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Short Communication

Effects at Nearctic north-temperate latitudes of indoor versus outdoor overwintering on the microsporidium *Nosema ceranae* and western honey bees (*Apis mellifera*)

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ABSTRACT

In northern temperate climates, western honey bee (*Apis mellifera*) colonies can be wintered outdoors exposed to ambient conditions, or indoors in a controlled setting. Because very little is known about how this affects the recently-detected microsporidium *Nosema ceranae*, we investigated effects of indoor versus outdoor overwintering on spring *N. ceranae* intensity (spores per bee), and on winter and spring colony mortality. For colonies medicated with Fumagilin-B® to control *N. ceranae*, overwintering treatment did not affect *N. ceranae* intensity, despite outdoor-wintered colonies having significantly greater mortality. These findings suggest that *N. ceranae* may not always pose the most significant threat to western honey bees, and that indoor-wintering may ensure that a greater number of colonies are available for honey production and pollination services during the summer.

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Winter poses a significant challenge to beekeepers in Nearctic northern temperate climates, where western honey bee *Apis mellifera* colonies face months of sub-zero temperatures during which they have to survive on honey and pollen reserves. In these regions, colonies can be wintered outdoors in sheltered locations with insulated wrapping. Alternatively, they can be wintered indoors in complete darkness in buildings maintained at ~5 °C and equipped with air circulation and ventilation systems that exchange heat and carbon dioxide produced by colonies with fresh outdoor air (Currie et al., 1998; Shimanuki et al., 2007). Outdoor wintering affords bees opportunities to leave the colony to defecate (cleansing flights), and to rear brood during mild temperatures; however, it also exposes colonies to long periods of sub-freezing temperatures during which bees must form a tight, ball-shaped cluster to maintain warmth. This cluster restricts movement of bees, making them vulnerable to starvation because they cannot leave to feed (Shimanuki et al., 2007). Conversely, indoor wintering reduces nutritional requirements (Genc and Kaftanoglu, 1997), and allows colonies to be moved outdoors when environmental conditions are ideal; however, it reduces brood-rearing and prevents cleansing flights because of constant cool temperatures and complete darkness. For much of the 20th century, beekeepers in Nearctic northern temperate regions did not overwinter their colonies, but rather imported packages each spring consisting of 0.9–1.4 kg of bees and a mated queen. Because of the recent threat of introducing parasites (e.g. the parasitic mite *Varroa destructor*) from countries exporting packages, as well as high import costs, beekeepers are now encouraged to overwinter their colonies (Currie et al., 1998).

Another potential disease that can be introduced is *Nosema*, caused historically in western honey bees by the microsporidium *Nosema apis*. Infection often results in reduced longevity and degeneration of infected queen ovaries, and ultimately colony death during winter, or slow spring build-up of survivors (Fries, 1993). In 2005, a second microsporidium, *Nosema ceranae*, was discovered parasitizing western honey bees in Taiwan (Huang et al., 2007), and has since been found distributed almost globally (e.g. Klee et al., 2007; Williams et al., 2008a; Higes et al., 2009). *N. ceranae* has likely parasitized western honey bees for decades (Paxton et al., 2007; Chen et al., 2008), but due to its recent detection, very little is known about its biology and management (Fries, 2009). In Europe, it has been associated with colony collapse (Higes et al., 2008) and high pathogenicity in caged bees (Higes et al., 2007;

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Paxton et al., 2007), whereas in North America, it appears to be more benign (Williams et al., unpublished). Both species can be controlled using the antibiotic Fumagilin-B®; unfortunately, treatment never completely eliminates infection (Higes et al., 2008; Williams et al., 2008b). Because infection levels of *N. apis* typically increase rapidly in spring as brood-rearing starts but while cleansing flights are infrequent (Pickard and El-Shemy, 1989), indoor-wintering may reduce *Nosema* levels in spring by allowing beekeepers to move colonies outdoors when cleansing flights are possible. Here, we investigated effects of overwintering environment (indoor versus outdoor) on spring *N. ceranae* intensity and on winter and spring colony mortality.

