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Specific detection and quantification of three microsporidia infecting bees, *Nosema apis*, *Nosema ceranae,* and *Nosema bombi*, using probe-based real-time PCR

Aurélie Babin [a,*], Frank Schurr [a], Marie-Pierre Rivière [a], Marie-Pierre Chauzat [a,b,1], Eric Dubois [a,*,1]

[a] *ANSES, Sophia Antipolis Laboratory, Unit of Honey Bee Pathology, F-06902 Sophia Antipolis, France*
[b] *Paris-Est University, ANSES, Laboratory for Animal Health, F-94701 Maisons-Alfort, France*

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Abstract

Among stressors affecting bee health, *Nosema* microsporidia are prevalent intracellular parasites. *Nosema apis* and *Nosema ceranae* have been described in honey bees (*Apis* spp.), while *Nosema bombi* has been described in bumble bees (*Bombus* spp.). Although available molecular methods serve as a complement to microscopic diagnosis of nosemosis, they do not enable accurate quantification of these three *Nosema* species. We developed three quantitative real-time PCRs (qPCRs) starting from *in silico* design of specific primers, probes, and recombinant plasmids, to target the RNA polymerase II subunit B1 (*RPB1*) gene in the three species. The complete methods, including bee grinding, DNA purification, and qPCR, were validated in honey bee (*Apis mellifera*) homogenate. Specificity was assessed *in silico* and *in vitro* with several types of bee samples. The limit of detection was estimated at 4 $\log_{10}$ copies/honey bee. A small, systematic method bias was corrected for accurate quantification up to 10 $\log_{10}$ copies/honey bee. Method accuracy was also verified in bumble bee (*Bombus terrestris*) and mason bee (*Osmia bicornis*) homogenates in the range of 5 to 10 $\log_{10}$ copies/bee. These validated qPCR methods open perspectives in nosemosis diagnosis and in the study of the parasite's eco-dynamics in managed and wild bees.

Keywords: *Nosema apis*; *Nosema ceranae*; *Nosema bombi*; Honey bee; Real-time qPCR; *RPB1* gene

Introduction

Pathogens and parasites, and their associated diseases, are major threats among the multiple interacting stressors responsible for recent honey bee colony losses and wild bee population decline (Goulson et al., 2015). Bee populations and species naturally harbour pathogens and parasites, and likely exchange them via shared floral resources (Alger et al., 2019; Durrer and Schmid-Hempel, 1994; Mazzei et al., 2014; McMahon et al., 2015) and pollinator trade (Gamboa et al., 2015; Goulson et al., 2015; Graystock et al., 2013a; Hedtke et al., 2015; Parmentier et al., 2016).

*Corresponding authors.
E-mail addresses: aurelie.babin@hotmail.fr (A. Babin), eric.dubois@anses.fr (E. Dubois).
[1] These authors contributed equally to the work.

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Among prevalent parasites, the unicellular eukaryote *Nosema* microsporidia (Nosematidae) are obligate intracellular parasites (Corradi, 2015). Two species have historically been described infecting honey bees: *Nosema apis* (Zander, 1909) and *Nosema ceranae* (Fries et al., 1996), and one species infecting *Bombus terrestris* bumble bees: *Nosema bombi* (Fantham and Porter, 1914). Honey bee nosemosis can strongly affect colony performance, jeopardising overwinter survival, and likely contributes to colony losses (Graystock et al., 2016; Pasca et al., 2019). Conflicting effects of *N. bombi* infection on individual bumble bees or colonies have been reported. These effects range from negative impacts to covert chronic effects, and even to positive fitness effects (Fries et al., 2001; Imhoof and Schmid-Hempel, 1999; Larsson, 2007; Otti and Schmid-Hempel, 2007, 2008; Schmid-Hempel and Loosli, 1998; van der Steen, 2008; Whittington and Winston, 2003). While *N. apis* has been detected only in *Apis mellifera* honey bees so far, *N. ceranae* infects *A. cerana*, *A. mellifera* (Fries et al., 2006; Gomez-Moracho et al, 2015; Martin-Hernandez et al., 2018) and a wide range of pollinators, including bumble bees and European mason bees (Arbulo et al., 2015; Gamboa et al., 2015; Graystock et al., 2013a; Graystock et al., 2013b; Li et al., 2012; Plischuk et al., 2009; Ravoet et al., 2014; Martin-Hernandez et al., 2018). *Nosema bombi* has been detected in commercially reared and wild bumble bees (Cordes et al., 2012; Graystock et al., 2013b; Kissinger et al., 2011; Tripodi et al., 2014), but not in *Apis* bees or mason bees so far.

