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**Original article**

# Onset of foraging and lifespan of Africanized honey bees (*Apis mellifera*) infected with different levels of *Nosema ceranae* spores in Neotropical Mexico

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**Abstract –** *Nosema ceranae* is a microsporidium pathogen widely spread around the world. Negative effects on foraging behavior and longevity of EHB colonies have been associated with this pathogen as well as possible population losses, but its effects have not been studied in tropical adapted honey bees. We studied the interaction between this pathogen and Africanized honey bees (AHB) in the Yucatan peninsula of Mexico where *N. ceranae* has only been detected since 2008. Non-infected and artificially infected workers with two different spore concentrations were introduced in observation hives to evaluate the onset and duration of foraging and longevity. The results showed precocious foraging, a reduction of the duration of foraging and a decrease in the longevity of infected bees compared with non-infected ones. However, the results indicate that although negative effects can be caused by *N. ceranae* in AHB, these were of a moderate magnitude compared with similar reports on EHB in temperate areas. Further research is necessary to evaluate the long-term effect of *N. ceranae* on AHBs in relation to colony dynamics to better understand the absence of significant colony losses associated with this pathogen in tropical and subtropical Mexico.

***Nosema ceranae*** **/ foraging behavior / longevity / Nosemosis / Africanized bees**

## 1. INTRODUCTION

The microsporidium *Nosema ceranae* is one of the most widely distributed honey bee pathogens (Goulson et al. 2015), causing severe damage to colonies in temperate areas. *N. ceranae* has successfully infected the Western honey bee *Apis mellifera* since at least 1998 (Paxton et al. 2007; Higes et al. 2006). Infections of the digestive tract occur with a rapid division inside the ventricular cells causing degeneration of the epithelium (Higes et al. 2007). Infected workers suffer nutritional stress (Mayack and Naug 2009) which affects several aspects of individual life; among the most important is a reduction in the lifespan (Higes et al. 2007; Paxton et al. 2007; Goblirsch et al. 2013). It also affects the orientation and homing skills of workers (Kralj and Fuchs, 2010; Wolf et al. 2014) and induces their early foraging (Goblirsch et al. 2013).

The effects of *N. ceranae* on honey bees from tropical regions have been poorly documented, mostly limited to reports of the presence of this microsporidium in different countries (Guzmán-Novoa et al. 2011; Teixeira et al. 2013; Santos et al. 2014). In tropical and subtropical Mexico, Africanized honeybee (AHB) colonies are predominant (Domínguez-Ayala et al. 2016). In Brazil, laboratory studies have confirmed a reduced survival of Africanized honey bee workers infected with *N. ceranae* under controlled conditions (Gregorc et al. 2016). However, there is no

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evidence of the effects of *N. ceranae* on whole colonies and under field conditions.

The Yucatan Peninsula is Mexico's leading beekeeping region with an estimated number of managed AHBs around 375,000 (Quezada-Euán 2007; SIAP-SAGARPA, 2016). The *N. ceranae* species in Mexico seems to be present since at least 1995 (Guerrero-Molina et al. 2016). In the state of Yucatan, the frequency of managed honey bee colonies positive to *Nosema* sp. infection significantly increased from 7.2–13.3% in the early 1990s, to 82% of prevalence, out of 165 colonies sampled from 13 different localities throughout the state in 2006 (García-Millán and Quezada-Euán 1993; Martínez-Puc et al. 2011).

There is evidence of detrimental effects of *N. ceranae* on the onset of foraging and longevity of EHBs (Goblirsch et al. 2013; Natsopoulou et al. 2016). These traits are important components of colony fitness which could affect survival (Higes et al. 2008; Higes et al. 2009; Botías et al. 2013). Nevertheless, it is yet unknown if compared to the situation in temperate areas, the interaction between *N. ceranae* and AHBs in the Neotropics could be of a similar detrimental magnitude. In this study, we performed an evaluation of the effects of *N. ceranae* on the onset of foraging, duration of foraging, and longevity in AHB workers in whole colonies under field conditions.

