A new duplex qPCR assay for the quantification of honey bee (*Apis mellifera*) parasites *Nosema ceranae* and *N. apis* tested with low dose experimental exposure

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Abstract

Nosema ceranae and *N. apis* are microsporidian parasites that cause disease in the European honey bee. *Nosema* infection is identified as a potential cause of colony loss by beekeepers. Given the importance of *Nosema* infection in colony mortality and productivity, developing new and improved ways of detecting and quantifying infection loads is vital. We designed and tested a new duplex qPCR assay for the accurate quantification of both *N. ceranae* and *N. apis* utilising TaqMan chemistry and the new gBlock® method for the standards. The assay showed good linearity with natural *Nosema* infection, and strong correlation with microscopic spore counts. This new assay has high sensitivity and repeatability and was used to investigate *Nosema* infection in hive surveys and following low dose experimental exposure. In local hives, we found relativity low levels of *N. ceranae* and very little *N. apis* across three sites in Blacksburg, VA. A survey of two bee yards in West Virginia showed much higher levels of *N. ceranae,* but consistent low levels of *N. apis*. For the experiment, caged bees from two different hives were fed 100 *Nosema* spores (or a control solution). Exposed bees were collected after 2 or 5 days, and infection was quantified using the new assay. Given the low dose, infection levels were not 100%, with some exposed bees remaining *N. ceranae* free, while others only developed low level infection. This low dose exposure and subsequent infection status (low level infection or infection free) could provide new understanding of *Nosema* infection establishment.

Keywords

Nosema, *Nosema ceranae*, qPCR, *Apis mellifera*, infection

Introduction

The European honey bee (*Apis mellifera*) is a globally important insect pollinator, contributing over €14.6 billion to the European Union and $15 billion to the US economy (Calderone, 2012, Leonhardt *et al.*, 2013). While honey bees are vital economic pollinators, globally, honey bees face a range of threats. These threats include pesticide exposure (Di Prisco *et al.*, 2013, van der Zee *et al.*, 2015), land use changes, including loss of forage (Naug, 2009), and interactions with parasites and pathogens (Rosenkranz *et al.*, 2010, Evans and Schwarz, 2011, Gisder and Genersch, 2017). Multiple pathogens infect honey bees, including viruses (Gisder and Genersch, 2017), bacteria (Forsgren, 2010, Poppinga and Genersch, 2015), and microsporidia (Milbrath *et al.*, 2015, Goblirsch, 2018, Martín-Hernández *et al.*, 2018).

Microsporidia are obligate intracellular fungal parasites that cause disease in many insect species, including honey bees (Martín-Hernández *et al.*, 2017). *Nosema apis* and *N. ceranae* are the two main microsporidian species that infect honey bees (Milbrath *et al.*, 2015, Goblirsch, 2018, Martín-Hernández *et al.*, 2018). A third species, *Nosema neumanni* was identified in Ugandan honeybees in 2017 (Chemurot *et al.*, 2017). *Nosema ceranae* successfully jumped host from the Asian honey bee (*A. cerana*) into the European honey bee (Klee *et al.*, 2007), and now co-infections of *N. ceranae* and *N. apis* can occur (Klee *et al.*, 2007, Traver and Fell, 2011, Copley *et al.*, 2012, Milbrath *et al.*, 2015). *Nosema* infection impacts honey bees on the cellular level (including gene expression changes), along with

more obvious physical symptoms (Martín-Hernández *et al.*, 2017). While infection mechanisms for both *N. apis* and *N. ceranae* are similar, some symptoms of infection are markedly different. *Nosema apis* infection is most commonly associated with crawling bees, faecal spots on the hive, and decreased honey yield, while *N. ceranae* is linked to increased weakness and colony mortality, along with lower honey yields (Martín-Hernández *et al.*, 2018). Part of the weakness caused by *N. ceranae* is reduced flying ability and increased energetic stress (Mayack and Naug, 2009, Mayack and Naug, 2010). *Nosema* infection can negatively affect the ability of honey bees to successfully return to hives following forage flights (Kralj and Fuchs, 2010), and is frequently given as a cause of death in overwinter colony loss in surveys of beekeepers (vanEngelsdorp *et al.*, 2012, Lee *et al.*, 2015, Kulhanek *et al.*, 2017). However, *Nosema* infections are frequently detected in hives that are also infected with other honey bee pathogens, with interactions between pathogens detected (Ryba *et al.*, 2012, Traynor *et al.*, 2016, Gajda *et al.*, 2021), indicating there are likely complex interactions of factors affecting overwintering loss.

Current methods of *Nosema* identification and quantification include both microscopic and molecular techniques. Microscopic analysis is often used in *Nosema* infection studies, as it allows the number of spores per mL of extracted bee material to be calculated using a haemocytometer (Human *et al.*, 2013, Roberts and Hughes, 2015, Kurze *et al.*, 2018). Molecular methods, including PCR and qPCR, are utilised to obtain current infection levels in surveys and infection studies (Martín-Hernández *et al.*, 2007, Bourgeois *et al.*, 2010, Forsgren and Fries, 2010, Traver and Fell, 2011, Copley *et al.*, 2012). A number of qPCR assays exist for *N. ceranae* and *N. apis* using both TaqMan (Bourgeois *et al.*, 2010, Traver and Fell, 2011, Copley *et al.*, 2012) and SYBRGreen chemistry (Forsgren and Fries, 2010). Several duplex assays have also been developed (Bourgeois *et al.*, 2010, Traver and Fell, 2011); however, for this study, we chose to develop a new assay using a gBlock® gene fragment standard. The gBlock® standard allows a standardised, custom-designed, synthetic nucleotide fragment to be used for the absolute quantification of *Nosema*, which is highly reproducible and time efficient when compared to usual cloning procedures (Conte *et al.*, 2018).

Our goals with this study were two-fold. First, we developed and optimized a probe-based qPCR assay using a gBlock® standard, and second, we used this assay to examine honey bee infection levels in a hive survey and following low dose (100 spores) exposure to *Nosema ceranae* in a lab study. Previous *Nosema* studies have used much higher spore doses ($>10^4$ spores), resulting in high infection levels (Antúnez *et al.*, 2009, Forsgren and Fries, 2010, Roberts and Hughes, 2015, Li *et al.*, 2018). Huang *et al*. (2015) found that 10 days after exposure to >3000 *N. ceranae* spores 50% of inoculated worker bees were infected. We are ultimately interested in studying physiological factors that determine whether infections will become established following exposure, and therefore we chose a low dose and short time frame, which could result in more variable infection outcomes across individuals. Thus, to begin to explore responses to low doses and to test our new qPCR assay, we designed a study exposing honey bees from two hives to 100 spores of *N. ceranae* and quantified infection at days 2 and 5 after exposure.

