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COMPARISON BETWEEN DIFFERENT FUMAGILLIN DOSAGE AND EVALUATION METHOD IN THE APIARY CONTROL OF NOSEMOSIS TYPE C.

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Sarlo E.G., Medici S.K., Porrini M.P., Garrido M., Floris I., Euguaras M.J. – Comparison between different fumagillin dosages in the apiary control of Nosemosis type C.

Fumagillin (dicyclohexylammonium) is an antibiotic with well-known microsporicidal activity widely used to control Nosema disease caused by *Nosema apis* in honeybees. Its use is permitted by law in Argentine and USA, but not in the EU countries, apart from specific cases under veterinary authorization. So far, the optimal dosage of this active ingredient in controlling *Nosema ceranae* has not been determined. The aim of the present work was to assess the efficiency of different fumagillin dosages on *Apis mellifera* colonies affected by different *N. ceranae* intensity. For this purpose, during April and May of 2007, in an apiary located near the Mar del Plata city (Buenos Aires Province, Argentine), forty eight *A. mellifera* colonies reared in Langhstroth hives, each containing 8 to 10 adult bee combs, were used in order to evaluate the effectiveness of different doses of fumagillin. The colonies were divided into those with mild or semi-severe disease intensity, based on the number of *Nosema* spores estimated in a sample of 60 returning bees (*arb,* spores abundance in returning bee). Then the colonies were randomly distributed forming 6 experimental groups (3 with mild intensity: one untreated left as control and the other two treated with 102 mg of fumagillin in two and three doses, respectively; 3 with semi-severe intensity: treated with 102 mg of fumagillin in two and three doses, and with 120 mg of fumagillin in four doses, respectively). Each dose was administered at intervals of 7 days. Before and during each administration, for a period of 35 days, the number of frames covered with adult bees was recorded in each hive and a sample of 100 workers per colony was collected from the hive entrance in order to determine the abundance (*arb*) and prevalence (*prb*) of spores. This alternative parameter (*prb*) was calculated by examining a sub-sample of 10 bees, crushing the propodeum of the bees in 0.5 ml distilled water, taking a drop of suspension and observing 20 fields under a optic microscope (450x) for spore absence (0) or presence (1); then the estimation of spores prevalence (*prb*) was recorded as percentage of spore presence in the 20 observed fields, and the average of two estimates were used. Moreover, spores from 5 randomly selected colonies were molecularly characterized to confirm the *Nosema* species. The sequencing results showed a 98% of homology with *N. ceranae*. The results obtained allow to establish that the efficiency of fumagillin in controlling Nosemosis type C caused by *N. ceranae* is affected by the intensity of the disease rather than from dosage or way of application. The best result was obtained when colonies were affected by a mild intensity and the drug was administered in two weekly doses of 51 mg per hive.

KEY WORDS: fumagillin; antibiotic; *Nosema ceranae*; microsporidian; *Apis mellifera*.

INTRODUCTION

Nosema ceranae (Microsporida, Nosematidae) is a microbial intracellular parasite of adult honeybees with worldwide distribution (KLEE *et al* 2007) known to infect the Asian honey bee, *Apis cerana*, and the European honey bee, *Apis mellifera*. It is responsible for the disease named Nosemosis type C (COLOSS Workshop, 2009) and for considerable economic losses among beekeepers (FRIES, 2010). Affected hives show low production and high bee mortality, and need to be treated with antibiotics for the control of this disease (FAUCON, 2002; HIGES *et al*., 2006a).

While in the EU countries fumagillin (dicyclohexylammonium) is generally not allowed (apart from specific cases under veterinary authorization), in the USA and Argentine it is regularly registered for use in beekeeping and represents a well-known and widely used antibiotic for the control of the honeybee Nosemosis (KATZNELSON and JAMIESON, 1952; GOCHNAUER and FURGALA, 1969; HARTWIG and PRZELECKA, 1971; WEBSTER, 1994; SARLO *et al*. 2002; SARLO *et al.* 2006). However, the efficiency of this active ingredient in the control of Nosemosis type C caused by *Nosema ceranae* remains unclear, varying either for pharmacological parameters and dosage or due to intrinsic factors like haplotype (WILLIAMS *et al.*, 2008) or the intensity of the disease in the honeybee colonies. HIGES *et al*. (2006b) reported a complete efficacy after the administration of 120 mg, while WILLIAMS *et al*. (2008) have established that higher doses (168 mg) gave only a temporary effect. The importance of unwanted side-effects that this antibiotic may exert on bees (LIU, 1990a, b; RADA *et al*. 1997) and humans (STANIMIROVIC *et al*., 2006) by the possible honey contamination is considered adverse (ASSIL and SPORNS, 1991, STEVANOVIC *et al*., 2000). However, it is essential a proper dosage optimization for this conventional antibiotic in the hive treatments.