Possible cross-infections by these *Nosema* parasites between bee species, their potentially high prevalence, and the ecological and economic impacts of infection on colony performance and bee diversity underlie the need for specific and accurate assessment of parasite loads. To date, only a few methods allowing accurate *Nosema* quantification have been developed, and data on infections by these three parasites in the same bee species are scarce (Erler et al., 2012; Fries et al., 2013). Used as a first-step tool for the detection of *Nosema* infection, spore counting on a haemocytometer by light microscopy lacks sensitivity and specificity for the discrimination between *Nosema* species (Stevanovic et al., 2013). Nosemosis diagnosis now relies mainly on molecular detection associated with the observation of clinical signs, when recordable. It targets the conserved and species-specific regions of the multicopy ribosomal RNA (rRNA) genes (Fries et al., 2013; OIE Terrestrial manual, 2018; Rivière et al., 2013). Many molecular methods yield only qualitative diagnosis with or without species discrimination, by conventional simplex/multiplex PCR (Carletto et al., 2013; Klee et al., 2006; Martin-Hernandez et al., 2007; Plischuk et al., 2009; Stevanovic et al., 2011), restriction fragment length polymorphism PCR (RFLP-PCR; Gisder et al., 2010; Klee et al., 2007) and sequencing (Chen et al., 2012; Cordes et al., 2012; Fries et al., 2001; Li et al., 2012). A few methods yield quantitative data by conventional duplex semi-quantitative PCR (Hamiduzzaman et al., 2010), or simplex/duplex real-time quantitative PCR with non-specific dye-based protocols (Burgher-MacLellan et al., 2010; Forsgren and Fries, 2013; Osterman et al., 2019; Wintermantel et al., 2018) and specific probe-based protocols (Bourgeois et al., 2010; Chen et al., 2009; Copley and Jabaji, 2012; Copley et al., 2012; Traver and Fell, 2011). Very recently, an ultra-rapid real-time quantitative PCR method has been developed for the sensitive detection within 20 min of low-intensity *N. ceranae* infections in *A. mellifera* (Truong et al., 2021).

Based on multi-copy rRNA genes, these molecular methods enable highly sensitive diagnosis, but this comes with several issues. The reliability of duplex PCRs for species discrimination was sometimes not confirmed (Erler et al., 2012), potentially leading to false-negative results and species misidentification, and hence to misestimation of infection prevalence (Gisder and Genersch, 2013; Stevanovic et al., 2011). This issue has been linked to the high intraspecies sequence variability (SNPs, indels, ITS tandem repeat numbers) reported for the three *Nosema* species, even within individual dikaryotic spores for *N. bombi* (Chen et al., 2013; Cordes et al., 2012; Cornman et al., 2009; Gatehouse and Malone, 1998; Ironside and Corradi, 2013; O'Mahony et al, 2007; Tay et al, 2005; Sagastume et al., 2011; Martin-Hernandez et al., 2018). Recombination between *N. ceranae* haplotypes adds further polymorphism (Sagastume et al., 2011, Sagastume et al., 2016), and opens the possibility that recombination may occur between *Nosema* species within infected host cells (Gisder and Genersch, 2013; Huang et al., 2008). Additionally, precise *Nosema* quantification in parasites per bee based on rRNA genes does not appear to be possible. Gene copy numbers are difficult to determine given the existing variability in *N. ceranae* and *N. apis* genomes (Chen et al., 2013; Sagastume et al., 2011). A preliminary unpublished study found 10 gene copies in the *N. ceranae* genome (Baffoni et al., 2016). For *N. bombi*, the assembled genome is not available, excluding the possibility of determining the number of gene copies.

