Fumagillin control of Nosema ceranae *(Microsporidia: Nosematidae) infection in honey bee (Hymenoptera: Apidae) colonies in Argentina*

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Keywords
Antibiotic control, Apiculture, *Apis mellifera*, Fumagillin, *Nosema ceranae*.

Summary
Information on the long-term consequences of *Nosema ceranae* to honey bee lifespan and effectiveness of *Nosema* control with fumagillin is scarce and not always consistent. Our objective in this study was to evaluate the effectiveness of the antibiotic fumagillin to control *N. ceranae* in hives in East-Central Argentina. Honey bee hives were assigned to 3 experimental treatments, a control group with un-treated hives, a preventive strategy group with hives treated monthly, and a monitoring strategy group with hives treated according to a *N. ceranae* threshold level. Apiaries were monitored monthly during Fall-Winter 2009 and 2010 and *N. ceranae* spore intensity and honey bee colony strength measures were estimated. Fumagillin-treated colonies had reduced *N. ceranae* spores load in 2010 compared to control colonies. However, there was no significant difference between treated and control groups for colony strength measures including adult bee population, bee brood availability, honey, or pollen. Fumagillin treatment reduced *N. ceranae* intensities but had little effect on colonies. The bee population during Winter was reduced in treated as well as in control colonies. Our results clarify that fumagillin treatment should be at least reviewed and that further research should be conducted to acquire a more complete perspective of Nosemosis disease.

Controllo dell'infezione da Nosema ceranae *(Microsporidia: Nosematidae) con fumagillina in colonie di api da miele (Hymenoptera: Apidae) in Argentina*

Parole chiave
Antibiotico, Apicoltura, *Apis mellifera*, Fumagillina, *Nosema ceranae*.

Riassunto
Le informazioni sulle conseguenze dell'infezione da *Nosema ceranae* sulla durata di vita delle api da miele e sull'efficacia della fumagillina sono scarse e non sempre coerenti. Lo studio ha avuto l'obiettivo di valutare l'efficacia dell'antibiotico nel controllo della micosi in alveari delle aree orientali e centrali dell'Argentina. Gli alveari sono stati suddivisi in 3 gruppi sperimentali: un gruppo di controllo costituito da alveari non trattati, un gruppo con alveari trattati mensilmente secondo una strategia preventiva e un gruppo di monitoraggio con alveari trattati secondo il proprio livello di infezione. Gli apiari sono stati monitorati mensilmente durante la stagione autunnale e invernale 2009-2010. In questo periodo sono stati misurati il numero di spore prodotte da *N. ceranae* e la resistenza delle colonie di api da miele. Confrontate con il gruppo di controllo, le colonie trattate con fumagillina nel 2010 hanno mostrato una riduzione della presenza di spore di *N. ceranae*. Non sono state tuttavia rilevate differenze significative tra le colonie trattate con fumagillina e i gruppi di controllo per quanto concerne: resistenza delle colonie, popolazione adulta, disponibilità delle larve, produzione di miele e raccolta di polline. Si è osservato che il trattamento con l'antibiotico pur riducendo la presenza di *N. ceranae* ha avuto un effetto ridotto sulle colonie. La popolazione delle api è diminuita durante l'inverno nel gruppo di controllo e in quello trattato con fumagillina. I risultati ottenuti mostrano che i trattamenti a base di fumagillina dovrebbero essere rivisti e che ulteriori studi consentirebbero di acquisire una conoscenza più approfondita sulla nosemiasi.

Introduction

Nosema ceranae (Phylum Microsporidia) (Fries *et al.* 1996), an intracellular parasite originally found in Asian honey bee *Apis cerana*, has recently been detected in European honey bee *Apis mellifera* L. (Fries *et al.* 1996, Fries *et al.* 2006, Higes *et al.* 2006, Martin-Hernández *et al.* 2007, Huang *et al.* 2007). Cross-infectivity with *N. ceranae* has been found in both hosts (Fries and Feng 1995). This microsporidium infecting *A. mellifera* should not be confused with *Nosema apis* (Zander, 1909), another microsporidium described more than 100 years ago, which is also commonly found throughout the beekeeping world (Klee *et al.* 2007, Chen *et al.* 2008, Williams *et al.* 2008a, Giersch *et al.* 2009). The pathology caused by *N. apis* in the ventricular cells of adult honey bees has been named Nosemosis type A, to distinguish it from the newly described disease, Nosemosis type C caused by *N. ceranae* (COLOSS workshop 2009, Higes *et al.* 2010). *Nosema apis* significantly reduces honey production, pollination effectiveness, and colony survival over the Winter (Malone and Gatehouse 1998). Nevertheless, a good degree of control is obtained with the antibiotic fumagillin dicyclohexylammonium (Webster 1984, Furgala and Sugden 1985, Szabo and Heikel1987).

