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NOTES AND COMMENTS

Nosema ceranae contamination in bee keeping material: the use of ozone as disinfection method

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The spore-forming microsporidian *Nosema ceranae* is considered an emergent and relevant parasite of western honey bees, *Apis mellifera*. It has been present in Europe since at least 1993 and has been spreading worldwide through commercial movements of bees, hive products, and bee keeping material. Wax and combs contaminated with spores of *N. ceranae* are an important source of infection. The goal of this work was to evaluate the resistance of *N. ceranae* spores to various ozone exposure times, both for experimentally inoculated and naturally contaminated wax combs. Spore viability was evaluated, by fluorescent microscopy, using Sytox-Green. The change in spore viability in artificially contaminated wax combs reached 78.18% after 10 h of ozone exposure. A significant reduction in spore viability was observed after the first 60 min of treatment (viability reduction of 20.25%). In naturally contaminated combs, 17.92% of spores were nonviable after a 6-h treatment period. Ozone has proven to be effective in reducing *N. ceranae* contamination on wax combs and could be proposed as method to minimize the risk of *N. ceranae* introduction into parasite-free areas. Future studies should focus on optimizing ozonation efficacy by maximizing the surface of combs directly exposed to ozone and by increasing the exposure time until a plateau in spore viability can be observed.

Keywords: *Nosema ceranae*; ozone; disinfection; wax combs; spore

Nosema ceranae is an obligate intracellular parasite that infects the epithelial cells of the ventriculus of adult honey bees *Apis cerana* and *Apis mellifera* resulting in gut tissue degeneration (Higes, Garcia-Palencia, Martin-Hernandez, & Meana, 2007; Huang & Solter, 2013). Immune suppression (Antúnez et al., 2009; Chaimanee, Chantawannakul, Chen, Evans, & Pettis, 2012), energetic stress (Mayack, Natsopoulou, & McMahon, 2015), and reduction of honey bee lifespan (Dussaubat et al., 2012) have also been associated to *N. ceranae* infection. Originally identified in the Asian honey bee *A. cerana* (Fries, Feng, da Silva, Slemenda, & Pieniazek, 1996), it has been present in Europe since at least 1993 (Ferroglio et al., 2013) and it is currently globally distributed, with higher prevalences than the native *Nosema apis* (Higes et al., 2007; Martín-Hernández et al., 2012). The impact of *N. ceranae* on honey bees varies across countries and climatic conditions (Gisder et al., 2017) as its implication in Colony Collapse Disorder has been confirmed in Spanish honey bees (Higes et al., 2010; Martín-Hernández et al., 2007). How this exotic parasite spread worldwide is unknown, but it is most likely through the trade of bees, bee keeping material and hive products (Ferroglio et al., 2013).

Microsporidian parasites produce an environmental spore that is transmitted between bees by oral-fecal route or by contaminated honey, pollen, bee keeping material, trophallaxis, and by regurgitated pellets of insectivorous birds (Higes at al., 2008). Environmental spores of *N. ceranae* are an important source of contamination as they were shown to be highly resistant to temperature, and desiccation (Fenoy, Rueda, Higes, Martin-Hernandez, & del Aguila, 2009, Sánchez Collado, Higes, Barrio, & Martín-Hernández, 2014). Their presence in bee keeping material, especially in wax and combs, which are frequently reused from year to year, may pose a serious threat to proper management of bee colonies. Studies related to the viability of microsporidia spores are scarce (Amigo et al., 1996; Fenoy et al., 2009; Shadduck & Polley, 1978) and spore resistance to disinfection was shown to be influenced by the suspension medium (Fenoy et al., 2009) making it necessary to assess viability on each substrate-type or medium. The action of ozone as an oxidizing compound is well known since the 19th century (Victorin, 1992; Vosmaer, 1916). The disinfection activity of ozone is due to its ability to attack the cell membrane and intracellular enzymes of microorganisms, as well as the viral capsids and DNA (Poulis et al., 2014) and is being used in a wide range of applications as a disinfecting agent (John, Haas, Nwachuku, & Gerba, 2005; Poulis et al., 2014; Seydim, Greene, & Seydim, 2004). Its efficacy on microsporidia has also been demonstrated (Fournier et al., 2002; John, Haas, Nwachuku, & Gerba, 2005). In this regard, the goal of the present study was to evaluate the use of ozone as a method for disinfecting wax combs contaminated with *N. ceranae* spores.

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Table 1. For artificially contaminated combs the mean (and standard deviation; sd) of nonviable and total number of *N. ceranae* spores (n/mL) are reported in the table together with the corresponding change in viability at the different ozone-exposure times. The statistical significance (*p* value) of the viability change at each exposure time in comparison to the control group is also reported in the table.

