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Effect of oxalic acid on *Nosema ceranae* infection

Antonio Nanetti [a,*,1], Cristina Rodriguez-García [b,1], Aránzazu Meana [c,1], Raquel Martín-Hernández [b,d,1], Mariano Higes [b,1]

[a] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Unità di Ricerca di Apicoltura e Bachicoltura, CREA-API, Via di Saliceto 80, 40128 Bologna, Italy
[b] Laboratorio de Patología Apícola, Centro Apícola Regional, Consejería de Agricultura, JCCM, 19180 Marchamalo, Spain
[c] Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain
[d] Instituto de Recursos Humanos para la Ciencia y la Tecnología (INCRECYT), Fundación Parque Científico y Tecnológico de Albacete, Spain

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ABSTRACT

Nosema ceranae is a honey bee pathogen parasitizing the ventricular epithelium and potentially causing colony death. The effect of 0.25 M oxalic acid solution administered to the bees in the form of sugar syrup was determined in laboratory and field trials. The spore numbers in an 8-day laboratory experiment were significantly lower when AO was administered (treated: 11.86 ± 0.94 s.e. × 10^6; untreated: 30.64 ± 0.31 s.e. x 10^6). When administered in autumn to free flying colonies twice, 3 weeks apart, the infection prevalence decreased in young (relative reduction of 53.8% ± 6.5 s.e.) and old bees (relative reduction of 44.4% ± 6.0 s.e.). Meanwhile increased prevalence in all the controls was detected (young and old bees: relative increase of 45.7% ± 22.8 s.e. and 10.2% ± 5.9 s.e., respectively). While all the treated colonies overwintered correctly, the untreated ones did not (3 out of 5 were dead).
In the absence of commercial products approved in several countries to control nosemosis, oxalic acid syrup appears promising in the development of alternative management strategies.

1. Introduction

Oxalic acid (OA) is one of the strongest organic acids. It is a natural constituent of living organisms and foodstuff, honey included, as free acid and oxalates. Toxicity of oxalic acid against *Varroa destructor* was shown in controlled laboratory experiments (Milani, 2001) and it has been used for years for control in the field in the form of sugar water syrups (Nanetti et al., 2003b). The treatments can be applied by trickling 0.3–0.5 M solutions (pH about 0.9–1) added with sucrose into the colonies (Nanetti and Stradi, 1997), where the substance is deemed to act as a contact agent. The bees do not show a tendency to feed on the liquid [unpublished observations], but pharmacokinetic studies revealed prompt absorption by the bees and distribution amongst the adult individuals of the colony after administration (Nanetti et al., 2003a). In spite of ingestion, therapeutic doses of OA are tolerated by the bees and are not associated with patent negative effects. In fact, although immunohistochemical analysis showed necrotic cell death in ventriculi of bees fed with oxalic acid (Gregorc and Škerl, 2007) and other studies put into evidence decreased bee longevity (Schneider et al., 2012), in an extensive investigation performed in several European countries obvious negative effects on the adult population of the colonies were not observed (Nanetti et al., 2003b).

In recent years, *Nosema ceranae* has become one of the most prevalent honey bee pathogens worldwide (Fries, 2010; Higes et al., 2010, 2013). In some regions (especially warm areas) it is reportedly implicated in the general phenomenon of honey bee colony losses (Bacandritsos et al., 2010; Bekele et al., 2015; Bromenshenk et al., 2010; Hatjina et al., 2011; Higes et al., 2008; Natsopoulou et al., 2015; Soroker et al., 2011; Suwannapong et al., 2011), but in colder areas its responsibility in events of widespread mortality has been ruled out (Gisder et al., 2010; Hedtke et al., 2011; Stevanovic et al., 2011). In spite of these discrepancies, it is generally accepted that better colony development can be expected following treatments against nosema (Botías et al., 2013; Higes et al., 2010, 2013). Finally, recovery was also observed by Van der Zee in *N. ceranae* infected colonies after OA treatments against *Varroa* infestations (personal communication).

With this study we wanted to create the basis to develop new control methods for *N. ceranae* treatment. For this purpose, we tested the efficacy of OA solution against artificial and natural infections.

* Corresponding author.
E-mail addresses: antonio.nanetti@entecra.it (A. Nanetti), crodriguezgarcia@externas.jccm.es (C. Rodriguez-García), ameana@ucm.es (A. Meana), rmhernandez@jccm.es (R. Martín-Hernández), mhiges@jccm.es (M. Higes).
[1] The authors contributed equally to this work.

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2. Materials and methods

2.1. Laboratory procedure

2.1.1. Preparation of N. ceranae spores

To obtain fresh *N. ceranae* spores, three infected colonies of *Apis mellifera iberiensis* located at the Regional Apicultural Center (CAR), Central Spain, were used. One hundred flying bees were collected from each colony and processed separately according to Botías et al. (2012) and Martín-Hernández et al. (2012). In short, the abdomens of each sample were pooled and macerated with a Stomacher 80 Biomaster (Seward, West Sussex, UK) provided with strainer bags (BA6040/STR, Seward) first in 25 ml and, after recovery, in 10 ml of PCRq water. A centrifugation (6 min at 800 rpm) allowed us to obtain a pellet, that was suspended into 1 ml of PCRq water and stored at room temperature until use.