Worker honey bees were collected from hive entrances of 61 commercial colonies from two beekeeping operations from the province of Nova Scotia and one from the province of New Brunswick between 26 August and 5 September 2009. Samples were stored at −20 °C until *Nosema* spore intensity could be estimated. We created suspensions for each colony by finely crushing 30 abdomens in 30 ml distilled water using a mortar and pestle, and then used light microscopy and a hemocytometer to count spores (see Williams et al., 2008b). Approximately half of the colonies sampled in each apiary remained outdoors until spring, with remaining colonies moved indoors between 4 and 15 December 2008 (Supplementary Table 1). Indoor-wintered colonies were moved outdoors between 27 March and 19 April 2009 (Supplementary Table 1), and for two beekeeping operations to the same location as the outdoor-wintered colonies; for beekeeping operation 1, indoor-wintered colonies were moved to a yard ~4 km away. Worker bees from hive entrances of surviving colonies were re-sampled for *Nosema* twice in spring 2009: first, within 4 weeks of moving indoor-wintered colonies outdoors and second, just prior to colonies being moved to blueberries for pollination. New Brunswick samples were collected from under the hive lid in April because very few bees were at the hive entrance due to cool temperatures. Since new bees had not emerged, those collected were likely wintered bees similar to those collected from the hive entrance at the other operations. *V. destructor* intensity was determined for a random subset of colonies (for beekeeping operations 1, 2, and 3, three, eight, and three colonies were sampled, respectively) in late summer 2008 by collecting ~200 bees from a brood frame. Bees were placed in a stainless steel mesh strainer and agitated in a basin containing windshield washer fluid (for use at −40 °C) and lined with a cotton sheet. After ~3 min, or when no more mites were observed, number of detached mites on the cotton sheet and number of bees washed were determined to calculate number of mites per 100 bees.

All colonies within each operation received the same medications, including in mid-September 2008 Fumagilin-B® (Medivet Pharmaceuticals Ltd., High River, Alberta, Canada) according to manufacturer recommendations for *N. ceranae* (one dose of 190 mg fumagillin per colony), and between spring sampling periods (four weekly doses of 30 mg fumagillin per colony), as well as acaricides for the parasitic mite *V. destructor* and Terramycin® for the bacterium causing American foulbrood, *Paenibacillus larvae*. Colony mortality was determined by the beekeeper; dead colonies were defined as those without a queen or those with <2 frames of bees during spring 2009 inspections.

Molecular analyses were performed on four randomly-selected *Nosema*-infected suspensions from each beekeeping operation from April 2009 using duplex PCR (Williams et al., 2008b) and primers 321APIS-FOR and 321APIS-REV for *N. apis* and 218MITOC-FOR and 218MITOC-REV for *N. ceranae* (Martín-Hernández et al., 2007).

Nosema and *V. destructor* intensity data were square-root transformed to improve fit to normality where appropriate. Split-plot ANOVAs tested for effects of beekeeper and overwintering treatment on *Nosema* intensity at each sampling period, and repeated measures ANOVAs tested for effect of overwintering treatment on *Nosema* intensity at all sampling periods simultaneously. We used logistic regression to test for a relationship between fall

