# Genetic detection and quantification of *Nosema apis* and *N. ceranae* in the honey bee

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ABSTRACT

The incidence of nosemosis has increased in recent years due to an emerging infestation of *Nosema ceranae* in managed honey bee populations in much of the world. A real-time PCR assay was developed to facilitate detection and quantification of both *Nosema apis* and *N. ceranae* in both single bee and pooled samples. The assay is a multiplexed reaction in which both species are detected and quantified in a single reaction. The assay is highly sensitive and can detect single copies of the target sequence. Real-time PCR results were calibrated to spore counts generated by standard microscopy procedures. The assay was used to assess bees from commercial apiaries sampled in November 2008 and March 2009. Bees from each colony were pooled. A large amount of variation among colonies was evident, signifying the need to examine large numbers of colonies. Due to sampling constraints, a subset of colonies (from five apiaries) was sampled in both seasons. In November, *N. apis* levels were 1212 ± 148 spores/bee and *N. ceranae* levels were 51,073 ± 31,155 spores/bee. In March, no *N. apis* was detected, *N. ceranae* levels were 11,824 ± 6304 spores/bee. Changes in *N. ceranae* levels were evident among apiaries, some increasing and other decreasing. This demonstrates the need for thorough sampling of apiaries and the need for a rapid test for both detection and quantification of both *Nosema* spp. This assay provides the opportunity for detailed study of disease resistance, infection kinetics, and improvement of disease management practices for honey bees.

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## 1. Introduction

The prevalence of the microsporidian *Nosema apis* has remained high in managed honey bee colonies throughout the US and Europe (Bailey, 1981; Matheson, 1993). Until very recently, all *Nosema* infections of managed honey bees were thought to have been caused by *N. apis*. A recent report identified *Nosema ceranae* in European honey bee samples across the US from 1995–2007 (Chen et al., 2008). Other reports have also identified *N. ceranae* in honey bees in much of the world (Fries et al., 2006; Higes et al., 2006, 2007; Huang et al., 2007; Topolska and Kasprzak, 2007; Chauzat et al., 2007; Williams et al., 2008) as early as 1998 (Paxton et al., 2007). *N. ceranae* had previously been thought to be host-limited to *Apis cerana* but is now found infesting *Apis mellifera* (Fries et al., 1984, 1996). This is problematic for beekeepers because *N. ceranae* has a different seasonal phenology than *N. apis*, causing more significant problems for beekeepers in summer months and in warm climates (Martin-Hernandez et al., 2007).

Symptoms of nosemosis caused by *N. apis* are more easily observed in honey bee colonies which show large numbers of dead bees and diarrhea spotting at hive entrances evidencing digestive

disorders of adults. Symptoms of *N. ceranae* infestations 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 productivity. For both species, spores are spread through infected feces, stored pollen, and corbicular pollen, which affect food supplies in the colony (Rinderer and Sylvester, 1978; Malone et al., 1995; Malone and Stefanovic, 1999; Higes et al., 2007; Martin-Hernandez et al., 2007; Higes et al., 2008a,2008b). Only adult bees are susceptible, and individual bees do not exhibit any external signs of infection. This makes rapid identification difficult for beekeepers. Species-specific identification is important for beekeepers to enable them to properly time control treatments. *Nosema* spp. spores are identifiable by microscopy. However, in order to distinguish between the two species, detailed and tedious morphological evaluation of spores is required.

Genetic assays have been developed that distinguish *N. apis* from *N. ceranae*, but do not quantify spore levels in a single reaction (Higes et al., 2006; Martin-Hernandez et al., 2007; Chen et al., 2008). Real-time PCR is a well-established technology for the use of pathogen detection and quantification in heterogeneous samples in a variety of taxa (Bilodeau et al., 2003; Yan et al., 2009; Kurkela and Brown, 2009). The goal of this project was to develop a real-time PCR assay that simultaneously detects both *N. apis* and *N. ceranae* and quantifies spore levels for individual bees or pooled

samples. A secondary goal was application of the assay to measure colony-level variation of *Nosema* spp. infestations over two seasons.

