



## Preservation And Processing Methods For Molecular Genetic Detection And Quantification Of *Nosema ceranae*

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### Summary

The prevalence of *Nosema ceranae* in managed honey bee colonies has increased dramatically in the past 10 - 20 years worldwide. A variety of genetic testing methods for species identification and prevalence are now available. However sample size and preservation method of samples prior to testing have not been thoroughly explored. Here, we test sample sizes ranging from 10 to 100 bees per colony and the suitability of ethanol, isopropanol, ice, and freezing as preservation methods prior to genetic testing. Larger sample sizes coupled with freezing/cold storage without the addition of chemical preservatives improve the reliability and suitability of samples for genetic testing and are recommending for diagnostic testing purposes.

**Keywords:** *Nosema ceranae*, real-time PCR, genetic testing

### Introduction

Prevalence of the microsporidian *Nosema ceranae* has increased in managed honey bee colonies globally since it expanded its host range from *Apis cerana* to include *A. mellifera* in the 1990's or perhaps earlier (Chen et al. 2008). Based on evidence from diagnostic sampling, it appears that *N. ceranae* has displaced *N. apis* as the dominant microsporidian parasite of honey bees in the United States (Chen et al. 2009). While both species of *Nosema* have deleterious effects on honey bee colonies, their seasonality and pathologies differ markedly. 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, mostly during winter and early spring. Symptoms of *N. ceranae* infestations are less detectable and consist primarily of poor colony growth and dwindling. *Nosema ceranae* can reach high infestation intensities

in both winter and summer. Both species cause decreases in honey production, foraging activity, and pollination productivity. For both species, spores are spread through infected feces which can contaminate combs, stored food, and even corbicular pollen (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, Higes et al. 2008b). Individual bees do not exhibit any external signs of infection. Even examination of the mid-gut without a microscope is inconclusive. This makes diagnostic sampling in a laboratory important for beekeepers to enable them to properly time control treatments. Diagnostic testing methods use either microscopy or molecular genetic detection and molecular quantification.

Microscopic analysis of the midgut contents is the traditional method for the detection and quantification of *Nosema* spores. Simple identification of *Nosema* sp. spores is probably of little value. Almost every honey bee colony seems to have some level of *Nosema ceranae* infection. Fortunately, honey bee colonies seem to tolerate a low level infection rather well. Beekeepers have learned that only when the number of spores rises to about 10 million do the colonies need treatment to prevent damage. Hence, meaningful microscopic analysis involves the use of a haemocytometer to quantify the intensity of infestations.

The availability of molecular diagnostics has increased very recently which introduces a need for preservation methods that will provide consistent and accurate results. Microscopy techniques are more forgiving of various preservation methods since the spores are difficult to inadvertently degrade with preservation methods. However, molecular tests require that moderately high quality DNA be extracted from *Nosema* spores and vegetative material. Preservation fluids like formalin or isopropanol may inhibit quality DNA extraction and amplification.

Another requirement of diagnostic sampling (whether it is for microscopy or molecular testing) is analysis of samples having an adequate number of bees. Variation in the intensity of *Nosema* infestations among individual bees within a colony is high. For colony-level diagnostic evaluations, individual bees are pooled together and then examined. Hence, the number of bees used in the pooled sample may affect diagnostic test accuracy. Highly infested bees may be less common but the sample size must be large enough to include them in the proportion that they exist in the colony.

Here, we test four methods of sample preservation to determine which method yields the most accurate and consistent results from molecular analysis. We also examine the levels of individual variation within colonies and test the repeatability and accuracy of pooled samples of different sizes.

## Materials and Methods

### Sampling

Eight colonies were tested for the presence of *Nosema* spp. with microscopy. The colony with the highest infestation level was chosen and sampled. This colony had an infestation level of 4.3 million spores/bee. Approximately 2000 adult worker bees were collected and placed on ice. Groups of 300 Individual bees were counted and were assigned to one of four different treatment groups. The treatment groups, with different methods of sample preservation were: FRZR – storage at -20°C, POST – placed in a Styrofoam cooler with packs of blue ice for 2 days and then stored at -20°C (simulating samples sent to a diagnostic laboratory for evaluation), ETOH – storage in 70 % EtOH and ISOP – storage in

70% isopropanol. All samples were maintained in their respective treatments for 1 week prior to DNA extraction.

