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Live and dead qPCR detection demonstrates that feeding of *Nosema ceranae* results in infection in the honey bee but not the bumble bee

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ABSTRACT

As the honey bee and bumble bee may suffer from the same or related microbial pathogens, cross contamination from commercially reared *Bombus* spp. to honey bees and wild bumble bees and vice versa is a major concern. Honey bee-collected pollen to feed commercially reared *Bombus* spp. is a potential risk. *Nosema* spp. is a fungal pathogen in bees. In this study, we developed new quantitative detection tools based on the detection of RNA using a TaqMan-based RT-qPCR for *Nosema ceranae* and *Nosema apis*, with extraction controls based on the actin gene of honey bees and bumble bees, respectively. These tools were subsequently applied to study the epidemiology of *N. ceranae*, a main disease in honey bees. We screened gamma radiation and cold treatment sterilisation for their efficacy to kill *N. ceranae* spores fed in sugar water and in pollen to honey bees and bumble bees, respectively. *N. ceranae* infection in adult bumble bees was checked. Spores passing the inter-alimentary track were found but no infection was observed. *N. ceranae* spores were fed to honey bees. Their presence and multiplication were demonstrated, showing the spores were both viable and infectious. Our results indicate that *N. ceranae* found in honey bees cannot infect commercially reared bumble bees (*Bombus terrestris*) and, that gamma radiation effectively kills *N. ceranae*. The highly specific and sensitive molecular assays developed, were exploited to detect *N. ceranae* in pollen and faeces, which would allow more comprehensive epidemiological studies on this important pathogen.

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Introduction

Entomopollination is of extreme ecological and agricultural importance as 87% of wild flowering plants and 75% of cultivated crops depend to a certain extent on insect pollinators (Garibaldi et al., 2013; Klein et al., 2007). Traditionally, the honey bee (*Apis mellifera*) has been the major, commercially available pollinator, but since the 1980s commercial bumble bee rearing (*Bombus* spp.) started and these non-apis bees now play an important role in fruit cultivation and horticulture, particularly in greenhouse tomato pollination (Heemert et al., 1990; Velthuis & van Doorn, 2006). In nature, bumble bee colonies collect nectar and pollen for food. Pollen provides all essential amino acids, minerals, fatty acids, and vitamins. Commercially reared bumble bees and bumble bee brood are fed on sugar and honey bee-collected pollen (Heemert et al., 1990; Plowright & Jay, 1966; Röseler, 1977).

Both honey bee and bumble bee may suffer from the same or related microbial pathogens. Therefore, cross contamination from commercially reared *Bombus* spp. to wild bumble bees and to honey bees and *vice versa* is a major concern, especially since they share food sources. In bumble bees, the fungus *Nosema bombi* and the trypanosome *Crithidia bombi* are causing serious problems: infection of these two organisms from reared bumble bee colonies to wild population was suggested as a factor in the decline of wild bumble bees (Colla et al., 2006; Otterstatter & Thomson, 2008). The trypanosome *C. bombi* may also be transmitted to honey bees (Durrer & Schmid-Hempel, 1994).

In honey bees currently, especially viruses from the Deformed Wing Virus (DWV) pool and the fungi *Ascosphaera apis, Nosema apis*, and *Nosema ceranae* are important diseases. Genersch et al. (2006) showed lethal infections with DWV are possible in *Bombus terrestris* and *Bombus pascuorum* and also surveys indicate the natural infection of bumble bees *B. terrestris* and *B. pascuorum* with DWV but not in other bee species (Evison et al., 2012). While chalkbrood-causing *Ascosphaera apis* has been

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detected in non-laying queens of three bumble bee species *Bombus griseocollis*, *Bombus nevadensis*, and *Bombus vosnesenskii* (Maxfield-Taylor et al., 2015). Both *N. apis* and *N. ceranae* cause nosemosis in adult honey bees. Several studies have detected *N. ceranae* in bumble bees (Arbulo et al., 2015; Evison et al., 2012; Graystock et al., 2014; Plischuk et al., 2009), however, these infections were found to be symptom-less (Fürst et al., 2014). *N. bombi* proved host specific and not to be infectious to honey bees (van den Eijnde & Vette, 1993).

Since the first detection of *N. ceranae* in Europe (Higes et al., 2006), its reported incidence increased and *N. ceranae* is currently one of the most common fungal pathogens in honey bees in Europe. In 2014, 2015 and 2016, surveillance study, in the Netherlands the prevalence of *N. ceranae* in the honey bee colonies was high up to 89% (Biesmeijer et al. 2015, 2016, 2017). High infection rates were also reported in Germany (Genersch et al., 2010) and Belgium (van der Steen, pers. communication). *N. ceranae* was also detected in corbicular pollen pellets (Higes et al., 2008), forming a possible infection route between bee species. Higes et al. (2008) hypothesised that Nosema spores from infected honey bees end up in the pollen pellets during the brushing and packing process from the hairs into the corbicula. Alternatively, *N. ceranae* spores could be collected from contaminated flowers as was shown for other microbial agents (Graystock et al., 2015). To prevent transmission via contaminated pollen, commercially reared bumble bees are fed with a combination of sugar water and honey bee-collected pollen that are gamma-irradiation treated (Graystock et al., 2016), where a dose of 5 kGy kills off fungi and coliform bacteria, but higher doses are needed to kill yeasts and aerobic bacteria (Álvarez Hidalgo et al., 2020).

For the detection of the biotrophic *N. ceranae* and *N. apis* spores, several methods have been described. These include microscopic analysis and visual inspection. The shape and size are relatively easy to detect particularly when present in high numbers. Staining techniques were developed to differentiate between viable and dead *Nosema* spores, based on viability dyes propidium iodide and SYTO16 in combination with microscopy or flow cytometry (McGowan, 2012; Peng et al., 2014). These microscopic methods are laborious particularly when quantitative data are needed and cannot be applied outside the host. In addition, DNA-based molecular tools like PCR and qPCR are used (Evans et al. 2013; Fries et al., 2013). These DNA-based methods are highly specific and sensitive, however, they cannot discriminate dead from life spores. Consequently, dead spores, that pose no threat, could lead to unnecessary concerns when DNA-based methods score positive for instance after gamma radiation treatments. RNA is degraded quickly in death or dying cells, therefore reverse transcriptase qPCR (RT-qPCR) allows the quantitative detection of viable cells specifically based on a reference gene or standard (Pfaffl, 2001), but this has not been applied to the detection of *Nosema* spp. To assess the viability of *N. ceranae* spores, bioassays are used for the virulence determination of *N. ceranae* spores (Huang et al., 2015; Natsopoulou et al., 2016).