Material and Methods

Nosema qPCR duplex assay, validation, and sample processing

Levels of *N. ceranae* and *N. apis* within pooled samples were calculated using a new qPCR TaqMan duplex assay adapted from Traver and Fell (2011). rRNA gene sequences for *N. ceranae* and *N. apis,* including the internal transcribed spacer and the small and large rRNA subunits, were downloaded from the GenBank database (Accession No. DQ486027.1 and U97150.1, respectfully) and used to design the qPCR assay and gBlock® standards. The duplex assay was designed using the PrimerQuest® Tool from IDT (Integrated DNA Technologies, Coralville, IA, USA). All primer and probe sequences were assessed for cross-dimers using the Multiple Primer Analyzer tool (ThermoFisherScientific, Waltham, MA, USA). *In silico* analysis was performed on all proposed primer and probe sequences to ensure chosen sequences did not cross-dimer, and sequences were checked to ensure only *Nosema* was detected using Blastn (NCBI).

For the *N. ceranae* assay, primers created a 133 bp amplicon, using *N. ceranae* F primer: GGTTGGGAGAAGCCGTTAC, and *N. ceranae* R primer: CGCTGACTCAATCGTCAGTTTA. A *N. ceranae* probe (CTGATCCAACGCAAATGCTACGGC) was labelled with a FAM reporter dye and was ZEN/Iowa Black FQ double quenched. The *N. apis* assay primers created a 118 bp amplicon, using the *N. apis* F primer: GTTATCCTTCGGGAAATCTCTAAAC, and *N. apis* R primer: AAATCGCCTGGTTCAATACAC. A *N. apis* probe (AGTGAGGCTCTATCACTCCGCTGA) was labelled with a Cy5 dye and was Iowa Black RQ quenched. Both assays' were produced by IDT, as PrimerTime® XL qPCR Assay's, with HPLC purification of the probe, and containing 12.5 nmoles of probe and 25 nmoles of each primer (Integrated DNA Technologies, Coralville Iowa, USA). A gBlock® gene fragment standard was designed for the *N. ceranae*/*N. apis* duplex assay comprised of both assay amplicon sequences (Integrated DNA Technologies, Coralville Iowa, USA). A small section of 22 bp is followed by the *N. apis* amplicon, including an additional EcoR1 restriction enzyme site (AA insert), followed by a 55 bp section, then the *N. ceranae* amplicon, again with an EcoR1 restriction enzyme site added (AT addition), followed by a final 11 bp sequence (Figure 1).

qPCRs were performed using a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) under the following conditions: 50 ºC for 2 minutes, 95 ºC for 10 minutes, followed by 40 cycles of 95 ºC for 15 seconds, 60 ºC for 1 minute. All qPCRs were performed in 20 µL reactions (10 µL PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies, Coralville, IA, USA), 1 µL of 20X *N. ceranae* custom PrimeTime XL qPCR Assay, 1 µL of 20X *N. apis* custom PrimeTime XL qPCR Assay, 3 µL molecular grade H_2O, and 5 µL template DNA/gBlock® standard. The gBlock® was serial diluted to give a linear curve from 100,000,000 to 100 genome equivalents per 5 µL. Assay linearity was assessed with 11 samples per dilution (except the 100 dilution, for which we had 10 samples). Linearity was also tested using honey bee DNA from hives that had natural *Nosema* infection, created by pooling 16 hive samples from West Virginia. Each West Virginia hive sample was created by pooling 5 whole bee guts and extracting the DNA (as described below), from samples collected in Fall 2018, from across five bee yards in West Virginia maintained by the Appalachian Beekeeping Collective (Hinton, WV, USA). This linearity pooled sample was serial diluted (1in 10 dilutions) to give five dilutions, with each dilution run in triplicate. The limit of detection (LOD) for each assay was calculated via the $LOD_{95\%}$ method, testing 75,

50, 40, 30, 25, 10, and 5 gBlock® genome equivalents per reaction based on the lowest quantity that can be detected in 95% of wells (Bustin *et al.*, 2009). LOD was tested on 20 samples of each dilution.

The correlation between microscopic *Nosema* spore counts and the qPCR values was assessed using West Virginia bee samples. A pool of five bee guts was created from a yard in West Virginia sampled in Fall 2018, as above. Individual bees were briefly thawed and surfaced- sterilized in 5% bleach followed by three sterile water rinses (Engel *et al.*, 2013), and whole guts were dissected. The 5 guts were then placed in a 1.5 mL microcentrifuge tube containing 500 µL MilliQ water. Guts were homogenized using a sterile plastic pestle and MilliQ water was added to a final volume of 1 mL. The initial solution was used to create five serial dilutions (1 in 10 dilutions) with initial volumes of 1 mL. Spore amounts in the initial solution and subsequent dilutions were calculated using an improved Neubauer 0.1 mm haemocytometer (ThermoFisherScientific, Waltham, MA, USA). Spore counts were calculated for each solution by counting all 25 squares in the central large square from three different views. Following microscopic analysis, all remaining liquid was used in DNA extractions, with the volume per dilution divided between two tubes for initial extraction steps. For DNA extraction, each tube had 180 µL DNA lysis buffer (containing 20 mM Tris-HCL pH 8, 2 mM EDTA pH 8, and 1.2% Triton-x-100) with lysozyme (20 mg lysozyme per 1 mL lysis buffer) added and was incubated at 37 ºC for 1 hour (adapted from Koch and Schmid-Hempel (2011)). Following incubation steps, the two tubes of total volume of each spore dilution were combined together into one DNeasy spin column per spore dilution and DNA extraction was carried out using an adapted Qiagen DNeasy Kit (Qiagen, Germantown, MD, UDA) protocol, with final DNA eluted in 100 µL molecular grade water. Each sample was run in triplicate using the new duplex *Nosema* assay. *Nosema ceranae* and *N. apis* average genome equivalents per sample well were calculated using the linear regression equation for the gBlock® standard curve (Log_{10} genome equivalents vs Cq values).

