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ORIGINAL RESEARCH ARTICLE

Long term effects of a food supplement HiveAlive™ on honey bee colony strength and *Nosema ceranae* spore counts

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The long term effect of HiveAlive™, a commercial food supplement, was evaluated with respect to colony population size and *Nosema ceranae* spore loads. The supplement was administered in sugar syrup at a dose of 2.5 ml per liter of syrup to up to 20 colonies per group for a period of 2 years. Its use before and after winter increased a colony's worker population size by 89% and reduced *Nosema ceranae* spores by 57% compared to control colonies. These results represent the first-known hive manipulation to have a lasting effect on nosema spore levels. The positive effect of HiveAlive™ may have been a result of its continued use over a two-year period.

Efectos a largo plazo del suplemento alimenticio HiveAlive™ sobre la fuerza de las colonias de abejas y el recuentos de esporas de *Nosema ceranae*

El efecto a largo plazo de HiveAlive™, un suplemento alimenticio comercial, se evaluó con respecto al tamaño de la población de la colonia y la carga de esporas de *Nosema ceranae*. El suplemento se administró en jarabe de azúcar a una dosis de 2,5 ml por litro de jarabe hasta 20 colonias por grupo en un período de dos años. Su uso antes y después del invierno aumentó el tamaño de la población de obreras de una colonia hasta en un 89% y redujo las esporas de *Nosema ceranae* en un 57% en comparación con las colonias de control. Estos resultados representan la primera manipulación de las colmenas con un efecto duradero sobre los niveles de esporas de *Nosema*. El efecto positivo de HiveAlive™ puede haber sido resultado de su uso continuado durante un período de dos años.

Keywords: HiveAlive™; feeding; food supplement; colony strength; *Nosema ceranae*; infestation; beekeeping; disease; population; natural; treatment

Introduction

The honey bee *Apis mellifera* L. was formerly considered to be parasitized by just one microsporidium *Nosema apis,* but in 1994 a second, *Nosema ceranae*, was detected in the Asiatic honey bee*, Apis cerana* (Fries, Feng, Da Silva, Slemenda, & Pieniazek, 1996), and since 1994, infection of *A. mellifera* by *N. ceranae* has been found to be spread worldwide (Chen, Evans, Smith, & Pettis, 2008; Higes, Martín-Hernandez, & Meana, 2006; Klee et al., 2007; Liu et al., 2008; Paxton, Klee, Korpela, & Fries, 2007). It is known that *N. apis* affects the epithelial cells lining the midgut of the adult bees (Bailey, 1955) and shortens the life span of both queens and adult bees (Wang & Moeller, 1970; Webster, 1994). Recent studies (Huang, Solter, Aronstein, & Huang, 2015; Natsopoulou, Doublet, & Paxton, 2015) suggest that *N. ceranae* is not more virulent than *N. apis,* in disagreement with older studies which suggested that it was associating it with colony depopulation and collapse (Hatjina et al., 2010; Higes et al., 2008; Paxton, 2010; Paxton et al., 2007) as well as colony losses in general, in particular in southern European countries (Botías, Martín-Hernández, Barrios, Meana, & Higes, 2013; Dussaubat et al., 2013; Globlirsch, Huang, & Spivak, 2013; Hatjina et al., 2011; Higes, Martín-Hernandez, & Meana, 2010, 2006; Higes et al., 2005, 2008; Soroker et al., 2011; Villa, Bourgeois, & Danka, 2013).

It has also been demonstrated that sublethal doses of pesticides highly increase the mortality of honey bees that have been infected by *N. ceranae,* suggesting that *N. ceranae* may weaken honey bees, or may increase the susceptibility of honey bees to stressors (Alaux et al., 2010; Aufauvre et al., 2012; Doublet, Labarussias, de Miranda, Moritz, & Paxton, 2015; Vidau et al., 2011).

As the above-mentioned effects have a negative direct and indirect influence on beekeepers' income (e.g., loss of animal capital and honey production, respectively), current work in this area is focused on the search for strategies, if not solutions, in order to control the disease. The high level of resistance of *N. ceranae* spores to temperature and to desiccation (Fenoy, Rueda, Higes, Martin-Hernandez, & Del Aguila, 2009) increases the severity of the disease for the beekeeping sector. Until recently, the only known effective substance for the control of nosema infection was the antibiotic fumagillin (Moffet, Lackett, & Hitchcock, 1969), but in some cases it has been shown to

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be less effective for *N. ceranae* as it was for *N. apis*, in particular in the long term (Huang, Solter, Yau, & Imai, 2013). Fumagillin has been withdrawn in the European Union for beekeeping use so there is a strong demand for a treatment that will reduce nosema loads on adult bee populations. Until now, no product has been shown to have a positive long term impact on nosema levels.