### DNA Extraction

All bees in each treatment group were thawed, degastered, and processed for DNA extraction either as individuals or as pooled samples. Individual DNA extraction followed the protocol described in Bourgeois et al. (2010). Briefly, this entailed grinding separated gasters in 1 mL dH<sub>2</sub>O per bee (i.e., making 1 mL of homogenate), concentration of 200 µL of the homogenate into a pellet, incubation of the pellet in H<sub>2</sub>O<sub>2</sub>, digestion in lysis buffer and proteinase K solution, precipitation of proteins with 7.5 M NH<sub>4</sub>OAc, and precipitation of genomic DNA with isopropanol. For the first 100 bees processed for each treatment, all extractions were processed individually (i.e., one 200 µL aliquot for DNA extraction) as per Bourgeois et al. (2010). The remaining homogenate of each bee was then divided into 4 additional 200 µL aliquots. These aliquots were used to generate pooled samples containing homogenates from 10, 30, 50, and 100 bees. Two hundred additional bees were homogenized in groups of 10. Sub-aliquots (200 µL per bee) of these samples were combined to generate additional pooled samples of 50 and 100 bees each. All pooled samples were centrifuged at 800 x g for six minutes. The supernatant was then removed and the pellet was resuspended in 5 mL dH<sub>2</sub>O. These samples were then processed in the same manner as the individual samples for DNA extraction.

### Molecular Genetic Analysis

Real-time PCR not only measures presence/absence of a target DNA sequence, but also quantifies the number of copies of that fragment. In the case of the *N. ceranae* assay, both vegetative and spore DNA are represented in the sample. Results are presented as “nosema/bee”, including both spores and vegetative material. 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 triplicate 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).