The aim of the present work was to asses the efficiency of Fumagillin dosage in relation to different Nosemosis type C intensity in *Apis mellifera* colonies.

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Table 1 – Environmental conditions prevailing during the experiment.

Month	Max. Temp.* (ºC)	Min Temp. * (ºC)	Medium Temp.* (ºC)	Med Rel. Hum..* (%)	Rainfall* (mm)
April	20.37	9.37	14.90	82.33	19.86
May	14.10	4.97	9.56	84.81	2.90

*Average monthly weather (Fuente EEA INTA Balcarce). The months correspond to autumn in the southern hemisphere.

MATERIAL AND METHODS

Experiments were conducted between April and May 2007, using 48 Langhstroth colonies of *A. mellifera*, located near Mar del Plata city, in the Buenos Aires Province, Argentine (37º 56' 02,29" S; 57º 40' 57,63" W). Environmental conditions are specified in table 1.

Forty eight colonies of *A. mellifera* in Langhstroth hives, each with 8 to 10 combs of adult bees, were first evaluated for the Nosemosis intensity. For this purpose, the mean number of spores per bee in a sample of 60 returning bees per hive was obtained using a haemocytometer (CANTWELL, 1970) and according to FRIES (1988) in order to obtain a 95 % of significance level. Because not all the bees in the sample are necessarily infected, it is proposed in this paper to consider the spore number obtained as the "spores abundance in the returning bees" (*arb*). After obtaining the *arb* values, the colonies were divided based on their infection level: 24 in the mild level and 24 in the semi-severe level according to JACOX scale (1980). Then the colonies were randomly distributed into six experimental groups (N=8), three of which with mild intensity (N=24) and the other with semi-severe intensity (N=24) (2).

The administration of fumagillin at various doses (2, 3 or 4), concentrations (0, 30, 34, 51 mg/dose per treatment) and total amounts (0, 102 or 120 mg/hive) was performed at 7-day intervals, supplied in 500 ml sugar syrup (66% w/v) according to GOCHNAUER and FURGALA (1969). The solution was given with an inner feeder at 16:00 (GTM). Total solution removal was complete after 24 hours. The treatment for each group is shown on Table 2.

During the trial (35 days), once a week, the number of frames covered by bees per each colony were recorded between 09:00 and 11:00 (GTM) and a sample of 100 returning bees was collected from each hive between 13:00 and 14:00 (GTM). The bee samples were conserved using a 4% formaldehyde solution until examination of bees to determine the abundance of spores (*arb, N 60*) and the prevalence of spores (*prb, N 10*). This last additional parameter was used as alternative evaluation of the effectiveness of the treatment by examining a sub-sample of 10 bees. The spore suspension for each bee was obtained by adding 0.5 ml distilled water to crushed abdomen collected after propodeum ablation. A drop of the suspension was examined under optic microscope (450X) for spores observation. Estimation of prevalence of spores (*prb*) was recorded as percentage of spore presence in 20 observed fields, and the average of two estimates were used.

After confirming the spores presence by microscopic analysis, purification was conducted according to COLE (1970) and molecularly characterized following the MARTIN-HERNÁNDEZ *et al.* (2007) method. The PCR fragments were purified and sequenced in the Pro-papa Laboratory EEA, INTA, Balcarce, Argentine. Afterward, they were sequenced and matched with respect to those published in the GENBANK (NCBI).

Finally, the data were analysed by non parametric Krustal Wallis and Mann Whitney test (XLSTAT® 2008).

