x parasites

Introduction

Evidence of reduced pollination in natural ecosystems is barely noticeable when compared to that in agricultural systems. The relevant consequences of pollination reduction are the extinction of plant species, the decline of animals that feed on fruits and seeds, insufficient regeneration of the flora, soil erosion and reduced water volume (Ahmad et al., 2006). In addition, the economic contribution of pollinators accounts for almost 30% (approximately $12 billion USD) of the total annual production of pollination-dependent crops (estimated at $45 billion USD) (Giannini et al., 2015). Prominently, *Apis mellifera* bees are considered the main pollinators of many species of cultivated plants resulting in increased crop productivity and quality (Mouga et al., 2012; Toledo et al., 2013).

Changes to agricultural production in recent decades, as characterized by large areas of monoculture with the use of many inputs, have led to negative environmental externalities owing to the incorrect application of pesticides to repel pests, diseases and invasive plants (Nunes, 2007). These practices have impacted the populations of numerous pollinator species on a global scale, especially bees, which are considered the most effective pollinators, leading to significant economic and environmental damage (Alves da Silva Cunha et al., 2014; Freitas et al., 2009). Many of these pesticides are not selective, such as neonicotinoids, which are associated with the reduction of pollinator populations in different countries (Di Prisco et al., 2013; Godfray et al., 2014; Henry et al., 2012; Whitehorn et al., 2012; Woodcock et al., 2017). It should be noted that their use was temporarily suspended by the European Union in 2013 (Commission Implementing Regulation (EU) No 485/2013). However, neonicotinoids can still be used as long as such use does not exceed levels that would otherwise result in harmful effects on bees (European Commission, 2020).

Because of their foraging activities, bees are chronically exposed to numerous agrochemicals, carrying them into the colony where residues can persist for variable periods (Desneux et al., 2007; Goulson, 2013). Importantly, the interaction between pesticides and parasites is also leading to the loss of bee colonies (Goulson et al., 2015), promoting immunosuppression in bees (Di Prisco et al. 2013), increasing the prevalence of *Nosema* spp. (Pettis et al. 2012) and inducing bee

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mortality by the presence of microsporidia (Abou-Shaara & Abuzeid 2018; Alaux et al. 2010).

Nosemosis, caused by the microsporidium *Nosema* sp., is one of the major bee diseases, having negative effects on both individual bees and entire colonies. Currently, *N. ceranae* is the dominant species in colonies with nosemosis in Brazil (Teixeira et al., 2013). It has been established that bee infection occurs by ingestion of mature spores, possibly during foraging, along with the ingestion of contaminated pollen, honey, water or through trophallaxis (Higes et al., 2009; L'Arrivee, 1965). Infection by *Nosema* spp. can also occur during hive cleaning, as well as cleaning the combs and removing dead or sick bees (Higes et al., 2009). In periods when bees have fewer opportunities for cleaning flights, such as in winter and periods of heavy rain, they are forced to defecate inside the hive, increasing disease rates, as spores can remain viable for up to a year (Fries, 1993). However, infection by *Nosema* spp. can also occur at the larval stage (Eiri et al., 2015). This disease causes such metabolic changes as decreased protein levels from the reduction of the hypopharyngeal glands (Wang & Mofller, 1970) and alteration in hemolymph composition at the fatty acids level (Antúnez et al., 2009), leaving bees weak and, consequently, reducing their lifespan. The interaction of this pathogen with different stressors, such as agrochemicals, contributes to increased mortality of individuals and loss of bee colonies (Goulson et al., 2015).

The effects of imidacloprid, a neonicotinoid insecticide, and pathogens on bee health were evaluated by Alaux et al. (2010). The authors verified that bees treated with Imidacloprid and fed with *Nosema* sp. spores showed reduced longevity and decreased activity of glucose oxidase. Studies evaluating the negative effects of neonicotinoids on long-term and realistic conditions have indicated that these insecticides have a negative effect on hives, in turn, resulting in a negative effect on social immunity (Tsvetkov et al., 2017), reproductive physiology and queen mortality (Tsvetkov et al., 2017; Williams et al., 2015). Similarly, studies by Pettis et al. (2013) showed that pollen contaminated with mixtures of insecticides and fungicides decreased the resistance of bees to *N. ceranae*.