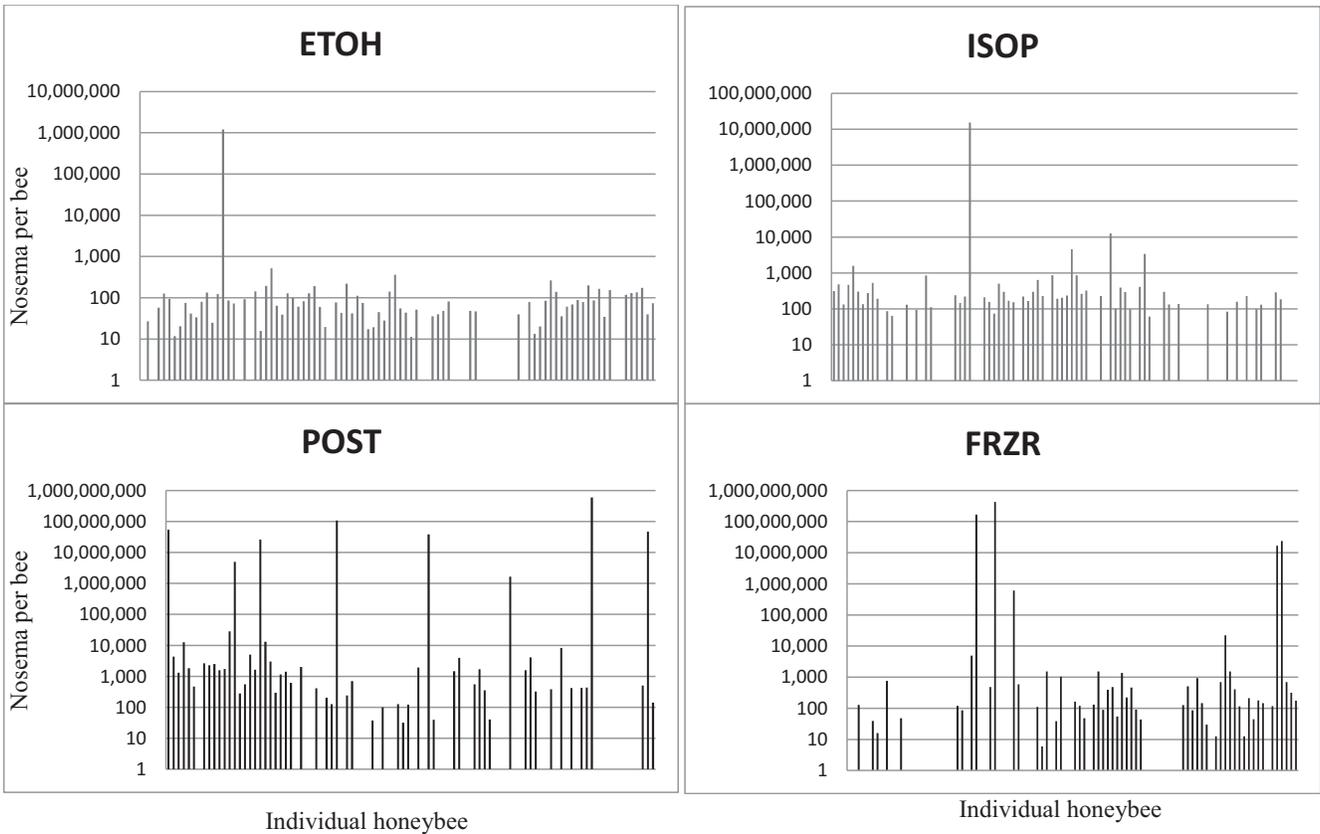
### Data Analysis

A Mixed Model (SAS v 9.1.3) ANOVA was used to compare results for honey bees preserved in the 4 treatments. Pooled data were also subjected to Mixed Model ANOVA, using treatment and pooling as fixed effects and Bonferonni correction for multiple comparisons. Data passed Levene’s test of homogeneity of variances ( $P > 0.05$ ). Correlations (Pearson) were determined in SAS v 9.1.3 with Proc CORR.

## Results and Discussion

Variation among individuals was evident for *N. ceranae* levels across all treatments (Figure 1). Of the individual bees tested for each treatment, only 1% in each of ETOH and ISOP treatments had *Nosema* levels greater than one million nosema/bee whereas POST and FRZR treatments yielded 8% and 5% of bees, respectively,

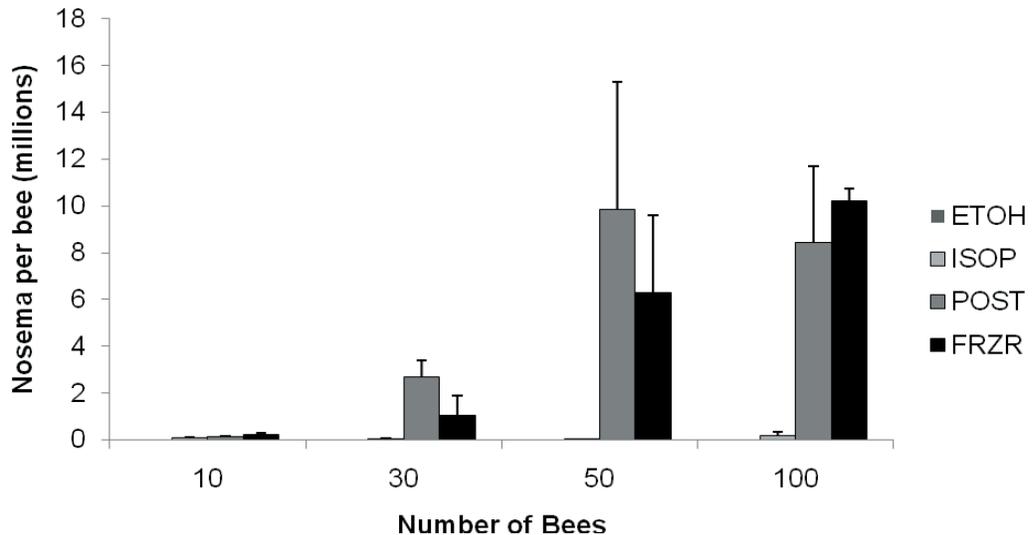
**Figure 1** - Levels of *Nosema ceranae* in individual bees preserved by four methods. Data were generated from molecular genetic analyses. Data are presented on a logarithmic scale to make lower concentrations visible. ISOP - isopropanol, ETOH, 70% ethanol, POST - postal shipping, FRZR - freezer.