## 2. MATERIALS AND METHODS

### 2.1. Study site, colony racial origin, and *Nosema* species

The study was carried out in the Department of Tropical Apiculture of the Faculty of Veterinary Medicine, of Universidad Autónoma de Yucatán at Xmatkuil, Mexico, between March 2014 and February 2015. Because *N. ceranae* and *N. apis* are both found in the study region, the species of *Nosema* infecting colonies was confirmed from worker samples (60 individuals) collected from each of 21 colonies of an experimental apiary. The samples were analyzed by conventional PCR based on the alignment of the preserved sequence of the small subunit 16S rRNA gene (Chen et al. 2008). Identification of *N. ceranae* and *N. apis* was based on the length of the amplified segment, 250 or 401 bp, respectively (Chen et al. 2008). The racial origin of the experimental colonies was determined with the standard molecular procedures where primers E2 and H2 were used for DNA amplification extracted from the thoracic muscles (one worker bee per colony) in order to identify the African mitotype (Garnery et al. 1993). The Fast Africanized Bees Identification System (FABIS) based on morphometrics was also used to confirm the origin of the colonies (Quezada-Euán and Medina 1998; Nielsen et al. 1999).

### 2.2. Establishment of observation hives

The molecular results identified ten colonies infected with *N. ceranae* only. Seven of these colonies were treated with Fumagilin-B® in order to reduce parasite loads using 1.2 g of the product dissolved in 1 l of sugar syrup (2:1 sugar:water). Colonies were treated every week during 1 month (four applications) and at the end of this period, the reduction of spore loads was confirmed showing an efficacy of 98.4%, similar to data reported elsewhere (Williams et al. 2011). Three AHB queen right colonies previously treated with Fumagilin-B® were placed in separate observation hives with two standard Langstroth frames 45 days before the beginning of the experiments. Workers in these hives were allowed to forage freely by connecting a plastic tube from the lower part of the observation hive to the exterior (Scheiner et al. 2013). Observation hives were separated from each other by two meters, and the external entrances of each observation hive were painted with different colored patterns to reduce drifting. The top frame of each observation colony contained a mixture of open and capped cells with honey and pollen, and the bottom frame contained brood in different stages; all observation hives had similar amounts of worker bees, pollen, and nectar-honey. Additionally, the observation hives were fed with sugar syrup twice a week and adapted with queen bee excluder mesh at the entrance to avoid swarming.

### 2.3. Infection procedure

From four colonies treated with Fumagilin-B®, we took frames with worker capped brood cells and maintained them in an incubation chamber at 34 °C and 70% RH for 24 h to obtain young bees non-infected with *N. ceranae*.

To infect recently emerged workers, fresh *N. ceranae* inocula were prepared using forager bees collected at the hive entrances from the three remaining colonies previously identified as infected with the parasite but that were not treated with Fumagilin-B®. The ventricles of 200 workers were dissected and crushed in distilled purified water to produce the inoculum (Fries et al. 2013). The number of spores was counted with the help of a hemocytometer grid. The amount of spores per microliter was determined, to finally calculate the volume of concentrate that was required for each dose for infection (Human et al. 2013; Fries et al. 2013). Additionally, we confirmed that only *N. ceranae* was present in the concentrate by means of PCR (Chen et al. 2008).

Newly emerged AHB workers from the four different colonies were mixed and randomly assigned to each treatment, to avoid possible colony effects. The treatments consisted on individuals fed with 10 μl sugar syrup (50% *w*/*v*) containing 10,000 spores (Treatment 1 = T1) or 50,000 spores (Treatment 2 = T2) of *N. ceranae*. As a control, cohorts of workers were fed 10 μl of sugar syrup only (Treatment 0 = T0). Three cohorts of 180 workers for each treatment were marked with numbered colored plastic tags to identify them individually and 1620 workers in total were marked for this study. After inoculation and marking, the different groups of bees were kept separately in wooden cages (10 × 10 × 8 cm) and maintained during 24 h in an incubation chamber (36 °C and 70% HR) before being introduced to the three observation hives. This period allowed discarding bees affected by handling (Scheiner et al. 2013; Fries et al. 2013).

In total, 1030 worker bees were introduced into the observation hives for the three treatments: 309 workers in T1, 300 workers in T2, and 421 workers in T0.

To confirm the rate of infestation, 40 workers of each treatment were maintained separately in wooden cages in an incubation chamber (36 °C and 70% HR) during a period of 12 days, after which all bees were sacrificed to determine the degree of infection of *N. ceranae* by counting the spores using a hemocytometer (Cantwell 1970; Fries et al. 2013).

