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Technical note

Efficacy of thymol in control of the fungus *Nosema ceranae* in Africanized *Apis mellifera*

Azucena Vargas-Valero [a]

Roberto C. Barrientos-Medina [b]

Luis A. Medina Medina [c]*

[a] Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias. Centro de Investigación Regional del Sureste, Campo Experimental Edzná, Campeche, México.

[b] Universidad Autónoma de Yucatán. Facultad de Medicina Veterinaria y Zootecnia, Departamento de Ecología, Yucatán, México.

[c] Universidad Autónoma de Yucatán. Facultad de Medicina Veterinaria y Zootecnia, Departamento de Apicultura, Yucatán, México.

* Corresponding author: mmedina@correo.uady.mx

Abstract:

Nosema ceranae is an obligatory parasite of the honeybee midgut. It destroys the epithelial cells, negatively affecting food digestion and assimilation, and impacting bee development and colony survival. Antifungals such as fumagillin effectively control *N. ceranae* but can be toxic to humans who consume honey from treated hives, and are prohibited in many countries, including Mexico. Essential oils from plants are promising alternative antifungals. An evaluation was done of the efficacy of the essential oil thymol in controlling *N. ceranae* in Africanized *Apis mellifera* colonies over a four-week period. A total of 56 colonies were distributed in three experimental groups: G1) 18 colonies treated with fumagillin (25.2 mg fumagillin/week); G2) 19 colonies treated with thymol (66 mg thymol crystals/week); and G3) 19 untreated colonies (control). Infection levels (*N. ceranae* spores/bee) were estimated in 60 adult bees from each colony. Fumagillin (G1) reduced infection levels from 123,529 to 1,805 spores/bee (95.2 % efficacy). Thymol (G2) reduced infection levels from 133,438 to

28,099 spores/bee (31.1 % efficacy). Infection levels also declined in the control group (G3), from 119,306 to 36,447 spores/bee. The clearly higher efficacy with fumagillin compared to thymol highlights the need for further trials to test different thymol concentrations, and administration frequencies and times. Under the present study conditions thymol was not effective against *N. ceranae*, but the pressing need for non-toxic antifungals for use in Africanized *A. mellifera* colonies in the tropics makes research on thymol and other essential oils imperative.

Key words: *Nosema ceranae*, Nosemosis, Fumagillin, Thymol, Efficacy, *Apis mellifera.*

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Nosemosis is a parasitic fungal infection affecting the digestive tract of honeybees *Apis mellifera*. It is caused by two species of the Nosematidae family: *Nosema apis* and *Nosema ceranae*. The former is associated with infection in *A. mellifera*, while the latter was originally associated with the Asian bee *Apis cerana*. First diagnosed infecting *A. mellifera* in central and northern Spain in 2006[1], *N. ceranae* infection causes reductions in honey production and high colony mortality in winter[2,3].

Both these microsporidia (*N. apis* and *N. ceranae*) reproduce rapidly inside the epithelial cells of the midgut (ventricle) of adult bees (queen, workers and drones). Infection causes destruction of the epithelial cells responsible for digestion and food assimilation[3], resulting in nutritional stress[4]. At an individual level, other damage includes reduced life span[3,5,6] and compromised orientation and foraging capacities in infected workers[7,8]. At the colony level, high *Nosema* prevalence and infection levels can cause serious damage, such as reductions in breeding areas and the adult population, consequent declines in honey production[9,10] and eventual collapse and loss[11,12].

Since its initial identification in *A. mellifera* in 2006, *N. ceranae* has become one of the most widely distributed bee pathogens worldwide[13,14]. It has been associated with high colony mortality in Europe[11], North America[15] and South America[16], with a much higher virulence than *N. apis*[5,17,18].

Apis mellifera colonies in the state of Yucatan, Mexico, are infected by *N. apis* or *N. ceranae* but no massive losses or high colony mortality have been reported as a result. Since 2008[19], high nosemosis prevalence (74 to 100 %) has been reported in commercial apiaries compared to previous years (7.2 %)[20]. This high prevalence has been attributed to the presence of *N. ceranae* in Africanized *A. mellifera* in Yucatan[21]. Moreover, under Yucatan's tropical conditions *N. ceranae* infection has been confirmed to negatively affect foraging initiation and duration, as well as worker longevity[22].

