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Effect of oxalic acid on *Nosema ceranae* infection



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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. $\times 10^6$; untreated: 30.64 ± 0.31 s.e. $\times 10^6$). When administered in autumn to free flying colonies twice, 3 weeks apart, the infection prevalence decreased in young (relative reduction of $53.8\% \pm 6.5$ s.e.) and old bees (relative reduction of $44.4\% \pm 6.0$ s.e.). Meanwhile increased prevalence in all the controls was detected (young and old bees: relative increase of $45.7\% \pm 22.8$ s.e. and $10.2\% \pm 5.9$ 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.

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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 The bees do not show a tendency to feed on the liquid [unpublished observations], but pharmacokinetic studies revealed prompt absorption by the bees and distribution amongst the adult individuals of the colony after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; Hedtker 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.

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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 mm²; 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 anaesthesia with CO₂ 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

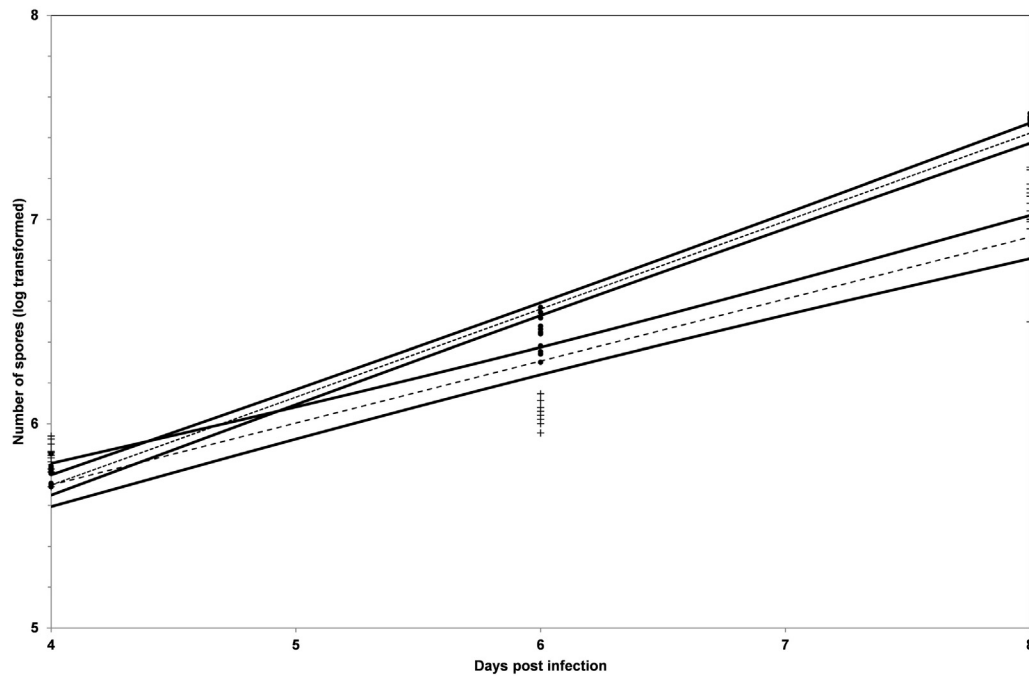


Fig. 1. Simple linear regression of the transformed spore counts measured in the artificially infected groups during the sampling period: regression lines (dotted: untreated; dashed: treated), individual data (dots: untreated; crosses: treated) and their 95% confidence intervals.

The relative change of prevalence ($100 \times [\text{PRE} - \text{POST}] / \text{PRE}$) in the three-week treatment interval was calculated for external and internal samples, and used as an indicator of the treatment efficacy in the field trial.

Small sample size suggested the use of Fisher's exact test instead of squared Chi to compare frequencies.

3. Results

3.1. Laboratory trial with artificial infections

N. ceranae infections were detected in all samples, except in the ones belonging to Group III. This confirms the lack of detectable infections in the colonies providing the samples and the reliability of the laboratory procedures. The average number of spores was respectively higher in group I at 4 d p.i. and in Group II at 6 and 8 d p.i. (Table 1).

