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Divya
Department of Entomology, Dr. Y S Parmar University of Horticulture and Forestry. Nauni, Solan, Himachal Pradesh, India

Sapna Katna
Department of Entomology, Dr. Y S Parmar University of Horticulture and Forestry. Nauni, Solan, Himachal Pradesh, India

Bachittar Singh Rana
Department of Entomology, Dr. Y S Parmar University of Horticulture and Forestry. Nauni, Solan, Himachal Pradesh, India

Harish Kumar Sharma
Department of Entomology, Dr. Y S Parmar University of Horticulture and Forestry. Nauni, Solan, Himachal Pradesh, India

Avinash Chauhan
Department of Entomology, Dr. Y S Parmar University of Horticulture and Forestry. Nauni, Solan, Himachal Pradesh, India

Correspondence
Divya
Department of Entomology, Dr. Y S Parmar University of Horticulture and Forestry. Nauni, Solan, Himachal Pradesh, India

Preliminary studies on *Nosema ceranae*: A microsporidian infecting *Apis mellifera* in India

Divya, Sapna Katna, Bachittar Singh Rana, Harish Kumar Sharma and Avinash Chauhan

Abstract
Five apiaries of *Apis mellifera* bees of Himachal Pradesh placed at Narayangrh, Haryana during autumn and winter were surveyed to study the symptoms of *Nosema ceranae*. Numerous dead bees were found lying on the ground in front of the hives. The infected bees were crawling in front of hives with swollen abdomens. They were unable to fly and walk properly. In diseased bees, the ventriculus was white with less constrictions. The microscopic studies revealed the presence of several, oval to sausage shaped spores (4.5 µm ± 0.109 x 2.1 µm ± 0.093). Pathogenic studies showed that 81.06% bees died at 16th day of post infection which confirms the virulency of the disease. Confinement of *A. mellifera* bees in hives was avoided by feeding sugar syrup (50%) to each colony in order to manage the disease. But this strategy was not found to be effective in our studies.

Keywords: Nosemosis, *Nosema ceranae*, *Apis mellifera*, symptoms, microscopy, pathogenicity

Introduction
Wide variety of bee fauna can be utilized for the development of honey industry in the world and can serve as an important source of income to the people in the rural areas of developing countries like India. *Nosema* is a pathogen of concern for beekeepers because it has been shown to adversely affect honey bee health. For decades, nosema disease was exclusively attributed to a single species of *Nosema* that is *Nosema apis*, which was first described in European honey bees, *Apis mellifera* [1]. In 1996, a new species of *Nosema* was discovered in the Asian honey bee, *Apis cerana*, thus named *Nosema ceranae* [2]. *N. ceranae* had previously been thought to be host – limited to *Apis ceranae* but is now found infesting *A. mellifera* [3]. Samples from across the world now demonstrate that the infection of *N. ceranae* in *Apis mellifera* is a worldwide phenomenon [4-9]. *Nosema ceranae* has become the dominant microsporidian infection in western honey bee colonies [10]. Microsporidia are single-celled organisms that parasitize animal hosts via the dispersal of spores. Unfortunately, there are no reliable field diagnostic symptoms of nosemosis but however in severe cases the abdomen of an infected worker bee become swollen and shiny in appearance. Seriously affected worker bees are unable to fly and may crawl about at the hive [11]. The ventriculus of heavily infected bees may appear whitish and wings are disjointed [12, 13]. Symptoms caused by *N. apis* are more easily observed in honey bee colonies which show large number of dead bees and diarrhea spotting at hive entrances evidencing digestive disorders of adults [14, 15]. Symptoms of *N. ceranae* infestation are more nebulous, consisting primarily of poor colony growth and dwindling. Infestations of either *Nosema spp*. result in decreased honey production, foraging activity, and hence reduced pollination. For both species, spores spread through infected feces, stored pollen, and corbicular pollen which affect food supplies in the colony [16, 17]. Very little work has been done on this disease in the country [18, 19, 13, 20] so therefore, this study aims to address the situation in India as well as globally.

Materials and methods
A. mellifera bee colonies from five apiaries of Himachal Pradesh, India viz., Kangra, Shimla, Una, Solan and Sirmour migrated to Narayangarh (Haryana) during October 2014- March 2015 were surveyed to study the symptoms of nosemosis. The colony level symptoms were recorded with respect to crawler bees, bees with swollen abdomen, bees with disjointed wings and dead bees in the diseased colonies.

To study the spores of *Nosema*, one hundred bees from each colony were examined and observed under light microscope at Dr. Y S Parmar University of Horticulture and Forestry Solan, Himachal Pradesh. To diagnose a nosema infection, the posterior pair of abdominal segments was removed with forceps to reveal the ventriculus, complete with the malpighian tubules, the small intestine and rectum. Before going for further investigations the gut was fixed in 70% ethyl alcohol or frozen in a standard freezer. The infected gut and feacal matter of the bees to be examined were separated and crushed in 2-3 ml of water. Drops of pooled suspension (guts and feacal matter separately) was placed on a slide under a cover slip and examined microscopically at 400-600X magnification under bright field. Observations were recorded on the basis of shape and size of the spores. The spore size was estimated by using the Axiovision software uploaded on Zeiss make Axioskop 40 compound microscope.