Nosema survey

Twenty-nine honey bee hives in the Blacksburg area (Virginia, USA) were sampled for both *N. ceranae* and *N. apis* levels. Seventeen hives were from the Hale Community Garden, seven hives from a Virginia Tech apiary, and five hives from a private garden. They were sampled over a two week period in late June/early July 2019. Two bee yards in West Virginia were also surveyed in April 2019; these yards are maintained by the Appalachian Beekeeping Collective (Hinton, WV, USA), with eight hives from each yard sampled. From each hive, worker bees were collected from internal frames and were stored at -70 ºC until DNA extraction. Five dissected whole guts were pooled per hive and DNA was extracted as described above, with guts homogenized using a sterile plastic pestle in the initial DNA lysis buffer solution, and with final DNA eluted in 200 µL molecular grade water. Each sample was run in triplicate using the new duplex *Nosema* assay. *Nosema ceranae* and *N. apis* average genome equivalents per sample well were calculated using the linear regression equation for the gBlock® standard curve (Log_{10} genome equivalents vs Cq values).

Honey bee collection

Honey bees used in the experiment were collected from two hives in August, 2019 from the Hale Community Garden showing low levels of *Nosema* infection following the

aforementioned hive infection survey. To allow the use of known-aged bees, emerging bees were marked by taking a single capped brood frame from each of the two hives and incubating them overnight in the laboratory at 30 ^{o}C inside emergence cages. The next day, newly emerged bees were collected and sedated following brief CO_2 exposure. Sedated bees were colour marked on the thorax using Uni Posca paint pens (bullet tip medium line), with each hive marked with a different colour. Marked bees and brood frames were returned to their respective hives for 7 days to facilitate the natural establishment of microbiomes (Powell *et al.*, 2014), as these bees were also used in a microbiome-*Nosema* study. After 7 days, marked bees were collected from the hives along with un-marked nestmates, and moved into plastic cages (9x9x8 cm). They were given access to *ad lib* 50% w/v sucrose and kept in a 30 ^{o}C incubator in the laboratory. For each hive, three marked bees were collected and immediately frozen at -70 ^{o}C to provide information on pre-exposure background levels of *Nosema* infection. Bees were kept in cages for 2 days prior to experimental infection, with a density of around 50 bees per cage (two cages per hive of marked bees and two cages per hive unmarked bees).

Exposure experimental setup

Nosema spore collection

Two hives from the surveyed West Virginia yards with high *N. ceranae* loads, and no *N. apis,* were selected as the source of spores for the experiment (Figure 4B). Guts from five foragers were dissected and put in 500 µL distilled H_2O. Dissected guts were homogenized, and filtered through a Millipore Nylon Net Filter (20 µm pore size) (Millipore Sigma, St Louis, MO, USA) using a method adapted from Fries *et al*. (2013). The filtered solution was then centrifuged at 5000 g for 5 minutes, the supernatant was removed and the pellet was resuspended in 1 mL distilled H_2O. This process was repeated twice (3 times total), with the pellet resuspended in 1 mL distilled H_2O after the final spin (Fries *et al.*, 2013). Spore concentration was calculated using an improved Neubauer 0.1 mm haemocytometer (ThermoFisherScientific, Waltham, MA, USA) to give a spore concentration of 16,820 spores per µL (Human *et al.*, 2013).

Experimental exposure procedure

Marked bees were fed either 10 µL 50% w/v sucrose (control) or 10 µL *Nosema* dose (100 spores in 50% w/v sucrose). This low spore dose of 100 spores was chosen as these bees were also used in a microbiome-*Nosema* study. Prior to experimental exposures, bees were starved for 5 hours to ensure the experimental bolus would be consumed. To allow for individual feeding, marked bees were placed at -20 ^{o}C until fully immobilised, then placed head first into 0.5 mL micro-centrifuge tubes with the ends cut off (Roberts and Hughes, 2015). Once bees had fully reawakened, they were fed either the sucrose (control) or *Nosema* dose. Only bees that consumed the entire bolus were included in the study. Following dosing, bees were kept in tubes for 15-30 minutes to ensure the liquid was fully ingested. Bees were separated into groups of 10 marked *Nosema* or sucrose-exposed bees and 10 un-marked non-exposed nest mates and put into clean cages with *ad lib* access to 50% w/v sucrose. For each hive, two control cages (20 sucrose-exposed bees in total), and three *Nosema* cages (30 *Nosema*-exposed bees in total) were established for the two day exposure experiment, and for one of the hives, an additional one control cage (10 sucrose-exposed bees total) and three

Nosema cages (30 *Nosema*-exposed bees total) were established for the five day exposure experiment. We chose these times points because we expected that the 2 day samples would provide information on *Nosema* survival following the initial exposure and the five day samples would provide more of an indication of successful parasite establishment and exposure outcome. Survival was checked twice daily, with all dead bees removed, and sucrose feeders re-filled.

Sample collection, processing and qPCR analysis

On sampling days (2 or 5 days post exposure), selected marked bees were snap frozen in liquid Nitrogen and stored in 1.5 mL micro-centrifuge tubes filled with 1 mL of RNAlater (Millipore Sigma, St. Louis, MO, USA). Tubes containing snap frozen bees in RNAlater were then stored at -70 ºC until sample processing. The bees collected at day 0 from each hive (to give pre-exposure background), and the snap frozen samples from days 2 and 5, were processed as follows. Bees were briefly thawed, and individually surface-sterilized, using 5% bleach followed by three sterile water rinses (Engel *et al.*, 2013). The whole gut was dissected using sterile technique, the mid-gut section was isolated from the whole gut, then individual mid-guts were placed into 1.5 mL micro-centrifuge tubes containing 300 µL 1X DNA/RNA Shield and homogenized using a sterile plastic pestle (Zymo, Irvine, CA, USA). Mid-gut DNA/RNA extraction was carried out according to the "solid tissue and blood cells" protocol with in-column DNase 1 treatment using the Zymo Quick-DNA/RNA Microprep plus kit (Zymo, Irvine, CA, USA). DNA was eluted in 50 µL molecular grade H_2O, and the RNA was eluted in 25 µL molecular grade H_2O. At the two day collection point, 38 *Nosema* exposed bees were collected (Hive A = 20, Hive B = 18), and 18 sucrose control bees (Hive A = 10, Hive B = 8), after five days 20 *Nosema* exposed bees and 10 sucrose control bees from Hive A were collected. For our experimental samples, mid-gut samples and gBlock® standards were run in triplicate. *Nosema ceranae* and *N. apis* loads per sample were calculated using the linear regression equation for the gBlock® standard curve (Log_{10} ge vs Cq values). *N. ceranae* load is given as the average amount of detected genome equivalents per sample well, either per spore solution, per group (survey data), or per individual (exposure experiment).