## 2. Materials and methods

### 2.1. Assay development and validation – DNA extraction

Worker bees experimentally infected with pure *N. apis* spores and other worker bees diagnosed with microscopy as positive for *N. ceranae* spores (i.e., from natural infection) were obtained from Kentucky State University (T. Webster). DNA was extracted from whole abdomens of individual bees or pooled bees with modifications to the protocol of Higes et al. (2006). Samples were initially homogenized with a mortar and pestle with 1000 μL deionized dH$_2$O per bee and vortexed. For pooled samples, the entire homogenate was centrifuged for 6 min. at 800g. The supernatant was then removed and the pellet resuspended in 5 mL dH$_2$O. For both individually processed and pooled samples, 200 μL of the homogenate or resuspended pellet was then transferred to a new tube and centrifuged at 14,000g for 2 min. The supernatant was removed and the pellet resuspended in 200 μL 0.3% H$_2$O$_2$ and incubated at room temperature for 15 min. Lysis Buffer (100 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 1% SDS) and 100 mg 1 mm glass beads were then added to each sample and homogenized for 3 min at speed 8 in a Bullet Blender (Next Advance, Inc., Averill Park, NY) and then treated with 80 μL Proteinase K (10 mg/mL) at 70 °C for 10 min. After Proteinase K treatment, 7.5 M NH$_4$OAc was added for protein precipitation, followed by isopropanol precipitation, 2 × 70% EtOH washes, and lyophilization. Pure genomic DNA was rehydrated in Millipore filtered and deionized dH$_2$O and stored at 4 °C.

### 2.2. Cloning

For real-time PCR analysis of *Nosema* spp. infestation levels, a recombinant plasmid containing the cloned PCR product of each species-specific product was generated using the appropriate primers for each gene (Table 1) and Taqman Fast Universal Master Mix (Applied Biosystems, Carlsbad, CA). The PCR products were inserted into the pZero-BluntII cloning vector (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Cloned PCR products were sequenced with the GenomeLab™ DTCS kit (Beckman Coulter, Fullerton, CA) for dye-terminator cycle sequencing as per manufacturer's instructions. Sequencing was performed on the CEQ 8000 Genetic Analyzer (Beckman Coulter). Sequences were then subjected to BLAST search in the GenBank database and showed 100% homology with the target sequences.

### 2.3. Fast real-time PCR amplification and calibration

The complete sequences of the small subunit ribosomal RNA, internal transcribed spacer and large subunit ribosomal RNA gene for both *N. ceranae* and *N. apis* were obtained from GenBank (Table 1). The two sequences were aligned in BioEdit v 7.0.9 (Hall, 1999) and primers and probes were selected in regions of the sequences that were species-specific using Beacon Designer software (Premier Biosoft, Palo Alto, CA; Table 1). The assay was developed for co-amplification of *N. ceranae* and *N. apis* DNA in each reaction. All real-time PCR reactions were performed on a StepOne™ real-time PCR System (Applied Biosystems, Carlsbad, CA). Each 12.5 μL reaction included 2 pmol of primers and probe (FAM/BHQ-1; Biosearch Technologies, Inc, Novato, CA) for *N. apis* and 3 pmol of primers and probe (JOE/BHQ-1; Biosearch Technologies) for *N. ceranae*, dH$_2$O, 1X Taqman Fast Universal PCR Master Mix (no Amperase® UNG) (Applied Biosystems, Carlsbad, CA) and 300 ng

**Table 1**
Primer and probe specifications for real-time PCR amplification of *Nosema apis* and *N. ceranae*.

| Species | Primers | Product size (bp) | Location |
|---|---|---|---|
| *Nosema apis* genbank: U97150 | For: GCCCTCCATAATAAGAGTGTCCAC Rev: ATCTCTCATCCCAAGAGCATTGC Probe: ACTTACCATGCCAGCAGCCAGAAGA | 142 | 4364 |
| *Nosema ceranae* genbank: DQ486027 | For: AAGAGTGAGACCTATCAGCTAGTTG Rev: CCGTCTCTCAGGCTCCTTCTC Probe: ACCGTTACCCGTCACAGCCTTGTT | 104 | 218 |

template DNA (1 μL of mixed honey bee, *Nosema* spp. and pollen DNA). The optimized amplification protocol was as follows: 20 s at 95 °C followed by 40 cycles of 1 s at 95 °C and 20 s at 63 °C. Amplification products were quantified by comparison of experimental $C_t$ (threshold cycle – defined as the PCR cycle where an increase in fluorescence first occurred) levels with those of a standard curve. The standard curve was generated from two replicates of serial dilutions of recombinant plasmid DNA as described above. Standards were run on each plate of samples as well as a no-template control. The coefficient of variation was calculated for replicates of all samples to ensure repeatability of amplification results (SAS v. 9.13). Amplification efficiency of each reaction was calculated based on the slope of the standard curve in the StepOne software.