Herbicides are not intended to kill insects, and for this reason, these pesticides do not carry label restrictions to reduce bee exposure (Johnson, 2015). Nonetheless, bees are often in contact with high concentrations of herbicides when applied to crop species that are attractive to pollinators during the flowering period (Pettis et al., 2013). In addition, commercial glyphosate-based herbicide formulations have inert ingredients that may be even more toxic than the glyphosate active ingredient alone (Mesnage et al., 2013; Sribanditmongkol et al., 2012; Williams et al., 2000). Some of these inert ingredients (preservatives, adjuvants, or stabilizing components) can also have toxic effects, contributing to the poor health of bee populations (Mullin et al., 2016; Zhu et al., 2014) and impairing their learning performance (Ciarlo et al., 2012).

The mechanisms of action of herbicides on bees are generally poorly studied. One exception is the work of Helmer et al. (2015) who reported a decrease in protein and β-carotene levels in worker bees that received food containing glyphosate, suggesting metabolic changes that could be attributed to herbicides. Adult workers of *A. mellifera* exposed to experimental diets contaminated with glyphosate presented reduced sucrose sensitivity, learning losses and difficulties in establishing associative memory, which could hinder the collection of resources for the colony, compromising its survival (Herbert et al., 2014) and impairing the cognitive capacities needed to retrieve and integrate spatial information for a successful return to the hive (Balbuena et al., 2015). In addition, Faita et al. (2018) observed that feeding bees that consumed pollen containing Roundup® residues showed early degeneration of the rough endoplasmic reticulum and morphological and structural alterations of mitochondria in cells of the hypopharyngeal glands. Moreover, sublethal effects of glyphosate can also alter gut microbiota in the chronically exposed honey bee (Blot et al., 2019).

Overall, herbicides induce metabolic changes that do not individually cause bee mortality but do impair colony survival. This is extremely detrimental given that many bee species are social insects. More in-depth studies with herbicides may lead to a better understanding of the events that can compromise hive viability.

In this sense, the objectives of this study were to evaluate the energetic food intake and the mortality of adult workers of *A. mellifera*, infected or not, with *Nosema* sp. spores and, at the same time, exposed to a diet containing Roundup® in concentrations below field dosage recommended by the manufacturer, but under laboratory conditions.

Materials and methods

Location

Two bioassays were developed in the Experimental Apiary of Bee City (27°32'12.28"S, 48°30'5.82"O), Federal University of Santa Catarina (Brazil). The apiary is surrounded east and north by a secondary forest of some 491.5 hectares and west and south by two small villages. No commercial agriculture is allowed in areas up to 10 km around the apiary, other than small family gardens where pesticides are not used.

Treatments

To test the interactions between *Nosema* microsporidia spores and Roundup® in the context of bee survival and food consumption index, four experimental dietary treatments were utilized: control, Roundup®, *Nosema* microsporidia spores, and both *Nosema* microsporidia

spores and Roundup®. The toxicity tests were conducted according to the methodologies proposed by OECD-213 (OECD GUIDELINES FOR THE TESTING OF CHEMICALS 213 – Honeybees, Acute Oral Toxicity Test, OECD, 1998).

Bioassays

We used recently emerged adult (0-6 h old) *A. mellifera* honey bees. All worker bees emerging from a B.O.D. incubator (Biochemical Oxygen Demand) were provided sealed brood frames of a healthy colony, not infected by *Nosema* spp., as previously determined by prevalence and PCR tests. The bees were maintained in perforated pots (volume of 1 L) for air circulation, in the dark, at ± 28 °C and 70% relative humidity during the entire experimental period. Depending on the experimental design, honey bees received both food and water *ad libitum* via 1.5 mL microtubes with holes drilled in the base. Each of the treatment plots consisted of 36 bees and four replicates, totaling 144 bees from each treatment. Bioassays were performed in both winter and spring and were monitored for a period of 120 h. Observations consisted of counting the number of surviving bees and the amount of consumed feed. At each 24 h interval, dead bees were counted and removed from the remaining bees of the plot, food and water microtubes were exchanged, and the amount of feed consumed was recorded, totaling five observation times. At the end of the experimental period, ventriculi of dead bees were individually processed for microscopic examination to verify the presence of *Nosema* spores by the OIE method, according to the applied treatment.

Herbicides used

The following commercial formulation of the herbicide Roundup® was used: (N- (phosphonomethyl) isopropylamine salt 480 g/L; N- (phosphonomethyl), glycine acid equivalent (GLYFOSATE) 360 g/L, with inert ingredients, 684 g/L). The feeding solution was prepared by mixing 1.5 μL of the commercial formulation of the glyphosate-based herbicide Roundup® with 200 mL of energy feed (50% sugar syrup, 50% distilled water), and stored under refrigeration (4 °C) for 2 h.