In this study we: (1) developed quantitative detection tools based on the detection of RNA using an RT-qPCR for *N. ceranae* and *N. apis* for the detection of viable spores. (2) Screened sterilisation techniques for their efficacy to kill *N. ceranae* spores. To this end performed bioassays with treated and untreated *N. ceranae*-spiked material on honey bee and bumble bee to test the virulence. To differentiate between living and dead *N. ceranae* spores, we thus applied bioassays, microscopy including viability stains, and generated new molecular assays based on the detection of RNA by RT-qPCR. Finally, we discuss the possibilities for a cross contamination of *N. ceranae* from commercially reared bumble bees to wild bees.

Materials and methods

Biological material

Preparation N. ceranae spores

N. ceranae spores were derived from naturally infected bee colonies from different beekeepers in the Netherlands. Worker bees were dissected and midgut (ventriculus) and hindgut (rectum) were ground with mortar and pestle. Tap water was added and the mixture was filtered over a 100 µM sieve. Filters were rinsed with water and the filtered suspension was centrifuged at 10.000 g for 20 minutes in the SL 40 R centrifuge (Thermo Fisher Scientific, Waltham (MA) USA) and the resulting pellet was collected and resuspended in 10 mL tap water. Subsequently, 2 mL of the concentrated spore suspension was pipetted on a 12 mL 100% Percoll (GE-Healthcare, Uppsala, Sweden) cushion in a 15 mL tube and centrifuged at 10.000 g for 20 minutes separating the spores from bee material. The purified *N. ceranae* spores were collected from the bottom of the 15 mL tube. The layers (approximately 2 mL) above the Percoll were removed and again 2 mL of the concentrated spore suspension was pipetted on the Percoll, again followed by a similar centrifugation step. Finally, 10 mL of the supernatant was removed and the bottom 4 mL with *N. ceranae* spores was washed with 10 mL sterile H_2O and centrifuged at 11.000 g for 15 minutes. The supernatant was removed and the *N. ceranae* spore pellet was resuspended in H_2O and stored for 1 to 5 days at room temperature.

The viability of the *N. ceranae* spores was determined according to the protocol by McGowan (2012); 20 µL propidium iodide (1 mg/mL) was added to 200 µL *N. ceranae* spores suspension containing 10E6 spores. The mixture was incubated in the dark for 20 minutes at room temperature and then centrifuged at 800 g for 6 minutes. The pellet with *N. ceranae* spores was dissolved in 100 µL sterile H_2O. This washing step was repeated two more times. Finally, 5 µL of the suspension was placed on a microscopic slide and checked under an epifluorescent microscope (Axioplan, Zeiss, Germany) for scoring live and dead *N. ceranae* spores as visualised by the ability of Propidium Iodide to penetrate the death *N. ceranae* spores.

Honey bee colonies

Honey bees were taken from the first frame without brood from three different colonies (A, B, C) at the WPR Droevendaal apiary (Wageningen, the Netherlands). Honey bees taken from these frames represent an average of age cohorts in the colony (van der Steen et al., 2012). Panels of 30 honey bee workers were kept in perforated 250 mL plastic containers in an incubator room at 27 °C, 60% RH, in the dark.

Bumble bee colonies

For the bumble bee tests, fifteen starting *B. terrestris* colonies containing a queen and approximately 10 workers were obtained from Koppert (Koppert Biological Systems, Berkel enRodenrijs, the Netherlands). Bumble bee colonies were kept in an incubator room at 27 °C, 60% RH, in the dark.

Pollen

Untreated pollen was obtained from the Koppert Biological Systems rearing facilities (Nove Zamky, Slovakia). To make palatable pollen dough for the bumble bee colonies, 70 g pollen was mixed with 30 g sugar solution 50% (w/w), also obtained from Koppert Biological Systems.

Total nucleic acid extraction

Honey- and bumble bees

For the honey bees, the ventriculus was dissected as described above and pooled in groups of five in 300 µL RNAlater™-solution (Invitrogen, Carlsbad (CA), USA). Similarly, the rectums of five honey bees were pooled in 300 µL RNAlater™-solution. For the bumble bees the ventriculus and rectum of three bumble bees were dissected, pooled, and stored in 300 µL RNAlater™solution. This sampling was performed in 2 mL screw-tap tubes with five ceramic beads and samples were stored at 4 °C before being homogenised in the Precellys Evolution (Bertin Instruments, Montigny-le-Bretonneux, France) for 1 minute at 6500 rpm. Total nucleic acid was extracted from a 30 µL sample with the Masterpure complete DNA & RNA purification kit (Epicentre, Madison (WI), USA) according to the manufacturer's instructions. Total nucleic acid was eluted in 35 µL TE buffer. For DNA-free RNA, 10 µL of the total nucleic acid sample was treated with the Turbo DNA-free™ Kit (Invitrogen, Carlsbad (CA), USA) incubated and deactivated as described in the manufacturer's instructions. The absence of DNA was verified by omitting the reverse transcriptase step in the TaqMan resulting in no amplification.

Pollen

For the nucleic acid isolation of pollen, 3 grams pollen dough was transferred to a 15 mL tube with five zirconia beads and homogenised at 6500 rpm for two times 30 seconds with a 45 seconds pause in between in the Precellys Evolution (Bertin Instruments, Montigny-le-Bretonneux, France). Subsequently, total nucleic acid was extracted from 10 mg subsamples using the Masterpure complete DNA & RNA purification kit as described above. Total nucleic acids were eluted in 35 µL TE buffer.

Faeces

DNA extraction from bumble bee faeces, deposited in the corners of the rearing boxes, was done with the Invimag Stool DNA kit (Stratec, Berlin, Germany) according to the manufacturer's protocol. Four samples of 250 mg were taken from each colony. DNA was eluted in 100 µL TE buffer.