Statistical analysis

Nosema ceranae and *N. apis* assay linearity across standard curve values and DNA serial dilution was assessed using regression analysis on Cq values and $\log_{10}$ transformed known values for both the duplex reactions, and the actual *N, ceranae* $\log_{10}$ values given for the DNA serial dilution analysis. Mean bias and linearity uncertainty (U_{LINi}) of the assays were determined for each primer/probe pair according to the methods of Blanchard *et al*. (2012). The relationship between the qPCR results and microscopic counts of *Nosema* were analysed using $\log_{10}$ transformed data, using Pearson's product-moment correlation. All *Nosema* levels given are mean ± standard error genome equivalents per sample well. All experimental analysis was done on *N. ceranae* using the $\log_{10}$ transformed load data. While many calculated *Nosema* quantifications are below the assay's 95% limit of detection (below 30 *N. apis* and *N. ceranae* genome equivalents), due to low levels of infection, we used these values in the analysis, as they still provide details about the different infection levels, and take into account that infection did occur, as even values below 5 genome equivalents had LODs above 50%. As the three day 0 samples per hive were collected only to provide the

potential range of background infection levels in the hives, they were not included in the statistical analysis; however, the infection intensity is presented in Figure 3B. From initial *Nosema* analysis of the *Nosema* source hives, showing only *N. ceranae* infection, we have focused solely on *N. ceranae* infection in the experimental study. To assess the effect of treatment (*Nosema* or control), hive (A or B), and their interaction on *N. ceranae* levels, a mixed linear model was used with Day 2 samples. To investigate the effect of treatment (*Nosema* or control), day (2 or 5 post exposure) and their interaction on *N. ceranae* levels, a mixed linear model was used on the samples from hive A (Day 2 and Day 5). All statistical analysis on experimental samples was carried out in R version 4.0.0 (2020-04-24) using the package lme4.

Results

Standard curves and assay linearity

Each assay within the duplex had good efficiency (98.1% (*N. ceranae*) - 97.1% (*N. apis*)) producing dynamic linear curves across a large range (100,000,000 to 100 genome equivalents per reaction) for both *N. ceranae* ($F_{(df=1,74)}$= 75960, p=<0.0001, R^2 = 0.999) and *N. apis* ($F_{(df=1,74)}$= 100900, p=<0.0001,R^2 = 0.9993) (Figure 2A and C). Mean bias and linearity of uncertainty (U_{LINi}) were determined to test the performance of the linear regression of each primer assay, with the absolute mean biases values at each dilution tested ≤ 0.25 $\log_{10}$, the critical bias value described by Blanchard *et al*. (2012) (Figure 2B and D). As described in Bustin *et* al. (2009), *N. ceranae* and *N. apis* had $LOD_{95\%}$ of 30 average genome equivalents. *N. ceranae* could also still detect 10 genome equivalents with an $LOD_{95\%}$, followed by a drop to an $LOD_{55\%}$ at 5 genome equivalents, while *N. apis* dropped to $LOD_{90\%}$ at 10 genome equivalents, and an $LOD_{75\%}$ at 5 genome equivalents. The serial diluted honey bee DNA did not contain any *N. apis*, however *N. ceranae* was present and diluted samples produced a linear curve ($F_{(df=1,16)}$= 13590, p=<0.0001, R^2 = 0.9988) (Figure 2E). To investigate the relationship between microscopic *Nosema* counts and qPCR data, a dilution curve was created using pooled guts. An initial pool was serial diluted (1 in 10 dilution) five times, with microscopic spore counts done on every sample. Positive spore counts were found in the initial sample and the first four dilutions, with no spore found in the final dilution. All dilutions were tested using the duplex qPCR, with *N. ceranae* detected in all dilutions, and no *N. apis* found. From the five samples that had both microscopic and qPCR counts, a significant correlation was found (Pearson, $T_{(df=3)}$=11.093, p=0.0016, R=0.988) between microscopic count and qPCR data (Figure 2F). With increasing qPCR values corresponding with higher microscopic spore counts. For the final dilution, no spores could be counted, however the qPCR showed 530 ± 106 *N. ceranae* genome equivalents.

Nosema survey data

The *Nosema* survey highlighted relativity low *N. ceranae* levels at all three locations in Blacksburg (Figure 3A). *Nosema apis* was also surveyed, but was only detected in two hives at very low levels (below 4 genome equivalents). The Hale community garden hives had the highest average loads with 412.95 ± 199.11 genome equivalents, followed by the private garden hives which had on average 260.07 ± 148.33 genome equivalents, and the Virginia Tech hives which had 163.12 ± 31.38 genome equivalents (Figure 3A). While the Hale hives

had higher average *N. ceranae* loads, there was a greater number of hives with low infection levels. Given the results of this survey, two hives from the Hale community garden were chosen as a source of bees for the *Nosema* lab exposure experiment. The hives chosen had low *N. ceranae* loads with 24.6 (Hive A) and 21.2 (Hive B) genome equivalents, respectively. While the hives chosen from the Hale community garden did have low *N. ceranae* loads and no *N. apis*, some natural variation in levels between sampled honey bees was detected in the Day 0 samples collected from the two chosen hives. Hive A Day 0 samples had 5.66 ± 3.16 average *N. ceranae* genome equivalents and 3.59 ± 2.06 *N. apis* genome equivalents, and Hive B Day 0 samples having 5.3 ± 2.4 *N. ceranae* genome equivalents, and 5.2 ± 2.1 *N. apis* genome equivalents.

The two bee yards sampled in West Virginia had high levels of *N. ceranae* and no detected *N. apis* (Figure 3B). Hives were chosen from within each yard, that had the highest levels of *Nosema* infection as our source of experimental spores for infection (WV yard 1 hive= $1.7x10^6 \pm 4.1x10^4$ *N. ceranae* and 0 *N. apis* genome equivalents and WV yard 2 hive = $2.0x10^6 \pm 4.0x10^4$ *N. ceranae* and 0 *N. apis* genome) (Figure 3C). Worker bees from these two hives were used to create the spore solution for the exposure experiment, as these yards had the highest level of *Nosema* infections from all the ones surveyed.