In both groups, the spore number increased by one and two orders of magnitude respectively in the first two sampling days (4–6 d p.i.) and in the overall four-day sampling period (4–8 d p.i.). The exponential-like trend suggested the transformation of the original infection data into logarithms, to allow for the adoption of a linear model approach in the statistical analysis. The analysis of variance for the regression lines separately drawn in Fig. 1 shows fit to the transformed data of Group I ($F(1, 43) = 222.9$, $p = 0.000$; adjusted squared $R = 0.835$) and Group II ($F(1, 43) = 2045.5$, $p = 0.000$; adjusted squared $R = 0.979$). Analysis of covariance was conducted to compare the two regression lines (Snedecor

and Cochran, 1967), showing results that were significantly different in slope and elevation (Table 2). This indicates more rapid prevalence increase in non-treated control cages in comparison to the treated ones, as shown also by the functions corresponding to the untransformed data:

- Group I: spore number = $30,576 \times e^{(0.699 \times \text{d p.i.})}$,
- Group II: spore number = $9474 \times e^{(0.992 \times \text{d p.i.})}$.

3.2. Field trial with natural infections

Fig. 2 shows the average prevalence after PCR analysis in each group of colonies ($n = 5$). A Mann–Whitney test was used to test whether the infection was homogeneous at the pre-treatment check of the two groups. Overall, the prevalence in samples collected at the hive entrance (old bees) was $85.5\% \pm 2.23$ s.e. ($U(5, 5) = 8.0$, $Z = 0.940$, $p = 0.337$) and 29.5 ± 5.60 s.e. in the young bees from the brood nest ($U(5, 5) = 12.0$, $Z = -0.104$, $p = 0.917$). The lack of evident between-group differences of pre-treatment prevalence validates the correct randomisation of the experiment. A Wilcoxon test for paired samples showed higher prevalence in external pre-treatment samples in comparison to the internal ones ($T(10) = 0.000$, $Z = 2.803$, $p = 0.005$).

In the untreated colonies, a Wilcoxon test evidenced a prevalence difference between pre and post treatment samples both in internal ($T(5) = 0.000$, $Z = 1.604$, $p = 0.109$) and in external bees, ($T(5) =$

Table 2

Test for slope and elevation homogeneity. The regression lines of log transformed spore counts of the laboratory experiment are compared (analysis of covariance from Statistica 7, elaboration according to Snedecor and Cochran, 1967).

	Slopes $\pm$ s.e.	C.I. (95%)	d.f.	S.S.	M.S.	F	p
Within control group	0.431 ± 0.010	0.412 ± 0.450	43	0.468	0.011		
Within treated group	0.304 ± 0.020	0.263 ± 0.345	43	2.135	0.050		
Regression residual			86	2.603	0.030		
Pooled slope	0.367 ± 0.015	0.337 ± 0.398	88	3.573	0.041		
Line parallelism			1	0.970	0.970	32.02	0.000
Common regression			88	5.027	0.057		
Line elevation			1	1.453	1.453	35.34	0.000