Development and validation of (RT-) qPCR assays

N. ceranae and N. apis

Single copy gene Elongation Factor 1-Alpha (*EF1α*) sequences of *N. ceranae* (XM_002995284.1) and *N. apis* (KE647072.1) were retrieved from GenBank. Sequence alignment was performed with the CLC-Genomics workbench tool from Qiagen (Aarhus, Denmark). Primers and probes (Table 1) were designed with the PrimerQuest tool from Integrated DNA Technologies (IDT, Coralville (IA), USA). Primers and probes were obtained from IDT (Leuven, Belgium). The amplicon length of the *N. ceranae* qPCR was 112 bp and for *N. apis* 105 bp. The hydrolysis probes were labelled with the 6-carboxyfluorescein (FAM) dye at the five prime end, and double quenched with an internal ZEN Quencher and a Iowa Black® FQ quencher at the three prime end (Table 1).

Honey -and bumble bees

The qPCR assay primers and probe on β– Actin in honey bees, developed by Chen et al. (2005) were used as extraction control for the honey bee

Table 1. Primer sequences of the primers and (RT-) hydrolysis probes.

Target	Primer/Probe	Sequence
β-Actin (*A. mellifera*) (extraction control) (Chen et al., 2005)	Apis-β-actin-Fw	5′-AGGAATGGAAGCTTGCGGTA-3′
	Apis-β-actin-Rv	5′-AATTTTCATGGTGGATGGTGC-3′
	Apis-β-actin-p	5′ FAM/ATGCCAACACTGTCCTTTCTGGAGGT/3′
B-Actin (*B. terrestris*) (extraction control)	F_Bbee_act R_Bbee_actP_Bbee_act	5′-TGCGATGTCGATATCCGTAAAG-3′
		5′-GGTGCTAGGGCAGTGATTT-3′
		5′ FAM/ACCTGTACG/ZEN/CCAACACTG/3IABkFQ 3′
Elongaton Factor *(N. ceranae)*	Ncer-EF-F1	5′-CCGGTTGATAATCCAGAGAG-3′
	Ncer-EF-R1	5′-ATCTACCAAGAGAAGGGAAATC-3′
	Ncer-EF-P1	5′ FAM/CCTCAGAAG/ZEN/CCTTGTGTTATGGAAACGGCA/3IABkFQ 3′
Elongaton Factor *(N. apis)*	Nap-EF-F1	5′-GATGCTTCTCCCGTTGATAA-3′
	Nap-EF-R1	5′-GAAATCTTTAGCTGTCTCCATAAC-3′
	Nap-EF-P1	5′ FAM/CCCTGAGAG/ZEN/AGTTGAACAAGGAGATAGTGC/3IABkFQ 3′

samples. For the development of a qPCR assay for bumble bees, an alignment was made between the β–Actin amplicon sequence by Chen et al. (2005) and the β–Actin sequence of bumble bees (Accession number XM003396941.2). As nucleotide differences were found in both primer and probe sequences, a new qPCR assay was developed for bumble bees. Primers and probe (Table 1) were designed with the PrimerQuest tool from Integrated DNA Technologies (IDT, Coralville (IA), USA). Primers and probes were obtained from IDT (Leuven, Belgium). The amplicon length of the β–Actin qPCR was 113 bp. The hydrolysis probe was labelled with the 6-carboxyfluorescein (FAM) dye at the five prime end, and double quenched with an internal ZEN Quencher and a Iowa Black® FQ quencher at the three prime end.

Assays

All qPCR assays were performed on the Quantstudio 12 K Flex (Thermo Fisher Scientific, Waltham (MA), USA). Reactions were performed with 1.0 µL target in a 25 µL volume, including 1x *Premix Ex Taq*™ DNA Polymerase (Perfect Real Time) (Takara BIO Europe, Saint-Germain-en-laye, France), 0.25x ROX reference Dye II (50×), 300 nM of forward and reverse primer, 100 nM double quenched fluorescence hydrolysis probe. Transparent AB17500 96 × 0.2 mL plates (Bioplastics, Landgraaf, The Netherlands) sealed with Microamp™ optical adhesive film (Applied Biosystems, Foster City (CA), USA) were used. The temperature profile consisted of an initial denaturation step of 95 °C for 2 min, followed by 40 cycles of 95°Cfor 15 sec and 60 °C for 1 min. Results were analysed with the Quantstudio 12 K Flex software. Threshold and baseline settings for each run were automatically set by the software. In each plate, a dilution series of the target (10E6 to 1 copies) and negative control (water) were analysed in parallel.

Assay validation

Specificity, sensitivity, and robustness of the assays were tested on artificial, double stranded DNA fragments (gBlocks) (Integrated DNA Technologies, Coralville (IA), USA) consisting of the amplicon sequence sometimes extended on the five prime end and three prime end as indicated (Supplementary material, Table S1). Specificity of the *N.ceranae* and *N. apis* assay was tested using *N. ceranae* primers and probe on the *N. apis* gBlock and vice versa. The sensitivity of both assays was tested with a gBlock dilution range from 10E6 to 10E1 copies. All tests were performed in eight replicates. Possible inhibition due to matrix effects was tested by adding 1000 copies of *N. apis* gBlock to extracted DNA from honey bee and bumble bee gut tissue and pollen and comparing the Ct with 1000 copies of *N. apis* gBlock in water. Specificity of the bumble bee β-actin qPCR assay was tested by using honey bee and bumble bee as DNA template. Sensitivity was tested with a gBlock dilution range from 10E6 to 10E1 copies.

To determine copies numbers of DNA (and RNA see below) per spore, a dilution series was made of 10E6–10E3 *N. ceranae* spores in pollen dough samples. The derived standard lines were used to calculate from copy numbers to numbers of spores in different sample types and to correct for nucleic acid extraction efficiency in these different matrixes.

