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Research article

Nosema ceranae Winter Control: Study of the Effectiveness of Different Fumagillin Treatments and Consequences on the Strength of Honey Bee (Hymenoptera: Apidae) Colonies

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Abstract

In Uruguay, colonies of honey bees moving to *Eucalyptus grandis* plantation in autumn habitually become infected with the microsporidian *Nosema ceranae*, a parasite that attacks the digestive system of bees. Beekeepers attributed to *N. ceranae* depopulation of the colonies that often occurs at the end of the blooming period, and many use the antibiotic fumagillin to reduce the level of infection. The aim of this study was to compare the effectiveness of four different fumagillin treatments and determine how this antibiotic affects the strength of the colonies during the winter season. The colonies treated with fumagillin in July showed less spore load at the end of applications, being the most effective the following treatments: the four applications sprayed over bees of 30 mg of fumagillin in 100 ml of sugar syrup 1:1, and four applications of 90 mg of fumagillin in 250 ml of sugar syrup 1:1 using a feeder. However, 2 month after the treatment applications, the colonies treated with fumagillin were the same size as the untreated colonies. In September, the colonies treated and not treated with fumagillin did not differ in colony strength (adult bee population and brood area) or spores abundance. Our study demonstrates that fumagillin treatment temporarily decreased the spore load of *N. ceranae*, but this was not reflected in either the size of the colonies or the probability of surviving the winter regardless of the dose or the administration strategy applied. Given the results obtained, we suggest to not perform the pharmacological treatment under the conditions described in the experiment.

Resumen

En Uruguay las colonias de abejas melíferas que se trasladan a las forestaciones de *Eucalyptus grandis* en otoño indefectiblemente se infectan con el microsporido *Nosema ceranae*, parásito que ataca el sistema digestivo de las abejas. Los apicultores atribuyen a *N. ceranae* el despoblamiento de las colonias que ocurre con frecuencia al terminar el periodo de floración y muchos emplean el antibiótico fumagilina para reducir el nivel de infección. El objetivo de este estudio fue comparar la eficacia de cuatro tratamientos diferentes con fumagilina y determinar cómo incide en la fortaleza de las colonias durante la invernada. Las colonias tratadas con fumagilina en julio presentaron una menor carga de esporas al terminar las aplicaciones, siendo los tratamientos más eficaces el de 4 aplicaciones mediante asperjado sobre las abejas de 30 mg de fumagilina en 100 ml de jarabe de azúcar 1:1, y el de 4 aplicaciones de 90 mg de fumagilina en 250 ml de jarabe de azúcar 1:1 utilizando un alimentador. Sin embargo, durante el período de experimentación, las colonias tratadas con antibiótico presentaron igual tamaño que las colonias no tratadas. En setiembre, las colonias tratadas y no tratadas con fumagilina no se diferenciaron en la intensidad de infección ni en su tamaño. En las condiciones en que se realizó el estudio, la aplicación de fumagilina disminuyó temporalmente la carga de esporas de *N. ceranae* pero esto no se reflejó en el tamaño de las colonias ni en la probabilidad de sobrevivir el invierno.

Key words: *Apis mellifera*, *Nosema ceranae*, nosemosis, antibiotic, *Eucalyptus*

Nosemosis is a disease of adult bees caused by the microsporidia *Nosema apis* and *Nosema ceranae* (Fries et al. 1996, Higes et al. 2006). The two *Nosema* species reproduce in the epithelial cells of the ventricle of bees, affecting the digestive and nutrient absorption functions, which leads to undernutrition, energetic stress, physiological aging, and premature death (reviewed in Holt and Grozinger, 2016). At colony level, the disease causes depopulation, replacement of queens, and lower production of honey (Fries 2010, Higes et al. 2013). While *N. apis* was described at the beginning of past century (Zander 1909), *N. ceranae* whose original host is the Asian honey bee, *Apis cerana* (F.) (Fries et al. 1996), was reported in European honey bees a decade ago (Higes et al. 2006). *Nosema ceranae* is currently distributed in five continents (Klee et al. 2007, Fries 2010, Higes et al. 2013). Regardless, the host switch of *N. ceranae* has happened in America at least 35 yr ago considering that Teixeira et al. (2013) detected its presence in honey bees collected in 1979 in Brazil, being the oldest record in Uruguay in samples of 1990 (Invernizzi et al. 2009).