The Roundup® treatment offered to bees gave a ratio of glyphosate/2.16 ug a.i. g^{-1} artificial food. This ratio is equivalent to (10^{-3} g/kg) herbicide ratios found in the experimental conditions conducted by Thompson et al. (2014) who identified 15.6 mg a.i./kg of glyphosate in the nectar and 310.1 mg a.i./kg in pollen of *Phacelia* at four days after the herbicide spraying. This amount also does not exceed the recommended values (1.4 to 7.6 mg and L^{-1}) for the control of aquatic and terrestrial weeds or those measured in natural environments (Feng & Thompson 1990; Giesy et al., 2000; Goldsborough & Brown 1988).

Obtaining Nosema *spores*

The spore solution was prepared following the method proposed by Fries et al. (2013). Briefly, bee ventricles infected with *Nosema* spp. (100 bees), obtained from Bee City Experimental Apiary hives were macerated and diluted in water (1 mL per bee ventricle). The obtained solution was filtered through a 74 μm sterile mesh and mixed with buffered ammonium chloride (NH_4Cl) (2 mM, pH 9.0) and centrifuged (5 min for 5,000 $\times$ *g*). The supernatant was discarded and the pellet was resuspended in distilled water and vortexed (5 s). This procedure was repeated and the obtained solution was evaluated in a hemocytometer to determine spore concentration (23 $\times$ 106 per mL). This solution was kept under refrigeration (4 °C) for 2 h. Subsequently, a 0.5 mL aliquot was mixed with 0.5 mL sugar syrup and provided to the bees. Identification of *Nosema* spores was carried out by light microscopy and PCR according to methods adapted from the "OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals," 2019, Chapter 3.2.4 (Fries, 2019).

Exposure of bees to treatments

The contamination of bees by Nosema microsporidia spores was adapted from Higes et al. (2007). Bees receiving *Nosema* treatment were maintained without food for 2 h and then given the syrup sugar mixture with 0.5 mL of spore containing solution, as described above. During the bioassay, food not consumed was replaced by a new sugar syrup solution containing spores in the case of *Nosema* treatment.

To better understand the effects of exposure of *A. mellifera* adult workers to Roundup®, two bioassays were performed in two distinct seasons. In the first (IS-W), bees were collected in winter, and in the second (IS-S), bees were collected in spring.

DNA extraction of Nosema *spores*

To determine the species of *Nosema* microsporidia in worker bees used to prepare the spore solution for treatments, DNA extraction was performed using the method proposed by Fries et al. (2013), as also recommended by the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals noted above. Briefly, approximately 100 worker bees were randomly collected at the entrance of all hive shives and stored in flasks with alcohol 70%. In the laboratory, they were dissected, and the posterior section of the alimentary duct of 30 worker honey bees was macerated using a pestle and mortar, adding 0.5 mL of distilled water per intestine. Afterward, the solution was centrifuged at 800 g for 6 min at 4 °C to allow the spores to precipitate. The supernatant was discarded, and the pellet was macerated in liquid N_2 and transferred to a Polypropylene microtube with 1 mL of CTAB (2%) and 4 μL proteinase K (0.3 mg/μL). The microtubes were