RT-qPCR

For RT-qPCR analyses total nucleic acids were DNase treated, since only RNA is used as a target. During the one step RT-qPCR. RNA of the target is specifically reverse transcribed to cDNA which is then amplified during the qPCR. For RT-qPCR, reactions were performed with 1.0 µL target RNA in a final volume of 25 µL including One Step RT-PCR Buffer III, 2.5 U*TaKaRaEx Taq* HS, 0.5 µL PrimeScript RT Enzyme Mix II, 0.125 µl ROX Reference dye II (50× conc.), 300 nM each of forward and reverse primers, 100 nM of the hydrolysis probe. The cycling conditions were an initial 42 °C 10 min, 95 °C 10 sec, followed by 40 cycles consisting of 95 °C for 10 sec and 60 °C for 1 min in a Quantstudio 12 K and analysed as described above.

Exposure experiments N. ceranae

Honey bees

Worker bees from the three distinct colonies, were split into five groups of 30 bees which received one of the five following treatments: 1.13E6 *N. ceranae* spores/mL spores (untreated; 1) γ-irradiation with 1 kGy (2) or 10 kGy (3), freezing at −18 °C (4) or no *N. ceranae* spores (5) as a negative control. The spores were administered in sucrose solution 50% (w/w) in which 1 mL spiked sucrose solution was offered in a punctuated 1.5 mL Eppendorf tube, functioning as a mass feeder for the 30 bees. By trophallaxis the test solution is divided amongst the bees, similar to laboratory first tier oral toxicity tests (OECD, 1998). The test groups had 24 hours to 48 hours, to take in the test solution. Next, every other day new sugar water without *N. ceranae* spores was offered to each group. On day 0, prior to administrative exposure, three bees per group were dissected to check for background *N.ceranae* contamination. After 14 days post administrative exposure, between 7 and 17 (average 12.7 ± 3.3) bees per treatment were alive.

Bumble bees

The bumble bee test colonies started with 10 workers, brood in all stages of development, and one queen. Colonies received pollen dough spiked with 125.000 *N. ceranae* spores per gram dough. Decontamination of the spiked pollen dough was performed by γ-irradiation applying a 1 or 10 kGy dose, or freezing at −18 °C for four days. An amount of 60 gram pollen dough administered, was based on *ad libitum* daily consumption of 60 mg per worker per day (van der Steen, 2008), the estimated colony growth of at least 10 workers, and 10 larvae per week and 29 days exposure. In the egg stage (6 ± 1 days) and pupal stage (6 ± 1 days), no food is consumed, while in the sealed larval stage (8 ± 1 days), open larval stage (6 ± 1 days), and adult stages (>100 days) pollen dough is consumed (van der Steen, 2008). The consumption rate was checked by weekly weighing the pollen dough. Each treatment was replicated in three (3) colonies.

In order to determine the stage of development at *N. ceranae* spore exposure (larval and adult), weekly all adults were taken from the colony, narcotised with CO_2, marked with paint on the dorsal side of the thorax, and returned to the colony. Each week, a different colour coding was used (Supplementary material, Table S2).

Bumble bee sampling and dissection

On day 0, 8, 15, 22 and 29 per colony three bumble bees of each adult week cohort were sampled for individual dissection and molecular detection of *N ceranae* in the ventriculus and in the rectum separately. This consecutive sampling of the coloured age cohorts allows studying the impact of prolonged exposure durations in successive bumble bee development stages. Theoretically, at a chronic exposure during four weeks, workers marked green were already in the colony prior to exposure. In the weekly sampling, these workers were non-exposed on day 0, exposed for one week at sampling after one week, exposed for two weeks at sampling at week two, and exposed for three weeks at sampling at week three. Workers, marked yellow, were first exposed in the last 8 days of their larval development, non-exposed during the pupal development, and finally exposed for 0 − 7 days after emergence when sampled in week three (Supplementary material, Table S2).

Data processing and statistical analysis

For the analytical sensitivity of the qPCR assays for *N. ceranae* and *N. apis* data were evaluated based on eight replicates based on spore numbers or gblocks. At the start of the exposure experiments, 3 bees per population were tested for background infections. Per treatments, on average 29 bees were analysed. This is the sum of the bees that died in the 14 days period and the ones that were alive at the end of the period. For every bee, the ventriculus and the abdomen were analysed separately. This resulted in two samples per honey bee. In total, we analysed 315 honey bee samples of which about half was a ventriculus – and a half was the abdomen analysis.

The number of bumble bees' samples in the test weeks is presented per colour code (Supplementary material, Table S2) and test week. Per sampling, based on treatment and colour code, three (3) bumble bees were sampled and pooled for analysis. Prior to treatment, three workers per colony were sampled and pooled. Of each pooled sample the ventriculus and the rectum were analysed separately.

Of the green marked bumble bees, in weeks 0, 2, 3 and 4, a number of 36, 20, 13 and 0 pooled samples were analysed, respectively. Of the red marked bumble bees these numbers were the same range of weeks, 0, 24, 24, and 0. Of the white marked bumble bees the number of samples was 0, 6, 6, 4; of the blue was this was 0, 0, 6, 4, and of the purple ones 0, 0, 0, 2. In total 145 pooled bumble bee samples were analysed. These analyses included approximately half ventriculus – and half rectum analyses. Calculated by the number of bumble bees analysed this is (145/2)*3 = 218.

For DNA and RNA copy numbers of *N. ceranae* measured based on Ct values and calculated number

of actual spores based on these, t-tests between treatments/populations were performed on log transformed (data +1). P-values <0.05 were taken as significantly different. In the Figures, significantly different treatments/populations are depicted with a different letter.

Results

Development and validation of (RT-) qPCR assays

The analytical specificity of the developed *N.ceranae* and *N. apis* qPCR assays was assessed *in silico* by using the amplicon sequence of both as a query in a BLASTn search on non-redundant sequences in the NCBI database. The second best hit for *N. ceranae* was a *Nosema pernyi* sequence (KJ210747.1) showing only 84% sequence identity (E-value of 2e-10). For the *N. apis* amplicon, the sequence showing the most similarity was *N. ceranae* BRL01 with 80% sequence identity (E-value of 2e-11). The homology of the primer and probe region clearly indicated that the TaqMan assays for both species do not cross react. The specificity for *N. ceranae*/*N. apis* was further verified by negative cross-species PCR: No signal was observed when using the *N. ceranae* primers and probes on 10E6 copies of the *N. apis* gBlock and vice versa.