Some studies involving *N. ceranae* with colony losses occurred in recent years, especially in some European countries (Hatjina et al. 2010, Higes et al. 2013, Lodesani et al. 2014). However, the role of *N. ceranae* in the loss of colonies has been questioned in many countries (Cox-Foster et al. 2007, Chen et al. 2008, Gómez Pajuelo et al. 2008, Mayack and Naug 2009, Forsgren and Fries 2010, Fernandez et al. 2012, Stevanovic et al. 2013), also in Uruguayan territory (Invernizzi et al. 2009). Different results could be explained by the existence of different strains of *N. ceranae* (Dussaubat et al. 2013, Branchiccela et al. 2014, Van der Zee et al. 2014), different viruses or other pathogens associated with microsporidia (Genersch and Aubert 2010, Runckel et al. 2011, Ravoet et al. 2013, Toplak et al. 2013, Doublet et al. 2015), exposure to insecticides (Alaux et al. 2010), differences in vitellogenin concentration in bees (Antúnez et al. 2013), and differences in the susceptibility to the pathogen between bee subspecies or biotypes of bees (Mendoza et al. 2014, Huang et al. 2014, Huang et al. 2015).

Fumagillin dicyclohexylammonium is the only commercially available antibiotic to control *Nosema* disease. The drug is effective in temporary control of the parasitosis (Williams et al. 2008, Sarlo et al. 2011), given that it stops the intracellular replication of the parasite (Gisder and Genersch, 2015). However, resistant forms, called spores, are not affected by this drug, which also causes consequences on bee health (Botías et al. 2013, Van den Heever et al. 2015a). In addition, *N. ceranae* apparently escapes from the suppressive effects of fumagillin at concentrations that continue to impact honey bee physiology (Huang et al. 2013). Also, its use is not recommended due to its high residues in honey, which could affect human health (Stanimirovic et al. 2007, Van den Heever et al. 2015b).

In Uruguay, nosemosis is caused only by *N. ceranae*, a species that is present in the country at least since the 1990s. The pathogen is always present in colonies that beekeepers move to the plantation of *Eucalyptus grandis* in late summer (February–March), taking advantage of the great potential as nectar source of this introduced species (Invernizzi et al. 2011; Mendoza et al. 2012, 2013; Antúnez et al. 2013).

While the harvests of honey are usually very important, beekeepers often found that at the end of flowering (May), the colonies are very depopulated, registering significant losses if they are not removed from the plantations, or even after moving the colonies to other locations (Invernizzi et al. 2011, Mendoza et al. 2013).

However, the colonies that were heavily infected by *N. ceranae* and removed from *E. grandis* plantation did not show a greater population size after fumagillin treatment when compared with non-treated colonies (Mendoza et al. 2012). It is possible that the magnitude of damage caused by *N. ceranae* during the winter is very dependent on the particular conditions of each year as well as the drug administration methods. In reference to this, different treatments have been recommended by laboratories who commercialize the drug, varying in the number of applications, the total amount of fumagillin employed, and how to supply the sugar syrup used as substrate to administrate the antibiotic. Furthermore, photosensitivity and heat sensitivity characteristics of fumagillin directly influence its antibiotic activity and must be very carefully prepared and transported (Higes et al. 2011).

The aim of this study was to analyze the effectiveness of different treatments with fumagillin in colonies infected with *N. ceranae* after removing them from plantation of *E. grandis* and determine how they affect the *Nosema* levels, the population size, and brood rearing during winter.

Materials and Methods

The study was conducted between 25 June and 25 September, 2013, in Langstroth hives retired 15 d before from the same plantation of *E. grandis* (placed at Rivera Province). On June 25, at the end of flowering period, 72 colonies of similar size, with young queens from the same origin and without symptoms of brood diseases, were selected. Colonies received amitraz treatment to control varroosis and then, were moved to Colonia Province and placed in two nearly located apiaries (34° 6′20.9″ S, 57° 18′17.3″ W and 34° 8′29.4″ S, 57° 18′26.1″ W). The apiaries were exposed to the same environmental conditions and hives were randomly selected (from two apiaries) in order to confirm the experimental groups.

In each colony, the adult population, the area, and the level of infection with *N. ceranae* was estimated following the recommendations of BEEBOOK (Williams et al. 2012). *Nosema ceranae* was confirmed by molecular method according to the Martín-Hernández et al. (2007) protocol. In order to estimate adult population, hives were carefully opened with very little smoke, spaces between frames were inspected in detail, and frames completely covered by adult bees were recorded. Once the adult population was registered, combs were removed from the hive and the brood area was estimated as quarters of frame faces covered by brood (with 1/4 frame face = 210 cm^2). The area occupied by honey, pollen, or empty was not included in the estimation. All determinations were visually performed by the same operator. To estimate the level of *Nosema* infection, 60 bees were collected from nest edge and their abdomens were macerated together with 60 ml of water. The concentration of spores in the suspension was measured with a hemocytometer and the average number of spores per bee was determined by following the technique of Cantwell modified by Fries et al. (1984).

