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Short Communication

# External and internal detection of *Nosema ceranae* on honey bees using real-time PCR

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ABSTRACT

Numerous methods exist for molecular-based detection of *Nosema ceranae*. Here we determine location of parasite loads, the optimal tissue for pathogen detection, and the likely sources of variability among assays. Bee washes and head/thorax samples revealed substantial *N. ceranae* loads ($2.67 \times 10^4 \pm 1.12 \times 10^4$ and $1.83 \times 10^4 \pm 4.14 \times 10^3$). Midgut samples carried the highest parasite loads ($3.42 \times 10^6 \pm 1.84 \times 10^6$), followed by the hindgut ($5.50 \times 10^5 \pm 3.24 \times 10^5$). We recommend using midgut samples for molecular-based detection and quantification of *N. ceranae* because of the low variability among samples.

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The range of the microsporidian *Nosema ceranae* has expanded in recent years to a nearly global scale where it has largely displaced *Nosema apis* as the predominant microsporidian parasite of honey bees (Anderson and Giacon, 1992; Chen et al., 2008, 2009; Fries, 2010). Because of the recency of the host and range expansion of *N. ceranae*, the pathogenicity and long-term effects on colonies is not yet clearly defined (Fries, 2010). A number of molecular-based diagnostic assays have been developed to detect and quantify *N. ceranae* DNA (Higes et al., 2006; Klee et al., 2007; Martin-Hernandez et al., 2007; Chen et al., 2008, 2009; Bourgeois et al., 2010; Hamiduzzaman et al., 2010; Traver and Fell, 2011a,b). However, collectively these studies use varied tissues for DNA extraction and comparisons between studies are difficult. Also, no direct comparisons of tissues for the same bee have been made in an attempt to determine which tissue is optimal. Hence, we sought to determine where *N. ceranae* can be detected on and in honey bees by combining techniques used in the various pathogen assays that have been published.

We measured *N. ceranae* levels in both individual bees ($n = 30$ from each of 3 colonies) and pooled samples ($n = 30$ bees from each of 30 colonies) in order to determine levels of individual and colony variation. Four types of samples were taken from each bee: whole bee wash water, head/thorax, midgut, and hindgut. Forceps were washed in 100% ETOH between samples to remove any potential sample to sample contamination.

For individual bee samples, each bee was placed in a separate tube with 250 μL $dH_2O$ and agitated in a TissueLyser II (Qiagen, Inc.) at 9 rev/s for 1 min. The bee was removed and de-gastered and the wash water was stored on ice for later processing. The head and thorax were placed into a new tube on ice and cut into approximately 2 mm pieces with dissecting scissors. The midgut and hindgut were dissected and placed separately into two additional tubes on ice. A total of 600 μL $dH_2O$ was added to each tissue sample along with 50 μL 1 mm zirconium silicate grinding beads (Next Advance, Inc., Averill Park, NY). Concentrated (3.0%) $H_2O_2$ was added in volume appropriate to achieve a final concentration of 0.3% to facilitate spore germination during a 15 min incubation at room temperature. To avoid spore settling and clumping, samples were agitated on an orbital plate shaker 15 min at 250 rpm.

In pooled samples, 30 bees were placed in a 50 mL conical tube with 15 mL $dH_2O$ (i.e., wash water) and agitated at 175 rpm on an orbital shaker at room temperature. Bees were then removed, degastered and dissected as described above. Concentrated (3.0%) $H_2O_2$ was added to the wash water in volume appropriate to achieve a final concentration of 0.3%. The solution was then incubated at room temperature with agitation on an orbital shaker at 175 rpm for 15 min. A 100 μL aliquot of wash water was then transferred to tubes with 50 μL beads. Pooled heads and thoraces for each set of bees were homogenized in 30 mL $dH_2O$ in a glass homogenizer. Addition of $H_2O_2$ and incubation were the same as described above. A 100 μL aliquot of the homogenized solution was transferred to tubes with 50 μL beads. Midguts and hindguts were pooled in groups in sets of 15 in individual tubes. A total of 600 μL $dH_2O$ was added to each set of 15 tissue samples as described above. Addition of $H_2O_2$ and incubation were also the same as described above.

After incubation in $H_2O_2$, all samples (pooled and individual) were homogenized in the TissueLyser II for 4 min at 30 rev/s, as per manufacturer instructions. A 100 μL aliquot of each sample was transferred to a 1 mL deep-well plate for further processing

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according to Bourgeois et al. (2010) with the following exceptions. Reagent volumes were scaled to the 100 μL starting sample volume (i.e., half volumes from published protocol) and spin times were changed to 20 min @ 4800*g* to accommodate sample processing in 96-well format.