Experimental *N. ceranae* exposure experiment

Two days post inoculation, *N. ceranae* levels in *Nosema* exposed bees had increased to 111.5 ± 71.7 average genome equivalents, while the sucrose control bees had on average 12.4 ± 4.1 genome equivalents for hive A bees (Figure 4A). After 2 days, *Nosema* exposed bees from hive B had on average 162.6 ± 69.2 *N. ceranae* genome equivalents, and sucrose control bees having on average 33.9 ± 25.8 *N. ceranae* genome equivalents (Figure 4A). While levels of *N. ceranae* increased after 2 days in honey bees from both hives after the bees were exposed to the *Nosema* dose, treatment (*N. ceranae* or sucrose bolus) had a marginally significant effect on *N. ceranae* levels ($F_{(df=1)}$=3.949, p = 0.052), however any detected treatment effect was not dependant on which hive the bees came from ($F_{(df=1)}$=0.608, p = 0.439). We also found that initial hive did not have any significant effect on *N. ceranae* ($F_{(df=1)}$=0.224, p = 0.638).

Nosema exposed bees had on average 236,398 ± 128,801 genome equivalents of *N. ceranae* after 5 days of exposure (Figure 4B). Sucrose control bees had on average 4.4 ± 1.29 *N. ceranae* genome equivalents after 5 days (Figure 4B). The day 5 samples allowed us to investigate the impact of the exposure dose on *N. ceranae* levels over time. While the average *N. ceranae* load is higher at day 5 compared to day 2, this is mainly due to a few individuals with no overall significant effect of day found ($F_{(df=1)}$= 0.0360, p = 0.8502). Treatment (*N. ceranae* or sucrose control) also did not have a significant effect on *N. ceranae* levels after 5 days ($F_{(df=1)}$= 2.487, p = 0.120), and the treatment type did not depend on which day the samples were collected ($F_{(df=1)}$= 0.664, p = 0.419).

Discussion

The accurate and sensitive quantification of *Nosema* infection levels in honey bees is of vital importance when looking at both disease incidence and more complex host-pathogen

relationships. We developed and tested a new duplex qPCR assay for quantifying both *N. ceranae* and *N. apis*. This new assay utilizes TaqMan chemistry and an external standard. The use of TaqMan chemistry allows a high level of specificity to be achieved between *N. ceranae* and *N. apis* within a duplex reaction. This specificity is due to the use of probes within the reaction, which therefore requires binding at three different unique regions for a positive fluorescent to be detected. Many pathogen detection assays use external standards to aid in quantification, including a known amount of positive PCR product (Kukielka *et al.*, 2008, Kukielka and Sánchez-Vizcaíno, 2009), a plasmid standard (Bourgeois *et al.*, 2010, Forsgren and Fries, 2010, Traver and Fell, 2011, Copley *et al.*, 2012) and gBlock® gene fragments (Alger *et al.*, 2018, Alger *et al.*, 2019). We chose to use a gBlock® design for several reasons, including the ability to decide every base within the sequence, the option to insert a restriction digest site, ease in obtaining them, no laboratory biosecurity certification requirement, and stable long-term storage. The ability to include a restriction digest site is useful for ensuring that contamination can be easily detected in potentially contaminated wells, with the qPCR product being used in a restriction digest assay. This method of contamination checking is similar to what can be used with plasmid standards, which can utilize either restriction digest sites or primers that cover the plasmid itself. As far as we are aware, this new duplex *Nosema* qPCR is the only published *Nosema* method that utilizes a gBlock® design for its external standard. Given this assay is a duplex, the ability to specify each base allows the target sequences of both *N. ceranae* and *N. apis* to be combined into a single sequence without the need for further downstream modification. Other ways to combine two separate sequences like this would involve using other methods, such as Gibson Assembly or GenScript to combine DNA sequences in a plasmid prior to cloning (Carrillo-Tripp *et al.*, 2016, Bradford *et al.*, 2017). As the gBlock® is manufactured, a known concentration can be ordered, which is easily checked when eluting the received dry material, allowing an easy calculation to produce consistent standard curves.

The range and efficiency of the assay was tested using both a linear standard curve, linearity of uncertainty analysis, and limits of detection. A wide and dynamic range was tested in the standard curve from 100 genome equivalents to 100,000,000 genome equivalents per reaction well, with each assay giving high qPCR efficiency (97-98%). This large linear range was used to calculate the linearity of uncertainty and mean bias values for each dilution for both *Nosema* species. These analyses give an indication of the reliability of the assay, with each dilution giving a result below the critical value (≤ 0.25 $\log_{10}$ (Blanchard *et al.*, 2012). The limit of detection is calculated so that the confidence in low detected values can be assessed. Both assays have low 95% limit of detections ($LOD_{95\%}$) of 30 genome equivalents for each assay, with *N. ceranae* still having an $LOD_{95\%}$ of 10 genome equivalents in a well. We also found that 5 genome equivalents could be accurately detected in 55% of *N. ceranae* wells and 75% of *N. apis* wells. The assay was tested with samples of known *Nosema* spores following microscopic counts. This allowed the relationship between qPCR values and microscopic counts to be analysed. *Nosema* values were significantly correlated with higher qPCR results correlating with higher microscopic spore counts. The assay's ability to detect lower infection levels was highlighted in that no spores were counted using the microscopic method at the maximum dilution, while 530 genome equivalents were detected in those samples with qPCR. The lower sensitivity of microscopic counts has been previously seen, with Traver and Fell (2011) finding 51.1% of microscopic spore negative counts to be *N. ceranae* positive

after qPCR analysis, while Copley *et al*. (2012) found 61.5% microscope negative samples were positive after qPCR analysis.

The various methods of assay validation highlight the high sensitivity of the duplex assay and why we used the low infection load data from the experimental study in analysis. The design of this assay allows it to detect the combined active and vegetative load of *Nosema* in honey bee samples. This combined approach would be highly beneficial for large scale survey data collection. This method could be easily combined with haemocytometer analysis of other samples from a hive. qPCR methods of *Nosema* load quantification are also used in many studies focusing on microbiome-*Nosema* interactions (Maes *et al.*, 2016, Li *et al.*, 2017, Rubanov *et al.*, 2019), as DNA extraction is required for assessment of the bacterial community, and you can thus collect both *Nosema* infection data and microbiome data from a single extracted sample.