The colonies were randomly divided into seven groups, verifying later the size homogeneity (adult bee population and brood area). Each group of colonies received one of the following treatments: Treatment 1 (T1): four applications of 30 mg of fumagillin in 250 ml of sugar syrup 1:1 ($n = 11$), Treatment 2 (T2): one application of 120 mg of fumagillin in 5,000 ml of sugar syrup 2:1 ($n = 11$), Treatment 3 (T3): four applications of 30 mg of fumagillin in 100 ml of sugar syrup 1:1 sprinkled on bees ($n = 11$), Treatment 4 (T4): four applications of 90 mg of fumagillin in 250 ml of sugar syrup 1:1 ($n = 11$), Control 1 (C1): four applications of 250 ml of sugar syrup 1:1 ($n = 9$), Control 2 (C2): one application of 5,000 ml of sugar syrup 2:1 ($n = 9$), and Control 3 (C3): four applications of 100

ml of sugar syrup 1:1 sprinkled on bees ($n = 10$). Fumagillin was obtained as commercial formulation (Nosemix-B, Kinter S.A. Laboratories, Montevideo, Uruguay).

To supply not sprinkled treatments, Doolittle internal feeders were used. The treatments were administered on days 1, 6, 15, and 19 July. The colonies were inspected on 29 July and 25 September (early spring), making the measurements described above. The samples of adult bees were obtained on 25 June, 29 July, and 25 September.

Measurements made in the colonies of different groups were compared using the Kruskal–Wallis since data was not normally distributed. Then, Mann–Whitney U-tests were used for pairwise comparisons. The proportion of dead colonies in each group was compared with chi-square test. Spearman correlations were made to analyze possible associations between variables. The significance value considered in all cases was 0.05. The R free software was used to perform the analysis.

Results

On 25 June, at the beginning of the experiment, all the colonies had *Nosema* spores and the selected ones for the study showed an average of 6.60 ± 1.98 frames covered by bees, 2.78 ± 1.14 sides box occupied by brood cells, and $2.34 \times 10^6 \pm 1.61 \times 10^6$ spores per bee. Colonies selected to integrate the treatment groups (7) showed no significant differences in colony strength ($H = 1.44$; $P = 0.96$) or the brood area ($H = 3.63$; $P = 0.73$), but they showed marginal differences in the level of *N. ceranae* infection ($H = 12.45$; $P = 0.053$). Control groups 1 and 2 showed the highest differences in average of spores per bee (1,661,000 vs. 3,758,000, respectively; Fig. 1).

Ten days after finishing the application of fumagillin (29 July), the colonies showed an average of 5.80 ± 2.04 frames covered by bees and 3.02 ± 1.89 sides box occupied by brood. Colony groups showed no significant difference in the size of its population ($M = 10.29$; $P = 0.11$) and in the brood area ($H = 3.92$; $P = 0.69$). However, differences were observed in the level of infection with *N. ceranae* ($H = 41.80$; $P < 0.001$) being the colonies of the groups T1, T3, and T4 treatments, which showed lower abundance values (Fig. 1).

The colonies came to the spring (25 September, 68 d after the end of treatments) with an average of 8.16 ± 2.27 frames covered by bees, 8.16 ± 3.81 sides box occupied by brood, and $750{,}806 \pm 763{,}592$ spores per bee. Colonies groups showed no significant differences in population size ($F = 4.50$; $P = 0.61$), brood area ($H = 6.58$; $P = 0.36$), or the level of *N. ceranae* infection ($H = 5.89$; $P = 0.44$; Fig. 1).

Finally, loss of 10 colonies (14% of total) was verified, corresponding to two dead colonies in each T1, T2, T3, T4, and C3 groups. No significant differences in death colonies between groups were found (Chi-square = 3.89; $P = 0.69$).