Consequently, molecular diagnosis methods targeting conserved single-copy coding genes have been developed to overcome these issues. A duplex PCR method improved the discrimination between *N. apis* and *N. ceranae* by targeting the RNA polymerase II subunit B1 (*RPB1*) gene (Gisder and Genersch, 2013). This housekeeping gene codes for the DNA-dependent RNA polymerase II largest subunit, frequently preferred to rRNA genes for microsporidia phylogenetic analyses because of its conserved sequence that facilitates the analysis of genome diversity between species (Hirt et al., 1999; Ironside, 2007; Maside et al., 2015). Recently, the development of a probe-based real-time quantitative PCR method targeting another single-copy gene, the heat-shock protein *Hsp70* gene,

yielded parasite loads in honey bee samples from Italy for *N. ceranae* (Cilia et al., 2018a; Cilia et al., 2018b). These studies open promising perspectives for the improvement of nosemosis molecular diagnosis. Nevertheless, there remains a need for a harmonised and accurate method that allows the detection and quantification of the three main bee microsporidia, *N. apis*, *N. ceranae*, and *N. bombi*, which can be applied to several bee pollinators. This article describes the first probe-based real-time quantitative simplex PCR method that relies on the single-copy gene *RPB1* for the independent detection and quantification of the three *Nosema* species in honey bees, bumble bees, and mason bees. It also reports validation parameters, which include PCR efficiency, specificity, limit of detection, limits of quantification and quantification accuracy.

Material and methods

Bee sample preparation for PCR

Bees were ground in 10 mM phosphate buffer for tissue disruption (pH 7.0; 1 mL buffer/honey bee or mason bee, and 2 mL/bumble bee). Coarse homogenates (1 mL) were transferred to microtubes containing 750 mg of 0.1–0.25 mm glass beads and 5 inox beads of 3 mm for fine grinding on a Mixer Mill MM400 (Retsch, Haan, Germany) to disrupt *Nosema* spores. After two centrifugations (10 min, 8000×*g*, 4 °C), nucleic acid purification from a 150 µL homogenate was performed on silica membranes with a NucleoSpin RNA Virus kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions, including proteinase K lysis at 56 °C for 10 min. Elution was performed in 100 µL of 5 mM Tris-HCl buffer (pH 8.5).

qPCR primers and probes, and recombinant DNA plasmids

The design of qPCR primers and probes, and of standard recombinant plasmids used to establish the standard curves, relied on the retrieval of DNA sequences of the *RPB1* gene available in the GenBank database (https://www.ncbi.nlm.nih.gov/). A set of 61 *N. apis* sequences (21 isolates from 13 countries, 1–3 clones per isolate) and a set of 59 *N. ceranae* sequences (21 isolates, from 19 countries, 2–3 clones per isolate) were retrieved from those sequenced by Maside et al. (2015) (sequence details in Supplementary information S1). Sequence alignments using Mega (version 7.0.26; ClustalW method, default parameters; Kumar et al., 2008; Kumar et al., 2016) yielded an *N. apis RPB1* consensus sequence of 992 nucleotides and an *N. ceranae RPB1* consensus sequence of 1,056 nucleotides (Supplementary information S2). For *N. bombi*, since the parasite genome has not yet been sequenced, primer-probe trios were designed on the single DNA sequence available, of 615 nucleotides (Supplementary information S2). Specific primer-probe trios were designed using Primer3 (version 4.1.0; Koressar and Remm, 2007; Untergasser et al., 2012; Kõressaar et al., 2018), targeting the conserved parts of the *N. apis* and *N. ceranae* consensus sequences. The quality parameters of each primer-probe trio were evaluated in Primer3 and OligoCalc (version 3.27; Kibbe, 2007) (melting temperature, complementarity, self-priming, potential hairpins, and primer-probe hybridisation). The *in silico* analytical specificity was verified with BLAST® (Altschul et al., 1990).