Three hoarding cages kept under room temperature, each having 30 adult young bees of age 10 -14 days were used in the pathogenicity experiment. Diseased gut suspension (2 ml) mixed with 50% sugar syrup was fed to the bees. In control, only 50% sugar syrup was fed to the healthy bees in three different cages. Then the experimental cages were observed regularly until all bees died. The experiment was repeated two times. Finally, dead bees were collected from the cages for the confirmation of nosema disease through light microscopy.

Confinement of *A. mellifera* bees in hives was avoided by feeding sugar syrup (50%) to each colony in order to manage the disease. Fifteen diseased colonies of *A. mellifera* already having 25 – 30% nosema infection were selected. Out of these selected colonies, three colonies were fed with 500 ml of 50% sugar syrup at the interval of two days and another three diseased colonies were fed with 500 ml of 50% sugar syrup at the interval of 5 days. In another set of experiment, three diseased colonies were fed with 200 ml of 50% sugar syrup at the interval of two days and another three colonies were fed with sugar syrup solution at the interval of five days. All the diseased colonies were fed up to one month. In control, the three diseased colonies were fed with 400 ml of 50% sugar syrup at 6 days interval. All the experimental colonies were observed regularly for one month. The data was subjected to statistical analysis using CRD (completely randomized block design).

Results and Discussion

Different symptoms of nosema disease were recorded in the observed apiaries. The infected bees were found with swollen abdomens (Fig. 1a). The bees were crawling as they were unable to fly and walk properly. Dead bees were found lying on the ground in front of the hive. (Fig. 1b). In diseased bees, colour of the ventriculus was white while healthy ventriculus appeared yellow due to the presence of the food material (Fig. 1c). For *N. ceranae* no specific colony level symptoms of infection have been described [10]. In Spain, infected colonies have been associated with gradual depopulation, higher autumn and winter colony death and a decrease in honey production. Strikingly, no dysentery is reported to be associated with infections of *N. ceranae* [21]. This is in contrast to our observations where diarrhea spots were noticed at the hive entrance of the colonies of Sirmour apiary (Fig. 1d). Therefore, it was speculated that feacal matter spots on the hive cannot be considered as a reliable symptom of disease due to *N. ceranae*.

Fig 1: Symptoms of Nosemosis a) *Apis mellifera* bees with swollen abdomen b) Dead bees c) Healthy and infected ventriculus d) Spots of faecal matter on the hive

The gut suspension of infected bees examined under light microscope at 400-600X magnification revealed the presence of several, oval to sausage shaped spores having thick wall. These spores varied in size with a length ranging from 4.2 – 5.3 µm (4.5 µm ± 0.109) and width 1.5 – 2.7 µm (2.1 µm ± 0.093). Our findings support the similar spore shape and size range reported for *N. ceranae* worldwide (Fig.2). Studies by Fries *et al.* [2, 22], who was the first to isolate *N. ceranae* from Asian honey bee describe the morphological observation under light microscopy as oval or rod, measuring 4.4 µm in length and 2.2 µm in width. Similarly, spore size to be 4.5×2.4 µm was reported earlier [6]. Chen *et al.* [23] also studied the ultrastructure of the *N. ceranae* spores revealed that the size of fresh spores from *A. mellifera* ranges between 3.9 - 5.3 µm in length and 2.0 - 2.5 µm in width. Thus, on the basis of morphology, our study concludes that nosema spores as observed under light microscope are of *N. ceranae* species.

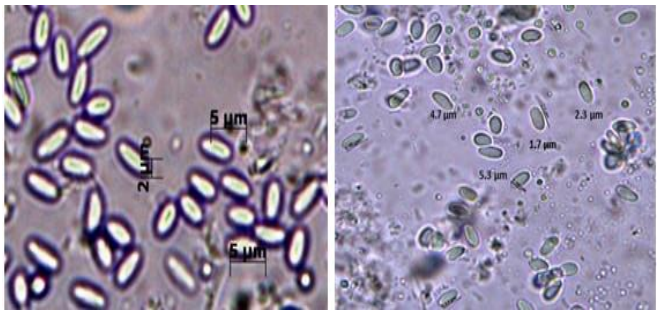

Fig 2: Spores of *Nosema ceranae*

To confirm the virulence of the microsporidia, diseased gut suspension was fed to the healthy bees in 50% sugar syrup in three different cages. Disease gut suspension was consumed within 1-2 days after feeding. No symptoms of disease were recorded till 3rd day after consumption of syrup. Bees started dying from 4th day onwards. All the bees were found dead at 16th day of treatment. Finally, the samples of diseased bees were collected from the treated cages for the confirmation of *Nosema* infection through light microscopy. The mortality due to *Nosema* infection was 6.67% at 4th day of post infection and reached up to 31.10% at 8th day after treatment.

