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AN ENVIRONMENTALLY FRIENDLY APPROACH TO THE CONTROL OF *VARROA DESTRUCTOR* MITE AND *NOSEMA CERANAE* DISEASE IN CARNIOLAN HONEYBEE (*APIS MELLIFERA CARNICA*) COLONIES

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Abstract - This paper presents data following the periodic checking of fallen mites *Varroa destructor* to determine the mite fall, and the presence and number of *Nosema ceranae* spores in samples of bees, determined by counting prior to and after BeeCleanse treatments. Because of the possibility of chemical resistance development, the variable efficiencies of current varroosis and nosemosis treatments and consequent contamination of honeybee products create a need for alternative treatment methods and the use of natural phytopharmacological preparations. BeeCleanse is a natural preparation containing different herbal, vitamin, mineral and essential oil recipes. The aims of the study were to establish the dynamics of the fallen mites and determine *Nosema* spores before and after treating honeybee colonies in order to establish the effectiveness of BeeCleanse for the control of varroosis and nosema disease in colonies during the brood season in order to reduce parasite populations to tolerable levels. In addition, the strength (number of populated and brood frames) of treated and untreated honeybee colonies was checked during the clinical examination in field conditions.

Key words: Varroa destructor, *Nosema ceranae*, honeybee, alternative control, BeeCleanse

INTRODUCTION

Varroosis is a disease of honeybee colonies (*Apis mellifera* L., 1758) caused by the haemophagous mite *Varroa destructor* (Anderson and Trueman, 2000). Nosemosis is a disease of adult honeybees caused by two described species of the microsporidia, *Nosema apis* (Zander, 1909) and *N. ceranae* (Fries, Feng Feng, da Silva, Slemenda and Pieniazek, 1996). The obligate ectoparasitic mite *V. destructor,* as well as the microsporidium *N. ceranae*, were originally confined to the Eastern honeybee *Apis cerana* F., 1793, and dispersed worldwide. At present, it is very hard to find a honeybee colony free of mites (Martin, 2001). Thus, varroosis is considered a major threat for apiculture and needs to be controlled because untreated colonies typically die within a few years due to damage to both pupae and adult bees, as well as secondary virus infections (Elzen et al., 2000). Varroa feed on the hemolymph of immature and adult bees during their reproductive and phoretic life stages, respectively. A high rate of mite infestation in a honeybee colony and poor colony management have an important influence on beekeeping because damage to the host caused by varroosis results in decreasing numbers of honeybee colonies, so that it is necessary to evaluate

chemical and biological methods of treatments. In general, regular treatment of honeybee colonies with acaricides allows productivity to be maintained. The control of this mite is obtained by the use of several acaricides. Little has been reported on the side effects of these treatments on honeybees (Haarmann et al., 2002; Gregorc et al., 2007). Continuous use of acaricides of the same generic group can result in the development of resistance and reduce their effectiveness against the mite. Infections with *N. ceranae* induce a nutritional stress, suppression of the bee's immune functions, and cause changes in behavior whereby infected bees tend to forage at cooler temperatures (Mayak, 2009). Bees infected with this "new parasitic pathogen" starve to death due to the lack of digestive function. This leads to an increased number of honeybee colony losses, the destruction of plant communities and low production in the same areas, which consequently cause a significant loss of beekeeper's income (Stefanidou et al., 2003).