Real-time PCR measures copies of a particular DNA fragment. In the case of the *Nosema* spp. assay, both vegetative and spore DNA are represented in the sample. Spore equivalents were determined by calibrating the real-time PCR results to actual spore counts achieved with microscopy on an Olympus BH-2 microscope (Olympus America, Inc., Center Valley, PA) at 400× magnification using a Bright-Line haemocytometer (Hausser Scientific, Horsham, PA). A total of 46 samples were quantified with real-time PCR and microscopy. The data were then subjected to linear regression (SAS v. 9.1.3) and the subsequent equation was used for the calibration.

### 2.4. Application of assay – survey data

A total of 50 bees per colony were collected from 104 colonies from commercial beekeepers in MS and AR in November 2008 and from 48 colonies from the same states in March 2009. Samples were placed on ice, then stored at −20 °C until processing. All 50 bees sampled from each colony were degastered and pooled. Each colony sample was then tested for the presence of *N. apis* and *N. ceranae* with the real-time PCR assay. A Mixed Model (SAS v 9.1.3) ANOVA was used to compare results for colonies collected in November and March, using apiary as a fixed effect.

## 3. Results

DNA extraction and real-time PCR amplification required 2.5 h per 24 samples. A BLAST search (Altschul et al., 1997) of each primer and the probe were homologous to only *N. ceranae* and *N. apis*. Amplification efficiencies of *N. apis* reactions were 92% and *N. ceranae* were 100.0% when run in single species reactions and when multiplexed (Fig. 1). Sensitivity of the detection assay was determined by amplification with real-time PCR. A set of 10-fold serial dilutions of purified *N. ceranae* and *N. apis* DNA (concentrations ranging from 500 pg/L to 50 ag/L) was amplified yielding amplifi-
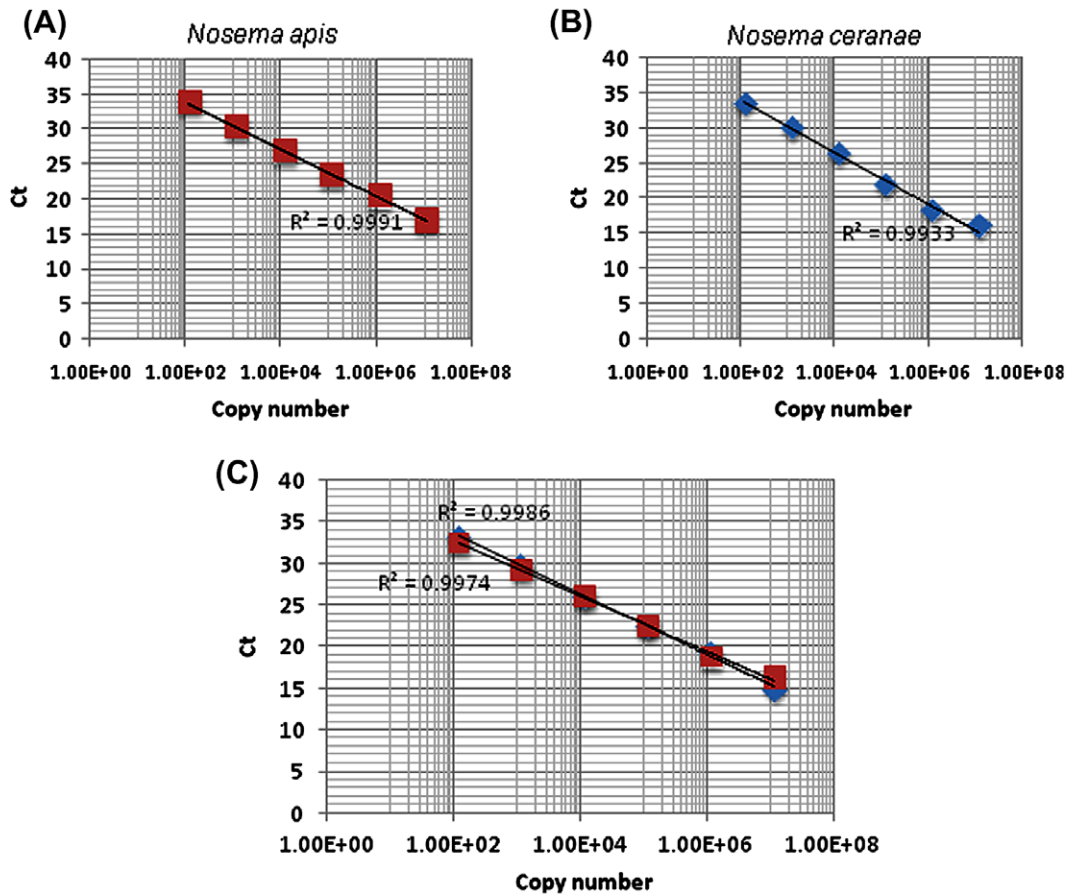
**Fig. 1.** Standard curves for real-time PCR assay developed for detection of *Nosema apis* and *N. ceranae*. (A) Standard curve based on six standards for *N. apis*. (B) Standard curve based on six standards for *N. ceranae*. (C) Standard curves for *N. apis* and *N. ceranae* co-amplified in the same reaction (i.e., multiplexed). $C_t$ is the cycle at which fluorescence was above background levels.
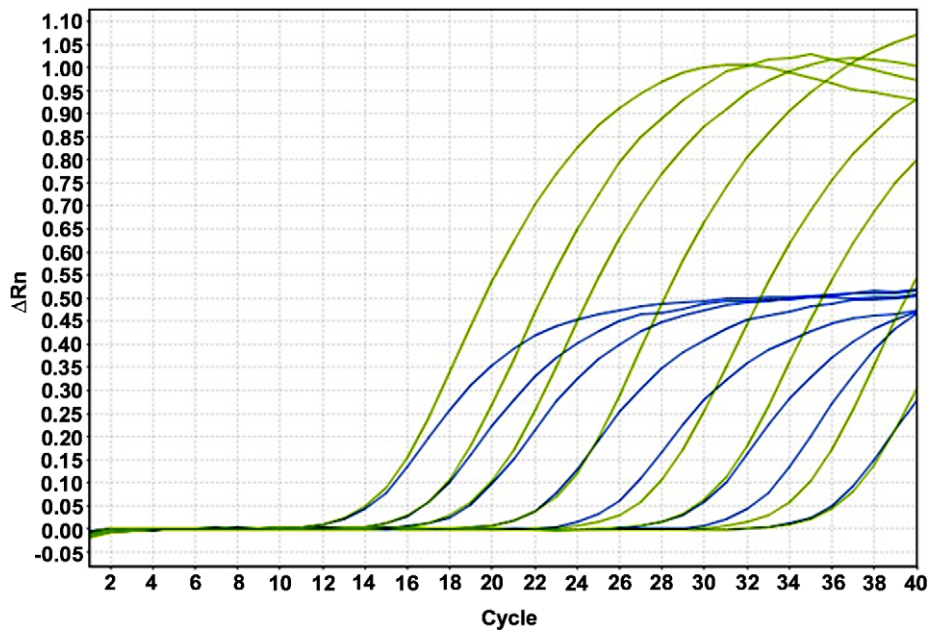