### 2.4. Assessment of the onset and duration of foraging behavior and longevity

A scan observation was used to record the onset of foraging behavior in all marked bees (infected and non-infected) introduced in the three observation hives (Kolmes 1984); this scan method consisted of carefully spotting and observing marked bees in both sides of each comb of the three experimental colonies. The behavior of the marked workers was recorded every day between 08:00 and 11:00 h in the morning with three observations of 20 min (1 hour per colony) in each observation hive in a random order. The onset of foraging behavior in a marked worker was considered as the first time the worker returned to the hive with pellets of pollen in the corbicula or when it transferred nectar to receiver bees (Hart and Ratnieks 2001), or when it was observed performing waggle or circle dances to recruit nestmates. These are behaviors typically related to foraging activity (Seeley 1995). The duration of foraging activity (days that the workers spend as foragers) was recorded as the time at which marked bees started foraging activities until they were last seen in the hive.

To evaluate longevity, the presence of each marked bee was registered daily through three additional observations of 20 min (1 h per colony) in each hive. These observations were carried out at the end of the day (between 17:00 to 20:00 h in the afternoon), when most field bees were inside the hives. Longevity was considered as the number of days elapsed between the introduction of each bee to the observation hive until the day they were last spotted (Rueppell et al. 2007).

### 2.5. Data analysis

We compared the effect of infection on the onset and duration of foraging behavior using a generalized linear mixed model (GLMM) with R

package "glmerMod." The treatments were used as fixed factors and observation hive as random factor. The longevity of the workers from non-infected and both infected treatments was evaluated using a Cox proportional hazard model (McMahon et al. 2016) with the R packages "coxme" (Therneau et al. 2003) and "coxph" (Therneau and Lumley 2015). We stablished the observation hive as a random factor.

## 3. RESULTS

*N. ceranae* spore load recorded in worker cohorts maintained under laboratory conditions confirmed the infection in the bees treated with two different spore concentration. At day 12 post infection, workers infected with 10,000 spores had an average of 532,400 ± 107,000 spores per bee while workers infected with 50,000 spores had an average of 1,312,600 ± 243,600 spores per bee. We did not detect the presence of spores in workers from the control group (T0).

The onset of foraging was registered during a period of 50 days in 1030 marked workers, of which 421 belonged to the non-infected treatment (T0 = non-infected workers), 309 belonged to T1 (workers infected with 10,000 spores), and 300 bees belonged to T2 (workers infected with 50,000 spores).

The results showed statistical differences between the two infected treatments compared with non-infected treatment (for T1-T0, GLMM Poisson error distribution $Z_{3\ 1030} = -23.36$, $p = 0.001$ and for T2-T0, GLMM Poisson error distribution $Z_{3\ 1030} = -23.92$, $p = 0.001$), showing that infected workers (T1 and T2) started to forage prematurely compared to non-infected ones (T0). On the other hand, we did not find differences between both infected treatments (T1-T2, GLMM Poisson error distribution $Z_{3\ 1030} = -0.802$, $p = 0.7$). Indeed, AHB workers infected with 10,000 spores started foraging at 14.9 ± 1.91 ($n = 309$) days of age while workers infected with 50,000 spores started this activity at 14.7 ± 2.07 ($n = 300$) days of age. In comparison, non-infected AHB workers started foraging until day 22.7 ± 2.82 ($n = 421$) (Figure 1). These results indicate that AHB workers infected with *N. ceranae* began their foraging activities precociously and at similar ages under tropical conditions regardless of the number of spores inoculated.

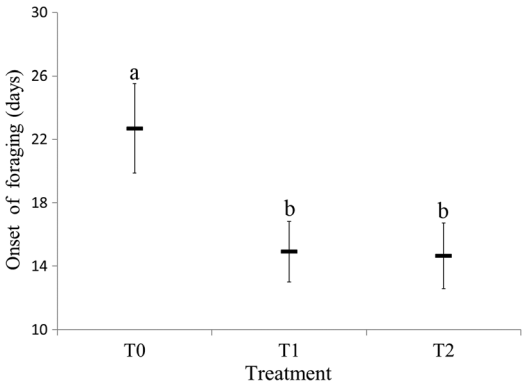


**Fig. 1** The onset of foraging behavior (days) for the three treatments: T0 = non-infected workers, T1 = workers infected with 10,000 spores, and T2 = workers infected with 50,000 spores. Dots and error bars represent the mean and standard deviation. Different letters indicate significant differences among treatments ($p < 0.001$).