Because of the possibility for chemical resistance development (Floris et al., 2001; Spreafico et al., 2001), the variable efficiencies of current varroa and nosema treatments and consequent contamination of honeybee products (Wallner, 1999) create a need for alternative treatment methods using natural phytopharmacological preparations. Also, the effects of residues and their by-products in honey and wax (Wallner, 1999) present environmental concerns and are another reason for reducing the use of conventional chemical mite- and parasite-control methods in bee-keeping (EU 3/01/081). During the search for naturally occurring environmentally friendly acaricides, organic acids (Milani, 1999), plant essential oils and their derivates have been investigated as possible controls for varroa (Calderone et al., 1997; Mutinelli et al., 1997; Gregorc and Poklukar, 2003) and nosema spore (Tlak Gajger et al., 2009a,b) levels in treated honeybee colonies. Listed natural preparations do not accumulate in wax and their residue build-up in honey and other bee products is limited and toxicologically insignificant (Imdorf et al., 1996). Many tested plant oils and their derivates have been shown to dislodge *V. destructor* in field studies (Sammataro, 1998; Colin, 1990; Harborne et al., 1991; Imdorf et al., 1995). The advantage of plant oils is that they contain structurally diverse compounds, including monoterpenoids (McUurry, 2000). They were developed by plants as defenses against insects as natural insecticides. Improvements for parasiticide activity are hydrophilic monoterpenoid alcohols, phenols and their lipophilic derivatives, which is a possible hindrance of penetration of the mite cuticular layers (Tsao et al., 1995).

BeeCleanse is natural phytopharmacological preparation compound made up of different herbal, vitamin, mineral and essential oil recipes traditionally used in beekeeping in North America. The producer recommendation is for its use against most of the known bee diseases and for better honeybee colony vigor. The most common method for assessing the level of mite infestation that is not destructive to bees entails counting the mites that drop from a colony onto a bottom board and it presents a reliable diagnostic method to evaluate the efficacy of an acaricide treatment (Ritter, 1981; Fries et al., 1991; Poklukar, 1999).

The aims of the study were to establish the dynamics of the fallen mites after treating honeybee colonies and to establish the effectiveness of BeeCleanse for the control of varroa in colonies during the brood season in order to reduce varroa populations to tolerable levels. This study presents data from the periodic checking of the number of mites that had fallen onto the bottom of hives in the pre-treatment periods and after treatments. In our field experiment, conducted on honeybee colonies, the acaricidal efficacy and the dynamics of mite mortality after base recipe BeeCleanse treatments, with five different strengths (100%, 95%, 90%, 85% and 80% water solution) in each one, were evaluated and compared.

MATERIALS AND METHODS

Twenty-four *Apis mellifera* honeybee colonies, populated in standard LR hives with ten combs (41 × 26 cm) in each brood chamber and honey super, were located at one site in the continental part of Croatia, which has a mild Mediterranean climate. At the end