In the experimental study, the *Nosema* dose chosen was low (100 spores) compared to other previously published *Nosema* infection studies that generally used infective doses ranging from $10^4 – 10^5$ spores (Antúnez *et al.*, 2009, Forsgren and Fries, 2010, Roberts and Hughes, 2015, Li *et al.*, 2018), resulting in higher infection loads. Most *Nosema* infection studies also investigate infection over longer periods of time, often between 6 and 21 days (Forsgren and Fries, 2010, Roberts and Hughes, 2015, Li *et al.*, 2018). There is limited information about infection intensity during early infection. A previous dose response study showed that after 14 days an infective dose of 100 will give around 30-60% infected bees (depending on whether either *N. apis* or *N. ceranae* spores were ingested, respectfully), while a dose of 10,000 results in 100% infection (Forsgren and Fries, 2010). While the time frame of that study is longer than the 2 and 5 days used in our study, it confirmed that infection can be achieved with a low 100 spore dose. Compared to Forsgren and Fries (2010), we had between 90-95% *N. ceranae* infection after 2 days and 75% infection after 5 days, with the 100 spore dose. While we did have high infection prevalence, the levels of infection were low regardless of collection day or original hive identity. The low infection loads seen in the bees did, however, correspond to the low detected loads in the hives found in that apiary, and in the hive prior to the experiment. These low natural hive *Nosema* infection loads also correspond to the low levels of *Nosema* detected in the control individuals at all time points. Mulholland *et al*. (2012) have also shown natural levels of individual *Nosema* variation within hives. These low levels of *N. ceranae* infections were seen in all three sites in Blacksburg, VA, potentially indicating a low natural prevalence in the area. A previous study in Virginia showed a high prevalence of *N. ceranae* in hives, with peak loads seen in March/April, followed by decreasing levels through to September (Traver and Fell, 2011), which corresponds to when our experimental samples were collected in late August. The low to non-existent *N. apis* levels seen during our hive survey in both Blacksburg, VA, and the yards in West Virginia, was expected based on the Virginia survey results from Traver and Fell, (2011). In this study *N. apis* infections were low level coinfections when detected, but extremely rare, only being found in 2.7% of hives surveyed (Traver and Fell, 2011).

This failure of *N. ceranae* establishment following low spore exposure could be due to the impact of physiological factors within the individual honey bees, such as their gut microbiome or their ability to evade the immune response. Li *et al*. (2017) found that levels of *N. ceranae* infection were higher in bees that were subsequently exposed to antibiotics, which significantly disrupted their gut microbiome, compared to bees not treated with

antibiotics. Individual gut bacterial strains may also be important in initial infection supplemental hive feeding of the gut bacterium *Parasaccharibacter apium* resulted in lower *N. ceranae* spore loads following individual treatment of a 10,000 spore dose compared to bees from a hive with no supplemental hive feeding (Corby-Harris *et al.*, 2016), and two *Gilliamella* strains were positivity associated with higher *N. ceranae* levels in nine study hives (Rubanov *et al.*, 2019). Antúnez *et al*. (2009) showed that 7 days post infection with *N. ceranae* downregulation in the expression of abaecin and hymenoptaecin, which is not detected after 4 days or seen in *N. apis* infection which sees upregulation of abaecin, defensin and hymenoptaecin after 4 days, and defensin after 7 days. These potential physiological impacts on *Nosema* infection success highlight the need to further investigate the initial stages of infection and individuals with low level infection, which can be achieved in the laboratory following low dose exposure, such as the 100 spore dose we explored.

Conclusion

In conclusion, our study presents a new duplex *N. ceranae*/*N. apis* qPCR assay, using a newly-designed combined *Nosema* gBlock® standard for accurate quantification. The assay shows high sensitivity and repeatability across a linear dynamic range, with a low limit detection, and strong correlation with microscopic spore counts. We also provide evidence that a low 100 spore *Nosema* dose can result in increased *Nosema* infection intensity in some caged honey bees within two days of exposure. While the low dose is capable of infection establishment, some bees were able to resist infection, which may provide future insights into other aspects of honey bee biology that are involved in host defence against pathogens.

Disclosure statement

The authors declare that there is no conflict of interest.

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Figure Legends

Figure 1: Nucleotide sequence for gBlock® synthesis for the *N. ceranae*/*N. apis* duplex qPCR assay. Nucleotides in bold represent primer sites, with the *N. apis* amplicon first, followed by

the *N. ceranae* amplicon. Nucleotides in *italics* represent the qPCR probe sequences. The underlined nucleotides represent the EcoR1 restriction digest sites, with the added nucleotides shown in lower case.

Figure 2: Standard curves across a dynamic linear range (100 – 100,000,000 genome equivalents) for the *N. ceranae* (A) and *N. apis* (C) assays using the gBlock® standard. Each dot indicates average Cq of each dilution. Linear regression performance of each assay was assessed using the mean bias and linearity uncertainty (U_{LINi}) for each dilution for each assay [*N. ceranae* (B) and *N. apis* (D) using the gBlock® standard]. Linearity uncertainty is shown by the bars, with the dots representing the mean bias. Standard curve produced using serial diluted *A. mellifera* DNA with known *N. ceranae* infections (1 = pooled samples, 2 = 1/10 dilution, 3 = 1/100 dilution, 4 = 1/1000 dilution, 5 = 1/10000 dilution) (E.). Each dot represents average log *N. ceranae* genome equivalents, with standard error bars. Relationship between microscopic spore counts (spores per mL) and duplex *N. ceranae* qPCR values (genome equivalents per sample well), from a serial diluted gut solution (F.) Each dot represents average log *N. ceranae* or spore count, with standard error bars.

Figure 3: A.) Levels of *Nosema ceranae* found during the Blacksburg, Virginia hive survey using three locations: Hale community garden (N=17), a private garden (N=5), and a Virginia Tech apiary (N=7). Points in the plot represent individual hives. B.) *Nosema ceranae* infection loads from two bee yards in West Virginia (N=8 hives/yard). Boxplots show median load along with the 25th and 75th percentiles, with dots representing individual hives. C.) Levels of *Nosema ceranae* infection in the two chosen hives from each yard that were chosen as the source for *N. ceranae* spores for the experiment. Bar charts show mean and standard error. No *N. apis* were detected.