Significant differences were also observed for the duration of foraging activity between bees of both *N. ceranae* infected treatments and no-infected treatment (for T1-T0, GLMM Poisson error distribution $Z_{3\ 1026} = -7.210$, $p = 0.001$ and for T2-T0, GLMM Poisson error distribution $Z_{3\ 1026} = -6.01$, $p = 0.001$). In contrast, infected treatments were not different from each other (T1-T2, GLMM Poisson error distribution $Z_{3\ 1026} = 1.175$, $p = 0.47$). AHB workers infected with the two levels of spores exhibited shorter duration of foraging activity (T1 = 7.7 ± 6.01 days; $n = 308$ and T2 = 8.3 ± 6.06 days, $n = 297$), compared with non-infected workers (9.59 ± 6.42 days, $n = 421$) (Figure 2). The difference between both infected treatments and non-infected workers was 1 to 2 days on average (Figure 2).

For longevity, we found statistical differences among infected and non-infected bees ($X^2_{3,\ 1470} = 449.28$, $p = 0.001$, Cox proportional hazard). AHB workers infected with *N. ceranae* (T1 and T2) had a reduced longevity compared to non-infected workers, but were not different from each other. AHB workers from T1 survived on average 21.6 ± 6.6 days ($n = 483$), bees from T2 did so for 21.3 ± 6.8 days ($n = 484$) and the

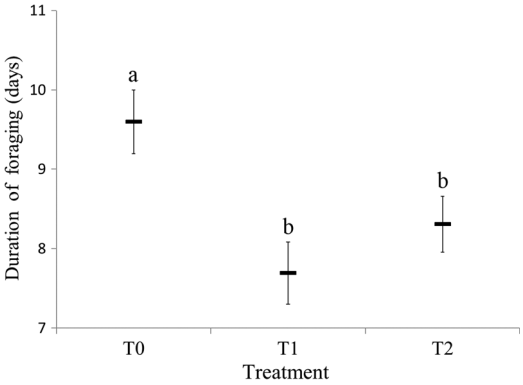


**Fig. 2** The duration of foraging behavior (days) for the three treatments: T0 = non-infected workers, T1 = workers infected with 10,000 spores, and T2 = workers infected with 50,000 spores. Dots and error bars represent the mean and standard error. Different letters indicate significant differences among treatments ($p < 0.001$).

non-infected bees survived 29.5 ± 8.4 days ($n$ = 503) (Figure 3).

For any of the former comparisons, we did not find significant statistical differences due to random effects (observation hive) (SD = 0.3141 Variance = 0.0986).

## 4. DISCUSSION

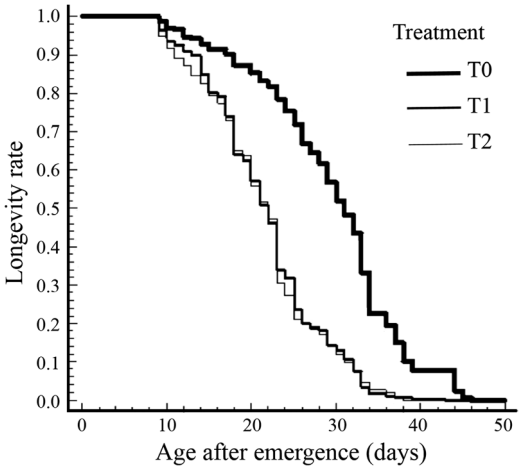


**Fig. 3** Longevity rate curves of AHB workers for the three treatments: T0 = non-infected workers, T1 = workers infected with 10,000 spores, and T2 = workers infected with 50,000 spores.

In this study, we evaluated the effects of *N. ceranae* on the onset and duration of foraging activity and longevity in AHBs under tropical conditions. A negative effect of *N. ceranae* infection was found on both foraging activity and longevity. Workers infected with two levels of spores started foraging precociously and the duration of foraging was reduced compared with non-infected bees. In addition, longevity was diminished in both infected treatments compared with non-infected workers.

### 4.1. Onset and duration of foraging in *N. ceranae* infected AHBs

We found that AHB workers infected with *N. ceranae* under tropical conditions, experienced similar behavioral changes in foraging activity to those of EHBs in temperate regions (Goblirsch et al. 2013; Natsopoulou et al. 2016). Our AHBs infected with two different concentrations of *N. ceranae* spores (10,000 and 50,000) started to forage on average 8 days earlier than non-infected bees. In EHB workers infected with *N. apis*, foraging started on average 10 days earlier (onset at 17.4 days old) than non-infected workers (Woyciechowski and Moron 2009). A negative influence of *N. ceranae* on the onset of foraging behavior in EHBs infected with 10,000 spores per bee was found by Goblirsch et al. (2013) under field conditions. They found that significantly more infected workers engaged in foraging at younger ages compared with non-infected workers (Goblirsch et al. 2013). On the other hand, Natsopoulou et al. (2016) also found that EHBs infected with *N. ceranae* had a tendency to start foraging earlier than control bees, but no statistical differences was observed between both groups.