with greater than one million nosema/bee. While isopropanol and ethanol are commonly used as preservatives, they may affect the recovery of high quality DNA from both sporulated and vegetative *Nosema*. Both types of preservatives at 70% concentrations may have deleterious effects on DNA quality and subsequently on amplification of DNA from samples that are used for quantification purposes. This is due to the high stringency and specificity that is inherent to real-time PCR technology. Any DNA degradation or inhibition of amplification will have a negative effect on the

results. Ethanol and isopropanol at 70% concentrations were selected because these are the concentrations commonly available and hence used by beekeepers. For research specimens, 95 - 100% ethanol is preferred for sample preservation to minimize DNA damage. However, this is cost prohibitive for beekeepers. Both POST and FRZR samples had higher levels of *N. ceranae* than either of the alcohol treatments (ETOH:  $P = 0.0016$  and  $P = 0.0142$ , respectively or ISOP:  $P = 0.0019$  and  $P = 0.0166$ , respectively; Figure 2) treatments. Immediate placement of samples on ice and

**Figure 2** - Variation among bees from four preservation treatments. Data were generated from molecular genetic analyses. ISOP - isopropanol, ETOH, 70% ethanol, POST - postal shipping, FRZR - freezer.



then storing at cold temperatures may have prevented degradation. No inhibitors were present in these samples, as no preservatives were used other than freezing temperatures.

Among POST and FRZR treated samples, the variation among individual bees was high. Levels of *N. ceranae* ranged from 0 to 59.6 million nosema/bee, with the majority of bees having levels below 10,000 nosema/bee. Individual-level variation within a colony may be related to the age of sampled bees and other factors. Older, foraging bees have higher levels of *N. apis* and *N. ceranae* than younger bees (Martin-Hernandez et al. 2007). However younger bees may still be exposed to contaminated materials such as fecal matter and retrieved pollen. Spores of *N. apis* are primarily transmitted through fecal matter and soiled comb (Higes et al. 2008b). Specific data on spore transmission of *N. ceranae* are not yet available (Fries 2010), but fecal matter and self-contaminated pollen are two likely sources .

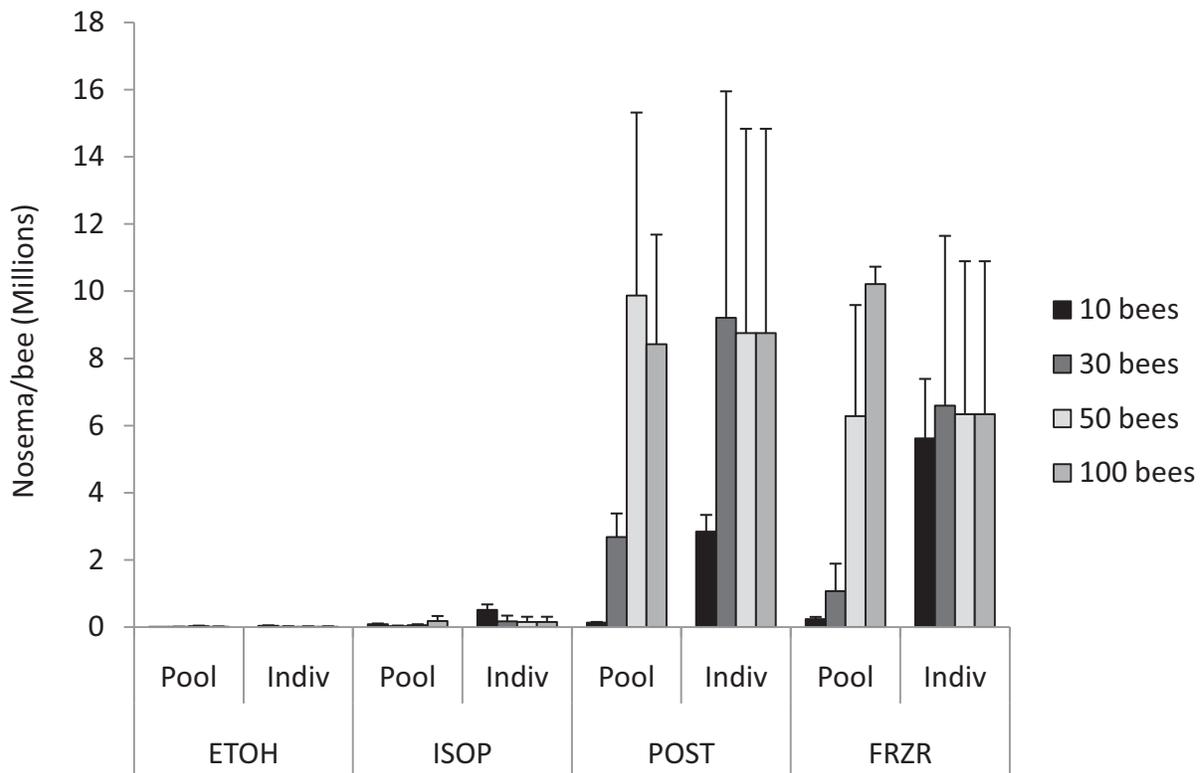
With high levels of individual variation within a colony, the question arises of how to adequately sample a colony for diagnostic purposes. Processing large numbers of individual bees is not time or cost effective. Pooled samples are typically used for diagnostic samples, however the number of bees in these pooled samples is different between laboratories. The first step in our evaluation of the sample size question was to test the reliability of pooled samples, i.e., how well do pooled samples represent the individuals from which they are taken. Levels of *N. ceranae* in bees used for both individual and pooled samples were highly correlated ( $R^2 = 0.7881$ ,  $p < 0.0001$ ). The second step was to test the consistency among different sample sizes and how different sets and sizes of pooled samples represented the colony. Among treatments, bees pooled in groups of 10 ( $1.13 \times 10^5 \pm 6.01 \times 10^4$  nosema/bee) had lower levels