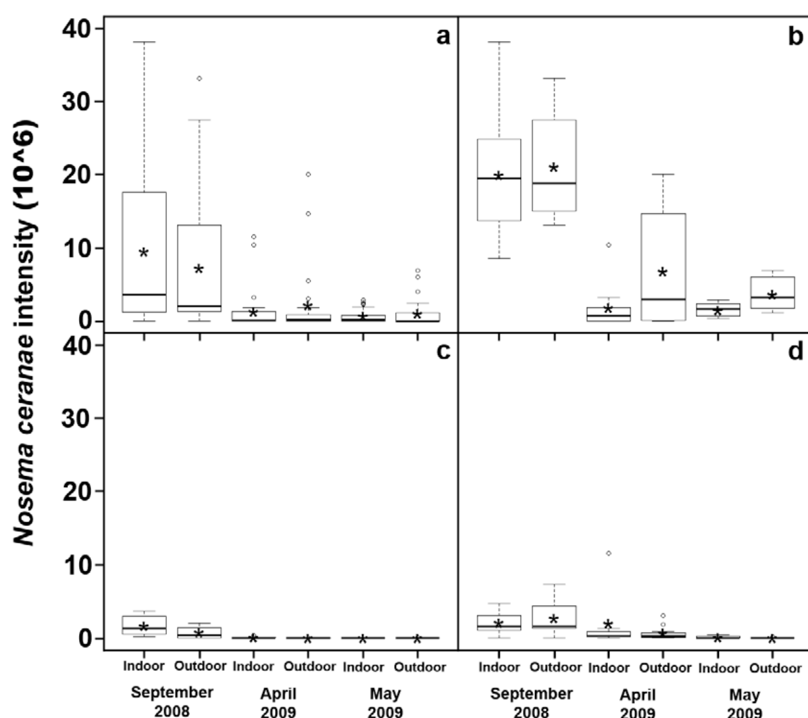

Fig. 1. Comparisons of *Nosema ceranae* intensity (in millions of spores/bee) between indoor- and outdoor-wintered western honey bee colonies in September 2008, April 2009, and May 2009 for: (a) all beekeeping operations, (b) beekeeping operation 1, (c) 2, and (d) 3. Boxplots show interquartile range (box), median (black line within interquartile range), data range (dashed vertical lines), and outliers (open dots). Asterisks represent means.

Table 1
Summary statistics of mean *Nosema ceranae* intensity (in millions of spores/bee) for indoor (Ind.) – and outdoor (Out.) – wintered western honey bee colonies, as well as colony mortality, from three beekeeping operations in Maritime Canada.

Province	Operation	Total	Ind.	Out.	Mean *N. ceranae* intensity (millions spores/bee)									Mortality (colonies)			
					August 2008			April 2009			May 2009			April 2009		May 2009	
					Average	Indoor	Outdoor	Average	Indoor	Outdoor	Average	Indoor	Outdoor	Ind.	Out.	Ind.	Out.
NS	1	21	11	10	21.0	20.0	22.2	3.6	1.8	6.8	2.3	1.6	3.7	0	4	0	4
	2	21	10	10	1.4	1.8	1.0	0.1	0.1	<0.1	<0.1	0	<0.1	2	3	2	5
NB	3	20	8	12	2.7	2.6	2.7	1.1	2.0	0.7	0.1	0.2	<0.1	1	0	1	1

2008 *Nosema* intensity and winter mortality. Differences in colony mortality between overwinter treatment groups were compared using a χ^2 test. All statistical analyses were performed in R version 2.9.0.

Nosema intensity was not significantly different between indoor- and outdoor-overwintered colonies for August, April, or May, regardless of whether we controlled for beekeeper (August, April, and May split-plot ANOVAs for beekeeper and overwinter treatment: $F_{1,44} < 0.1$, $P = 0.93$; $F_{1,44} = 0.5$, $P = 0.47$; $F_{1,44} = 2.4$, $P = 0.13$, respectively) or did not (repeated measures ANOVA: $F_{1,46} = 0.1$, $P = 0.74$) (Fig. 1; Table 1); however, *Nosema* intensity was significantly higher in outdoor-wintered colonies for one of the three beekeepers (beekeeping operation 1 repeated measures ANOVA: $F_{1,15} = 0.5$, $P = 0.04$).