The consensus sequences were cloned in vector Amp+ pUC57 (GeneCust) for the synthesis of three standard plasmids (one per targeted species: pNa for *N. apis*, pNc for *N. ceranae*, and pNb for *N. bombi*). For polymorphic sites, the most frequent nucleotide was included in the sequence (Supplementary information S3). Lyophilised plasmids were rehydrated in Tris-EDTA buffer (100 mM Tris, 50 mM EDTA, pH 8.0), then quantified by UV spectrophotometry for concentration adjustments.

Harmonised real-time qPCR

Primer and probe concentrations in the reaction mix were adjusted in order to optimise PCR efficiency between 80 % and 120 % (AFNOR, 2015) and to harmonise the qPCR methods. Reactions were performed in duplicate in 96-well MicroAmp™ optical reaction plates (Applied Biosystems, Waltham, MA, USA) on a QuantStudio 5 thermocycler (Applied Biosystems). The 20-µL final PCR mix included 5 µL of DNA template, 1X LightCycler® 480 Probes Master (Roche), 50 nM of the passive reference dye ROX (Life Technologies), 500 nM of each primer, and 200 nM of probe. Thermal conditions were 95 °C for 3 min (polymerase activation), 45 PCR cycles at 95 °C for 10 s (denaturation), 60 °C for 30 s (primer annealing), 72 °C for 25 s (elongation), and final cooling at 40 °C for 10 s. Specific standard curves were established by linear regression analysis of the Cq values *vs* the $\log_{10}$ standard plasmid copy numbers (from 8.0 $\log_{10}$ to 2.0 $\log_{10}$ copies/PCR). For statistical analysis, all loads were transformed into decimal logarithm ($\log_{10}$). The coefficient of variation (CV) of each plasmid dilution was below 5 %.

qPCR performances

Several parameters assessing the performance of the three qPCRs were recorded following French standard NF U47-600 (AFNOR, 2015), by running three independent plates each loaded with standard plasmid dilutions, in duplicate for within-run repeatability. For each qPCR, six standard curves were established and used to evaluate the following parameters:

i. qPCR efficiency (E_{PCR}) calculated for each replicate with the slope of the linear regression formula: Cq = a × lo

g_{10} (plasmid copy number) + b, where a is the slope and b the intercept. E_{PCR} was calculated with the formula E_{PCR} (in %) = $(10^{(-1/a)} - 1) \times 100$. This parameter should range from 75 % to 125 % for the qPCR to amplify the target DNA sequence satisfactorily.

ii. intercept of the qPCR standard curve showing Cq precocity.

iii. R^2 of each standard curve measuring the relevance of the linear adjustment.

iv. coefficient of variation (CV, in %) for the Cq of each dilution point of the standard curve measuring intra-laboratory reproducibility.

v. mean linear qPCR uncertainty (U_{LIN}) obtained by calculating the $\log_{10}$ plasmid loads from the Cq and comparing these values with the theoretical $\log_{10}$ loads of the quantification standard (AFNOR, 2015).

vi. limits of quantification of the qPCR (LOQ_{PCR}) tested on the seven dilution points of the standard curve, and corresponding to the minimum and maximum concentrations of the quantification range with the maximum bias set at ±0.25 $\log_{10}$.

The limit of detection of the PCR (LOD_{PCR}), which is the lowest DNA concentration detected in ≥95 % of the replicates, was assessed on 24 replicates of the lowest concentration of the standard, evenly distributed on three independent PCR runs. The LOD_{PCR} was valid if a specific amplification signal was detected in at least 23 replicates (AFNOR, 2015).