The only substance considered effective against this microsporidium is fumagillin (dicyclohexylammonium salt). Since its initial evaluation in the early 1950s[23], this antimicrobial, derived from the fungus *Aspergillus fumigatus*, has been used to control *N. apis* in *A. mellifera*. Its action mechanism is inhibition of microsporidium DNA replication, which suppresses its reproduction, resulting in lower spore counts in the bee ventricle[24,25].

Fumagillin is approved in the United States and is widely used to control *N. apis* and *N. ceranae* infections. However, in many European countries[26], as well as in Mexico, it is prohibited for nosemosis control due to its toxicity in humans; any residue remaining in honey from colonies under treatment represents a direct risk to the consumer[27]. Alternative nosemosis control products have been proposed. These include essential oils from plants, such as thymol from *Thymus vulgaris* and vetiver from *Chrysopogon zizanioides*, as well as resveratrol, a natural polyphenol presents in numerous plants and fruits such as grapes. These essential oils, particularly thymol, have been shown to exercise some control of nosemosis[28,29]. When treated with thymol (0.44 mM) administered via sugar syrup, *Nosema*-infected colonies are reported to exhibit reduced spore counts per bee, expansion of brood areas, increased adult bee populations and greater honey production compared to untreated infected colonies[28]. These natural-source alternative essential oils products represent no toxicity risk for bees, a low probability of leaving residues in honey[30], and are less expensive than commercial pharmaceuticals.

As part of the search for alternative products for nosemosis control, the present study objective was to compare the efficacy of the essential oil thymol in controlling nosemosis caused by *N. ceranae* infection in Africanized *A. mellifera* under tropical conditions in Yucatan, Mexico.

The experiment was carried out in two apiaries at the Faculty of Veterinary Medicine and Zootechnics of the Autonomous University of Yucatan (Universidad Autónoma de Yucatán - UADY), Xmatkuil, Yucatan (20º51'51" N, 89º36'45" W; 20º51'55" N, 89º36'46" W). Regional climate is warm sub-humid with summer rains (Awo). Average annual rainfall is 985 mm, average annual temperature is 26.8 ºC and average annual relative humidity is 78 %[31].

The colonies in both apiaries were double colonies (brood chamber and super) housed in Langstroth-type hives. All hives had naturally-fertilized Africanized queens, an adult population covering 7 to 9 combs in the brood chamber, 6 to 8 combs containing brood at different stages (eggs, larvae and pupae), as well as combs containing honey and pollen. The colonies were distributed in a similar way among the experimental groups. Infection with *N. ceranae* was identified by endpoint PCR[32] of forage bees collected at the entrance to each experimental colony.

Before starting the evaluations, a preliminary diagnosis was made in both apiaries to quantify *N. ceranae* infection level (spores/bee) in all colonies. This ensured that all three experimental groups had a comparable initial infection level. Adult bees (~100 to 150 workers) were collected from the entrance of each experimental colony. Sixty individuals from each sample were analyzed to identify the presence of *N. ceranae* spores and quantify infection severity based on spore count per bee in the digestive tract. The abdomen was removed from each of the 60 bees, placed in a mortar and 60 ml distilled water added[9,33]. The abdomens were macerated until creating a homogeneous mixture and this was filtered through gauze to remove impurities. One drop of the resulting solution was placed in each reticule of a Neubauer chamber and viewed at 400x magnification. The spore counts were used to calculate average infection level (i.e. spores/bee).

Efficacy of the fumagillin (Fumagilin-B®) and thymol crystals (Sigma-Aldrich; ≥99.5% purity) in control of *N. ceranae* was evaluated over a period of four weeks with treatments applied once a week. The colonies in both apiaries were divided into three experimental groups:

Group 1 (G1): 18 colonies treated with fumagillin (Fumagilin-B®) administered at 1.2 g product (25.2 mg fumagillin)/colony/week in one liter sugar syrup (2:1, sugar:water);

Group 2 (G2): 19 colonies treated with 66 mg thymol crystals (99.5% purity)/colony/week in one liter sugar syrup (2:1, sugar:water);

Group 3 (G3): a control consisting of 19 colonies administered only one-liter sugar syrup (2:1, sugar: water), and no antifungals, per colony/week.