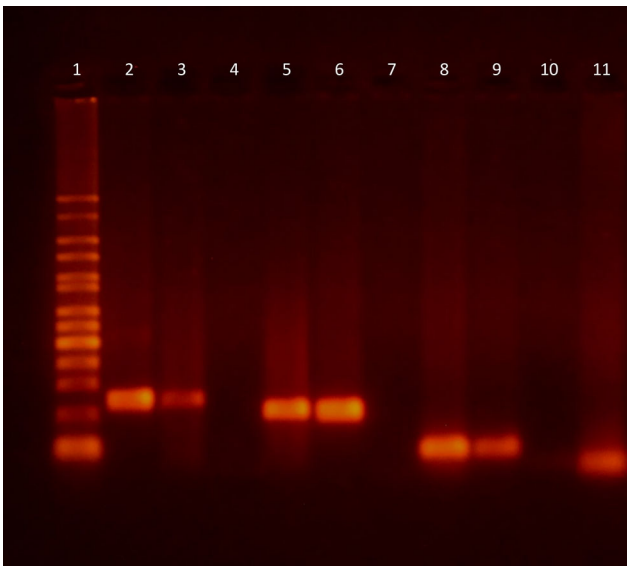

Figure 1. Agarose gel (1.5%) showing amplification of the small subunit portion of ribosomal RNA using primers 218MITOC (218 bp) and QNoC (97 bp) for *N. ceranae* and QNoA (77 bp) for *N. apis*. Lines 1 and 14 - 100 bp ladder DNA marker. Lines 5, 6, 8 and 9 – PCR product of samples of *N. ceranae*-positive bees and lines 11 and 12 are bees positive for *N. apis*. Line 2 and 3 are the positive PCR product for the Family Nosematidae; Lines 4, 7, 10 and 13, without amplification is the negative control.

incubated for 60 min at 65 °C. Subsequently, the suspension was washed with 600 μL of chloroform and isoamyl alcohol (24:1), centrifuged at 7500 rpm for 5 min, and the supernatant transferred to a new microtube. This step was done three times. Then, we added cold isopropanol to the solution and incubated at −18 °C overnight. Following that, the microtube was centrifuged, the supernatant discarded, and the pellet washed with alcohol 100°GL. After drying, 2 μL of RNAse (10 mg/μL) were added to the microtube at 37 °C for 30 min. Finally, 50 μL of ultrapure water were added. The DNA quality and quantity were evaluated in nanodrop and agarose 0.8% gel, respectively.

PCR reaction for molecular identification of *Nosema* species: DNA samples were challenged with primer sequences used to amplify the 218 bp and 97 bp fragments corresponding to the 16S ribosomal gene of *N. ceranae*, 218MITOC (Forward – 5′-CGGCGACGATGTGATATGAAAATATTAA-3′; Reverse – 5′-CCCGGTCATTCTCAAACAAAAAACCG-3′) (Martín-Hernández et al., 2007) and QNo (UF – 5′-GGATTGTGCGGCTTAATTTGA-3′; CR 5′-ACCACTATTATCATTCTCAAAC-3′) (Chemurot et al., 2017), respectively. To amplify the 321 bp and 77 bp fragment of the same 16S gene in *N. apis*, we used primers 321 APIS (Forward 5′-GGGGGCGGTCTTTGACGTACTATGTA-3′; Reverse 5′-GGGGGGCGTTTAAAATGTGAAACAACTATG-3′) and QNo (UF – 5′-GGATTGTGCGGCTTAATTTGA-3′; AR 5′-CCTCAGATCATATCCTCGCAG-3′) (Chemurot et al., 2017), respectively. In addition, a negative control (absence of DNA) and positive controls were used with the primer NOS (Forward 5′-TGCCGACGATGTGATATGAG-3′; Reverse 5′-CACAGCATCCATTGAAAACG-3′), which is specific for the Nosematidae family (Higes et al., 2006). The PCR reaction was performed in a Veriti thermocycler (Applied Biosystems, Foster City, CA, USA), containing 2.5 μL 10X buffer, 1 μL 10 mM dNTP mix, 1.25 μL of primer (100 μM), 0.25 μL of HotStart Taq polymerase (5 u/μL), and ultrapure water to reach the final volume of 20 μL. The following cycling was used: (1) one cycle of 5 min at 94 °C, (2) 35 cycles of denaturation for 30 s at 94 °C, annealing 30 s at temperatures suggested by each primer developer, extension for 30 s at 72 °C, and (3) a final extension for 3 min at 72 °C. The amplified PCR products were electrophoresed for 60 min at 100 volts through 1.5% agarose TBE gel in standard TBE buffer, stained with GelRed® (Biotium), and visualized using UV illumination. All analyses were done in triplicate.

Data analysis

The data of each bioassay (IS-W and IS-S) were submitted to analysis of variance using a randomized block design in a 4×5 factorial scheme (food types X times) in the ASSISTAT® program. In both analyses, when the food intake (volume) and survival rate presented significant differences among treatments, at the probability level of $\alpha = 0.05$ by the F test, were submitted to the Scott Knott or Tukey test, respectively, at the same level of significance.