**Fig. 2.** Real-time PCR fluorescence curves for eight *Nosema apis* and *N. ceranae* standards co-amplified. Standard template was added in 10-fold serial dilutions from 500 pg template to 50 ag template DNA, representing $1.18 \times 10^8$ to 11.8 copies of the target fragments.

cation products for all concentrations (Fig. 2). Hence, the lower detection limit for each primer set was 50 ag which is the equivalent of 11.8 copies of *N. ceranae* or *N. apis* DNA fragment. Copy number per genome was determined based on amplicon size and

preliminary evidence for 10 copies of the gene in the *N. ceranae* genome (J. Evans, personal communication.). Either this set or a subset of standards was used in all subsequent real-time PCR reactions (Fig. 2).

Due to the protocol used for DNA extraction, conversion factors from copies/µL to spore equivalents/bee were calculated for both individual and pooled samples as follows:

Individual samples:

$$\frac{\text{Num. Individual } Nosema}{\text{bee}} = \frac{\left(\frac{a\ Nosema\ \text{copies}}{\mu\text{L PCR}}\right)\left(\frac{25\ \mu\text{L}}{0.2\ \text{bee}}\right)}{10\ \text{copies per genome}}$$

where $a$ = copy number from real-time PCR, and 0.2 bee represents the 200 µL aliquot taken from the 1 mL homogenate.

Pooled samples:

$$\frac{\text{Num. Individual } Nosema}{\text{bee}} = \frac{\left(\frac{a\ Nosema\ \text{copies}}{\mu\text{L PCR}}\right)\left(\frac{25\ \mu\text{L}}{(0.04)\ (b\ \text{bees})}\right)}{10\ \text{copies per genome}}$$

where $a$ = copy number from real-time PCR, b = number of bees pooled in the DNA extraction.