Experimental qPCR specificity

Two parameters assessed experimental specificity: inclusivity, which verifies the amplification in all positive samples by the PCR method; and exclusivity, which verifies that samples free of the targeted *Nosema* species are not assigned as positive by the PCR method (AFNOR, 2015). Inclusivity and exclusivity were evaluated on two sample types: infected honey bee and bumble bee samples that have already been characterised with a diagnostic method validated by the European Reference Laboratory for Bee Health (S. Franco and V. Duquesne, personal communication), and recombinant plasmids with *N*osema sequences. To address *N. apis* genetic variability, three new recombinant plasmids were designed in addition to the standard plasmid pNa (Supplementary information S3). Similarly, one new recombinant plasmid was designed for *N. ceranae* in addition to the standard plasmid pNc (Supplementary information S3).

Method performances

For practical reasons, the method performance parameters were assessed by spiking bee homogenates devoid of the target parasites with defined loads of the standard plasmids. The spiked bee homogenates went through the procedure of nucleic acid purification described earlier prior to the qPCR. Final results were expressed in $\log_{10}$ copies/bee after the conversion of qPCR results in copies/PCR. The conversion factor for each bee homogenate was based on the volume yielded and sampled at each step of the entire method; this factor was 133 for honey bee and mason bee homogenates, and 267 for bumble bee homogenate.

The LOD_{METHOD} is the lowest DNA concentration reliably detected positive and was preliminarily evaluated at 4 or 5 $\log_{10}$ copies/bee. Following the French standard (AFNOR, 2015), it was verified on eight samples derived from the two independent plasmid spikes, each spiked sample being purified four times.

The limit of quantification of the method (LOQ_{METHOD}) and the mean method bias were assessed by building and interpreting an accuracy profile for each qPCR that estimates the precision and reliability of the qPCR values (Feinberg, 2007; AFNOR, 2015). The accuracy profiles were built independently for the three bee species. The profiles contained three or four plasmid loads, ranging from the LOD_{METHOD} (corresponding to 4 or 5 $\log_{10}$ copies/bee) up to 10 $\log_{10}$ copies/bee. On three independent serial plasmid dilutions per bee species, the results for each plasmid load were compared to the expected theoretical load. The accuracy profiles were built with the mean bias (trueness error) between the mean result and the theoretical load, and the reproducibility of the data (SD_R, precision error) (AFNOR, 2015). Interpretation of the accuracy profiles enabled us to define the lower and upper LOQ_{METHOD}, which are the lowest and highest plasmid loads quantified with acceptable accuracy of ±1 $\log_{10}$ copies/bee (i.e. between the acceptable accuracy limits displayed on the profiles).

Results

Primers and probe design

For each *Nosema* target, the primer-probe trio showing optimal characteristics *in silico* (specificity, amplicon size, melting temperature, no cross-match) and *in vitro* (specificity, PCR performance, fluorescence increase amplitude, Cq of the standard curve) was selected (Table 1). *In silico*, the *N. apis* primers and probe matched on 217 *RPB1* gene sequences with 100 % coverage and 100 % identity. The amplicon sequence contains five polymorphic sites that are not the target of the primers or the probe, allowing the detection of within-species diversity (see Supplementary information S4). Among the other organisms' matched sequences, the probe partially matched (82 % coverage) on a few sequences of *Paenibacillus polymyxa* and *P. peoriae*, two plant-beneficial soil bacteria unlikely to be present in bee homogenates. For *N. ceranae*, the primers and the probe matched on 84 sequences available with 100 % coverage and 100 % identity. Only one mismatch on the 3rd nucleotide at the 5′ end of the reverse primer was found

Table 1. Trios of primers and probe selected for each target *Nosema* species based on the results of the *in vitro* assay, and some of their characteristics.

Species	Primers and probe (5′-3′)	Melting temp. (°C)	Amplicon size (bp)
N. apis	Fwd: TGCAGATTTTGACGGAGATGA	57.5	138
	Rev: TGTACAATACCCATTATAGGACGAPr: TGAATTTACACATGCCACAATCA	60.3	
	(6FAM, MGB-Eclipse®)	57.6	
N. ceranae	Fwd: TCTTGTTCCTCCACCATCAGT	59.5	75
	Rev: TGTGTCAAATCATCTTCTGCTCTPr: ATCTATTGTTATGGAAGGGATG	59.2	
	(6FAM, MGB-Eclipse®)	56.4	
N. bombi	Fwd: GGAGAAATCTGTGAAAGTGGGT	60.1	81
	Rev: GGCTACTAGTCCCATTCCTTCTPr: TGTGGGAATAAACAGCCTGCT	62.1	
	(6FAM, BHQ1®)	59.5	