Results

Nosema *species identification*

It was first verified if the bees were carrying *Nosema* microsporidia spores in the intestine. When the presence of *N. apis* and *N. ceranae* spores was detected with light microscopy, DNA of the spores was extracted and challenged with the referenced primers described above. Beehives with positive results for the presence of *Nosema* provided bees to prepare the feeding solution for *Nosema* treatments of the bioassays. Beehives that did not show any microsporidian infection furnished bees for survival tests. To precisely identify the *Nosema* species, as revealed by light microscopy, PCR amplicons that corresponded to the size of DNA fragments expected for *N. ceranae* and *N. apis* were used (Figure 1). Specific amplicons were revealed in the DNA analysis; therefore, in this article, we will use *Nosema* spp. to represent the two identified species.

Survival rate

The analysis of variance indicated significant differences in the survival rate 72 h and 96 h after the start of the IS-W and IS-S bioassays, respectively. In IS-W and IS-S, the cumulative survival rate decreased with time in all treatments (Tables 1 and 2). However, the treatment

Table 1. Survival rate (%) of the IS-W bioassay for adult worker bees in winter, emergence in the BOD and submitted to the following dietary treatments: control, Roundup®, *Nosema* spp., and Roundup® + *Nosema* sp. during a 120-h period. Total of 144 bees from each treatment.

	Time													
Treatment	24 h			48 h			72 h			96 h			120 h		
Control	93.8	a	A	91.0	a	A	91.0	ab	A	86.8	ab	A	84.7	a	A
Roundup®	97.9	a	A	97.2	a	A	96.5	a	A	94.4	a	A	90.3	a	A
Nosema sp.	97.2	a	A	90.9	a	AB	90.3	ab	AB	88.9	ab	AB	75.0	a	B
Nosema sp. + Roundup®	95.8	a	A	88.2	a	AB	77.1	b	B	75.0	b	B	56.3	b	C
CV%	9.42														

Table 2. Survival rate (%) of the IS-S bioassay for adult worker bees in spring, emergence in the BOD and submitted to the following dietary treatments: control, Roundup®, *Nosema* spp., and Roundup® + *Nosema* spp. during a 120-h period. Total of 144 bees from each treatment.

	Time													
Treatment	24 h			48 h			72 h			96 h			120 h		
Control	100	a	A	95.8	a	A	95.8	a	A	93.8	a	A	91.7	a	A
Roundup®	100	a	A	95.1	a	A	93.1	a	A	92.4	ab	A	92.4	a	A
Nosema sp.	100	a	A	95.8	a	AB	95.8	a	AB	93.8	a	AB	86.1	a	B
Nosema sp. + Roundup®	100	a	A	93.1	a	AB	91.0	a	AB	83.3	b	B	70.8	b	C
CV%	5.48														

with simultaneous exposure to Roundup® + *Nosema* spp. presented the lowest cumulative survival rate, followed by the treatment with exposure to *Nosema* spp. Additionally, a significant statistical difference was verified when means of the *Nosema* spp. + Roundup® and *Nosema* spp. treatments, both of which showed lower survival rates 120 h after the start of the experiment, were compared with the control and Roundup® treatments.

Dietary treatments and evaluation times were statistically significant at 1% probability ($p < 0.01$). The interaction between diets and times was significant at 5% probability ($0.01 \leq p < 0.05$). The averages followed by the same lowercase letter in the column and uppercase in the row do not statistically differ from each other by the Tukey test at 1% probability.

Dietary treatments and evaluation times, as well as the diet x time interaction, were statistically significant at 1% probability. The averages followed by the same lowercase letter in the column and uppercase in the row do not differ statistically from each other by the Tukey test at 1% probability.

The simultaneous Roundup® + *Nosema* spp. treatment presented significant statistical difference from all other treatments indicating that this interaction has a stronger effect on bee mortality than individual treatments, suggesting that the herbicide decreases resistance to the microsporidian.

Food consumption

Food consumption measurements performed during the survival tests showed significant differences among the treatments and time of evaluation. Because of the similarity between data obtained for food consumed during the winter (IS-W) and spring (IS-S) bioassays, the data for food consumption of both assays were grouped and analyzed together (Table 3).

Dietary treatments and evaluation times, as well as diet x time interaction, were statistically significant at 5% probability. The averages followed by the same lowercase letter in the column and uppercase in the row do not differ statistically from each other by the Scott Knott test at 5% probability.