There were no significant relationships between fall 2008 *Nosema* spore intensity and winter mortality by April or May when all colonies (logistic regression: April, $Z = -0.9$, $P = 0.38$; May, $Z = -0.1$, $P = 0.91$), indoor-wintered colonies (April, $Z = 0.7$, $P = 0.48$; May, $Z = 0.7$, $P = 0.48$), or outdoor-wintered colonies (April, $Z = -1.6$, $P = 0.12$; May, $Z = -0.7$, $P = 0.48$) were pooled. There was no significant relationship between overwintering treatment and mortality ($\chi^2_1 = 1.5$, $P = 0.22$) in April, with 3 of 29 and 7 of 32 indoor- and outdoor-wintered colonies dying, respectively; however, significantly more outdoor-wintered colonies died (10 of 32) by May than did indoor-wintered colonies (3 of 29; $\chi^2_1 = 4.0$, $P = 0.05$).

Eight of twelve samples molecularly analysed had *N. ceranae*, and one had both *N. ceranae* and *N. apis*. The remaining three samples failed to amplify a PCR product, possibly due to DNA degradation. These data support previous studies (e.g. Williams et al. 2008b) that show a predominance of *N. ceranae* in honey bees in Maritime Canada.

V. destructor intensity was above the recommended treatment threshold of 3.3 mites per 100 bees for 2 of 14 samples (McRory et al., 2009); both were from beekeeping operation 2. There was no significant difference in *V. destructor* intensity between overwintering treatment groups ($F_{1,12} = 0.8$, $P = 0.39$; mean ± SD for indoor and outdoor: 1.2 ± 1.2 and 2.3 ± 2.3, respectively).

Overall, overwintering treatment (indoor versus outdoor) did not affect *N. ceranae* intensity the following spring. It is possible that potential differences in *N. ceranae* intensity between indoor- and outdoor-wintered colonies were removed by application of Fumagilin-B® to all colonies in fall because fall application is effective at reducing *N. ceranae* intensity the following spring (Williams et al., 2008b). Future experiments should investigate effects of overwintering treatment on *N. ceranae* in the absence of Fumagilin-B® treatment, as well as investigate why *N. ceranae* intensity was higher in outdoor-wintered colonies in spring for beekeeping operation 1 only. Possibly, differences in spring colony locations of indoor and outdoor-wintered colonies affected results for beekeeping operation 1; however, this is unlikely, at least during April sampling, since samples were collected from colonies within 10 days of being moved outdoors. Fumagilin-B® may on its own be effective at reducing spring *N. ceranae* intensity when fall intensity is below an unknown threshold; however, when intensity is high, such as was the case in colonies belonging to beekeeping operation 1 (Table 1), indoor-wintering may aid the antibiotic in reducing *N. ceranae* the following spring. It is also likely that length of time spent indoors influences *N. ceranae*, although outdoor winter conditions were not considered particularly challenging to colonies during the study. Colonies belonging to beekeeping operation 1 remained indoors >3 weeks longer than operations 2 and 3; moving colonies outdoors too early in spring may encourage brood-rearing during nectar and pollen dearth, possibly also encouraging *Nosema* development.

Although overwintering treatment had no significant effects on *N. ceranae* intensity, mortality was significantly higher in colonies wintered outdoors. This, coupled with an insignificant relationship between fall *N. ceranae* intensity and colony mortality, supports data that *N. ceranae* may not always pose the most significant threat to western honey bees (Guzmán-Novoa et al., 2010; Williams et al., 2010). We can only speculate, but it is possible that *N. ceranae* present in Maritime Canada (Williams et al., 2008b) differs in virulence from strains found in other regions of the world, or that experimental colonies were not sufficiently stressed, and therefore not particularly susceptible to *N. ceranae* disease. More work is needed to investigate damaging thresholds of *N. ceranae*, as well as factors influencing *N. ceranae* pathogenicity, such as haplotype and overall colony health. Although overwintering treatment did not affect *N. ceranae* intensity, the ultimate measure of colony strength, colony survival, benefitted from indoor wintering likely because winter food reserves lasted until nectar and pollen dearth ended, thus ensuring a greater number of colonies were available for pollination services and honey production throughout the summer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jip.2010.01.009.

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