Figure 4: Levels of log *Nosema ceranae* genome equivalents in caged honey bees (A.) 2 days post exposure from two different original hive stock (Hive A or Hive B), and (B.) from Hive A, 2 and 5 days post exposure to either *Nosema* or sucrose control doses. Boxplots show median loads along with the 25th and 75th percentiles.

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724 *of Apicultural Research,* **51**(1)**,** pp. 115-124. 10.3896/IBRA.1.51.1.14

725

Figure 1

CCATTGCCGGATAAGAGAGTCC**GTTATCCTTCGGGAAATCTCTAAAC**TTTGACC
TGAGGGAGGTCAGGCATGAT*AGTGAGGCTCTATCACTCCGCTGA*ACTGAGTTGTGa
aTTCTTAAATG**GTGTATTGAACCAGGCGATTT**TGTTCCTATACTCTTAACTGAGT
TGTATCCTAAGGGAACACCTTTTCTATCCAGTGCC**GGTTGGGAGAAGCCGTTAC**
CCTTCGGGGAATCTTCAAAAAACACACAACCTGGGGGAGGGTTTGGCAAGCTGC
TGAC*GCCGTAGCATTTGCGTTGGATCAG*GTCAGAatTCCT**TAAACTGACGATTGAG**
TCAGCGTGTTATCCCTA

Figure 2

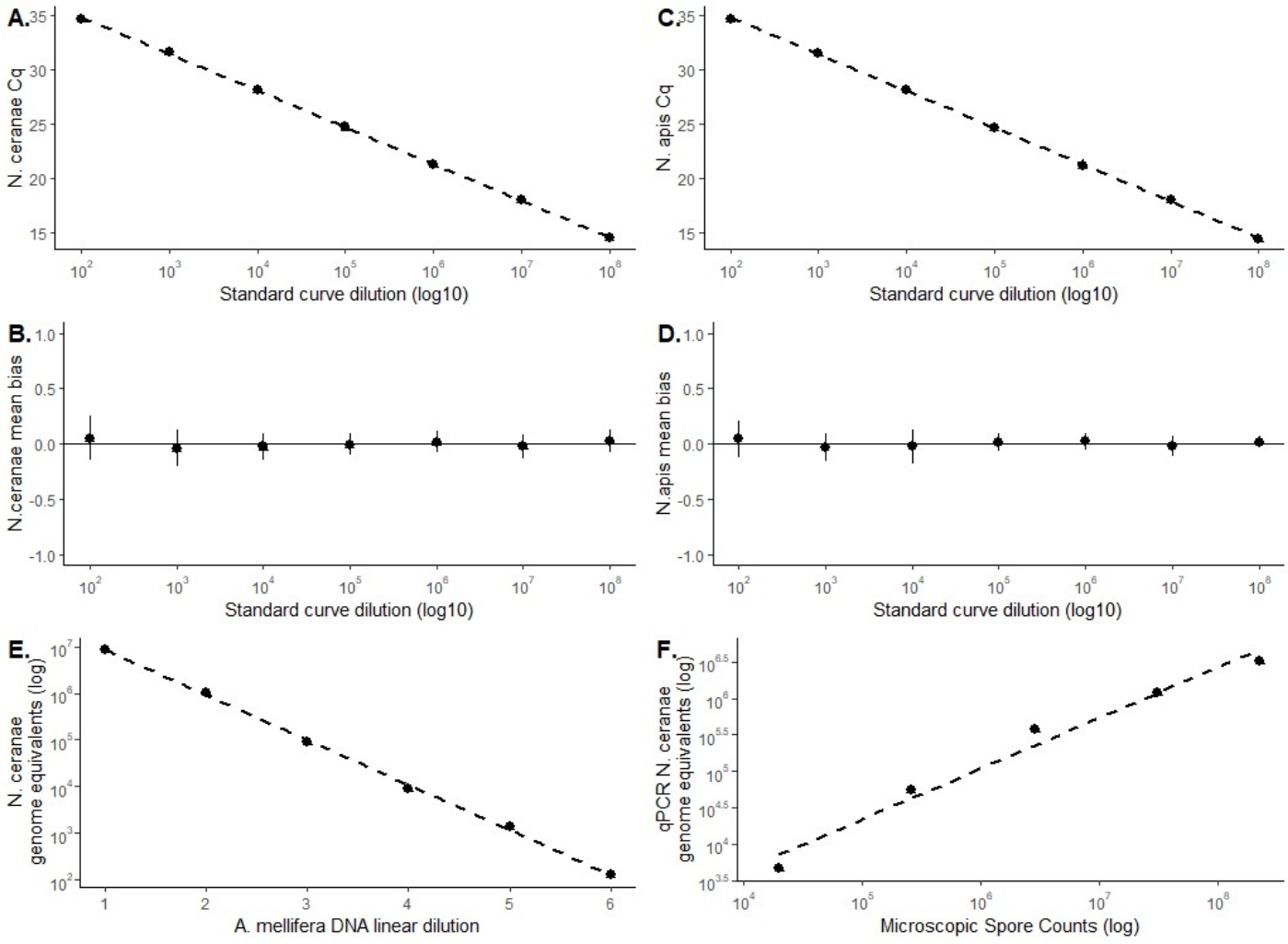

A.
N. ceranae Cq
Standard curve dilution (log10)
C.
N. apis Cq
Standard curve dilution (log10)
B.
N.ceranae mean bias
Standard curve dilution (log10)
D.
N.apis mean bias
Standard curve dilution (log10)
E.
N. ceranae genome equivalents (log)
A. mellifera DNA linear dilution
F.
qPCR N. ceranae genome equivalents (log)
Microscopic Spore Counts (log)