Nosema ceranae is widely distributed (Klee *et al.* 2007), particularly in Argentina (Sarlo *et al.* 2008, Medici *et al.* 2012) and in bordering countries, such as Uruguay (Invernizzi *et al.* 2009), Chile (Martinez *et al.* 2012), and Brazil (Texeira *et al.* 2013). However, information on the long-term consequences of *N. ceranae* in honey bee lifespan is scarce and not always consistent (Higes *et al.* 2007, Higes *et al.* 2008, Martín-Hernández *et al.* 2007, Paxton *et al.* 2007, VanEngelsdorp *et al.* 2009, Fries 2010, Gisder *et al.* 2010, Stevanovic *et al.* 2010, Williams *et al.* 2010, Forsgren and Fries 2012). Particularly, few studies on chemical and alternative control strategies can be found (Williams *et al.* 2008 a, b, Williams *et al.* 2010, Higes *et al.* 2011). Moreover, there is no consensus with regards to the effectiveness of fumagillin to control *N. ceranae* infection (Kochansky and Nasr 2004, Gomez-Pajuelo *et al.* 2008, Williams *et al.* 2010, Higes *et al.* 2011), especially because it has not proved to be efficient with *Nosema bombi*, a closely related species (Whittington and Winston 2003). Additionally, even when fumagillin is the only commercial medication available to control both *N. apis* and *N. ceranae*, some European countries do not allow to treat hives with antibiotics. Yet, it is necessary to carry out a proper control of diseases in food producing animals, due to its great relevance to human and environmental health.

Recently, a possible mechanism through which *N. ceranae* escapes fumagillin control has been described suggesting that field studies are necessary to determine whether the fumagillin use has value in specific situations (Huang *et al.* 2013). The objective of the present work was to evaluate the effectiveness of the antibiotic fumagillin to control *N. ceranae* in hives in East-central Argentina.

Materials and methods

Study design

Experiments were conducted in hives located at INTA Rafaela Experimental Station (Santa Fe province, Argentina) from March to August 2009 and from March to July 2010. Trials were designed in accordance with CONSORT guidelines (Moher *et al.* 2010). Samples from regional hives were tested for the presence of *N. ceranae* using multiplex polymerase chain reaction (PCR), as described by Martín-Hernández and colleagues (Martín-Hernández *et al.* 2007). To detect a reduction of 200,000 spores (SD 120,000) in *N. ceranae* spore counts with a two-sided 5% significance level and a power of 80%, a sample size of 8 hives per group was necessary. Twenty-four hives were randomly assigned to 3 different experimental treatments (8 hives/treatment).

Treatment groups

Hives were randomly assigned to 1 of 3 treatment groups (Moher *et al.* 2010). The first group (henceforth referred to as CG) was a control group with untreated hives, the second group (henceforth referred to as MG) underwent a preventive treatment strategy, with hives treated with fumagillin once per month, and the third group of hives (henceforth referred to as NLG) was treated when *N. ceranae* counts were higher than 350,000 spores/ml, following an integrated pest management strategy (Signorini *et al.* 2010). The antibiotic fumagillin (FUGIPRIN®) was applied according to label instructions; mixing 2.25 l of 2:1 sugar syrup with fumagillin antibiotic (102 mg per colony). The untreated colonies received 2.25 l of 2:1 sugar syrup without fumagillin.

N. ceranae spore counts and honey bee colony strength measures

Once a month, *N. ceranae* spore intensity and colony strength measures were estimated. Worker honey bee samples were collected from the hive entrance using a portable vacuum device. A minimum of 60 bees was gathered and placed in labelled plastic flasks containing 96 ml of water and 4 ml of formaldehyde. At the laboratory, for each colony, spore suspensions were made by adding 60 ml of distilled water to crushed abdomens of

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60 randomly-selected individuals. This sampling size can detect a 5% of infected bees by 95% of confidence (Fries *et al.* 1984).