in two additional sequences. The reverse primer and the probe partially matched (up to 95 % coverage and/or identity) on a few sequences of *Nosema* moth parasites (*N. lymantriae*, *N. fumiferanae*, *N. bombycis* and *N. tyriae*). The *N. bombi* primers and probe, designed with the single available *RBP1* sequence, partially matched (72 % to 76 % coverage) on a few sequences of bacteria unlikely to be present in bee homogenates: *Paenibacillus odorifer* (responsible for milk spoilage) and *P. baekrokdamisoli* (isolated from soil and crater lakes).

In vitro, the test of the primer-probe trios on the standard plasmids (pNa, pNc, and pNb) showed no cross-specificity on the two non-target *Nosema* species, and a sharp fluorescence increase during amplification on the specific standard. PCR efficiency (E_{PCR}) on logarithmic serial dilutions of the standard plasmids ranged from 95 % to 110 %. Cq of the lowest concentration (2.0 $\log_{10}$ plasmid copies/PCR) was below or close to 40 PCR cycles.

qPCR performances

For *N. ceranae* and *N. bombi* qPCRs, the linear domain covers 6 $\log_{10}$, the minimum and maximum LOQ_{PCR} were 2.0 $\log_{10}$ and 8.0 $\log_{10}$ plasmid copies/PCR, respectively (Fig. 1). For *N. apis* qPCR, the lowest plasmid concentration (2.0 $\log_{10}$ copies/PCR) was not systematically detected (positive for four technical replicates among six tested), consistent with the higher standard curve intercept (Table 2). Consequently, the linearity domain was reduced to 3.0 to 8.0 $\log_{10}$ plasmid copies/PCR (Fig. 1). Between-replicate variability was low (absolute value of U_{LIN} below 0.25 $\log_{10}$). All the assessed qPCR parameters were satisfactory and fulfilled the French standard requirements (Table 2).

The LOD_{PCR} differed between target parasites. For both *N. ceranae* and *N. bombi* qPCRs, the LOD_{PCR} equalled the minimum LOQ_{PCR}, 2.0 $\log_{10}$ plasmid copies/PCR (24/24 detected). For *N. apis*, consistently with the linearity domain results, the lowest standard concentration was detected in less than 95 % of the replicates (14/24 detected at 2.0 $\log_{10}$ plasmid copies/PCR). The validated LOD_{PCR} was 3.0 $\log_{10}$ plasmid copies/PCR (24/24 detected).

Experimental qPCR specificity

Nosema apis inclusivity was tested on the four recombinant plasmids (including the pNa standard plasmid) and on one honey bee sample diagnosed positive for *N. apis*. *Nosema ceranae* inclusivity was tested on the two recombinant plasmids (including the pNc standard plasmid) and nine natural honey bee samples diagnosed positive for *N. ceranae*. *Nosema bombi* inclusivity was tested on the standard plasmid and two natural bumble bee samples diagnosed positive for this parasite. For all these samples, each specific qPCR yielded a detectable signal and loads relatively similar to, but not below, those expected (Table 3).

For each *Nosema* qPCR, exclusivity was tested on the heterologous *Nosema* samples described above, and the test yielded no amplification (Table 3). In addition, exclusivity was tested on natural bee samples that were previously diagnosed positive for *Paenibacillus larvae* (n = 4) and *Melissococcus plutonius* (n = 5), two honey bee pathogenic bacteria, and the test also yielded no amplification.