The species assessment of the spores was made by PCR according to Martín-Hernández et al. (2012). Concisely, aliquots of the macerates were shaken at 9500 rpm for 95 s with ceramic beads (MagNA Lyser Green Beads, Roche 03 358 941 001). The DNA was extracted with MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche 03 730 964 001) in a MagNA pure compact machine (Roche). The PCR amplification was performed with 218MITOC FOR/218MITOC REV and 321APIS FOR/321APIS REV primers, specific for *N. ceranae* and for *N. apis* respectively and COI as an internal control. The PCR products were analysed in a QIAxcel System (Qiagen, Hilden, Germany) using a QIAxcel DNA High Resolution Kit (Qiagen, No. 929002) to detect positive and negative reactions. Negative controls were included to detect and exclude possible contaminations.

Once the presence of *N. ceranae* spores and the absence of *N. apis* spores were ascertained, the suspensions were mixed, purified by Percoll density gradient centrifugation (Higes et al., 2008; Martín-Hernández et al., 2011) and maintained at room temperature for 5 days or less. Finally, counts in Neubauer haemocytometer chamber under phase contrast microscope allowed us to calculate the spore concentration in the purified suspension (Higes et al., 2007).

2.1.2. Experimental infections

Brood combs were taken from three *A. m. iberiensis* colonies found free of *N. ceranae* and *N. apis* infections by PCR (Higes et al., 2007) and maintained in an incubator at 34 ± 1 °C. On hatching, new adult bees were carefully transferred into nine cylindrical mesh cages (base: 71 mm^2; length: 175 mm) and stored at 33 ± 1 °C for five days. Syrup was provided *ad libitum* (50% sugar water with 2% Promotor L, Calier Lab as pollen substitute) by plastic feeder.

One hundred and twenty bees, five days old, were starved for 2 h. After slight anesthetisation with CO_2 to make handling easier, they were artificially fed 2 μl each of 50% sugar water containing 150,000 *N. ceranae* spores. As needed, the mouth parts were stimulated to induce the ingestion of the droplet from the micropipette. Individuals not consuming the entire dose were discarded (Higes et al., 2007, 2010). The same procedure was used to administer sugar water not containing spores to 60 other honey bees.

Three groups of 60 adults each were formed from the infected and uninfected bees above. Groups I and II were composed of infected bees and group III of uninfected controls. The individuals of each group were split into three subunits of 20 and transferred into 9 mesh cages. To avoid cross contaminations, the 6 cages containing infected bees and the 3 containing the uninfected ones were stored separately at 33 ± 1 °C into two Memmert incubators of same age and model (Mod. IPP500).

Each cage of groups II and III was provided with 2 ml syrup (50% sugar water with 2% Promotor L, Calier Lab) for eight days, via feeder. The cages of group I received by the same method 0.25 M oxalic acid syrup, the last being composed as described above. Ecoxal® (Ceva) was used as source of oxalic acid.

On days 4, 6 and 8 post-infection (p.i.), five bees were removed randomly from each cage, pooled in samples of 15 individuals and dissected. To assess their degree of infection, the abdomens were introduced into sterile Eppendorf microtubes filled with 200 μl of PCRq water. After thorough grinding, the spores were counted using a haemocytometer and a phase contrast microscope. PCR was then used to confirm that the spores belonged to *N. ceranae* species.

2.2. Field trial

In May, ten colonies of the apiary at CAR were equalised to 10 combs covered with bees and 5 with brood. At that time and until the end of the experiment none of them showed symptoms of American foulbrood, European foulbrood and chalkbrood. Symptomatic *Nosema* spp. infections and *V. destructor* infestations were not present.

Samples of flying bees were collected from each colony and analysed by PCR (Martín-Hernández et al., 2012) to confirm that they were infected by *N. ceranae*. Two groups of 5 colonies each were then formed by simple randomisation without replacement.

On 26 October the colonies of one of the groups were treated with 50 ml of 0.25 M oxalic acid syrup (50% sugar water with 2% Promotor L, Calier Lab) that was dispensed by trickling onto the combs with a syringe. The same day, the colonies of the other group received syrup without oxalic acid. On 16 November the administration was repeated according to the method described above.

Samples of 20 bees were collected on 25 October (pre-treatment) and on 23 November (post-treatment) from the entrance and from the brood chamber of each colony (assumed to represent the young and the old colony population, respectively). They were analysed individually to quantify the infection prevalence according to Higes et al. (2008) and Botías et al. (2013). In short, 20 abdomens of each sample were dissected and introduced in 96-well plates (Qiagen) containing glass beads (2 mm diameter; Sigma) and 300 μl of PCRq water. Once shaken in TissueLyser® (Qiagen) for 6 min at 30 Hz, the DNA was extracted and analysed by PCR as described previously (Martín-Hernández et al., 2012).

Three months after the second administration, the colonies were inspected to evaluate their general conditions and the number of combs covered with the bees and the ones containing brood.

2.3. Statistical analysis

The package Statistica 7.0 was utilised for the statistical analysis of data. Non parametric approach (Mann–Whitney *U* test and Wilcoxon test for paired samples) was used, except for the calculation of regression parameters and for the comparison of regression lines (analysis of covariance).

Table 1
Count of *Nosema ceranae* spores in the artificially infected groups at the three sampling points (in millions).

d p.i.	Group I (treated infected)				Group II (untreated infected)				Mann–Whitney U-test	
	N	Mean ± s.e	s.d.	C.I. (95%)	N	Mean ± s.e	s.d.	C.I. (95%)	U	p
4	15	0.69 ± 0.03	0.10	0.63 ± 0.74	15	0.58 ± 0.01	0.05	0.55 ± 0.61	39.0	0.002
6	15	1.11 ± 0.04	0.16	1.02 ± 1.20	15	2.77 ± 0.13	0.50	2.49 ± 3.05	0.0	0.000
8	15	11.86 ± 0.94	3.66	9.84 ± 13.89	15	30.64 ± 0.31	1.18	29.99 ± 31.30	0.0	0.000