Analytical sensitivity of the qPCR assays for *N. ceranae* and *N. apis* was evaluated in eight replicates on gBlock dilution series from 10E6 to 10E1 copies (Supplementary material, Figure S1). The Ct was automatically set by the Quantstudio 12K Flex software. For both *N. ceranae* and *N. apis* we were unable to quantify up below 10 copies at a Ct 38 and Ct 35, respectively. The PCR efficiency for the *N. ceranae* assay was 96.36% and for the *N. apis* assay 97.71%. Negative controls (water) showed no amplification signal.

N. ceranae infection in pollen samples

The developed *N. ceranae* RT-qPCR assay was used to detect the natural infection in the pollen samples used in the bumble bee-feeding experiment. To correlate DNA/RNA copy numbers to actual spore numbers, we spiked pollen dough with a dilution series of *N.ceranae* spores in the range of 10E3 to 10E6. Ct values show that detection of the less stable RNA which involves a RT-step is less efficient than DNA detection (Supplementary material, Figure S2). Based on these dilution series we can now calculate the spore numbers in samples, although at high Ct values and thus low concentrations of pollen, – typically found in natural contaminated pollen – these calculations have low accuracy.

In the natural pollen samples, the numbers of spores based on the DNA and RNA measurements ranged from 0 to 100–1000 spores per 10 mg sample (DNA Ct values 33.1–38.5, RNA Ct values 34.1–40; taking into account dilutions). The ratios between RNA and DNA in a sample were low, on average 30×.

N. ceranae in honey bee

To check the virulence of collected *N. ceranae* spores and to test the efficacy of sterilisation procedures, honey bee workers were fed during 24 hours with 50% sugar water with 1 kGy irradiated *N. ceranae* spores, 10 kGy irradiated *N. ceranae* spores, freeze-treated *N. ceranae* spores, alive untreated *N. ceranae* spores ("spiked") or nospores ("control"). In each group the 1500 µL test suspension was consumed within 24 h therefore on average every honey bee consumed 37,500 *N. ceranae* spores.

Prior to the administration of the test solution, three individuals from each of the populations, A, B, and C were tested for natural *N. ceranae* infections. In populations A and C neither DNA nor RNA could be detected in ventriculus and rectum samples. However, in population B, one of the individuals proved naturally infected with *N. ceranae*. Detected copy numbers in both rectum and ventriculus of this individual were low (1–12 copies of DNA/RN Ain the test tube, respectively). Taking into account the weight of the sample analysed relative to the ventriculus, this individual bee of the B population would harbour 7.8E4 spores in the ventriculus (based on DNA). The RT-qPCR detection of RNA indicates active infecting *N. ceranae* spores. Apparently, healthy populations can harbour symptom-less but infected individuals. When this population was monitored over the course of the experiment this natural infection of population B became more and more prevalent, although a large variation from individual to individual was found. As population B had a natural infection that could be obscuring treatments, data from this population was discarded in further analyses. Nevertheless, because of the high variation from individual to individual, we cannot exclude that also in populations A and C natural infections with *N. ceranae* were present.

The Ct values obtained for the actin gene from honey bee rectum and ventriculus, used as a positive extraction/amplification control, ranged from 20 to 24 with a clear amplification curve indicating efficient extraction and amplification in all samples. After two weeks of feeding, the highest spore numbers were detected in the ventriculus of the honey bees fed with the life *N. ceranae* spores (in the range of 10E9–10E11 based on DNA and RNA, respectively), while numbers in the rectum were lower (10E7–10E9) (Figure 1). In the treatments with 1 kGy

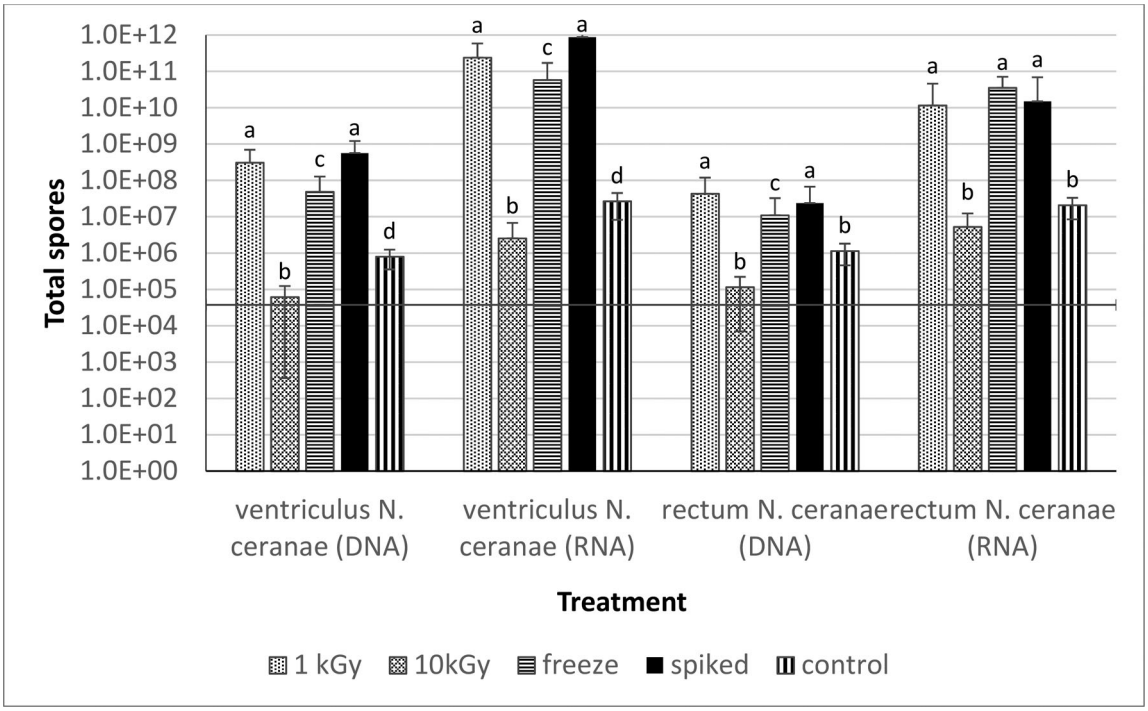

Figure 1. Infection loads measured as total *N. ceranae* cells based on DNA and RNA copy numbers in the whole ventriculus and rectum of honey bees, respectively. Average numbers on log scale are given for honey bees fed for two weeks with (1) 1 kGy irradiated *N. ceranae* spores, (2) 10 kGy irradiated *N. ceranae* spores, (3) freeze-treated *N. ceranae* spores, (4) alive *N. ceranae* spores ("spiked") or (5) control. The horizontal line indicates the average number of *N. ceranae* spores fed to individual bees. Different letters above the columns indicating significant differences are given per location and nucleic acid types as based on t-tests.