Thus, we found that *N. ceranae* induced a similar precocious start of foraging in AHBs with a similar number of days (8 days on average) compared with EHBs.

It seems that AHB workers infected with *N. ceranae* transit from nest duties to foraging activities with evidence of an accelerated behavioral maturation similar to that found in EHBs (Holt et al. 2013; Lecocq et al. 2016). In other studies, precocious foraging in *Nosema* infected

bees has been attributed to the destruction of the midgut tissue (Higes et al. 2007), which causes starvation, and a decrease in the levels of vitellogenin (Goblirsch et al. 2013), affecting age polyethism and inducing precocious foraging and premature senescence (Antúnez et al. 2009; Holt et al. 2013; Goblirsch et al. 2013).

We also found that *N. ceranae* infected AHBs foraged 1 to 2 days less than non-infected bees. Despite these results, apparently do not indicate big differences in the duration of this activity, the reduction of the duration of foraging could affect whole colony traits such as honey yield. Fries et al. (1984) reported that Nosemosis caused by *N. apis* has negative influences on honey yield due to the reduced lifespan of infected workers.

In addition, Naug (2014) found that EHB worker bees infected with *N. ceranae* perform longer foraging trips, spending less time in the nest between foraging trips but return with less amount of sucrose per trip that results in a less efficiency of energetic gain that probably explain the reduction of honey yield.

Recently, a negative relationship between honey production and the numbers of workers infected with *N. ceranae* was observed in AHBs from the Yucatan in Mexico (Martin and Medina unpubl. data). Our findings on the reduction of foraging in infected workers could be related to colony dynamics that need to be evaluated in the future.

### 4.2. Worker longevity in *N. ceranae* infected AHBs

*N. ceranae* infection reduced AHB worker longevity 8 days in average compared to non-infected bees under field conditions.

A similar negative effect of *N. ceranae* on worker longevity was found in EHBs under laboratory conditions, resulting in a reduction of lifespan ranging between 8 and 14 days (Higes et al. 2007; Paxton et al. 2007; Dussaubat et al. 2013). Our results (8 days of longevity reduction) are in general similar to those registered by Goblirsch et al. (2013) on EHB workers at similar levels of *N. ceranae* infection (10,000 spores per bee), resulting in a 9-day reduced lifespan compared with non-infected bees under field conditions. These authors assumed that the longevity reduction in infected bees could affect colony development (Goblirsch et al. 2013). Our results suggest that a similar reduction in lifespan occurs in AHBs infected with *N. ceranae* in the field. Similarly, EHB workers infected with *N. apis* also lived significantly less under both laboratory and field conditions compared with non-infected workers (Woyciechowski and Moron, 2009).

In summary, our results showed that AHB workers are susceptible to *N. ceranae* when infected artificially with this microsporidium which results in diminished in foraging activity and longevity under field conditions, as has been observed in EHBs. Nevertheless, massive colony losses due to *N. ceranae* in Neotropical regions seems rare (Vandame and Palacios 2010). This indicates the need of further research to evaluate the effects of this parasite in managed colonies as well as in wild swarms considering the effect of seasonality and other aspects of AHBs colony dynamics in Neotropical regions.

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## AUTHORS' CONTRIBUTIONS

All authors have contributed equally to the work.

## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of interest** The authors declare that they have no conflict of interest.

**Début de butinage et durée de vie des abeilles africanisées (*Apis mellifera*) infectées par différents niveaux de spores de *Nosema ceranae* dans le Mexique néotropical**

***Nosema ceranae* / Comportement de butinage / longévité / nosémose / Abeilles africanisées**

**Der Beginn des Sammelverhaltens und die Lebenserwartung von Afrikanisierten Honigbienen (*Apis mellifera*), die im neotropischen Mexiko mit unterschiedlichen Dosierungen von *Nosema ceranae* Spores infiziert wurden**

***Nosema ceranae* / Sammelverhalten / Lebenserwartung / Nosemosis / Afrikanisierte Bienen**

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