In the present study, we evaluated the long term effect of a product with the trade name HiveAlive™ (Advance Science Ltd., Ireland) on colony population and *N. ceranae* spore loads. HiveAlive™ uses natural active ingredients, in particular a proprietary blend of seaweed extracts, and is available worldwide. HiveAlive™ is HACCP controlled, its ingredients and process are EU approved, and the ingredients are generally regarded as safe (GRAS) establishing a high level of protection for animals and humans. Seaweeds are now routinely used in animal feeds for weight gain and general health, due to their vitamins, minerals, antioxidants, and bio-active compounds (Brownlee, Fairclough, Hall, & Paxman, 2012; Fleurence, 1999; Holdt & Kraan, 2011; Kovač, Simeunović, Babić, Mišan, & Milovanović, 2013; Mayer & Hamann, 2004; Mayer, Rodriguez, Berlinck, & Hamann, 2007; O'Sullivan et al., 2010). Polysaccharide seaweed extracts have recently shown their potential to prevent or control nosema in honey bees (Roussel et al., 2015). According to the manufacturer, each seaweed species used in HiveAlive™ has been chosen for its specific properties, in particular antifungal, antibacterial, antiviral, and immune stimulatory.

Materials and methods

Experimental design

The study began in November 2012, and was completed in June 2014. All colonies were naturally infected with *N. ceranae* as previously demonstrated in other studies (Hatjina et al., 2010, 2011; Meixner et al., 2014) and were maintained at the apiary site of the Division of Apiculture, Institute of Animal Science, Chalkidiki, Greece for the duration of the experiment. At the start of the study in November 2012, all colonies were equalized to ensure each colony had approximately six frames of bees and sister queens. All colonies were re-queened in spring 2013, and the same colonies were used for both years. Each treatment group was comprised of 10 colonies during the first experimental year (Table 1). For the second year, the two control groups were combined to facilitate a comprehensive study of 20 colonies. Additionally, the two treatment groups that had been fed with the supplement in the first year were also combined to make a treatment group of 20 colonies in the second year (Table 1).

All colonies were evaluated for the number of adult bees and nosema spore levels before the experiment began in November 2012. The same measurements were taken each spring, summer, and autumn over the two years of the study, giving a total of six sampling points, providing a complete picture of spore levels throughout the seasons. The evaluation of colony strength was performed according to the standardized "Liebefeld" method as follows: the adult bee population was evaluated by visually assessing the percentage of the comb surfaces densely covered by bees. Each percentage value was then quantified into an actual bee population based on the fact that each side of the Langstroth frame has 8.8×10 cm^2 surface area and that each 10 cm^2 contains approximately 130 bees (see Costa et al., 2012; Delaplane, van der Steen, & Guzman, 2013). Colonies that did not survive the winter or were too weak to be maintained were given a 0 value.

Table 1. Formation of feeding groups and doses of HiveAlive™ and fumagillin administered during the 1st and the 2nd experimental years.

	1st year				2nd year			
	Colony #	Autumn feeding	Winter feeding		Colony #	Autumn feeding	Winter feeding	Spring feeding
Control Positive Control	1–10 11–20	Syrup Fumidil & syrup	Candy Candy	→	Control Group 1–20	Syrup	Candy	Syrup
Treatment Group 1	21–30	HiveAlive™& syrup	Candy	→	HiveAlive™ Group 21–40	HiveAlive™& Syrup	Candy	HiveAlive™& syrup
Treatment Group 2	31–40	No Feed	HiveAlive™& Candy					
Treatment Group 3	41–50	Trickling HiveAlive™	HiveAlive™& Candy					

Notes: All syrup fed at 2 l per week for 2 weeks (total 4 L).
Candy fed at 6 kg per colony.
HiveAlive™ fed at 2.5 ml per l liter (syrup) or 5 ml per kg (candy).
HiveAlive™ trickled at 0.5 ml in 50 ml, twice per week, for 2 weeks.
Fumidil-B fed at 2.5 g per liter.
#=Number

For the calculation of nosema spore levels, 60 bees were collected from the outer frames of each colony (assumed foragers). Subsequent detection and counting of nosema spores were performed using a modification of the official method described by Cantwell (1970) and Fries et al. (2013) as follows: the abdomens of the 60 bees were macerated with 5-ml distilled water, filtered through a thin mesh and rinsed with another 4 ml of distilled water. Then a subsample of 3 ml was taken and centrifuged for five minutes at 800 g. Finally, the supernatant was discarded, 3 ml of water was added to the pellet, and the sample was homogenized. The spores were counted with a hemocytometer slide (on 0.1-l volume). One nosema spore observed in the hemocytometer's entire grid (25 × 16 = 400 small squares) was equal to an average of 1,500 spores per bee. The reported information was the number of spores per bee.