irradiation and freezing, abundant DNA and RNA, was detected comparable or only slightly lower than in the spiked, untreated material. Hence, neither 1 kGy radiation nor freezing kills off *N. ceranae* spores sufficiently to prevent infections. In contrast, in the bees fed with 10 kGy treated *N. ceranae* spores the numbers of spores in the ventriculus were significantly lower compared to the non-spiked control group (10E5 to max 10E7). The number of spores in the rectum was also lower, though not significant.

The ratio between the calculated number of present spores based on RNA and DNA in the 1 kGy irradiated *N. ceranae* spores, freeze-treated *N. ceranae* spores, and untreated spiked samples are on average 1280× (range 270–3244×) indicating living, active cells, while for the 10 kGy irradiated (dead!) *N. ceranae* spores and control treatments this ratio is only 35× (21–45×). The higher amounts of RNA and RNA-based calculated spore numbers 1 kGy irradiated *N. ceranae* spores, freeze-treated *N. ceranae* spores, and untreated spiked samples indicate active, infective cells. In contrast, the lower ratios in the 10 kGy irradiated *N. ceranae* spores and the negative control treatments (ratio 50–100×) are indicative of non-active, dead cells.

Bumble bees

The pollen dough offered to each bumble bee colony was infected with *N. ceranae* spores from the same batch as administered to the honey bees. To reduce evaporation of moisture from the pollen dough it was administered divided over three plastic tubes with openings at the front- and rear end. After irradiation, freezing or non-treatment, the pollen dough was placed in the colony in the direct proximity of the brood nest. Pollen dough consumption in the different colonies was roughly similar (Supplementary material, Table S3). However, monitoring of the colony size indicates that colonies fed with 1 or10 kGy irradiated pollen, stay significantly smaller (Supplementary material, Figure S3). In bumble bees that were fed for two-three weeks with pollen spiked with *N. ceranae* spores during their adult life, no accumulation of *N. ceranae* spores was found. In honey bees detected levels of *N. ceranae* spores were on average 10^4-10^7 times higher than in the bumble bees (Figure 2). On average per bumble bee population/treatment combination 12.3 ± 4.8 bees that were fed between 2 and 3 weeks with contaminated pollen were tested, while the Ct values based on the actin gene from bumble bee used as extraction controls, resulted in Ct values in the range of 20–24.

As the bumble bee colonies consumed pollen dough with (1) 1 kGy irradiated *N. ceranae* spores, (2) 10 kGy irradiated *N. ceranae* spores, (3) freeze treated *N. ceranae* spores, (4) untreated *N. ceranae* spores ("spiked") or (5) untreated *N. ceranae* spores,

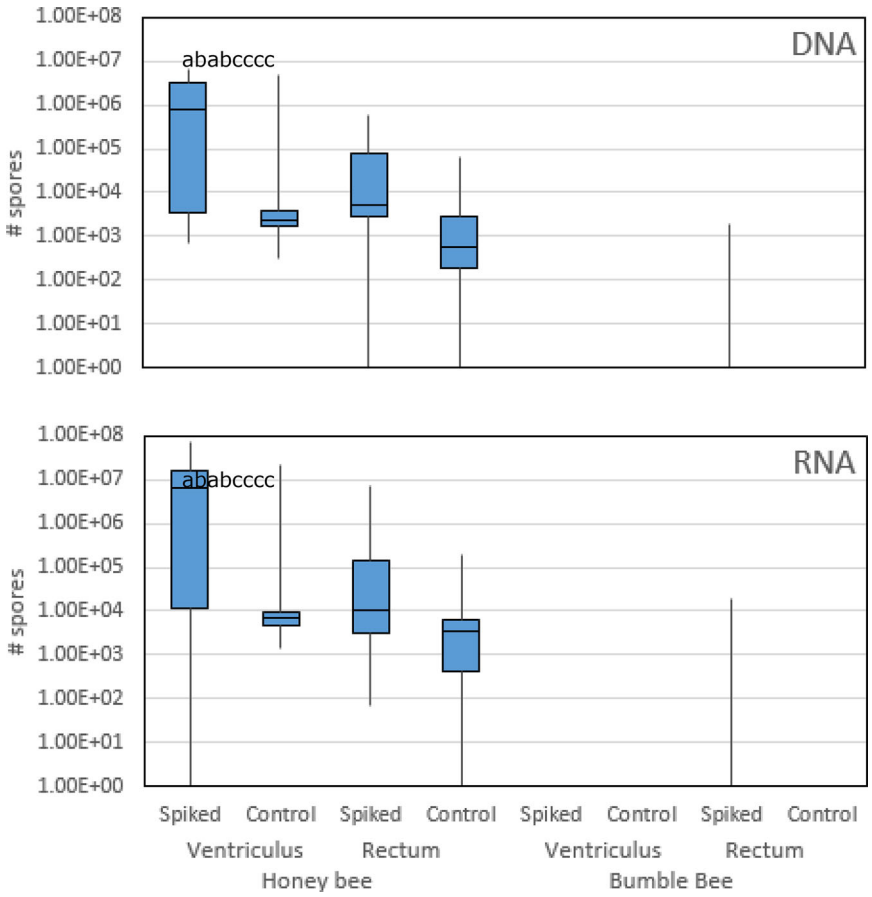

Figure 2. Boxplot for *N. ceranae* spores in both honey bee (left) and bumble bee (right) as detected by DNA (top panel) or RNA (bottom panel). Only the spiked (positive) and the negative control samples are shown. For honey bees, clear differences are observed in the spiked versus the control (1000 fold in DNA and 10,000 fold in RNA) in the ventriculus and 10 fold in the rectum. In bumble bees no accumulation of *N. ceranae* spores was found. Letters a, b and c indicate groups that show significant differences. Both in DNA and RNA content in both ventriculus and rectum in bees, spiked and negative control samples differ significantly (a and b, respectively) these amounts in turn differ significantly from the low amounts observed in bumble bee (c).

weekly, of each colony three workers per coloured age cohort, exposed during successive stages of development were dissected and individual molecular analyses of the bumble bee workers were performed.