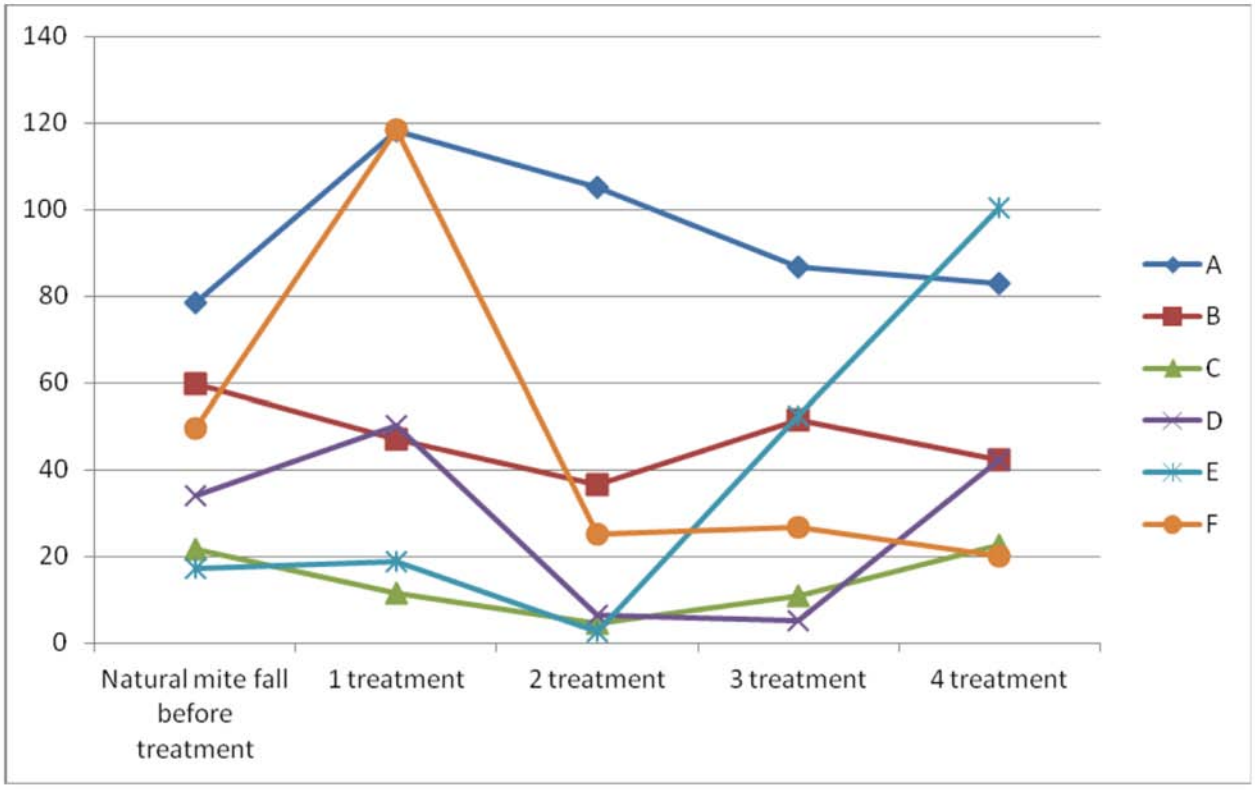

Fig. 1. *Varroa destructor* mite fall before and after BeeCleanse treatment.

of spring of 2010, paper sheets were placed on the floors of each of the hives in order to record the hives' natural mite mortality. Wire screens above the sheets prevented the bees from coming into contact with the debris. The number of mites was recorded on five occasions in the pre-treatment period, and then recorded regularly once a day for 16 days after treatments began. The treatments were performed four times with four days apart using the "drench method" according the manufacturer's instructions (four teaspoons of BeeCleanse mixed with 1 L sugar syrup in proportion 1:1): the treatments were applied to the experimental colonies by trickling the prepared syrup over the combs *in situ* with 0.25 L of prepared phytopharmacological preparation, respectively. The control group of honeybee colonies was treated with sugar powder (Aliano and Ellis, 2007). Each frame covered with bees was taken out of the hive and directly dusted over the bees. 200 g of powdered sugar (Franck, Croatia) were used per hive. The mite drop during the treatment period was recorded after each application.

Every time before the next treatment we took 60 bees per colony from the hive entrance and examined them under a microscope for the presence of *Nosema* spores. The number of spores were determined by counting in a Bürker-Türk hemocytometer (Cantwell, 1970). Determination of *Nosema* species (extraction of genomic DNA and further molecular analysis) was performed as follows: for each of the selected suspensions of isolated *Nosema* spores, an aliquot of 50 µl was transferred to a fresh tube, boiled at 100°C for 30 min and centrifuged at 14 000 x g for 10 min. 30 µl of supernatant was removed and supplemented with 10x TE buffer to a final concentration of 10 mM Tris and 5 mM EDTA, pH 8. This supernatant served as a source of template DNA and was