RESULTS

After multiplex PCR, the fragments obtained from the spore samples, exhibited only a band corresponding to 218 bp in agreement with MARTÍN-HERNÁNDEZ *et al.* (2007). Sequencing results were entered in the GENBANK BLASTn, which yielded 98% homology with *N. ceranae* (accession N° FJ425736).

The fumagillin treated groups showed a significant reduction in the *arb* of spores after 35 days of treatment [Mann-Whitney, p-value (bilateral) < 0.0001, a = 0.05] (fig. I). The effectiveness estimated by this indicator was calculated as a percentage of decrease for each group. This percentage was over 91% for every groups, while the control group showed an increase of the *arb* spores, with final values of 10.707.500 ± 2.188.331 SD spores per bee (Table 3). More precisely, final *arb* values of groups with mild degree (2a and 2b) resulted significantly lower than semi-severe degree groups (3a and 3b) [Mann-Whitney / p-value (unilateral) < 0.0001 a = 0.05). These groups (3a and 3b] did not present significant differences respect to Group 4 (dose of 120 mg) at the end of the

Table 2 – Treatment groups formed from mild degree colonies (1 - 2 x10^6 spores/ bee) and semi severe (10 - 15 x10^6 spores/ bee) according to Jaycox scale.

Group	Number of colonies	Nosemosis intensity (abundance of spores/bee)	Number of doses per hive	mg of fumagillin / dose / hive	Total mg. of fumagillin/ hive.
Control (1)	8	mild	4	0	0
2a	8	mild	2	51	102
2b	8	mild	3	34	102
3a	8	semi-severe	2	51	102
3b	8	semi-severe	3	34	102
4	8	semi-severe	4	30	120

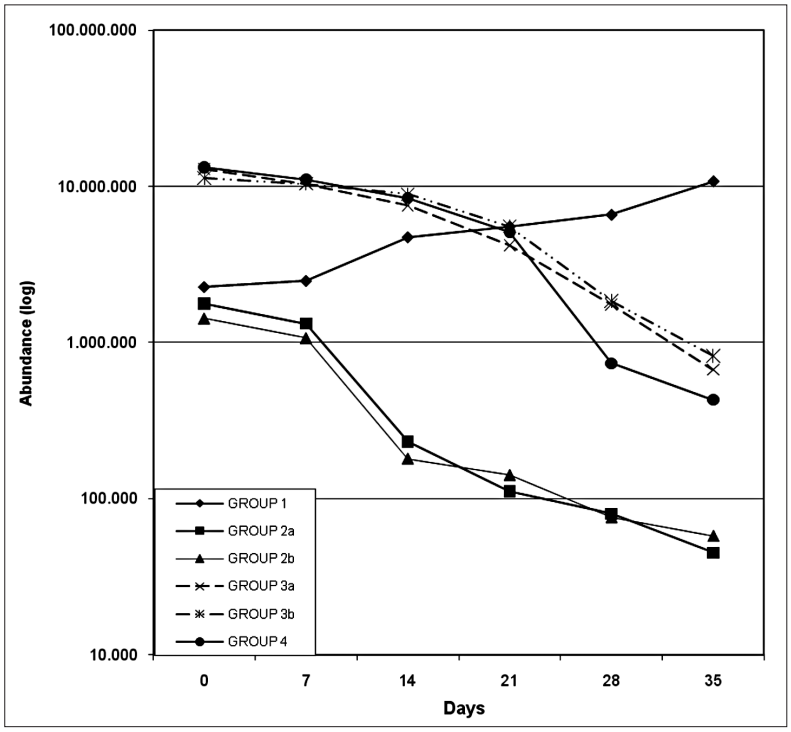

Figure I – Mean abundance of spore per bee (*arb*) recorded weekly during a period of 35 days. *Y* axis is represented in logarithmic scale.

Table 3 – Mean abundance of speres (*arb*) and Fumagillin effectiveness of the groups obtained at 0 and 35 days from the beginning of the treatment.