Administration of HiveAlive™ food supplement

Details of the diets and treatments administered to the experimental groups during the two years are described in Table 1. Sugar candy was prepared by mixing 15% honey (previously heated to destroy yeasts and possible spores) +5% water +80% icing sugar). In November 2012 and 2013, the syrup administered to the colonies was 2:1, sugar to water dilution, in order for bees to store the syrup. In April it was a 1:2, sugar to water dilution, for colony stimulation. The experimental groups had supplement added, as per label instructions, to candy or syrup as indicated in Table 1. Control groups were given plain sugar syrup or candy as indicated. During the first year, one group was also administered fumagillin (in the form of Fumidil-B) in the sugar syrup which was used as a positive control. All groups fully consumed the same amount of food syrup/candy.

Statistical analysis

A repeated measures design was used for the statistical analyses of the data in order to examine the differences between the two main experimental groups (control and treated) in terms of colony strength and nosema infestation, during the course of the six sampling periods. Before analysis, normality and homoscedasticidy of the data were assured.

Results

Starting and ending measurements for each year are presented in Figures 1 and 2. During the first year of the experiment, 2012–2013, no differences were noted between the groups in colony strength between November 2012 and June 2013 (Figure 1). However, some differences were observed among the groups regarding the number of nosema spores per bee. By June 2013, nosema spore levels decreased in all supplement-treated groups, more so than the control and fumagillin groups. In June, the control group had on average 50% fewer nosema spores than the previous autumn and the fumagillin group had a 33% decrease over the same time period. However, the three supplement-treated groups had a considerably larger reduction in nosema spore counts: food supplement in syrup 92%, food supplement in candy 79%, and food supplement trickled and in candy 82% (Figure 1).

Continuing the experiment into its second year (November 2013 to June 2014) demonstrated greater differences among the groups in terms of increased colony strength and nosema spore reduction (Figure 2). The supplement-treated group showed increased adult bee population from 8,500 ± 600 (Mean ± SE) bees in November 2013 to 14,500 ± 850 bees in June 2014

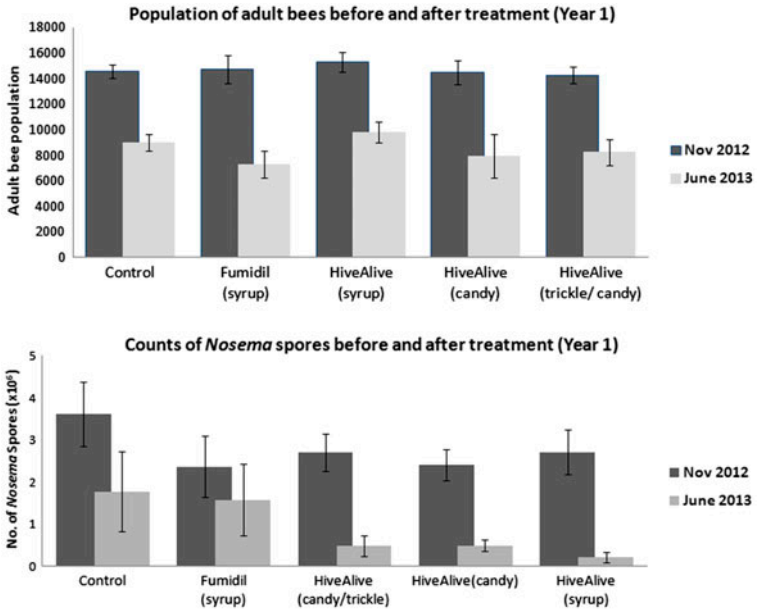

Figure 1. Colony strength and nosema spore counts at the start and end points of the 2012/2013 study.