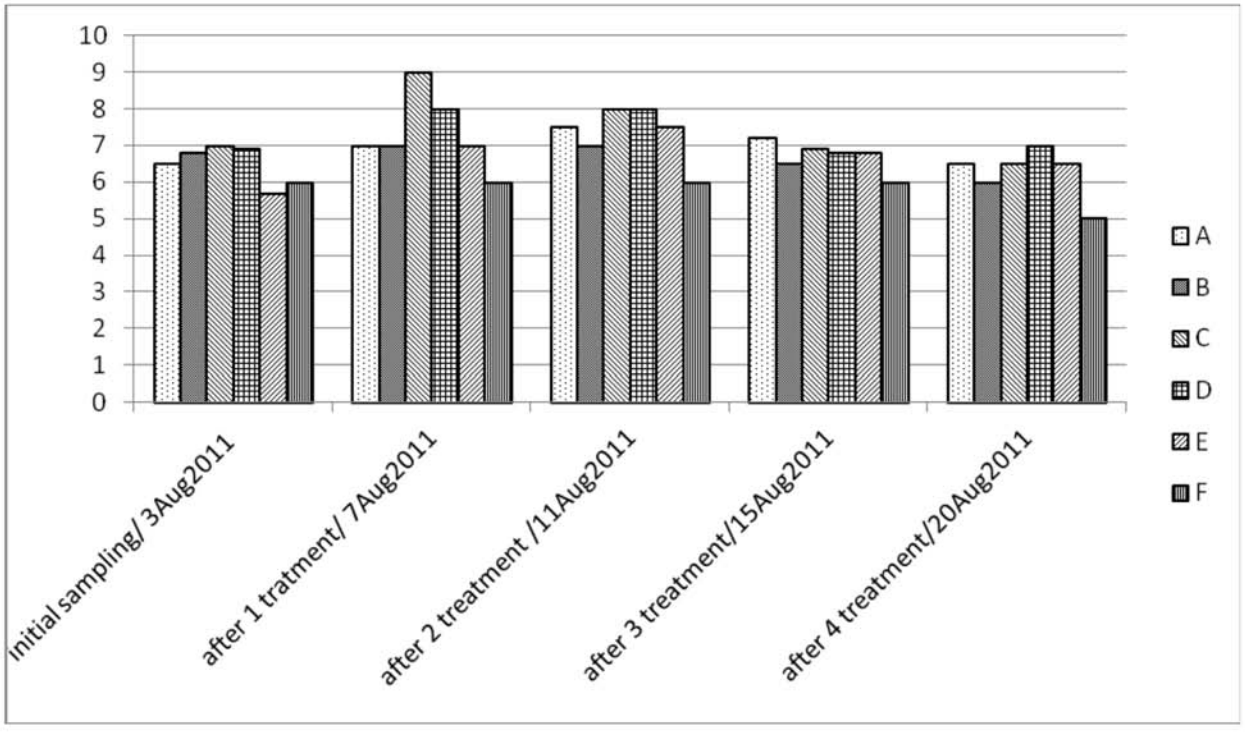

Fig. 2. Strength of honeybee colonies treated with BeeCleanse (number of frames populated with bees and containing honeybee brood).

stored at -20°C, or used immediately for multiplex polymerase chain reaction. Primers used for specific amplification of *N. apis* DNA were 321APIS-FOR (5'-GGGGGCATGTCTTTGACGTACTATGTA-3') and 321APIS-REV (5'GGGGGGCGTTTAAAAT-GTGAAACAACTATG-3') and the expected size of amplicon was 321 bp. Primers for *N. ceranae* were 218MITOC-FOR (5'CGGCGACGATGTGA-TATGAAA-ATATTAA-3') and 218MIT OC-REV (5'-CCCGGTCATTCTCAAACAAAA-AACCG-3'). The expected size of the amplicon was 218-219 bp. Primers were selected taking into account that primer sequences were specific to each of the two species, and that both amplicons could be simultaneously amplified and separated using agarose gel electrophoresis for visualization of results. The PCR conditions the manufacturer of Taq polymerase instructions (Sigma, USA). The molecular size of PCR products were determined by electrophoresis in a 2% agarose TAE (Tris-acetate ethylene diamine tetra-acetic acid) gel in standard TAE buffer, stained with SYBR green, and visualized using the UviTec gel documentation system.

The efficacy of the BeeCleanse and sugar powder treatments, expressed as percent mite mortality, was established after each treatment, and the mean mite drop values of the treated groups were compared. The efficacy of the treatments was also estimated by comparing the numbers of mites that fell before and after the treatments between experimental and control groups. The data analyses were performed by ANOVA (analysis of variance) with the use of the Statistica Data Miner v.7 (Stat Soft, 2004).