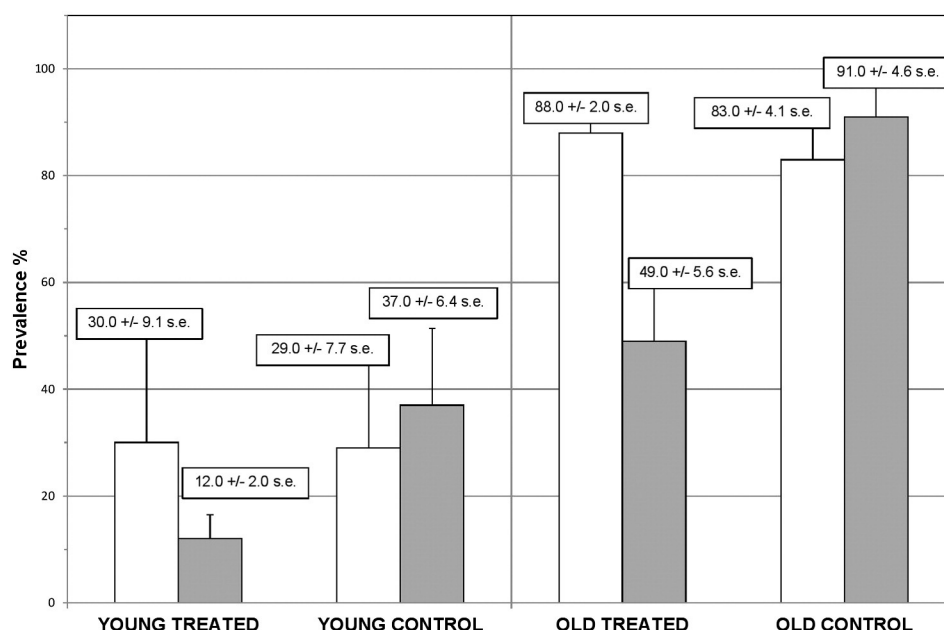


Fig. 2. Prevalence of infected bees in the honey bee samples: average (white: pre-treatment; grey: post-treatment) and standard deviation (pins).

0.000, $Z = 1.826$, $p = 0.068$). However, it should be noted that the observed increases are supported by probability coefficients slightly higher than the conventional threshold $\alpha = 0.05$.

Conversely, in the treated group the prevalence of infected bees decreased both in samples from the hive entrance ($T(5) = 0.000$, $Z = 2.023$, $p = 0.043$) and in the ones from the brood nest ($T(5) = 0.000$, $Z = 2.023$, $p = 0.043$).

The relative change of prevalence in the three weeks of treatment was compared with a Mann–Whitney test (Fig. 3). This was negative in all samples from the treated colonies and positive or null in the controls, both in old ($U(5, 5) = 0.0$, $Z = -2.61$, $p = 0.009$) and young bee samples ($U(5, 5) = 0.0$, $Z = -2.61$, $p = 0.008$).

The colony checks made in February following the treatment aimed at evidencing whether the treatments improved the health condition in comparison to the control administrations. As shown in Fig. 4, whereas the control group lost three out of five colonies, all the treated ones survived the winter, (one-tailed Fisher's exact test: $p = 0.083$). However, the bad conditions of the surviving colonies made further recovery unlikely in the next spring.

4. Discussion

As reported, therapeutic OA solutions are hardly ingested by the honey bees. Although this is likely to play little influence on the effect against the *Varroa* mite, it should be noted that *N. ceranae* is a ventricular parasite that must be reached once established in its target organ. Palatability is then important if the honey bee ventriculum has to be reached, and this is why this study was conducted by using relatively low concentrations, that preliminary observations proved acceptable for the bees.

Based on polyethism, bees taken from the hive entrance were considered as representative for the older population of the colony, while bees from the brood nest represented the young population.

In agreement with Higes et al., 2008 the infection prevalence of untreated colonies was 2.9 times higher in old than in young bees as a consequence of higher chances of contact with infected substrates and of protracted infection growth. This stresses the need to sample old bees to spot subacute infections, and individuals of both ages for quantitative studies (Chen et al., 2012; Meana et al., 2010).

In November, the prevalence of infected young bees sampled from the untreated controls averaged 37%. All of them failed to overwinter correctly (Fig. 5). The reported value is very close to the limit mentioned by Higes et al., 2008, who observed detrimental effects on colonies when the samples of young bees contained 40% of infected individuals or more. Similar conclusions were substantiated by other authors reporting decreased lifespan in *N. ceranae* infected bees (Dussaubat et al., 2013; Goblirsch et al., 2013).

In the pre–post-treatment period, the proportion of infected bees increased both in old (10.2%) and in young bees (45.7%) of untreated colonies, but decreased in the treated group (44.4% and 53.8% respectively). All the colonies receiving oxalic acid survived the winter in fair and uniform condition as a likely consequence of the treatment efficacy.