Nosema spores/ml were determined using light microscopy 400X and an improved Neubauer haemocytometer (1/10 mm Boeco, Hamburg, Germany®). For each sample, the number of spores was counted in 80 haemocytometer squares (Cantwell 1970).

The populations of adult bees and the amount of brood, pollen, and honey reserves in the hives were measured by estimating the total area of frames covered by adult bees (FWB), brood (FWBr), sealed honey (FWH), and pollen (FWH) (exceptionally, no pollen registration was performed during 2010). Once each hive was opened, each frame was sequentially removed and the percentage of coverage in both sides was estimated (VanEngelsdorp *et al.* 2009, Delaplane *et al.* 2013).

Researchers involved in this study were not informed of the treatment group assignment. Moreover, researchers who conducted the treatments did not take outcome measurements.

Table I. *Summary statistics of* Nosema ceranae *infection intensity (1,000 spores/ml) for control and fumagillin-treated groups (monthly and according to* N. ceranae *level) in Rafaela during Fall-Winter 2009 and 2010.*

Year	*Nosema ceranae* spore intensity (in thousands)						*P*
	Control		*N. ceranae* level		Monthly		
	Mean	SD	Mean	SD	Mean	SD	
2009	187[a]	61	46[a]	73	45[a]	61	0.22
2010	1,433[a]	232	587[b]	344	528[b]	291	0.04

[a,b] Different letters indicates signficance difference for each year ($P < 0.001$, ANOVA repeated measures).

Statistical analysis

Repeated-measures ANOVA and Duncan test were used to compare spore intensity and hive strength variables (FWB, FWBr, and honey, and pollen stores) in control and fumagillin treated groups. The effect of treatment strategies was considered significant when $P < 0.05$. All statistical analyses were performed using Infostat software (Universidad Nacional de Córdoba 2009).

Results

In 2009, no significant difference between treatments ($P = 0.22$) was found in the spores load (mean ± SD). However, both fumagillin-treated groups showed lower loads when compared with the control group, which presented the highest value. In 2010, *Nosema* was lower in fumagilling-treated colonies than in control colonies ($P = 0.04$) (Table I and Figure 1). There was no significant difference between fumagillin-treated and control groups as regards the number of frames covered with bees (2009: $P = 0.99$ and 2010: $P = 0.45$), with brood (2009: $P = 0.57$ and 2010: $P = 0.59$), honey (2009: $P = 0.72$; 2010: $P = 0.52$) or pollen (2010: $P = 0.14$) (Figure 2).

Discussion

Fumagillin treatment reduced *N. ceranae* intensities. Nevertheless, *N. ceranae* seems to have little effect on colonies given that no difference was found in colony strength variables when fumagillin-treated