RESULTS

During the pre-treatment periods, between June 30 and August 3, 2011, the average daily natural mite

Table 1. *Varroa destructor* mite fall before and after 1, 2, 3 and 4 treatment with BeeCleanse in consecutive day periods.

		Natural daily mite fall before treatments	*Varroa destructor* mite fall after BeeCleanse treatment in consecutive day periods			
			1 treatment	2 treatment	3 treatment	4 treatment
A	mean	78.33	118.16	105.00	86.83	82.83
	SD	22.07	24.23	15.83	11.25	14.40
	range	50-109	88-154	89-134	76-106	62-100
B	mean	59.60	47.00	36.40	51.40	42.40
	SD	24.30	27.69	14.18	25.84	13.04
	range	26-88	16-83	16-53	25-84	33-65
C	mean	21.60	11.40	4.40	10.80	22.60
	SD	14.72	7.40	4.39	5.26	11.84
	range	3-43	4-22	1-12	5-19	7-38
D	mean	34.00	50.25	6.25	5.00	42.25
	SD	22.70	27.42	2.21	2.16	27.89
	range	2-54	26-74	4-9	3-8	15-81
E	mean	17.25	18.75	2.50	52.50	100.25
	SD	2.75	7.36	3.31	49.40	29.71
	range	14-20	10-28	0-7	3-113	56-120
F	mean	49.50	118.50	25.25	26.75	20.00
	SD	14.61	94.77	9.17	5.73	10.16
	range	30-65	15-224	17-37	20-32	12.34

A – E = base recipe BeeCleanse like five different strenth water solutions in each one.
F = control group, treatment with powder sugar as a biological way of varroa supression.

drop was estimated to be 91.50 (± 28.20). Mite mortality after BeeCleanse application in the period from 4-19 August 2011, was higher (P <0.05) compared to natural mite mortality in the pre-treatment period. The total number of fallen mites during the experiment was significantly (P >0.05) different between the treated A, B, C and D experimental groups. Relative mite fall is shown in Table 1 and 2, and Fig. 1.

Results regarding the efficiency of honeybee colonies treating against *N. ceranae* with BeeCleanse are shown in Table 3 and Fig. 2. The results of PCR amplification with a generic *Nosema* primer pair perfectly matched the results of amplification with a specific *N. ceranae* primer pair.

DISCUSSION

It is essential to ensure standard honeybee colony development during the brood season, which is important for successful overwintering. Because of this, varroa control during spring and summer is important and treating colonies is a necessity. Alternative treatments have gained more acceptance in recent years as they are perceived to be more natural in origin, cheaper and less harmful to environment (Mutinelli et al., 1997; Howis and Nowakowski, 2009; Skubida and Semkiw, 2009), but less effective. The number of fallen mites from our experimental colonies during the present study indicates a moderate level of overall infestation, thus allowing the use of less effective control treatments in the active season. In addition, the increased resistance of mites to synthetic active ingredients has been observed and the improper use of acaricides could result in increased mortality in honeybee colonies (Spreafico et al., 2001). In this study, the relative mite mortality in the period between 4 and 19 August, 2011, during the brood period as a result of BeeCleanse treatment in colonies of Group

Table 2. *Varroa destructor* total mite fall before and after BeeCleanse treatments from July 30 to August 19, 2011.

Group of colonies	Total natural mite fall before treatments (July 30 to August 3)	Total mite fall after treatments (1+2+3+4) (August 4 - 19)
A (100%)	470	2436
B (95%)	298	846
C (90%)	108	227
D (85%)	136	341
E (80%)	69	836
F	198	762

Table 3. Spore counts of *Nosema ceranae* (per 0.04 mm) before and after multiple treating of honeybee colonies with BeeCleanse.