N. ceranae in bumble bee faeces

With the low numbers of *N. ceranae,* DNA and RNA detected in the bumble bees, after the termination of the test, the presence of *N. ceranae* in the faecal pellets of the bumble bees was screened. In all treatments, only low copy numbers were detected of up to 370 spores/mg of faecal matter much lower than the amounts added to the pollen (125.000 *N. ceranae* spores per gram dough)fed to the bumble bees.

Discussion

In this study, we developed life/dead RT-TaqMan assays based on the single copy *EF1α*-gene for the detection of *N. ceranae* and *N. apis*, respectively for use in different sample types. These tests can be combined with Taqman assays based on the actin gene of honey or bumble bee hosts as extraction controls. The *N. ceranae* test was used for the further analyses described in this paper: When we tested decontamination techniques only the 10 kGy irradiation effectively kills *N. ceranae* cells. Most importantly, bumble bees are not a host to *N. ceranae* and seem to purge spores from their gastro-intestinal tract efficiently and may even reduce the infection load. A drawback of feeding irradiated pollen dough is the slower growth of the bumble bee colony, which may be due to a missing microbiome that when alive may add to the intestinal microflora and make the pollen better digestible and more nutritious.

Commercially reared *Bombus* spp. are important for the pollination of many of our crops. Global dispersal may lead to the inadvertent spread of bee diseases and parasites (e.g., Goka et al., 2001). Using honey bee-collected pollen as a food source in the rearing of *Bombus* spp. could create a risk of cross contamination of the microsporidia *N. ceranae*, which was reported to occur both in *Bombus* spp. and *Apis*

spp. (Fürst et al., 2014). Previously, cross contamination of pathogens from commercial bumble bees to wild bumble bees and honey bees was suggested as a factor in the decline of wild bumble bees (Colla et al., 2006; Otterstatter & Thomson, 2008). In our study, we show that in bumble bees *N. ceranae* spores only pass through their gut, but bumble bees do not get an infection.

One of the aims of this study was to develop a quantitative detection tool for *N. ceranae* and *N. apis* based on RT-qPCR to allow the distinction between alive and dead fungal cells. The essential Elongation Factor 1-alpha (*EF1α*) gene was chosen as a specific region for primers and probes for both species. qPCR tests on the actin gene of honey bee and bumble bee, respectively can be used as tests for successful extraction of nucleic acids. The mean of the actin Ct values of the bumble bees and honey bees was between 21.6 and 24.6.

The developed RT-qPCR tests proved both specific and sensitive and can detect up to 10–100 spores of the target genes. Although the RT-qPCR is a quantitative method, quantification becomes inaccurate with low numbers of copies/spores. The ratio of RNA and DNA in a sample proves an indication of the activity of these spores. In pollen, the ratios between RNA and DNA are low, indicative of dormant or possibly dead cells, while during infection these ratios are 10-100× larger in active cells. Ratios are thus also an indication of cellular activity.

The second aim was to test the efficacy of freezing and gamma irradiation sterilisation techniques for killing *N. ceranae* spores in spiked sugarwater (honey bee experiment) and pollen dough (bumble bee experiment). In captivity reared bumble bees are fed with honey bee collected pollen. Treatment of pollen to avoid cross-infection with honey bee pathogens like *N. ceranae* is standard practice in the commercial production of bumble bees for pollination. Especially gamma irradiation is proposed and used as a method to decrease the infectivity of *N. ceranae* spores (Graystock et al., 2016; Simone-Finstrom et al., 2018). Our experiments show that 10 kGy irradiation kills *N. ceranae* spores efficiently, but that a lower dose (1 kGy) may not be sufficient. This is in accordance with the recent results of Álvarez Hidalgoand co-workers (2020) who find that at least 5-7 kGy is needed to kill fungi. Additionally, freezing pollen appeared only partially effective and should not be recommended to kill the *N. ceranae* spores.

N. ceranae infection test with caged honey bee workers

Honey bees were fed treated and un-treated *N. ceranae* spores. The honey bee infection was conducted to prove the viability and infectivity of the *N. ceranae* spores. The 14-days response to the different treatments varied greatly within and between the different colonies. This is in line with the finding of Porrini et al. (2011) that the parasite response depends on the host conditions. The lower amount of spores per mg tissue in the rectum, compared to the numbers found in the ventriculus, indicate that that the number of released spores is lower than the number that is still dividing in the ventriculus, possibly because the inter-alimentary track transport to the rectum has not yet fully commenced or this phase is shorter in time/more diluted with other material. The ratios between the amount of RNA and DNA indicate high activity of the *N. ceranae* spores. In heavily infected individuals this ratio goes up to a factor 1000. It is important to note that in this heavily infected stage, the honey bees were still not showing visual symptoms of infection.

In the negative control bees of one colony, the dissection 14 days after infection revealed RNA in the ventriculus. Although these bees were not exposed to *N. ceranae* spores and no *N. ceranae* has been detected in the colony prior to the infection test. This shows that asymptomatic honey bees may carry latent infections that can remain unnoticed. However, these latent infections increased in a 14 day period which may lead to misinterpretation of infections. We recommend careful checking of starting material with sensitive techniques to prove absence of *N. ceranae* in the control group. In free living bees the number of infected honey bees may be higher than expected based on traditional PCR methods that are less sensitive than qPCR.

Group feeding of honey bees in laboratory cages of on average 10,000 viable *N. ceranae* spores per bee, results in infection (Fries et al., 2013). Individual feeding of 4600 spores per bees also induces an infection. The honey bees in our study, were administered a relatively high dose of 37,500 *N. ceranae* spores per bee. In this way decontamination with efficacy of approximately 90% would result in spores numbers that would be sufficient to induce an infection. This is required to critically assess the decontamination steps.