Detection and quantification were performed on a StepOne™ Real-time PCR System (Applied Biosystems, Carlsbad, CA). Protocols for FAST PCR and reagent specifications followed those described by Bourgeois et al. (2010). Modifications to the protocol were made by elimination of primers and probe reagents for *N. apis* and addition of distilled water to adjust the reaction volume to 12.5 μL. All other reagents and specifications remained the same. All samples were run in duplicate and were directly quantified by comparison to a standard curve of known levels of *N. ceranae* DNA copy number. Standards were generated from serial dilutions of a cloned PCR product of the target sequence from *N. ceranae* (Bourgeois et al., 2010).

Data were converted from copy number to Nosema individuals per bee based on the formula defined in Bourgeois et al. (2010). Data were tested for homogeneity of variance using Levene's test in PROC ANOVA with SAS 9.2 (SAS Institute, Inc., Cary, NC), variances were unequal, and were subsequently log-transformed. Transformed data were analyzed using PROC GLM in SAS 9.2. *P*-values were calculated for comparisons by colony and sample type ($p_{diff}$) when the GLM results were significant. Correlations between tissue types were calculated using Pearson's correlation coefficient in PROC CORR.

Infestation levels of *N. ceranae* varied among sample groupings (individual bees versus pools of 30 bees; $P < 0.0001$) and among sample types (Fig. 1; $P < 0.0001$). Surprisingly high levels of *N. ceranae* were found in both wash water and head/thorax samples among both individual and pooled samples, although the levels were lower than those found in midgut and hindgut samples. Wash water removed *Nosema* DNA (presumably spores) that was located externally on the bees. These spores are likely obtained by the bee through incidental contact with fecal matter on comb and other bees. To our knowledge, this is the first report of *N. ceranae* levels measured from external samples of honey bees. After finding high levels of *N. ceranae* in the wash water, we sampled an additional 3 colonies (both pooled and individual samples) and washed the bees three times (as described above) to determine how effective the washing procedure was at spore removal. No significant differences were found ($P = 0.8574$) although overall concentrations did decrease in the second wash. Although not significantly differentiated, the third wash samples showed an increase in *N. ceranae* levels. The majority of these samples also had some cloudiness in the post-wash water likely due to partial disruption of the abdominal contents which include considerably higher levels of *N. ceranae*.

The primary infection route of *N. ceranae* is via the midgut after ingestion, hence head/thorax combined samples could contain recently ingested spores. Mesenteric samples contained the highest levels of *N. ceranae* overall, with the least amount of variance among sampling groups (Fig. 1). The molecular assay used here is capable of detecting DNA from both vegetative cells and spores. Vegetative cells proliferate and sporulate in the ventricular tissue (Higes et al., 2007), so higher levels in this tissue were expected. The hindgut is expected to contain primarily mature spores, but the spore content is highly dependent upon the recency of defecation and hence is more likely to show greater variation compared to that of the midgut (Fig. 1).

Differences among individual and pooled samples were evident ($P < 0.0001$; Fig. 1). There were differences in the concentration of the initial homogenates (i.e., the homogenization step for each sample type; water, head/thorax, midgut, and hindgut), however this was accounted for when sample values were converted from PCR results (number of target sequence copies/PCR reaction) to number of *Nosema* genomes/bee (hereafter denoted as Nosema/bee). Midgut homogenates were proportionately more (10X) concentrated in pooled samples (30 bees/1.2 mL 0.3% $H_2O_2$) than individual bees (1 bee/600 μL 0.3% $H_2O_2$). This could potentially affect the downstream chemistry of the extraction; however the protocol was designed such that large ranges in tissue sizes are equally efficiently extracted. Hindgut homogenates also differed in concentration, but were not as markedly differentiated between the sample groupings. The real-time PCR assay amplifies a wide range of sample concentrations and should have been unaffected.

In all cases, midgut and hindgut infestations were correlated (C1: $R^2 = 0.50$, $P = 0.005$, C2: $R^2 = 0.72$, $P < 0.0001$, C3: $R^2 = 0.64$, $P = 0.0002$, Pooled: $R^2 = 0.73$, $P < 0.0001$), but were also differentiated among the pooled samples ($P = 0.001$). A relationship between midgut and hindgut samples is logical, as Nosema spores are shed