Microscope-based spore counts correlated well ($R_2$ = 0.7431, $P < 0.0001$) with real-time PCR results. The mean coefficient of variation for genomic PCR products was 2.50% and 2.91% for *N. apis* and *N. ceranae*, respectively. The equation used for calibration of real-time PCR results to spore counts is: $y = 0.0265x + 0.0791$ where $x$ = spore equivalents per bee from the real-time PCR results.

Mean levels of *Nosema* spp. levels in commercial colonies were 303 ± 38 and 12,768 ± 8106 spore equiv. per bee (*N. apis* and *N. ceranae*, respectively). Colony to colony variation was large for *N. ceranae* only in November (Fig. 3) and April. Mean spore levels varied by collection date. In November, *N. apis* levels were 1212 ± 148 spores/bee and *N. ceranae* levels were 51,073 ± 31,155 spores/bee.
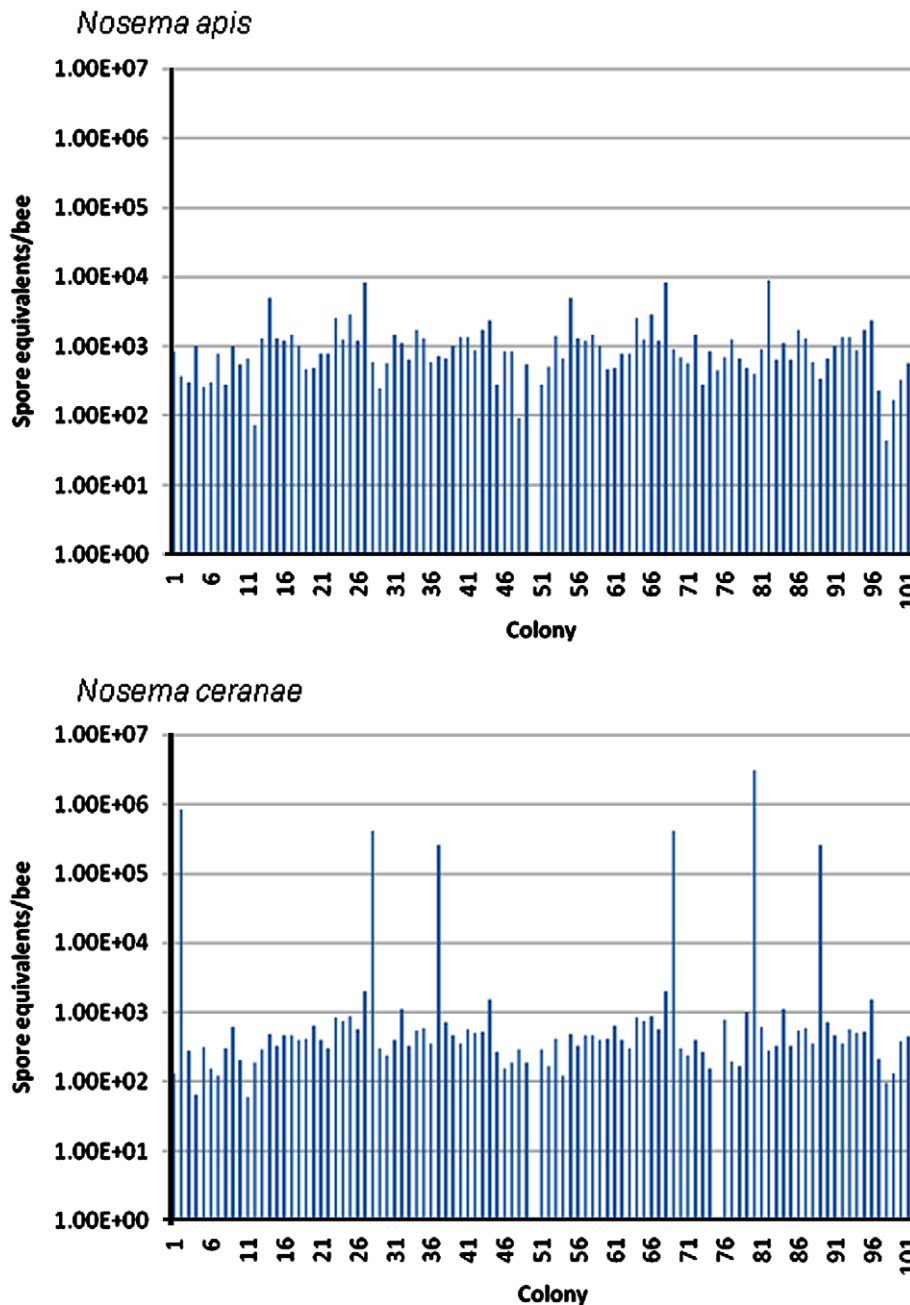


**Fig. 3.** *Nosema ceranae* spore equivalents per bee generated from real-time PCR for 104 honey bee colonies collected in November 2008. Data have been calibrated with spore counts generated from microscopy data.