The dissection of the bees was done 14 days after the initial *N. ceranae* spores feeding. This 14 days period is long enough for *Nosema* to cause an infection and reproduction as shown by Fries (1988) for *N. apis* and in 2012 by Pettis et al. (2012) for *N. ceranae*. The study by Porrini et al. showed, that spore development in the ventriculus epithelium was between 6 and 9 days and peaked before 12 days. Consequently, the dissection in our study, 14 days after infection was induced should be long enough for reproduction. In our study, on average every honey bee took in 37,500 *N. ceranae* spores

resulting after 14 days in 10^6 spores in the ventriculus. The number of spores transferred to the rectum was lower with around 10^4 spores. As honey bees do not defecate in cages the spores are not expected to get out of the gastrointestinal tract and therefore expected reproduction factor is 30 times in 14 days. This number is expected to further increase as the spores in the ventriculus are highly active and probably still dividing.

The 1 kGy irradiation – and freeze treatment of the *N. ceranae* spores, prior to the administration had only a minor, non-significant reducing effect. Only the irradiation of 10 KGy resulted in a non-infection. Gisder et al. (2010) demonstrated a marked reduction of the germination after four days, 4 °C treatment of the *N. ceranae* spores. One week in the freezer resulted in 12.5% mortality and still 87.5% viable spores and this mortality slightly increased in a three-week treatment study (Fenoy et al., 2009). Our freezing treatment showed no significant effect on the *N. ceranae* infection, as would be expected for an efficacy of 10 to even 90% given the high dose of spores applied in our study. Only efficacies close to 100% are expected to result in significant changes as was observed for the 10 KGy radiation. Interestingly in all cases, lower infection levels were observed in the 10 KGy radiation treatment compared to non-spiked control. Possibly the high number of dead *N. ceranae* spores trigger the immune system of honey bees resulting in lower reproduction rates of *N. ceranae*.

N. ceranae infection is very common in honey bee colonies in Europe. The data of the Dutch surveillance study shows a great variation in infection levels, both temporally and spatially. In our study, also colonies of honey bees that appeared healthy had a low level of natural infection demonstrated by the background detection in population B at the start of the experiment and also the low detected levels in the 10 kGy-pollen fed and negative controls after 14 days. The natural untreated pollen actually proved to test positive when tested directly in the qPCR, while colonies fed with the naturally infected pollen (the "control" groups) actually resulted in intermediate levels of infection. As the technical controls, the negative controls of the TaqMan assays, were all negative and the specificity of the test is high, we hypothesise that *N. ceranae* may be more prevalent and often undetected and/or latent. This complicates epidemic studies and highlights the importance of the immune system and physiological factors to combat this disease in honey bees.

N. ceranae infection tests in bumble bee colonies under indoor rearing conditions

In the study by Higes et al. (2008) *N. ceranae* spores were detected in bee collected pollen. Our results corroborate these findings, low numbers of spores were detected in the honey bee-collected pollen. Therefore, the mutual food source for both honey bees and bumble bees could be a potential infection route of the foraging bumble bee worker. It is certain that honey bee collected pollen, as it is applied in the bumble bee rearing facilities can be contaminated with *N. ceranae*.

The exposure tests are conducted in bumble bee colonies in which weekly the number of workers was counted, but no calculations have been done with the number of eggs, larvae, and adults. This was a deliberate study set-up because any exact counting would impact brood development due to distribution and on-exact counting would only result in rough estimations. The study set-up represents the indoor rearing practise. A bumble bee colony, starting with about 10 workers and ending with about 120 workers consumes in a three-week period about 60 grams pollen dough in which the majority was swallowed in the first two weeks. This consumption rate is in line with the findings in the Tasei and Aupinal study (2008). In this study, the mean daily consumption of a bumble bee colony after oviposition started was 2.8 grams per day. This included adult feeding, larval feeding, and nest construction. However, as discussed above the different disinfection methods led to different nutritious values of the pollen: colonies fed irradiated pollen stopped growing after four weeks while the colonies fed non-irradiated or frozen pollen continue to grow although the growth flattens at about 120 bumble bees.

The actual exposure duration via the pollen route to bumble bee colonies was three weeks in which during week three, the last experimentally contaminated pollen dough was consumed. In week four, the colonies were fed unspiked, untreated pollen. In this way on average an amount of 7.5E6 viable *N. ceranae* spores were presented to the colony. After three weeks all *N. ceranae* spores were taken up by the colony. Individuals were exposed during different stages of their development. Chronic feeding of living *N. ceranae* to bumble bee colonies, during which adults were exposed for minimally two and maximally three weeks and larvae during the entire larval period, did not result in infection. *N. ceranae* could only be detected in very low numbers in the bumble bee workers and in low numbers in the faeces deposited in the nest, showing no sign of infection/multiplication of *N. ceranae* in the bumble bee colony. On the contrary, these findings indicate actively purging of the *N. ceranae* spores and possibly degeneration by the bumble bee workers. This hypothesis is interesting to be tested in the future, in controlled feeding and faeces collection experiments, combined with dissection.

In contrast to the results of *N. bombi* infection tests (van der Steen, 2008), this study shows that

chronic exposure to adults, as well as exposure of these individuals in the larval stage, does not result in *N. ceranae* infection.

Feeding bumble bees with gamma irradiated pollen has the drawback that the feed proves less nutritious and the colony grows less well. This may be due to the loss of the natural microflora on the pollen: A healthy microbiome is important for the development of bee development and health (Raymann & Moran, 2018).

In conclusion, the low detected numbers of *N. ceranae* spores based on DNA and RNA, in our bumble bees clearly suggests that *N. ceranae* is not pathogenic to bumble bee and explains why supposedly infected bumble bees show no symptoms (Fürst et al., 2014). Sensitive molecular assays like qPCR are required to eliminate possible latent infection as the cause of nosemosis in feeding experiments. Unfortunately, these molecular methods are destructive and a large variation between individuals was found. Potentially the use of faecal material can be helpful. In addition rearing honey bees in the lab may eliminate the problem of latent infection present at the start of the experiment. The molecular methods described in this study including the procedure to test for RNA can assist epidemiological studies in the future. In particular, for these quantitative tests, it would be useful to study reproduction rates of *N. ceranae* and *N. apis* under various developmental, physiological and stress conditions.

Disclosure statement

The authors declare to have nothing to disclose.

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