According to the results, food consumption increased over time and was higher in treatments where individuals were contaminated with *Nosema* spp. In treatments where bees were not contaminated by microsporidia, the average food consumption was 25 uL/bee (control 20 μL/bee and Roundup® 30 μL/bee), while in bees contaminated with *Nosema* spp., the average consumption was 38 μL/bee (*Nosema* 40 μL/bee and *Nosema* + Roundup® 36 μL/bee) 120 h after the start of the bioassay. This line of evidence suggests that infected bees eat more in an attempt to compensate for the energy lost by parasitic stress. The decrease in food intake from 96 to 120 h by bees treated with Roundup® + *Nosema* spp. simultaneously probably resulted from the higher mortality in comparison with the other treatments.

Infection by Nosema *spp*

The identification of infection caused by *Nosema* spp. was performed on bees that died during the bioassay and those that remained alive at the end of the bioassay. Bees receiving spores of the microsporidium were positive for infection. This is an important result since it associates mortality with a pathogenic effect.

Table 3. Average consumption of feed (mL) of the two bioassays for adult worker bees in the BOD and submitted to the following dietary treatments: control, Roundup®, *Nosema* spp., and Roundup® + *Nosema* spp. during a 120-h period.

	Time														
Treatment	24 h			48 h			72 h			96 h			120 h		
Control	0.73	a	A	0.43	b	A	0.41	a	A	0.55	ab	A	0.56	A	A
Roundup®	0.56	a	A	0.48	b	A	0.48	a	A	0.69	ab	A	0.80	A	A
Nosema sp.	0.80	a	A	0.82	a	A	0.69	a	A	0.93	ab	AB	0.73	A	B
Nosema sp. + Roundup®	0.76	a	B	1.10	a	A	0.80	a	B	1.01	b	B	0.50	B	C
CV%	38.23														

Harm caused by Roundup®

For Roundup® and Roundup® + *Nosema* spp. treatments, the IS-W bioassay showed statistical differences between the means 72 h after initiating the experiment (Table 1). Similarly, the higher effect on mortality was verified 96 h after bioassay IS-S was initiated (Table 2). However, Roundup® alone caused less mortality than when it was combined with *Nosema* infestation, owing to the sub-lethal dose tested during the bioassays.

Discussion

The presence of the microspore *Nosema* in apiaries in the state of Santa Catarina (SC) is well known by beekeepers and bee researchers. However, the scientific reports of this pathogen in the state are scarce. In this work, two species of microsporidians were identified to infect bees from the experimental apiary of the Federal University of Santa Catarina. Because of the differences in pathogenicity and virulence between *N. apis* and *N. ceranae*, we carefully identified which species was present in solutions provided to the tested bees. This made it possible to attach and accurately report cause and effect to the precise parasite. Using molecular biology, we are the first to determine the presence of *N. apis* and *N. ceranae* in the municipality of Florianópolis. Previously, Wiese (1974) drew the first sporulation curve for *N. apis* indicating the presence of this microsporid in SC. Both *N. apis* and *N. ceranae* are present in other municipalities of Santa Catarina, but the prevalence of the second species was higher than the first species (Teixeira et al., 2013).

The microsporidia *N. ceranae* and *N. apis* have simple cellular organization and no mitochondria, allowing them to perform oxidative phosphorylation (Burri et al. 2006; Cornman et al. 2009; Forsgren & Fries 2010). These parasites interfere with the metabolism of the host by decreasing protein levels, altering hemolymph composition, and competing for ATP (Antúnez et al., 2009). *Nosema* microsporidia also affect the nutritional needs of bees, inducing them to a state of energy stress (Forsgren & Fries, 2010; Mayack & Naug, 2009; Naug & Gibbs, 2009), favoring the germination of their spores by decreased efficacy of the host immune response (Mayack & Naug, 2009).

The energy stress detected in bees infected by *Nosema* microsporidia was higher compared to uninfected bees that consumed less food compared to the infected ones. Microsporid parasitism promotes changes bee behavior and results in death (Martín-Hernández et al., 2011); in particular, *N. ceranae* directly affects the immune system (Antúnez et al. 2009; Chen et al. 2009). The parasite's dependence on the host's energy is manifested by the increase in syrup consumption by infected bees as demonstrated in previous studies (Alaux et al., 2010; Mayack & Naug 2009). Higher consumption of syrup probably provides additional energy to the host but it is also sufficient to support the reproduction of microspores (Chen et al., 2009). In addition, energy is expended by the host to activate its immune system allowing the insect to fight infections (Schmid-Hempel, 2005).