In the laboratory oxalic acid ingestion hindered the development of *N. ceranae* infections compared to untreated controls, confirming the results obtained in the field. Nonetheless it must be considered that 0.25 M concentration was selected based on practical reasons rather than on the infection inhibition. Actually, full control could not be attained and this makes it advisable to investigate further to optimise concentration and use in the field. Coformulants different from sucrose should be tested as well.

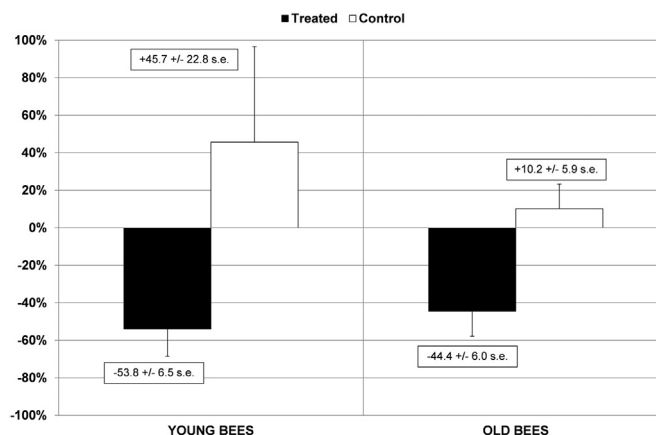


Fig. 3. Relative change of prevalence measured in the pre–post-treatment period: average (white: untreated; black: treated) and standard deviation (pins).

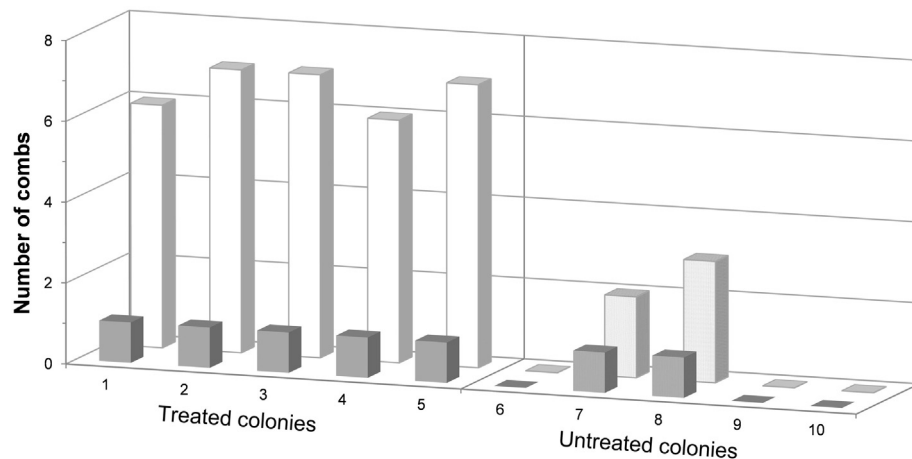


Fig. 4. Number of combs with bees (background columns) and brood (foreground) at the post-winter check.

The mode of action of oxalic acid against *N. ceranae* is still unknown and should be elucidated by taking into consideration the eminent chemical characteristics of the substance. In effect, the high dissociation constant and the aptitude to chelating metallic cations might change the chemical environment of the ventriculum, impairing the intraluminal cycle of the parasite. Our results make realistic the future availability of double action medicaments, controlling both *N. ceranae* and *V. destructor*.

The fact that oxalic acid is unevenly used to treat *Varroa* infestations in different regions might explain conflicting records on the importance of *N. ceranae* on honey bee health (Dainat et al., 2012; Gisder et al., 2010; Hatjina et al., 2011; Huang et al., 2012; Martínez et al., 2012; Martín-Hernández et al., 2012).

Competing interests

The authors declare that they have no competing interests.

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Fig. 5. Conditions of the untreated colonies that were found dead (above) and weak (below) at the post-winter check.

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