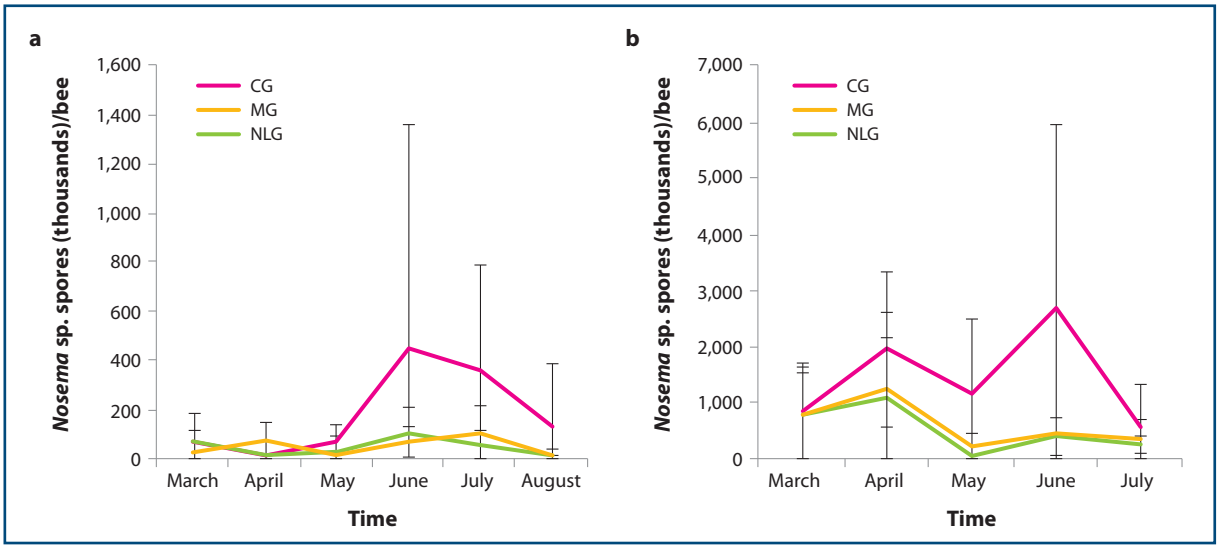

Figure 1. *Comparisons between control (CG), monthly fumagillin-treated (MG), and according to* Nosema *level fumagillin-treated (NLG) in Fall-Winter 2009 (**a**) and 2010 (**b**), for* Nosema ceranae *spore intensity.*

