To Investigate the effects of Hive Alive on Spore viability

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Abstract.

An experiment was carried out to investigate the efficacy of Hive Alive to disrupt the plasma membrane/spore coat of *Nosema ceranae* thereby killing the spores of this microsporidian parasite of honeybees. To determine if spores were dead, the sytox green staining approach using fluorescent microscopy was employed. After incubation in Hive Alive for a period of three months spores were beginning to fluoresce suggesting that the spore coat had been compromised. The majority of spores stored in distilled water for the same length of time were not visible under the fluorescent filter employed suggesting that they were alive. A number of difficulties encountered with the approach are provided.

Introduction:

Fenoy et al., (2009) described a method to determine the viability of *Nosema ceranae* spores utilising DAPI and sytox green stains. The rationale is that all spores would be visible under white light. Unextruded spores would fluoresce with DAPI, which stains nuclear material under the 395- to 415-nm excitation wavelength filter and dead spores would absorb sytox green and fluoresce under the 470- to 490-nm excitation wavelength filter. In this experiment we attempted to repeat the approach of Fenoy et al (2009) and use it to investigate the efficacy of Hive Alive in killing *Nosema* spores by compromising their spore coat or by disrupting their plasma membranes, Rice (2001). Therefore, after treatment with Hive Alive *N. ceranae* and *N. apis* spores should fluoresce under a under the 470- to 490-nm filter after staining with sytox green. Previous attempts to stain *Nosema* spores with DAPI were unsatisfactory and

therefore this step was excluded from the following experiment.

Methods:

Materials: For this experiment a concentrated spore suspension (presumed to be *N. ceranae*) provided by M Kamler was utilised.

Experimental Approach: Three 500µl aliquots of spore suspension were pipetted into each of nine eppendorfs creating three replicates each of two concentrations of Hive Alive (x1 dilution and x2 dilution of recommended volume added to feed) and three replicates of a control (containing no Hive Alive). All tubes were placed on a shaking thermoblock at 25 ^{o}C for three months

After 3 months 50 µl of spore suspension from each tube was transferred to a clean eppendorf, washed in 500 µl distilled water, pelleted by centrifugation and resuspended in the same volume of distilled water. Spores were stained by adding an equal volume of sytox green solution, placing at room temperature for 20 min, followed by a single wash using 200 µl distilled water. The final pellet was resuspended in 10 µl water. This spore suspension was placed on a microscope slide in its entirety before being viewed on a fluorescence microscope under white light and under fluorescence (green filter used, red fluorescence). Photographs were taken under white light (to identify location of spores) and under fluorescence. Spores were subsequently counted from two fields of view for each replicate, from the controls and x2 dilution, documenting which spores were visible under white light and fluorescence.

Results:

The approximate number of spores, counted in each field of view on each slide, is listed in Table 1. The majority of the spores visible under white light on the control slides were not visible under fluorescence. However, most of the spores on the treated slide were also visible under fluorescence. Figures 1-3 show the difference in fluorescence between spores in the control replicates versus those in the treatment replicates.

Table 1. Spore counts from control and treatment slides under white light and fluorescence. FOV = field of view

Slide	Number of spores –white light microscopy	Number of spores – fluorescence microscopy
Control 1: FOV 1	>100	0
Control 1: FOV 2	>50	>20 few flourescing
Control 2: FOV 1	>30	0
Control 2: FOV2	>30	0
Control 3: FOV1	>80	9
Control 3: FOV2	>70	0
Treated1: FOV1	>90	>40 difficult to make out spores due to movement. flourescene visible
Treated1: FOV2	>70	>70 very visible
Treated2: FOV1	>90	>40 difficult to make out spores due to movement. flourescene visible
Treated2: FOV2	>60	can see spores but unclear due to movement,
Treated3: FOV1	>110	>100 fourescing
Treated3: FOV2	>40	>40 flourescing

Discussion.

There was a very visible difference between the fluorescence of spores on the treated versus control slides. Due to the fact that sytox green stains dead spores, this result suggests that most of the spores in the treated samples were dead while those in the controls were alive. The only difference between the tubes was that Hive Alive was added to the 'treated' tubes.