Group	Initial mean abundance (*arb* ±SD)	Final mean abundance (*arb* ±SD)	Effectiveness (%) ± SD
Control (1)	1 976 625 ± 559 933 [a]	10 707 500 ± 2 188 331 [a]	-
2a	1 768 750 ± 573 384 [a]	45 000 ± 18 028 [b]	97.46 ± 0.89
2b	1 432 500 ± 235 160 [a]	57 500 ± 25 355 [b]	95.96 ± 1,67
3a	12 938 000 ± 1 434 727 [b]	669 375 ± 409.394 [c]	94.93 ± 2.80
3b	11 336 875 ± 4 532 981 [b]	820 000 ± 424 845 [c]	91.74 ± 5.36
4	13 233 125 ± 2 079 411 [b]	428 750 ± 119 006 [c]	96.63 ± 1.33

Different letters (a, b, c) indicate significant differences.

treatment [Kruskal-Wallis p-value (bilateral) 0.510, a = 0.05].

The administration of 102 mg of Fumagillin in 2 or 3 doses did not present significant differences in final *arb* values among the mild degree groups 2a and 2b [Mann-Whitney p-value (bilateral) 0.294 a = 0.05] and between 3a and 3b [Kruskal-Wallis p-value (bilateral) = 0.510; a = 0.05].

The effectiveness of treatments, estimated as the decrease or increase percentage of *prb* values obtained on days 0 and 35 are shown on table 4. Mild grade groups, treated with 102 mg (2a and 2b) did not show significant differences on final *prb* [Mann-Whitney p-value (bilateral) 0.732 a = 0.05), but there was a significant reduction comparing to initial *prb* [Mann-Whitney p-value (unilateral) < 0.0001 a= 0.05]. Groups 3a and 3b with semi-severe spore load, did not show significant differences on initial [Mann-Whitney p-value (bilateral) 0.198 a = 0.05] or final *prb* values [Mann-Whitney p-value (bilateral) 0.694 a = 0.05].

Table 5 shows the initial and final numbers of bee frames. Treated groups with mild intensity of disease (2a and 2b) did not suffer a reduction in their consistence, while groups with semi-severe intensity suffer of a sensible reduction in the adult bee consistence from day 21 onwards (fig. II).

DISCUSSION AND CONCLUSION

The reduction higher than 90% of *N. ceranae* spores upon the totality of the colonies treated with fumagillin appears to be significant regardless of dosage or the initial abundance of spores. However, the fact that all the groups, independently of the dosage and the initial parasitosis degree, showed a certain spore load at the end of the treatment (particularly the semi severe group), could indicate that despite the drastic reduction of the parasite development, the remaining spores will continue multiplying over time. This situation could explain the temporality of the treatment observed by WILLIAMS *et al.* (2008) or the effectiveness loss of the fumagillin as

Table 4 – Mean prevalence of spores (*prb*) and fumagillin effectiveness of the groups obtained at 0 and 35 days from the beginning of the treatment.

Group	Initial prevalence (*prb* ± SD)	Final prevalence (*prb* ± SD)	Effectiveness (%) ± SD
Control (1)	41 ± 12 [a]	97 ± 5 [a]	–
2a	40 ± 13 [a]	11 ± 2 [b]	67.41 ± 15.72
2b	34 ± 16 [a]	10 ± 5 [b]	59.54 ± 45.22
3a	72 ±18 [b]	81 ± 21 [c]	-14.11 ± 20.47
3b	98 ±7 [b]	81 ± 12 [c]	17.33 ± 9.96
4	96 ± 9 [b]	88 ± 17 [c]	6.31 ± 23.87

Different letters (a, b, c) indicate significant differences.