Exposure times	T0 control	T1 30 min	T2 60 min	T3 3 h	T4 6 h	T5 8 h	T6 10 h
Mean nonviable spores (sd)	37500 (12990.38)	150000 (63639.61)	303750 (86412.89)	371250 (51280.48)	622500 (90932.67)	840000 (203469.9)	645000 (82840.20)
Mean total spores (sd)	1050000 (259807.62)	1590000 (76485.29)	1500000 (11361.23)	1721250 (197115.92)	1657500 (166188.9)	1432500 (298024.75)	825000 (98361.58)
Change in viability	3.57%	9.43%	20.25%	21.57%	37.56%	58.64%	78.18%
p-value		0.811	0.0039	0.009	<0.001	<0.001	<0.001

A total of 126 squares (size 4×4 cm) were cut out of empty nest combs from *Nosema* spp. free-colonies previously analyzed by PCR (Martín-Hernández et al., 2007). Each square was artificially contaminated with 8.93×10^6 spores of *N. ceranae*. The spores of *N. ceranae* were obtained from freshly collected foragers with clinical Nosemosis (Higes, Martín-Hernández, & Meana, 2010). The abdomens of infected bees were manually homogenized in a mortar, suspended in sterile saline solution (0.9% NaCl solution, pH 5.0) and filtered to remove coarse bee parts (Paxton, Klee, Korpela, & Fries, 2008). Spores were recovered by centrifugation (3000 *g* for 5 min), resuspended in 10 mL of sterile saline and evenly sprayed on both sides of the wax comb. The wax squares were placed in the ozone disinfection chamber on top of a grid to maximize ozone circulation. Six periods of treatment were evaluated: 30 min, 60 min, 3 h, 6 h, 8 h, and 10 h, namely from T1 to T6, respectively. Eighteen comb samples were tested for each exposure time and 18 samples served as reference control (T0). These control samples were stored at the same conditions of temperature and humidity of the treated samples (Table 1).

Forty-two combs from naturally infected bee colonies were also included in the study. The bee colonies were naturally infected with *N. ceranae*. The actual identity of *N. ceranae* was confirmed by PCR (Martín-Hernández et al., 2007) on a portion of all wax combs (square 4×4 cm). The combs were placed in the disinfection chamber stacked inside a metal cage (treatment A) or piled staggered in groups of three (treatment B). A portion of each comb was removed before treatment, air-exposed to the same conditions of temperature and relative humidity as the treated samples, to establish the initial (T0) mortality rate for treatment A and B. The samples were exposed to ozone for 6 h (Schwartzbrod, Maux, & Chesnot, 2003). After treatment, a square of side 4 cm was cut out of each comb and stored until further analysis.

A corona-discharge ozone generator (O3 Power 16G, De Nora, Milan, Italy) was used to generate a constant ozone production of 15 g/h with a flow rate of 4 L/min into an airtight disinfection chamber. During the experimental procedure, 10 min were added to the exposure time for the ozone to reach the desired concentration at the onset of the real exposure time (Poulis et al., 2016). Ozone concentration inside the disinfection chamber was constantly monitored through a UV-photometric ozone analyzer (BMT Messtechnik Gmbh, DE). The environmental conditions of the chamber were continuously recorded during treatments using temperature and relative humidity data loggers (OM92 data logger, OMEGA Engineering, Ltd., Manchester, UK). The samples were placed inside the disinfection chamber of the ozone disinfection device and exposed to ozone for the specified time (Table 1). Control samples were subjected to the same environmental conditions of temperature ($T = 24 \pm 2\,^{\circ}$C) and relative humidity ($58 \pm 1\%$) as the treated samples.

After completion of the disinfection cycle, the samples were removed from the disinfection chamber and placed in sterile polyethylene containers. Ozone treated, and control samples were stored at room temperature until further analysis, which occurred within 5 days from treatment.

Each wax square (from artificially and naturally contaminated combs) was cut into smaller pieces ($\approx$0.5 cm) added to 50 mL of sterile physiological solution and stirred for 30 min to allow spore detachment. The resulting suspension was filtered and centrifuged at 3000 *g* for 5 min. The supernatant was removed, and centrifugation repeated twice by adding 1 mL of sterile saline before each centrifugation. The resulting pellet was resuspended in 500 μL of sterile saline and stained (1:1 ratio) with SytoxGreen 1 μM (S7020 LifeTechnologies, Milan, Italy) as previously described (Fenoy et al., 2009). Spores were incubated for 20 min at RT and loaded onto a Burker's hemocytometer (Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Non-viable spores were counted as yellow-green ovals ($3.6–5.5 \times 2.3–3.0$/μm) (Fries et al., 1996) through the 470- to 490-nm excitation wavelength filter used for viewing SytoxGreen staining and the total number of spores (viable and non-viable) was counted under white light (Figure 1). Sytoxgreen staining was preferred to alternative methods to assess spore viability (i.e. flow cytometry, infection or germination assays) due to its reliability, ease of use and low cost.