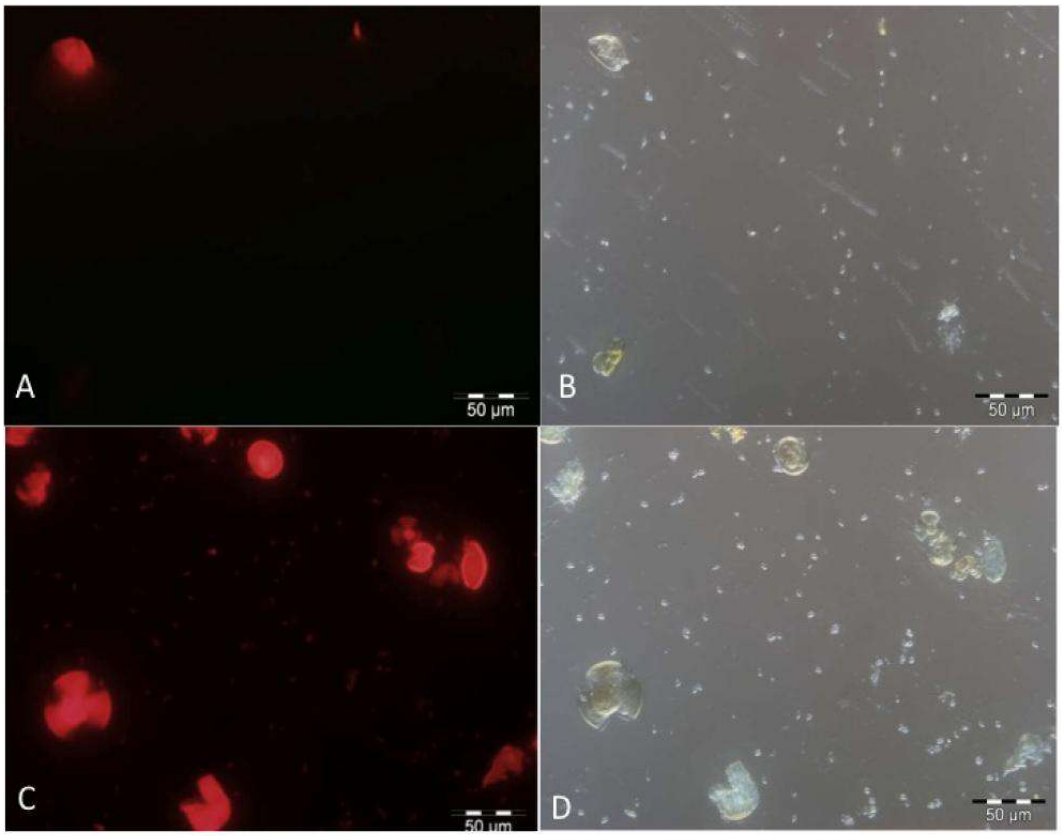
Difficulties experienced include that spores were difficult to photograph due to water movement. Fluorescence was variable in the slides suggesting variable uptake of dye. It is difficult to determine if the spores were truly dead and how the spores were uptaking dye but the results implies that the spore coat has been compromised in treated spore samples. The filters on the fluorescent microscope had been incorrectly placed and so the photographs were taken under green filter (red fluorescence) rather than under a blue filter (green fluorescence) as recommended by Fenoy et al. (2009).

In conclusion, this experiment gives a good indication that spores incubated in Hive Alive have been compromised but to generate data for publication the recommendation is to repeat the experiment after addressed some of the difficulties encountered here and including more incubation times and dilutions of Hive Alive.