Field testing of BeeCleanse efficiency against *Nosema ceranae*		Initial sampling (3.8.2011.)	Days after first treating			
			4. (7.8.2011.)	8. (11.8.2011.)	12. (15.8.2011.)	16. (19.8.2011.)
A	Mean value	37.61	30.85	28.15	20.20	15.86
	SD	9.08	7.89	9.06	10.77	1.31
	range	27.93-50.37	19.81-40.18	14.18-38.87	3.75-31.12	14.25-17.87
B	Mean value	12.94	7.02	23.74	11.64	6.78
	SD	1.95	2.00	4.27	1.06	2.64
	range	11.31-16.25	5.18-10.18	18.93-29.37	10.56-13.12	4.87-11.43
C	Mean value	79.24	24.95	20.87	11.72	10.45
	SD	18.39	20.04	3.29	1.88	4.04
	range	67.31-100.43	3.56-43.31	18.93-24.68	10.31-13.87	5.87-13.50
D	Mean value	43.43	16.43	21.62	9.87	9.03
	SD	17.23	4.59	6.45	7.33	3.93
	range	31.25-55.62	13.18-19.68	11.37-20.50	4.68-15.06	6.25-11.81
E	Mean value	52.93	14.02	15.49	13.46	9.03
	SD	2.02	7.20	6.18	4.81	2.78
	range	51.50-54.37	8.93-19.12	11.12-19.87	10.06-16.87	7.06-11.00
F	Mean value	25.74	62.03	77.28	52.06	56.68
	SD	20.32	31.77	36.72	43.48	49.49
	range	11.37-40.12	39.56-84.50	51.31-103.25	21.31-82.81	21.68-91.68

A ranged from 66.29% to 90.21% with a mean of 78.17% in comparison with pre-treating period. Results for Group B showed a total fall for 35.22%, and Group C, 47.57%. Group D, after the last treatment, showed an increase of 80.47% of mite fall in comparison with natural mite fall, and a total efficacy of 39.88%. In Group E, an increase of 92% mite fall after the first treatment was achieved compared with the pre-treatment period, and total efficacy was 8.25%. The control group of honeybee colonies was treated with powdered sugar as a biological approach to suppress varroosis (Aliano and Ellis, 2005). Our results show an increase in mite presence after the first treatment (41.77%); the total efficacy was 25.98%. Because of the very variable results of treating presented are similar to some other alternative options (Chuda-Mickiewicz et al., 2007; Sas et al., 2008). We assume that it is necessary to provide multiple treatments of honeybee colonies during the whole year, or to use some other "green" methods in combination for varroosis suppression. The efficacy of the BeeCleanse treatments that we

applied is underestimated because mite reproduction within the honeybee colonies and mite reinvasion from both control and neighboring colonies were not taken into account. Further experiments should be conducted in order to establish how to increase the efficacy of mite control in highly infested colonies during the period of brood rearing under continental climatic conditions.

Nosemosis is a parasitic disease affecting adult bees. Due to its inconspicuous signs and the need for eradication by the interchange of frames with broods in a disinfected hive, beekeepers apply insufficient attention or often neglect the disease. Because of EU normative regulations that prohibit the use of antibiotics for the curing of apian diseases, it appears to be necessary to introduce herbal preparations into the treatment of nosemosis. This experiment was designed to test the effectiveness of repeated treatments with BeeCleanse phytopharmacological preparation to control nosema disease in field conditions as well. Results demonstrated that the disease was not cured, but a considerable reduction in spore number was achieved, especially in the colonies of Group A that were treated with pure BeeCleanse preparations: 91.24% after the first treatment; 71.75% after the second treatment and 78.51% after the penultimate treatment if reduction is calculated in relation with the result achieved in the previous treatment; and 82%; 74.84%; 53.70%; and 42.16% after each treatment in relation to results of the initial sampling time, respectively. In Group B colonies, 89.95% of spore reduction was determined after the third treatment. For all other groups of colonies (C, D, E), spore reduction ranged from 17.06% to 49.78%; and in the control groups increases in spore count from 33.30% after second treatment to 49.44% after the penultimate treatment were observed. Based on the fact that the number of *N. ceranae* spores was considerably reduced after multiple treatments with BeeCleanse, we believe that the preparation has potential for mass use and deserves further studies.

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