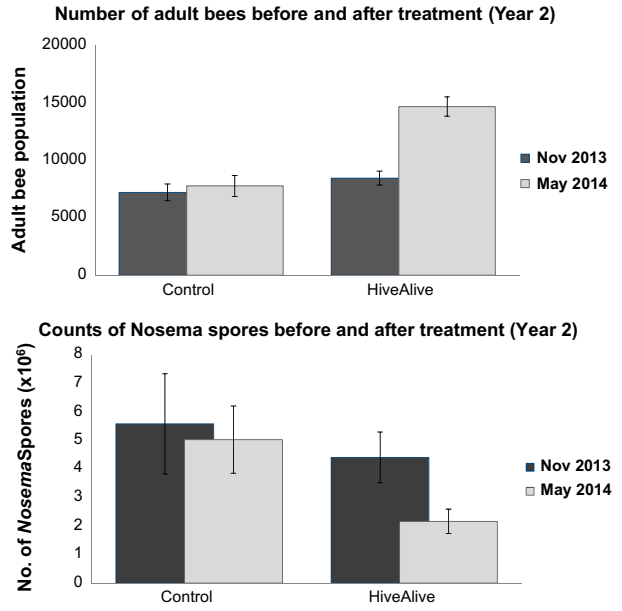

Figure 2. Colony strength and nosema spore counts at the start and end points of the 2013/2014 study.

(70% increase), while the population in the control group was increased from 7,200 ± 850 to 7,800 ± 900 bees (7% increase). The treated group had an 89% larger population than the control group when compared at the end of the second year (June 2014, Figure 3).

Reductions in nosema spore counts for the second year were as follows: the control group had a reduction of 10% between November 2013 and June 2014, while the supplement-treated group had a 51% spore reduction over the same period (Figures 2 and 4). At the end of the experimental period in June 2014, the supplement-treated group had 57% fewer spores than the control group.

Statistical analysis results of the pooled data (six measurements for both years) regarding the adult bee population are shown in Table 2. Significant differences between the seasons were observed (Multivariate Test Effects, 'Season': $F = 189.55$; $p < 0.001$), as expected. The interaction effect between treatment and season was also significant showing that the treatment had a differential effect on the population changes of the two groups across the seasons (Multivariate Test Effects, 'Season X Treatment': $F = 11.80$; $p < 0.001$). Most importantly, the overall increase in adult bee population, which was observed when HiveAlive™ supplement was administered, was significantly higher when compared to control (Tests of Between-Subjects Effects, 'Treatment': $F = 15.44$; $p < 0.001$).

Analysis of results for the nosema spore counts is presented in Table 3. Significant differences were observed between the seasons with respect to nosema spore counts (Multivariate Test Effects, 'Season': $F = 5.37$; $p < 0.005$). However, the treatment had no differential interaction with season (Multivariate Test Effects, 'Season X Treatment': $F = 1.57$; n.s). Nevertheless, the treatment with HiveAlive™ supplement demonstrated an overall significant reduction in nosema spore levels compared to control (Tests of Between-Subjects Effects, 'Treatment': $F = 5.98$; $p < 0.05$).

Discussion

N. ceranae spore loads have often been associated with honey bee colony depopulation and high colony losses in many parts of the world (Botías et al., 2013; Hatjina et al., 2010, 2011; Higes et al., 2010; Soroker et al., 2011; VanEngelsdorp, Evans, Saegerman, Mullin, & Haubruge, 2009). Consequently, professional beekeepers are looking for alternatives to treat their colonies and improve the overall colony health status. Nosema infection levels are naturally lower in the summer compared to spring and autumn (Meixner et al., 2014), which has also been demonstrated in this study, probably due to the more rapid replacement of the older bees with

Table 2. GLM-Repeated measures analysis of colony strength.

		df	*F*	Sig.
Multivariate test effects				
Season	Wilks' Lambda	5	180.55	<0.001
Season × Treatment group	Wilks' Lambda	38	11.80	<0.001
Tests of Between-Subjects Effects	Mean square	df	*F*	Sig.
Treatment group	271788444	1	15.44	<0.001

Table 3. GLM-Repeated measures analysis of nosema spores per bee.

		df	*F*	Sig.
Multivariate test effects				
Season	Wilks' Lambda	5	5.37	<0.01
Season × Treatment group	Wilks' Lambda	38	1.57	n.s.
Tests of Between-Subjects Effects	Mean square	df	*F*	Sig.
Treatment group	126545753910256	1	5.98	<0.05