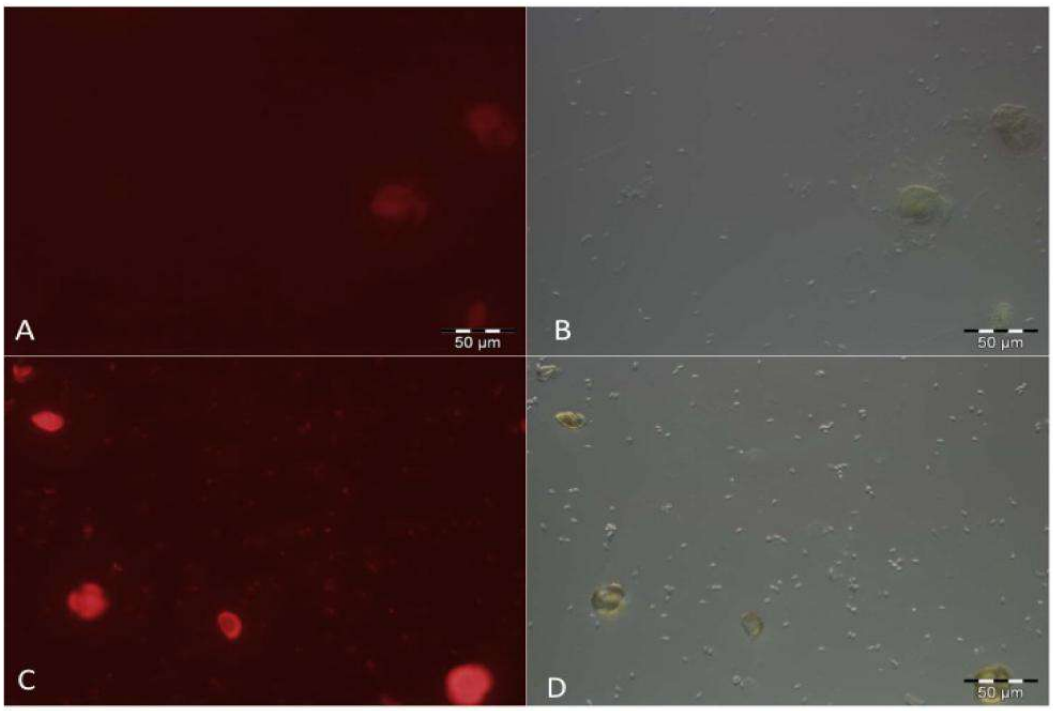
References

Fenoy S, Rueda C, Higes M, Martín-Herńandez R, del Aguila C. (2009). High-Level Resistance of *Nosema ceranae*, a Parasite of the Honeybee, to Temperature and Desiccation. *Applied and Environmental Microbiology*. 25(1):6886-9.

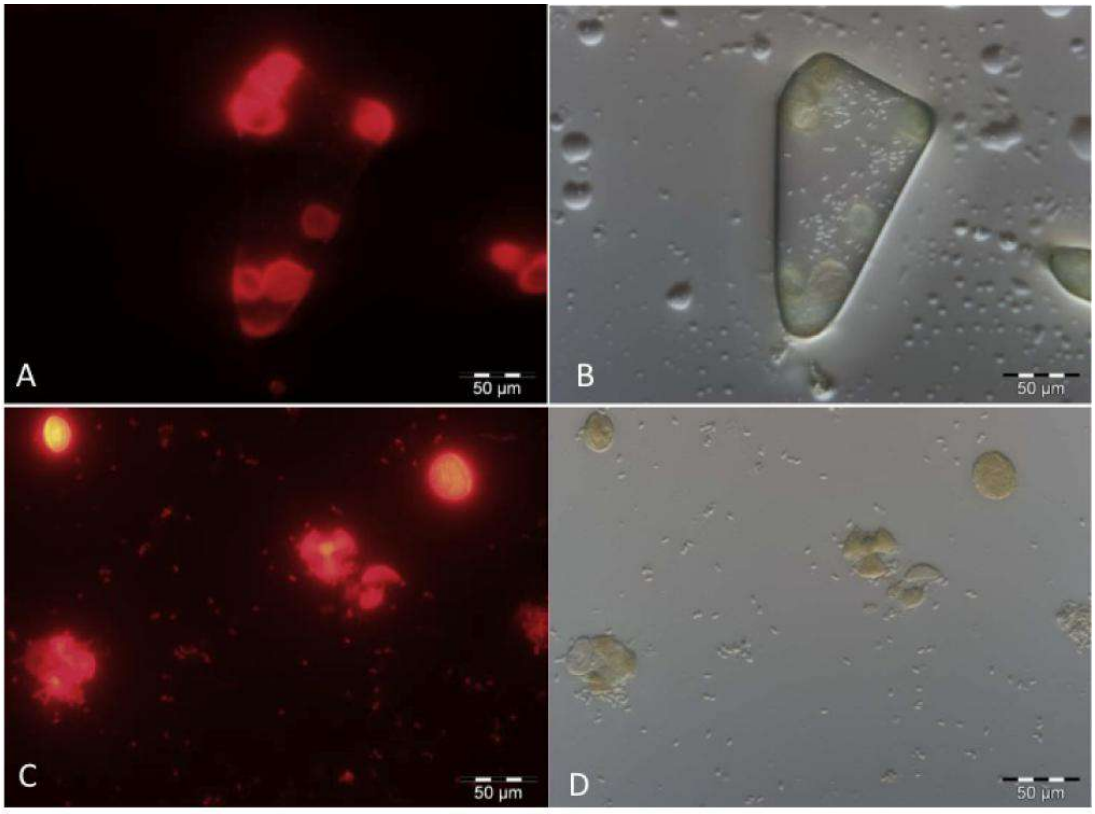
Rice RN (2001). *Nosema* Disease in Honeybees; Genetic Variation and Control. A report for the Rural Industries Research and Development Corporation. RIRDC Publication No 01/46. 1-36.



Fgure 1. Spores photogrpahed under fluorescence (A and C) and white light (B and D). A and B are generated from control replicate1 and C & D are generated from treatment replicate 1.



Fgure 2. Spores photogrpahed under fluorescence (A and C) and white light (B&D). A and B are generated from control replicate 2 and C & D are generated from treatment replicate 2.



Fgure 3. Spores photographed under fluorescence (A and C) and white light (B&D). A and B are generated from control replicate 3 and C & D are generated from treatment replicate 3.