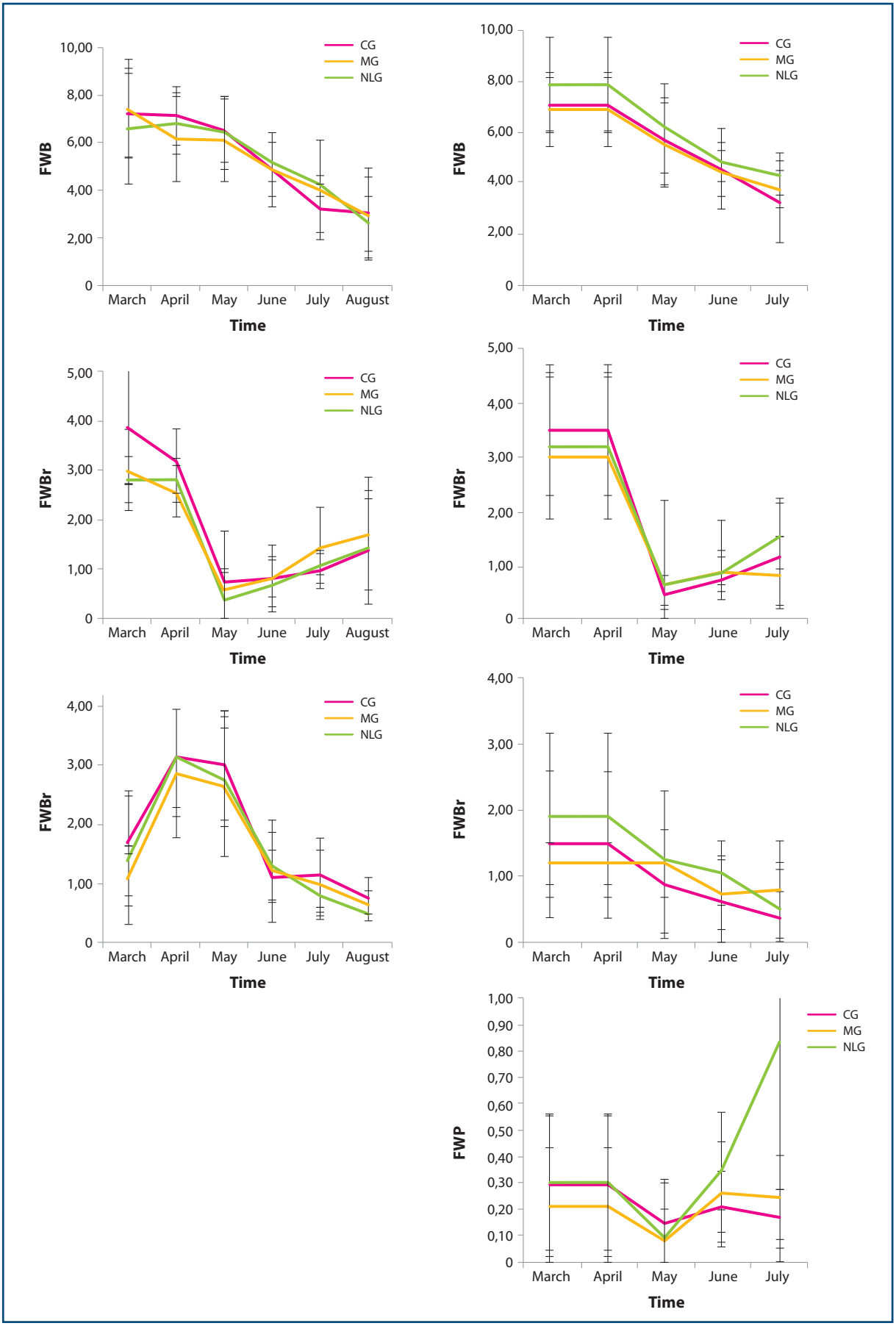

Figure 2. *Comparisons between control (CG), monthly fumagillin-treated (MG) and according to* Nosema *level fumagillin-treated (NLG) in Fall-Winter 2009 and 2010, for number of frames out of 10 of: adult bees (FWB), brood (FWBr), honey (FWH), and pollen (FWP).*

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and control hives were compared. Similarly, honey consumption and pollen storage were not affected by *N. ceranae* intensity either, as Williams and colleagues reported (Williams *et al.* 2010). We found that the colonies in the control group suffered the same bee population size reduction in Winter as in fumagillin treated hives. It has previously been found that hives which had experienced spore reduction also showed a decrease in the number of frames covered with bees (Signorini *et al.* 2010). This may show that spore intensity decline is not related to the fumagillin treatment *per se* but to bee renewal, and that infected colonies recover during the Summer in a natural way (Williams *et al.* 2008b).

This is important, since the use of ineffective antibiotic increases production costs and contamination risks (Gomez-Pajuelo *et al.* 2008). Moreover, given that *N. ceranae* showed less susceptibility and hyper proliferation in the presence of low residues of fumagillin, it may provide an advantage to *N. ceranae* infection instead of suppress it (Huang *et al.* 2013).

Previous studies not only found that fumagillin reduced or eliminated the infection with *N. ceranae* (Higes *et al.* 2008), but also confirmed the antibiotic was effective to prevent honey bee colony collapse when appropriate support and dosage was applied (Higes *et al.* 2011). Williams and colleagues reported a weak degree of control of fumagillin in commercial colonies in Canada (Williams *et al.* 2010). They suggested possible reasons to explain such high inconsistency in the results, for example, different *N. ceranae* haplotypes virulence, accurate damage thresholds, environmental conditions or an erroneous selection of the infection indicator. Specifically, the method used to determine *N. ceranae* spore intensity is one of the most controversial aspects discussed in this field (Fries *et al.* 2013), consequently, it is difficult to determine fumagillin effectiveness to control unknown levels of disease.

Monitoring results obtained by our research group showed that during four years and in two different regions, *N. ceranae* spore intensity increased and decreased but no detectable damage was observed in the hives. Furthermore, long term consequences of *N. ceranae* infection and its role in honey bee colony losses is significantly discussed in literature (Martín-Hernández *et al.* 2007, Gomez-Pajuelo *et al.* 2008, Higes *et al.* 2008, Higes *et al.* 2009, Higes *et al.* 2010, Invernizzi *et al.* 2009, VanEngelsdorp *et al.* 2009, Fries 2010, Genersch *et al.* 2010, Gisder *et al.* 2010, Williams *et al.* 2010). Probably, the severity of the disease impact depends on multiple factors that may occur simultaneouly. The presence of *N. ceranae* (even in high levels) would not necessarily imply pathological consequences for honey bee at colony level. Furthermore, the fact that no difference was found in strength measures may indicate that higher *N. ceranae* spore intensity does not necesarily have a negative impact on honey bees colonies.Thus, apparently, no harm is caused to hives by the elevated infection intesity itself. In this context, fumagillin treatment seems neither to influence Nosemosis nor does it reduce disease impact on honey bee colonies, therefore its relevance on *N. ceranae* control should be revised. Our study contributes to elucidating that the contribution of *N. ceranae* to the development of hive damage is questionable and that the role of fumagillin treatment might be overestimated. However, further research should be conducted in order to get a more complete perspective of the problem.

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