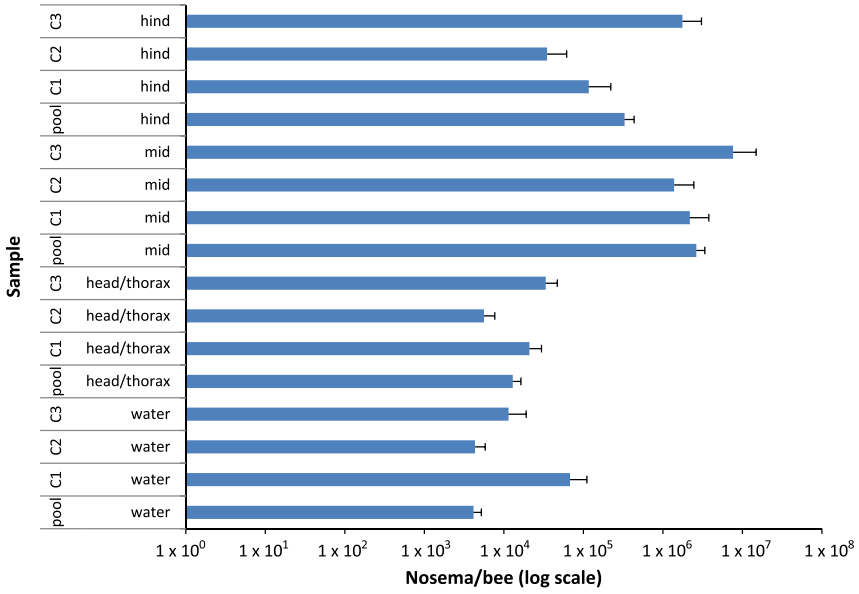


**Fig. 1.** Infestation levels of *Nosema ceranae* in honey bees processed as individuals ($n = 30$/colony) and as pooled samples ($n = 30$ colonies including 30 bees/colony pooled). Pool – pooled samples. C1, C2, and C3 – colony 1, 2, and 3, respectively. Bars represent mean ± SE.

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into the lumen of the midgut, enter the hindgut and eventually are dispersed via defecation.

Based on our results, we recommend that the first step in processing samples for molecular-based detection of *N. ceranae* samples be washing in $dH_2O$ to remove spores that may contaminate the external surface of the bee since dissected tissue may come in contact with the bee's external surface. Also, we recommend that the midgut be used as the tissue of choice. Midgut samples showed the least variance among samples and hence have a greater reliability. Standardization of sample preparation techniques can reduce the variability both within and among studies. This would provide necessary information for a more accurate comprehensive view of *N. ceranae* infestations of honey bee colonies and the relationship to colony losses.

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## References

Anderson, D.L., Giacon, H., 1992. Reduced pollen collection by honey bee (Hymenoptera: Apidae) colonies infected with *Nosema apis* and sacbrood virus. J. Econ. Entomol. 85, 47–51.

Bourgeois, A.L., Rinderer, T.E., Beaman, L.D., Danka, R.G., 2010. Genetic detection and quantification of *Nosema apis* and *N. Ceranae* in the honey bee. J. Invert. Pathol. 103, 53–58.

Chen, Y., Evans, J.D., Smith, I.B., Pettis, J.S., 2008. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J. Invert. Pathol. 97, 186–188.

Chen, Y.P., Evans, J.D., Murphy, C., Gutell, R., Zuker, M., Gundensen-Rindal, D., Pettis, J.S., 2009. Morphological, molecular, and phylogenetic characterization of *Nosema ceranae*, a microsporidian parasite isolated from the European honey bee, *Apis mellifera*. J. Eukaryot. Microbiol. 56, 142–147.

Fries, I., 2010. *Nosema ceranae* in European honey bees (*Apis mellifera*). J. Invert. Pathol 103 (Suppl. 1), S73–S79.

Hamiduzzaman, M.M., Guzman-Novoa, E., Goodwin, P.H., 2010. A multiplex PCR assay to diagnose and quantify Nosema infections in honey bees (*Apis mellifera*). J. Invert. Pathol. 105, 151–155.

Higes, M., Garnery, L., Martin-Hernandez, R., Meana, A., 2007. Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). J. Invert. Pathol. 94, 211–217.

Higes, M., Martin-Hernandez, R., Meana, A., 2006. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. J. Invert. Pathol. 92, 81–83.

Klee, J., Besana, A.M., Genersch, E., Gisder, S., Nanetti, A., Tam, D.Q., Chinh, T.X., Puerta, F., Ruz, J.M., Kryger, P., Message, D., Hatjina, F., Korpela, S., Fries, I., Paxton, R.J., 2007. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. J. Invert. Pathol. 96, 1–10.

Martin-Hernandez, R., Meana, A., Prieto, L., Salvador, A.M., Garrido, B., Higes, M., 2007. Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. Appl. Environ. Microbiol. 73, 6331–6338.

Traver, B.E., Fell, R.D., 2011a. *Nosema ceranae* in drone honey bees (*Apis mellifera*). J. Invertebr. Pathol. 107, 234–236.

Traver, B.E., Fell, R.D., 2011b. Prevalence and infection intensity of Nosema in honey bee (*Apis mellifera* L.) colonies in Virginia. J. Invert. Pathol 107, 43–49.

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