In March, no *N. apis* was detected, *N. ceranae* levels were 11,824 ± 6304 spores/bee. Spore levels of *N. ceranae* in colonies measured in both November and March varied by apiary ($P = 0.0087$).

## 4. Discussion

Development of a sensitive, quantitative, and multiplexed real-time PCR assay for both *N. apis* and *N. ceranae* provides a tool for assessing and monitoring levels of *Nosema* infection in honey bees in both a research and commercial environment. There are no previously published assays capable of simultaneous identification and quantification of both species in the same reaction (Higes et al., 2006; Chen et al., 2008). This capability reduces costs associated with PCR reagents and halves the time for processing, without compromising the sensitivity of detection of either species.

The genetic assay described here enables sensitive detection of as few as 1 *Nosema* spp. spore per bee which is well-beyond the capability of standard microscopy procedures. Microscopy results can be improved by increasing the concentration of the sample. However, this requires either more time for processing (i.e., dissection of the ventriculum) or results in increased levels of debris/pollen in concentrated homogenates which have the potential to obstruct a clear view of spores on the haemocytometer. Samples can also be processed much more quickly with real-time PCR than with a microscope. Fast Real-time PCR technology facilitates both rapid and higher throughput sample processing, allowing 48–96 samples to be processed completely within a few hours.

The high correlation between *Nosema* spp. counts generated with microscopy and with the genetic assay demonstrates the high accuracy of the assay. The PCR assay generated higher counts of *Nosema* spp. than did microscope counts. This is likely attributable to the presence of vegetative *Nosema* spp. parasites present in the gut epithelium of the bees (Higes et al., 2007, 2008a,b). Ratios of vegetative cells to spore levels can change throughout a microsporidian infection. Experimental infection with *N. ceranae* demonstrated that mature spores are evident by day 3 post-infection (Higes et al., 2007). In later stages of infection, mature spores will be produced in much higher proportion when compared to the vegetative cells. In samples used for development of this assay (individual and pooled), the ages of bees sampled varied. A random sample of the colony should give a random age distribution and a reasonable representation of colony-level infestation. However the random nature of the sample may also contribute to the variation seen among both individuals and colonies, as older bees are more likely to have been exposed to both *N. apis* and *N. ceranae* and the bees sampled may be have different stages of infection. The ratio of vegetative to spore material will vary with stage of infection. Further investigation into the kinetics of *Nosema* spp. infection will better elucidate this relationship.

When applied to a selective breeding program for disease resistance, this assay can be used to track the early stages of infection and provide the sensitivity necessary to detect resistance differences among bee stocks.

As is true for other pathogen detection assays, real-time PCR can be readily applied to disease management, in this case, for nosemosis by service laboratories for beekeepers. Monitoring of *Nosema* spp. levels throughout the year, or at least at times of likely high infestation may help to reduce the need for anti-microsporidian treatment. The example shown here of repeated samples of the same colonies in the Fall and Spring yielded varying results. Colony to colony variation was high, yet differences were apparent between apiaries. Hence, if treatment is warranted, the need may be specific to apiaries. This benefits the beekeepers as a cost savings and benefits the honey bees by reducing the amount of chemical residues in the hive. A shift from detection of both *N. apis* and

*N. ceranae* in the Fall to only *N. ceranae* in the Spring suggests that *N. ceranae* is increasing in prevalence. This is supported by data from other commercial colonies and research colonies sampled throughout the southern US (data not shown).

Real-time PCR has been used successfully for pathogen identification and quantification for numerous pathogens affecting a variety of host species. This technology enables simultaneous detection and quantification of *N. apis* and *N. ceranae* that cannot be accomplished by any other currently used detection assay. Large numbers of samples can be processed in a relatively small amount of time enabling more thorough examination of infestation levels among apiaries. This assay provides the opportunity for detailed study of disease resistance, infection kinetics, and improvement of disease management practices for honey bees.

## Acknowledgments

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