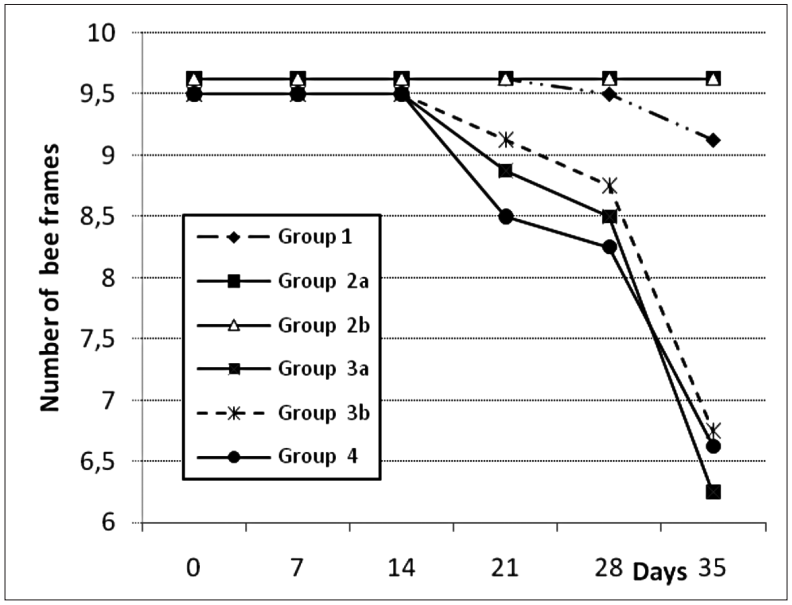

Figure II – Mean number of bee frames per colony of each group recorded weekly during a period of 35 days.

Table 5 – Mean values of number of bee frames per hive and related reduction percentage at the beginning and at the end of the treatment.

Group	Initial number of bee frames (average) ±SD	Final number of bee frames (average) ±SD	Decrease (%)
Control (1)	9.63 ±0.74 [a]	9.13±0.64 [a]	5.2
2a	9.63±0.52 [a]	9.63±0.52 [a]	0
2b	9.75±0.46 [a]	9.75±0.46 [a]	0
3a	9.5±0.53 [a]	6.25±1.16 [b]	34.2
3b	9.38±0.74 [a]	6.75±1.39 [b]	28.0
4	9.5±0.76 [a]	6.63±1.06 [b]	30.2

Different letters(a, b, c) indicate significant differences.

proposed in other investigations (COLOSS Workshop, 2009).

Anyway, based on our results, the experimental administration of 102 mg antibiotic is the most efficient amount of active ingredient in controlling this parasitosis after 35 days from the beginning of the treatment. On the other hand, if the administration of 102 mg on colonies with mild degree of parasitosis does not eliminate totally the parasite, causes reductions of spore load (*arb*) at levels harmless to the colonies. This temporality is essential to control the parasitosis in production apiaries, because if the Autumnal treatment is applied on mild level colonies, the lower spores load remaining probably will not generate the necessary number of parasites to affect adversely the colonies during Spring. Thus, spring treatments could be avoided, as suggested by BOTÍAS *et al.* (2009) for a rational use of this drug, allowing the natural colonies restoration during summer as observed by PICKARD and EL-SHEMY (1989) with *N. apis*.

The absence of differences in the abundance of spores (*arb*) (Tab. 3) relative to the different ways of application tested, could indicate the achievement of a higher efficiency

with two doses (51 mg fumagillin), regardless of the initial spores load, but related only to colonies with mild level of Nosemosis. Infact, if final prevalence of spores (*prb*) (Tabs 4 and 5) is considered as a parameter of evaluation, we can conclude that administering that dosage on colonies with semi-severe loads will not reduce the number of parasitized individuals with the subsequent bee population loss. Hence, the results of this investigation lead to conclude that the maximum efficiency of fumagillin is obtained treating the colony when parasitosis abundance does not exceed the mild degree (asymptomatic) in two applications of 51 mg. The Autumn treatment of colonies with semi severe grade has determined a significant reduction in *arb* values. However, it is necessary to consider the effect caused by the mass mortality of highly parasitized adult bees recorded 21 days after treatment (fig. I) as proved also by the reduction of bee frames (fig. II). The high prevalence of spores (*prb*) of those experimental groups which had bee loss, indicates that under these conditions, the information provided by this alternative estimator is better than the one given by the *arb* for assessing the parasitosis status. Infact, by this parameter we can show that colonies with semi-severe degree of Nosemosis are still exposed to serious risks beyond the apparent effect of the treatment on the reduction of spore load recorded in the bees, and the treatment is not able to prevent damages of the parasitosis, even temporarily.

Finally, based on the above results, it could be postulated that preventive evaluation of the parasitosis degree on returning bees is a valid and necessary tool in the pharmacological control of Nosemosis type C using fumagillin. The control efficiency is more related to the parasite load at the beginning of the treatment rather than the active ingredient amount or the way of administration.

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