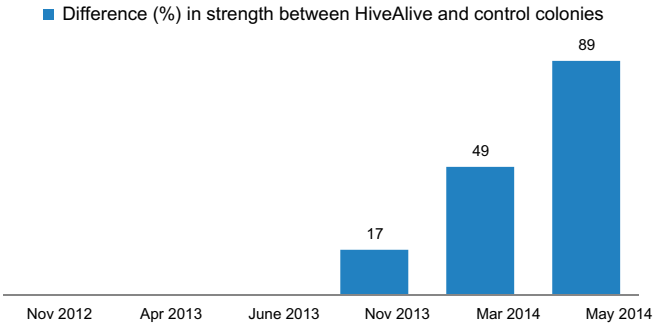

Figure 3. Long term effects of HiveAlive™ on colony strength compared with control (2012 to 2014).

emerging bees. It is also a common beekeeping practice to feed colonies in spring with sugar syrup for stimulation, which further facilitates population replacement and naturally reduces nosema spore loads.

During the first year of this study, administering HiveAlive™ food supplement in candy or in syrup before winter had no effect on colony population in spring compared to both control and fumagillin groups. However, a difference in nosem *a* spore loads was observed. Furthermore, when HiveAlive™ was administered for a second successive year in sugar syrup, before and after winter, nosema spores were significantly reduced throughout the winter and into the following spring and summer (Figures 2 and 4). Additionally, the adult bee population of the supplement-treated group increased considerably over the same period (Figure 3).

According to Mayack and Naug (2009) *N. ceranae* seems to increase hunger levels which induces a number of pathological effects (Dussaubat et al., 2012), a reduced level of vitellogenin in the bees (Antúnez, Mendoza, Santos, & Invernizzi, 2013) and lower survival of infected bees (Higes, Garcia-Palencia, Martín-Hernandez, & Meana, 2007). The administration of seaweed extract through HiveAlive™ before winter possibly strengthens the gut epithelium, which may minimize winter losses and improve the ability of the colony to build up in spring. However, no histological findings exist as yet to verify this hypothesis. As the parasite's development depends on host condition (Porrini et al., 2011) stimulating spring build up of colonies with healthier bees coming out of the winter could be a key factor for increased spring and summer populations with reduced nosema spore loads. Considering that nosema is associated with the presence of different viruses (Bailey & Ball, 1991; Bromenshenk et al., 2010; Toplak, Ciglenečki, Aronstein, & Gregorc, 2013) it is essential to keep the nosema loads low, reducing the probability of virus increase or vice versa. It is quite probable that the positive effect of HiveAlive™ shown during the second year is a result of its long term use (starting in autumn 2012 and finishing in spring 2014).

HiveAlive™ is not an antibiotic; it uses ingredients approved safe for humans and animals (GRAS) and therefore it can be fed as a natural food supplement with no apparent adverse effects on the bees themselves or humans. Furthermore, honey bees are frequently exposed to environmental pollution such as agrochemicals, and as previously noted, there is a synergistic effect between *N. ceranae* and pesticides (Aufauvre et al., 2012; Wu, Smart, Anelli, & Sheppard, 2012), resulting in detrimental health and lifespan of the bees. Reducing one of the stressor parameters might help to prevent colony losses.

From the above study, it would appear that seaweed extract (through HiveAlive™), fed in sugar syrup before and after the winter, can help maintain healthier bees during the winter, as well as stimulate population increase during the spring. Healthier colonies during the winter can potentially build up quicker in spring and can

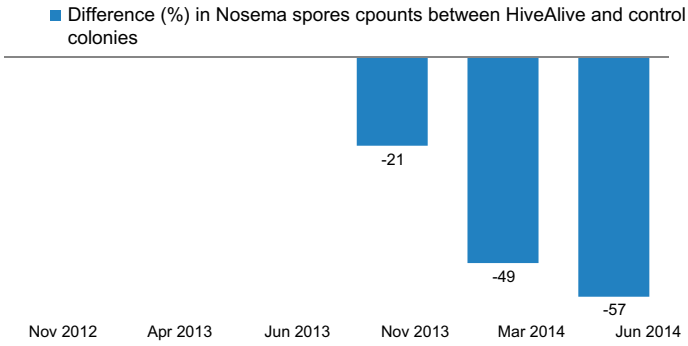

Figure 4. Long term effects of HiveAlive™ on nosema spore counts compared with the control (2012 to 2014).

maintain lower disease levels thereafter. It would appear that with continued use of HiveAlive™ food supplement, the benefits become more apparent. Further tests could possibly demonstrate the mode of action of HiveAlive™ food supplement on bees' gut epithelium or beneficial bacteria.

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Disclosure statement

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