54. 40% mortality was recorded at 12th day after treatment and maximum mortality of bees 81.06% was observed at 16th day of post infection (Fig. 3). These results confirm the virulence of *N. ceranae* infection. Earlier [10] 100% mortality of *A. mellifera* bees due to *Nosema* on 14th day after feeding the pure *Nosema* spores was recorded. Another caged experiment [16] reported that bees infected by *N. ceranae* died within eight days of infection and recorded total mortality of 94.10%. The difference in the per cent mortality of bees in present study at 16th day may be due to the variations in feeding and experimental conditions.

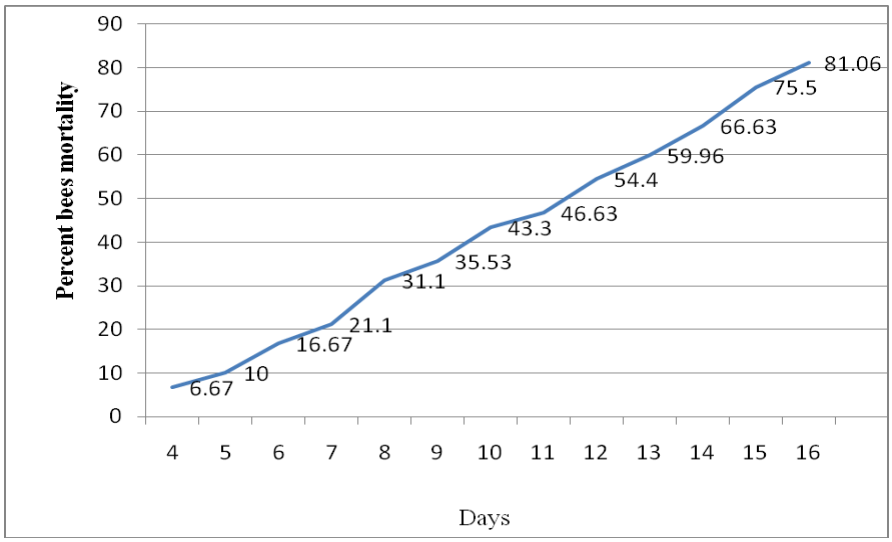

Fig 3: Pathogenicity of *Nosema ceranae* inoculated through feeding *A. mellifera* bees under caged conditions

The status of use of fumagillin for the control of nosemosis in India is unclear. Therefore, an attempt was made to manage the infected colonies by feeding sugar syrup so as to activate the bees during winters. Data in the Table 1 revealed that when 500 ml of sugar syrup at 2 days interval given to the colonies, minimum percent mortality of *A. mellifera* bees (32.55%) was recorded which was statistically at par when 500 ml sugar syrup at 4 days interval was fed to colonies (33.11%). Maximum percent mortality was recorded in control (38.00%) where bees were fed with 400 ml of sugar syrup at 6 days interval which was statistically at par with treatment four (36.55%) where 200 ml sugar syrup at 4 days interval was given to bee colonies. Minimum per cent mortality of bees was recorded on 10th day (29.66%) while maximum was recorded on 30th day (43.66%). These results showed that the effect of different doses of sugar syrup at different days interval was not effective in managing the disease. Such type of studies has been done by Higes *et al*. [21] and Williams *et al*. [24]. They also found in their studies that sugar is not effective in controlling *N. ceranae* infections.

Table 1: Effect of feeding sugar syrup to avoid confinement of *Nosema* infected *Apis mellifera* bees

Treatment	Dose	*Average bee mortality (%) – days after treatment			
		10 Days	20 Days	30 Days	Mean
Sugar syrup (50%)	500 ml at 2 days interval	27.33 (31.50)	30.33 (33.40)	40.00 (39.21)	32.55 (34.70)
Sugar syrup (50%)	500 ml at 4 days interval	28.00 (31.91)	30.33 (33.39)	41.00 (39.79)	33.11 (35.03)
Sugar syrup (50%)	200 ml at 2 days interval	29.66 (32.98)	32.33 (34.63)	44.33 (41.71)	35.44 (36.44)
Sugar syrup (50%)	200 ml at 2 days interval	31.33 (34.01)	32.66 (34.83)	45.66 (42.49)	36.55 (37.11)
Sugar syrup (50%)	400 ml at 6 days interval(Control)	32.00 (34.43)	34.66 (36.05)	47.33 (43.45)	37.99 (37.97)
	Mean	29.66 (32.97)	32.06 (34.46)	43.66 (41.33)	

*Mean of three replications
Figures in parenthesis are angular sin transformed.
C.D $_{(0.05)}$ Dose = 2.27
Days = 1.75
Dose × Days = NS

Conclusion

India ranks 6th in global honey bee production and is a key exporter of honey to countries like United States, Saudi Arabia, United Arab Emirates, Morocco and Bangladesh. Recent assessments and research propose *N. ceranae* to be fast emerging pathogen in the beekeeping agro-industry and thus needs to be addressed globally. The present studies based on the symptomatology, microscopy and pathogenicity gives an idea of the fungal microsporidia *Nosema ceranae* causing nosemosis in *A. mellifera*. The fungal microspores are otherwise difficult to distinguish using conventional microscopic technique. Advanced methods like electron microscopy and molecular tools are required to confirm the species causing nosemosis in honeybees.

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