of *N. ceranae* than groups of 50 and 100 bees ( $3.77 \times 10^6 \pm 1.83 \times 10^6$  nosema/bee;  $P = 0.0461$  and  $4.80 \times 10^6 \pm 1.60 \times 10^6$  nosema/bee;  $P = 0.0055$ , respectively after Bonferroni correction for multiple comparisons). Bees pooled in groups of 30 ( $9.47 \times 10^5 \pm 4.01 \times 10^5$  nosema/bee) also had lower levels than those grouped as 100 ( $P = 0.0308$ ; Figure 3). All other comparisons were non-significant ( $P > 0.05$ ). Ideally, the more bees sampled the better representation of infection levels within a particular colony. However, there were no differences between those bees grouped in lots of 50 or 100.

We used a colony that had an infection level of less than five million *Nosema*/bee. As the sample size increased, the standard error associated with multiple estimates decreased until, with 100 bee samples, the standard error was about 1/3 of the mean estimate. Probably, larger samples still would have a smaller standard error. However, for the purpose of determining whether apiaries should be treated for *N. ceranae* the accuracy obtained from 100-bee samples is sufficient while samples that are lower than 50 bees might lead to incorrect treatment decisions. With colonies that have levels of *N. ceranae* that meet the higher treatment thresholds of 10 million or 20 million nosema/bee, the standard error would be smaller since a larger proportion of the bees would be highly infested and the effects of individual bees on the pooled sample would be less. For colonies with fewer than five million nosema/bee the standard error may be larger but these colonies have infestations well below treatment thresholds.

Beekeepers and many bee scientists are accustomed making treatment decisions and recommendations based on nosema spores per bee rather than numbers of nosema DNA copies. Using the correlation between molecular and microscopic measures of nosema numbers in a previous study, a conversion factor (0.028) has been

**Figure 3** - Variation in levels of *N. ceranae* among pooled and individual honey bee samples, generated from molecular genetic analyses. ISOP - isopropanol, ETOH, 70% ethanol, POST - postal shipping, FRZR - freezer.



developed to convert molecular measures to spores/bee. However, the conversion from number of nosema/bee to spores/bee should be used cautiously, as the stage of infection in individual bees is unknown and the ratio of vegetative material to mature spores will vary with the stage of infection. A more conservative conversion factor that has been determined for samples consisting only of older/foraging bees (data not shown) is 0.195. We recommend using this conversion factor when samples are processed with molecular genetic analyses.

#### Conclusions and Recommendations

Placing bees on ice in the field and later moving them to a freezer is a cost-effective means of sample collection and provides high quality samples for molecular genetic testing.

Samples which are placed on ice in the apiary, then shipped cold with "blue ice" and frozen within two days, also will produce acceptable molecular genetic results.

Sample size has an effect on results. Larger samples provided more consistent estimates of colony infestation levels. For small colonies, we recommend sampling a minimum of 50 bees. For larger colonies we recommend 100 bees. One hundred bees will easily allow the determination of whether a colony meets economic thresholds for treatment that are established at 5 or more million spores per bee.

Use of these recommendations should ensure that sample size and sample quality are adequate for accurate determination of *N. ceranae* levels in managed honey bee colonies.

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