The Spearman correlation analysis reported a positive correlation between brood area and adult bee population at every sampling date and along the assay (June: rho = 0.35, $P = 0.002$; July:

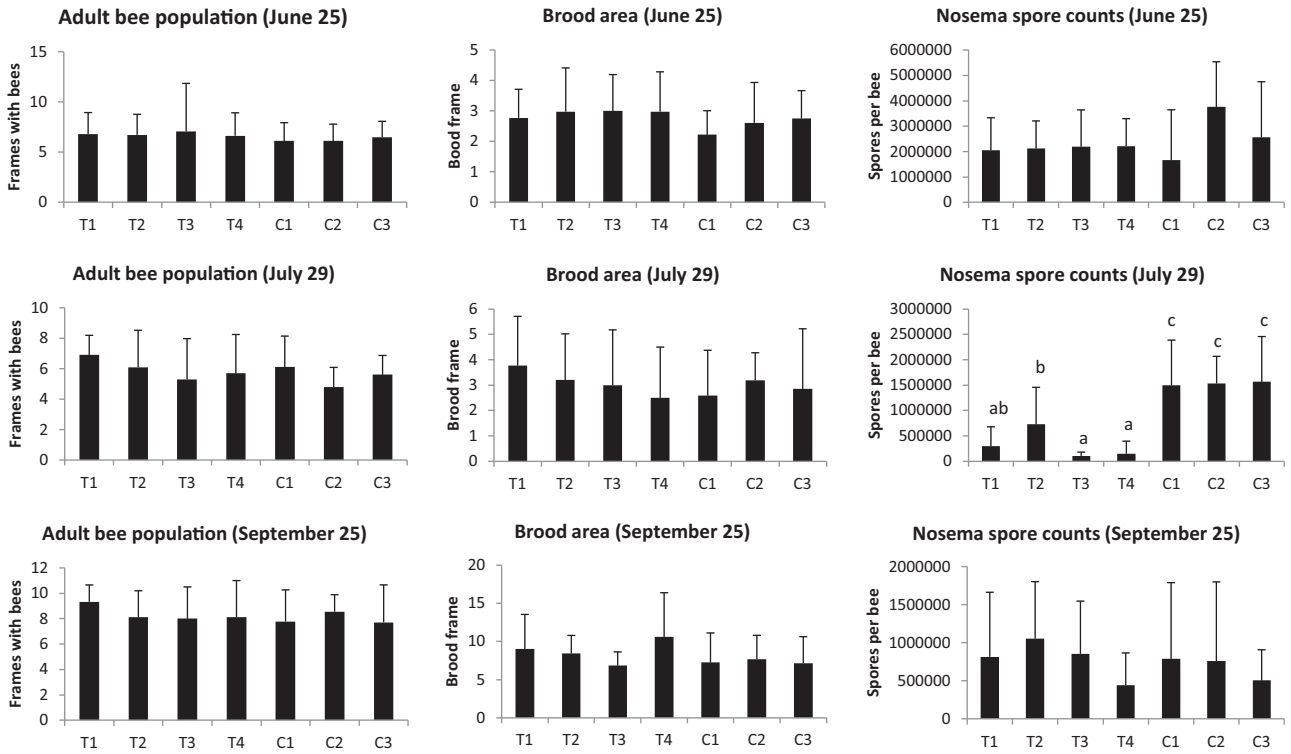

Fig. 1. Adult bee population, brood area, and level of *N. ceranae* infection in colonies that received different treatment with fumagillin. Using the Kruskas–Wallis test, we only detected significant differences in the level of *N. ceranae* infection on 19 July (last treatment administration). Different letters indicate significant differences ($P < 0.05$) for the Mann–Whitney test. June 25: at the beginning, before fumagillin application; July 29th: 10 d after the end of applications; September 25: early spring. Treatment 1 (T1): four applications of 30 mg of fumagillin in 250 ml sugar syrup 1:1 ($n = 11$), Treatment 2 (T2): one application of 120 mg of fumagillin in 5,000 ml of sugar syrup 2:1 ($n = 11$), Treatment 3 (T3): four applications of 30 mg of fumagillin in 100 ml sugar syrup 1:1 sprinkled on bees ($n = 11$), Treatment 4 (T4): four applications of 90 mg of fumagillin in 250 ml sugar syrup 1:1 ($n = 11$), Control 1 (C1): four applications of 250 ml sugar syrup 1:1 ($n = 9$), Control 2 (C2): one application of 5,000 ml of sugar syrup 2:1 ($n = 9$), and Control 3 (C3): four applications of 100 ml sugar syrup 1:1 sprinkled on bees ($n = 10$).

rho = 0.47, $P = 4.059e-05$; September: rho = 0.56, $P = 2.105e-06$). The test also showed no association between sampling date and *Nosema* spore counts (June–July: rho = 0.14, $P = 0.240$; June–September: rho = −0.02, $P = 0.857$; July–September: rho = −0.14, $P = 0.284$), or either between *Nosema* counts and adult bee population (June: rho = 0.05, $P = 0.650$; July: rho = 0.10, $P = 0.40$; September: rho = 0.22, $P = 0.08$), or brood area for any particular date (June: rho = −0.06, $P = 0.568$; July: rho = −0.10, $P = 0.39$; September: rho = −0.17, $P = 0.19$).