At the end of each week, adult bees were collected from the entrance of each colony and processed following the methodology described above to quantify infection level in each group.

Spore counts per bee were divided by one thousand to facilitate statistical analysis. The Box-Cox transformation[34] was applied to normalize variable distribution. Finally, a repeated measures analysis of variance was used to compare the means per treatment (i.e., experimental group) and week, and the interaction between both factors (treatment x week). Significance level was $P = 0.05$ and calculations were run with the PAST ver. 3.20 software program[35].

In Group 1 (colonies administered fumagillin) infection levels ($\overline{X}$±SE) dropped from 123,529 ± 41,200 spores/bee on d-0 to 1,805 ± 527 spores per bee on d-28, representing a 95.2 % overall efficacy against *N. ceranae* (Table 1). In Group 2 (colonies administered thymol crystals) infection levels declined from 133,438 ± 59,291 to 28,099 ± 17,574 spores/bee, representing 31.1 % efficacy. The decreases in infection levels in Group 3 (control) may be the result of seasonal fluctuations, as reported elsewhere[36].

Table 1: *Nosema ceranae* spore counts per bee ($\overline{X}$ ± SE) in response to administration of fumagillin or thymol

	Fumagillin G1	Thymol G2	Control G3	H
Day 0	123,529±41,220 [a]	133,438±59,291 [a]	119,306±53,100 [a]	0.5587
Day 7 (1st application)	104,688±42,293 [a]	67,812±14,426 [a]	80,394±22,381 [a]	1.8960
Day 14 (2nd application)	57,666±14,827 [a]	84,500±23,054 [a]	96,666±25,476 [a]	2.1100
Day 21 (3rd application)	30,468±11,190 [a]	27,058±9,779 [a]	42,631±9,055 [a]	4.9040
Day 28 (4th application)	1,805±527 [a]	28,099±17,574 [b]	36,447±17,554 [c]	24.0900
Overall efficacy, %	95.2	31.1	-	

$\overline{X}$ ± SE= Average ± standard error; H = Kruskal-Wallis test result.

[abc] Different letter superscripts in the same row indicate difference (P<0.05).

Initial *N. ceranae* infection levels did not differ between the three experimental groups based on a Kruskal-Wallis test (H= 0.5587, P= 0.7563) (Table 1); indeed, levels did not differ between the groups during the first three weeks of the experiment. It was not until the fourth week did differences become apparent, with G1 (fumagillin) exhibiting the highest efficacy. Clearly, administration of 100.8 mg fumagillin (4.8 g Fumagilin-B®), following manufacturer recommendations, effectively controlled *N. ceranae* reproduction in this group.

Application of fumagillin in G1 significantly decreased *N. ceranae* infection levels after four weeks at an efficacy over three times those of G2 (thymol crystals) and G3 (control). This coincides with previous reports indicating that fumagillin remains appropriate for control of *N. ceranae* infection[37-40]. Though efficient at temporarily reducing *N. ceranae* infection levels, it does not prevent reinfections within six months of the last application[38].

The 31.1 % efficacy of thymol observed in the present study was lower than the 40 % reported in a study of *N. ceranae*-infected bees fed sugar syrup containing thymol under laboratory conditions[41]. Of note is that thymol is reported to have greater efficacy in controlling nosemosis after three consecutive years of application[28]; this is much longer than the four-week period used in the present study. Continued application of thymol for an additional two years would be vital to verifying the effectiveness of the dose used here.

Fumagillin was effective in reducing *N. ceranae* infection levels in Africanized *A. mellifera* under tropical conditions. However, its use in bees is prohibited in Mexico, and its application at the doses used in the present study is only recommended when infection levels exceed two million spores/bee[28]. Considering that the highest initial infection level recorded in the present results was only 192,729 spores/bee, substantially lower than two million, application of thymol may yet exhibit a controlling effect against *N. ceranae* under the experimental conditions, just at higher infection levels. Further research will be needed to determine the potential of thymol as an alternative fungus control in Africanized *A. mellifera* under tropical conditions. Evaluations are needed in which higher doses and/or different application frequencies are tested. Because of its lower toxicity risk in both bees and humans, and the low residues it leaves in honey, the potential of thymol as an alternative antifungal in honeybees is well worth pursuing.

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