Thus, in the case of infection by *Nosema* spp., food consumption by bees tends to increase (Martín-Hernández et al., 2011) in an attempt to supply the ATP deficit. In addition, the interaction of the *Nosema* spp. pathogen with different stressors to which bees are exposed, such as agrochemicals, can contribute to the increased mortality of individuals (Alaux et al., 2010; Abou-Shaara and Abuzeid, 2018) and loss of bee colonies (Goulson et al., 2015). The results of the present work agree with those of previous studies even though distinct pesticides were utilized. Moreover, studies have shown that bees exposed to residual levels of pesticides during the larval phase (Wu et al., 2012), fed pollen contaminated with mixtures of pesticides and fungicides (Pettis et al., 2013) and chronically exposed to different concentrations of neonicotinoids (Alaux et al., 2010) are less resistant to infection by *Nosema* spp.

Additionally, honey bees that consumed pollen containing Roundup® herbicide showed morphological alterations of mitochondria and degeneration of the rough endoplasmic reticulum in cells of the hypopharyngeal glands (Faita et al., 2018). One of the effects of Roundup® herbicide on nontarget organisms (mammalian) involves alterations to the mitochondrial ridges, reducing the bioenergetic functions of these organelles (Peixoto, 2005). Thus, it is possible that the lower survival of bees exposed to microsporidia and also to Roundup® results from the impairment of mitochondria and consequent production of ATP.

In addition, it has been observed that glyphosate exposure leads to increased apoptosis in the intestine of larvae (Gregorc & Ellis, 2011), changes in the

carotenoid-retinoid system (Helmer et al., 2015), and increased lipid peroxidation (Jumarie et al., 2017). Moreover, a study verified that bees' microbial activity was significantly influenced by the presence of glyphosate in their diet (Motta et al., 2018).

The increase of reactive oxygen species can interfere with the physiology of the fat body of the bee, compromising their longevity (Ament et al., 2011; Corona et al., 2007). The function of the fat body is related to the production of proteins, lipids and carbohydrates which serve as precursors for the metabolism of several substances found in hemolymph (Arrese & Soulages, 2010), and it is involved in detoxification processes (Roma et al., 2006). Among the substances produced is vitellogenin which in *A. mellifera* was characterized as a multifunctional protein involved in several biological processes, such as reproduction, longevity and immunity (Amdam et al., 2004, 2006). According to Corona et al. (2007), the herbicide Paraquat induces oxidative stress in *A. mellifera* decreasing levels of vitellogenin and the longevity of bees. Thus, it can be hypothesized that a similar effect can occur in bees exposed to the herbicide Roundup® although vitellogenin protein quantification was not part of the objectives of this study.

The greater survival of bees in bioassay IS-S can be partly attributed to their larval diet of greater nutritional quality pollen. The bees used in the second survival bioassay (IS-S) had their larval development during a period of higher pollen availability (October) owing to full bloom of the native forest species adjacent to the apiary when compared to the period of the IS-W assay in August, or winter. Pollen is a food with antioxidant properties possibly by the presence of phenolic acids and ubiquitous compounds in plants that act to eliminate free radicals (Almeida-Muradian et al., 2005; Fatrcová-Šramková et al., 2013; Krishnaiah et al., 2011; Pascoal et al., 2014). The excess of food ingested in this period is usually stored in the fat body ensuring a reserve of nutrients necessary for metamorphosis (Cruz-Landim, 2009), which could, in turn, ensure less interference in the synthesis of vitellogenin or damage from the accumulation of reactive oxygen species in the post-emergence period.

The highest food intake observed by bees treated with *Nosema* spp. is related to the mechanism of action of the parasite which uses the energy produced by the host cells for their own metabolic processes (Burri et al., 2006). The data obtained in this study are in accordance with the findings of Alaux et al. (2010) who observed higher food consumption by bees infected with *Nosema* spp. when compared to those of control bees and bees exposed to imidacloprid. Mayack and Naug (2009) evaluated the proboscis extension reflex (PER) and feed intake in bees infected and not infected by the *Nosema* parasite. The authors confirmed that the infected group consumed more food and responded faster than other groups of bees in the PER test suggesting that infected bees tend to compensate for the imposed energy stress by feeding more.

In this study, we saw a combined effect of infection by *Nosema* spp. and the diet containing Roundup® by their significant contribution to the reduction of survival of adult *A. mellifera* workers. Similarly, in comparison with the other three treatments, this presented the highest increase in food consumption.