Figure 3

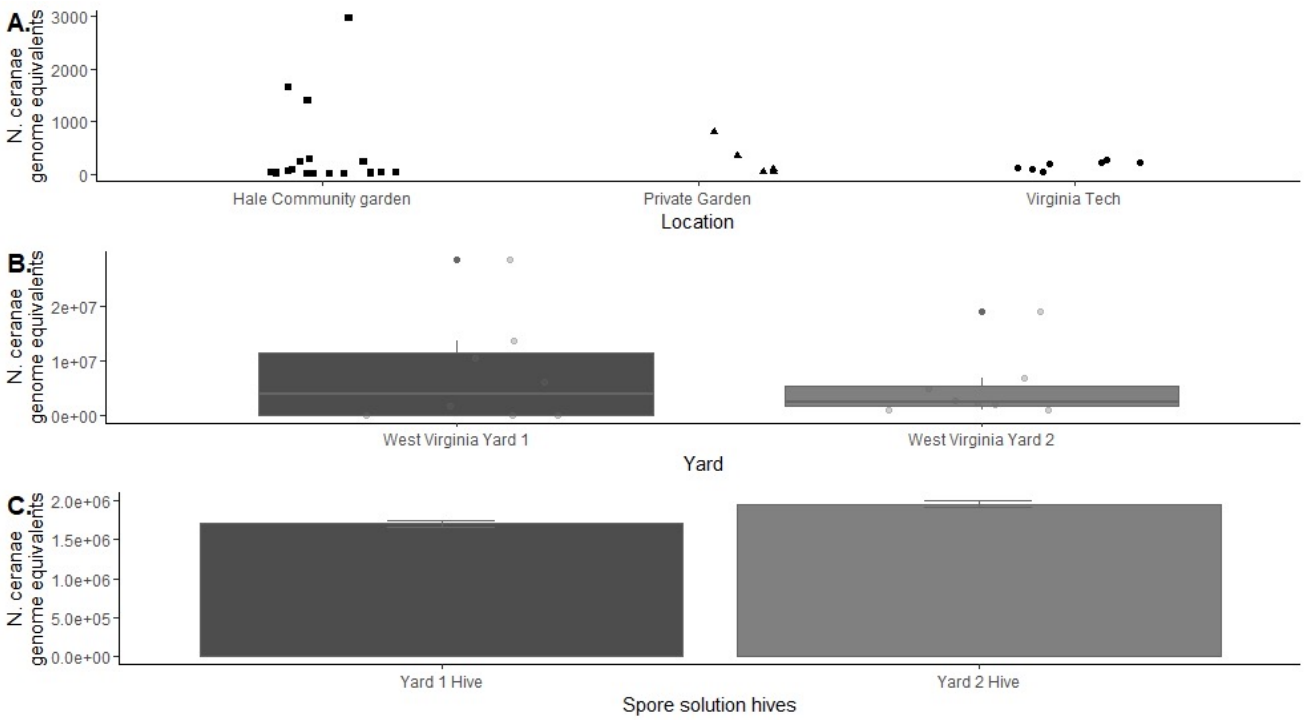
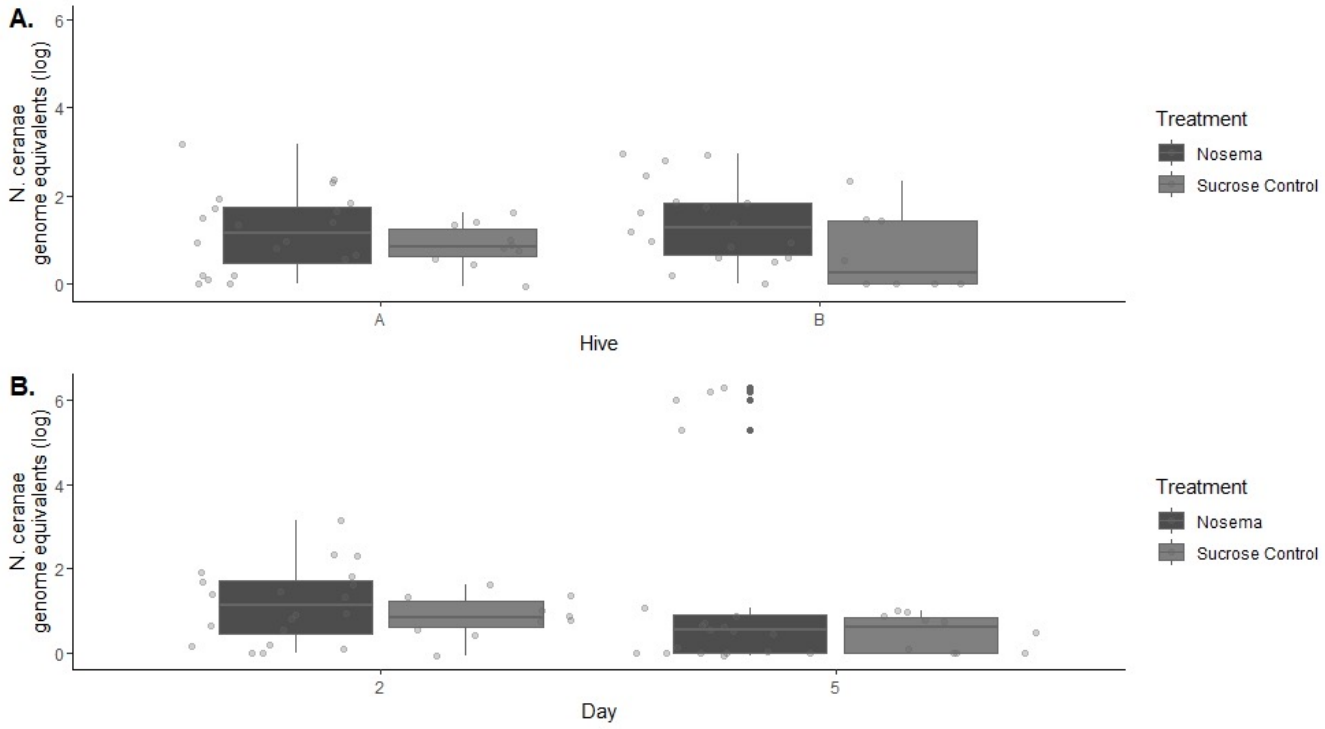

A.
N. ceranae genome equivalents
3000
2000
1000
0
Hale Community garden
Private Garden
Virginia Tech
Location
B.
N. ceranae genome equivalents
2e+07
1e+07
0e+00
West Virginia Yard 1
West Virginia Yard 2
Yard
C.
N. ceranae genome equivalents
2.0e+06
1.5e+06
1.0e+06
5.0e+05
0.0e+00
Yard 1 Hive
Yard 2 Hive
Spore solution hives

Figure 4


A.
N. ceranae genome equivalents (log)
0
2
4
6
A
B
Hive
Treatment
Nosema
Sucrose Control
B.
N. ceranae genome equivalents (log)
0
2
4
6
2
5
Day
Treatment
Nosema
Sucrose Control