Discussion

The results corroborated differential effectiveness of treatment with fumagillin on parasitosis; however, indicators of strength of the colonies were not significantly affected. The Spearman correlation analysis reported a positive correlation between brood area and adult bee population at every sampling date and along the assay, evidencing a reasonable development of hives, according to the end of winter season. Nevertheless, it is worth mentioning that we performed a short-term experiment and the effects of the treatments were only tested 2 mo after the last dose of the treatments, and during the period where honey bee colonies do not grow significantly.

All colonies included in the experiment left the plantation of *E. grandis* with a significant infection of *N. ceranae*, which corroborates previous studies indicating that plantation conditions during flowering favor the appearance of nosemosis (Invernizzi et al. 2011; Mendoza et al. 2012; Antúnez et al. 2013; Mendoza et al. 2013, 2014). The enormous nectar influx in the hives in a short period that force a continuous work of foragers, coupled with nutritional problems caused by the low quality of the Eucalyptus pollen, looms as possible causes of the rise in the abundance of spores in colonies placed in the plantation. At this time, different experimental approaches are carried out in order to test these hypothesis.

The three treatments with fumagillin that demanded four applications were the most efficient. Among them, it is worth noting that the application of 30 mg of fumagillin with the sprinkling method had the same effect as the application of a dose three times more concentrated but supplied in the feeder. Possibly, the administration of sprinkled treatment caused an immediate cleaning of the bees, facilitating the consumption of the treated syrup in a short time and allowing the antibiotic to reach the target site quickly. In this sense, it was found that bees who get syrup, cleanse their body within minutes. Colonies that received 120 mg of fumagillin (one application in 5,000 ml of sugar syrup), showed less effectivity reducing the spore counts than the other three treatments. It is possible that the volume of syrup used was too large for the sizes of the colonies at the time of treatment, leading to bees consuming low doses of the drug over an extended period, limiting its effectiveness. This has also been suggested by Botías et al. (2013) and Higes et al. (2011).

Even though treatments that demanded four applications were more efficient than the one needed for an application, it should be noted that the first requires a high economic cost because of the need to carry out several visits to the apiary at regular intervals.

Regardless of the effectiveness of each treatment, reducing *Nosema* spore levels had no impact on the population size of the colonies on a short-term period. Also, no correlations were detected between *Nosema* infection rate and population size (worker bees and brood). Furthermore, the reduction in the level of infection was temporary because in the early spring, the spore concentration in the colonies that received fumagillin and control colonies did not differ. This result reinforces the finding of Mendoza et al. (2012) who reported no differences in size in the spring season between treated and untreated hives with fumagillin after withdrawing from a *Eucalyptus* plantation. Instead, Mendoza et al. (2013) in an apiary located in a plantation of *E. grandis* reported differences in the proportion of strong, weak, and dead colonies from colonies that, in autumn, received 400 mg fumagillin and receiving 200 mg or not receiving the antibiotic. It is possible that the effect of fumagillin on the survival of the colonies during winter depends on the environmental conditions. Although we lack robust information about the effect of high humidity on nosemosis development, we can hypothesize that the disease causes damage in winter under a humid atmosphere as in *Eucalyptus* plantation, but not in open environments, as usually the beekeepers winter their hives.

The untreated colonies dropped to less than one-third the level of infection during winter. It is remarkable that in treated colonies, temporary reduction in spore load due to the application of fumagillin has not contributed to a marked decline in the level of infection. It cannot be ruled out that these colonies were again reinfected from the colonies that did not receive antibiotic or from infective loads of spores remaining in bee matrices. However, variables like drifting of infected bees or remaining spores inside the hive matrixes should be deeply studied in order to know their real impact on reinfection process.

The most important conclusion of this study is that the use of fumagillin in colonies heavily infected with *N. ceranae* in autumn–winter, as the colonies retired from the mounts of *E. grandis*, does not improve survival or size of the colonies during the winter, regardless of the dose or the administration strategy applied.

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