Pure glyphosate can also exhibit sublethal effects on the honey bee microbiota (Blot et al., 2019). However, these authors concluded that pure glyphosate did not enhance the effect of *N. ceranae* infection. In our study, we used the commercial herbicide formulation of Roundup®, which contains glyphosate as an active ingredient as well as adjuvants, which may have toxic effects on bees, contributing to decreased health of their populations (Mullin et al., 2016; Zhu et al., 2014). In addition, we tested the effects on both *N. apis* and *N. ceranae* performance. Our results for bee survival are in agreement with those found by Abou-Shaara and Abuzeid (2018), who evaluated bees infected with *Nosema* spp. and exposed to glyphosate-based herbicides for over 12 days. In addition, the authors also identified a lower survival of bees exposed to both stressors when compared to bees infected only with *Nosema* spp. and the control group. Thus, in addition to the interaction between both stressors, one hypothesis holds that adjuvants can also enhance *Nosema* spp. effects, thereby explaining the significant effects of both intestinal parasites on *A. mellifera*. Alternatively, the combined effects could be explained by the passive effects of glyphosate-based herbicides and *Nosema* spp. spores on the health of bees (Abou-Shaara & Abuzeid 2018).

Considering the mechanism of action of Roundup® and its active ingredient glyphosate against nontarget organisms, which can cause damage to bioenergetic synthesis, the formation of reactive oxygen species and inhibition of the synthesis of enzymes that degrade these compounds can also occur in pollinators. Thus, the reduction in bioenergetic synthesis caused by the herbicide Roundup® together with the effects of *Nosema* spp. infection induces higher food consumption by bees exposed to both stressors simultaneously, as an attempt to overcome the energy deficit and other adverse effects caused by such dual challenge.

An additional effect may be the increased level of reactive oxygen species (ROS) in bees contaminated by Roundup® + *Nosema* spp. The presence of reactive oxygen species promotes changes in the fat body of insects responsible for producing many compounds, including vitellogenin, a protein associated with longevity in bees, as noted above.

In Brazilian agroecosystems, glyphosate-based herbicides have been the major pesticides used in the past decade (Pignati et al., 2017), in part, due to the use of herbicide-tolerant transgenic varieties of soybean, corn and cotton. Thus, in these agroecosystem conditions, an

interaction may occur between a monofloral diet and Roundup® residues. Consequently, it cannot be ruled out that this interaction could have a significant effect on the mortality of adult worker bees in the field, with consequent commitment of colony homeostasis capacity owing to a gradual reduction of the population. It is still important to consider that social species, such as *A. mellifera*, present a unique challenge for toxicologic pathology studies because the conventional approaches are not designed to detect indirect effects on the colony caused by disruption of social interactions, only those caused by direct impacts on individuals (Berenbaum & Liao, 2019).

In addition, *N. ceranae* has an ample prevalence throughout Brazil. A previous study revealed that 79.9% of the 637 samples collected in 10 Brazilian states, including Santa Catarina were infected with *Nosema* of which 98.82% were *N. ceranae* (Teixeira et al., 2013). In this sense, this work has made a significant contribution. Specifically, we have revealed the presence of a sublethal dose of Roundup® in the food utilized by bees with nosemosis, two very common stressors in agroecosystems that potentiate the effects of both factors, Roundup® + *Nosema* spp., thus elevating the lethal potential of the exposed bees.

However, it is important to note that the field scenario may be even worse since the dosages used in cultivated crop species may be higher than those recommended by manufacturers based on the increased presence of weeds resistant to herbicides. Specifically, caution should be taken since the doses tested in this study do not represent the doses used by farmers. Therefore, the results obtained in this study may underestimate those doses that may, in fact, occur in Brazilian agroecosystems in that the current management practices may require that high doses and an increasing number of doses of glyphosate-based herbicide applications continue to be used.

Another issue involves the genetic background of the bees. We take the following into account: (i) random collection of bees eight times from six hives, (ii) no kinship among hives, and (iii) statistical significance between treatments, even in the presence of genetic variation among bees. This line of evidence from this study suggests that can be inferred further to one hive.

In conclusion, this study has advanced scientific knowledge about the effects of the most utilized herbicide in Brazil. Particularly, our results show that Roundup® herbicide in bees contaminated by *Nosema* spp. can increase their mortality rate compared to the effects of each individual stressor. Also, from a regulatory perspective, we recommend that sub-lethal dose studies be conducted and enacted as an additional criterion to be met before the use of this herbicide is allowed.

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Disclosure statement

The authors declare no conflicts of interest.

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