The analysis of data was carried out in the statistical environment R version 3.0.2 (R Foundation for

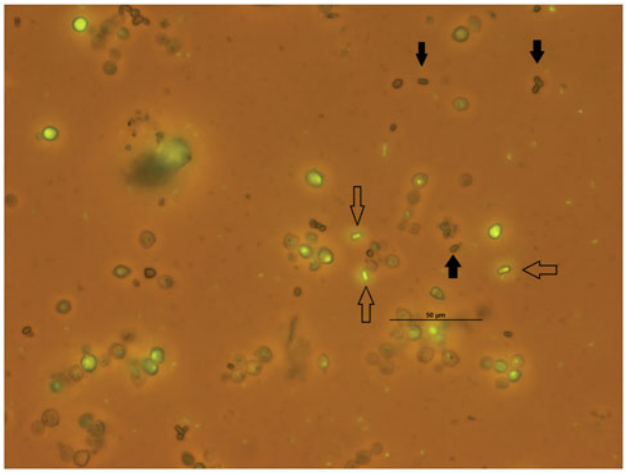

Figure 1. Nosema *ceranae* spores previously stained with SytoxGreen were identified by fluorescent microscopy to distinguish non-viable spores colored in green (hollow arrow) from viable spores (solid arrow).

Statistical Computing, Vienna, Austria). Student's T test for paired data was used to compare treatment methods to the relative control samples. Fisher's Exact Variance test was used to compare between-groups variance of spore viability. Results were considered statistically significant with $p < 0.05$.

The reduction of spore viability in artificially contaminated wax combs progressed with exposure time, reaching 78.18% at T6. Treatment started showing a significant effect on spore vitality after 60 min (significant difference between T0 and T2; *p*-value at T2 $p = 0.0039$) (Table 1).

Of the 42 naturally contaminated combs only 36 were suitable for further analysis. Five comb samples of treatment group A and one comb sample of treatment group B were excluded from the study because of the presence of pollen, which prevented a reliable visualization of spores. A significant reduction in the number of viable spores was recorded in combs subject to treatment A ($t = 3.24$, $p = 0.005$) and treatment B ($t = 5.15$, $p < 0.001$) compared to the corresponding control samples. The mean spore mortality in combs subject to treatment A was 17.92% while in combs subject to treatment B was 15.61%. A significant difference (F test $p < 0.001$) in the variance of spore mortality was recorded between the two treatment types.

Ozone is used as disinfecting agent in a variety of applications from medical (Poulis et al., 2014) to surface water treatment (John, Haas, Nwachuku, & Gerba, 2005) and in food and livestock industries (Seydim, Greene, & Seydim, 2004). Microsporidian spores are sensitive to ozone oxidizing capacity. *Enterocytozoon bineusi*, *Encephalitozoon cuniculi*, and *Encephalitozoon intestinalis* have all been demonstrated to be effectively inactivated by ozone (Fournier et al., 2002; John, Haas, Nwachuku, & Gerba, 2005). Our data confirm the efficacy of ozonation in significantly reducing the viability of *N. ceranae* spores on wax combs.

On artificially contaminated combs, ozone showed an efficacy that increased progressively over time. A significant reduction of spore viability was recorded after a minimum exposure time of 60 min and after the maximum exposure time considered (10 h) no plateau in viability change was observed. Even if the vital staining with SytoxGreen does not allow to determine the number of spores that are destroyed during the assay, we hypothesize that the lower number of dead and total spores at T6 had to be ascribed to the oxidizing activity of ozone which deformed the spores making them unrecognizable to microscopic observation. At T6, viability loss reached 78.18% which exceeded any other treatment applied to *N. ceranae* spores besides autoclaving which inactivates 96% of *N. ceranae* spores (Fenoy et al., 2009). Unlike autoclaving, ozonation is easily applicable on a large scale on bulky equipment and on materials susceptible to medium-high temperatures like wax combs. Other decontamination methods do not reach the level of decontamination provided by ozone, even with much longer exposure periods (71% viability loss after 12 months at 4 °C) (Fenoy et al., 2009).

On naturally contaminated combs, the mortality level reached by ozonation was lower than in artificially contaminated wax frames at the same time of exposure. Even if spore mortality in combs subject to treatment A and treatment B is comparable (17.92% and 15.61%, respectively) the variance of spore viability is significantly different in the two treatment groups. In treatment group A, where combs were piled together, spore viability was less uniform than in combs subject to treatment B. Considering the rapid decay of ozone (Bocci, 2002) and its slow penetration capacity (Kells, Mason, Maier, & Woloshuk, 2001), combs should be arranged to maximize the exposure to ozone and exposure time should be increased until a plateau can be observed in spore viability loss.

The use of contaminated wax, bee keeping material as well as bees and bee products are an important source of infection with *N. ceranae* which must be limited to effectively prevent infection. Ozonation is proposed as an environmentally friendly, residual-free and economic method for reducing *N. ceranae* spore viability in contaminated combs and frames. Ozone is extensively used as antimicrobial in industrial processes involving a variety of substrates from food to waste-water (John, Haas, Nwachuku, &, Gerba, 2005; Poulis et al., 2014; Seydim, Greene, & Seydim, 2004). It has the versatility for being used in apiculture, from small family based to large commercial apiaries, as ozone generators can be easily adapted to productive needs. Further research is necessary to increase the level of spore viability loss, standardize and maximize the efficacy of ozonation to propose ozone as an effective disinfection method for bee keeping material and bee products. If optimized, ozone use on wax and

wax combs could be suggested as viable method to minimize the risk of *Nosema ceranae* introduction into parasite-free areas.

Disclosure statement

No potential conflict of interest was reported by the authors.

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