Method performances

In honey bee homogenate, the LOD_{METHOD} was estimated at 4.0 $\log_{10}$ copies/bee for *N. ceranae* and *N. bombi* methods (8/8 samples detected positive). For *N. apis*, 7/8 spiked samples were detected positive, which meant that the LOD_{METHOD} was estimated to be slightly higher than 4.0 $\log_{10}$ copies/bee. The plasmid quantifications revealed systematic positive biases for the three methods. In detail, the method for *N. apis* quantification overestimated plasmid loads with a mean bias of 0.63 $\log_{10}$ copies/bee, that for *N. ceranae* quantification with a small mean bias of 0.16 $\log_{10}$ copies/bee, and that for *N. bombi* quantification with a mean bias of 0.37 $\log_{10}$ copies/bee. For all the *N. apis* plasmid loads except the lowest one, the tolerance limits of the accu-

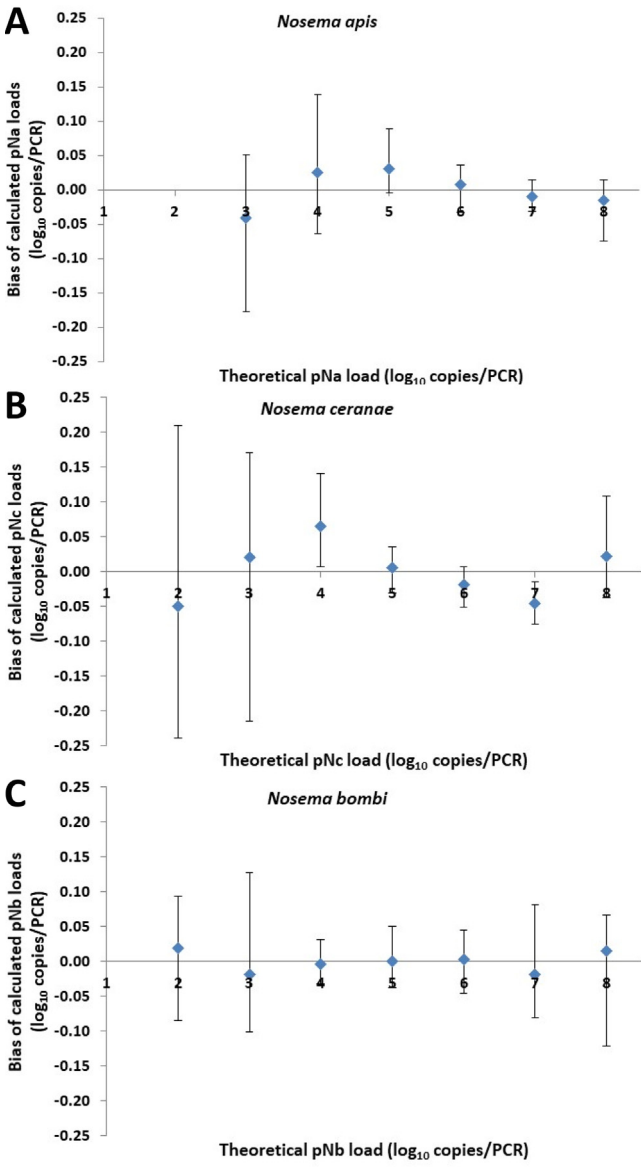

Fig. 1. Quantification bias of the three *Nosema* qPCRs between the detected loads and the theoretical loads, based on six standard curves from the duplicates of three independent serial plasmid dilutions. Bars stand for minimum and maximum biases.

racy profile fell inside the acceptable accuracy limits. Corrections of the mean bias improved the accuracy (trueness and precision errors) of the methods, especially for *N. apis* quantification (Fig. 2). The low and high LOQ_{METHOD} of the three methods were calculated and indicate that the plasmid loads were accurately quantified from 4.0 $\log_{10}$ to 10.3 $\log_{10}$ copies/bee.

Quantification of plasmid spikes in bumble bee homogenate yielded similar systematic positive biases for the three methods: 0.54 $\log_{10}$ copies/bee for the *N. apis* method, 0.11 $\log_{10}$ copies/bee for the *N. ceranae* method, and 0.49 $\log_{10}$ copies/bee for the *N. bombi* method. The tolerance limits of the accuracy profiles fell outside the acceptable accuracy limits for the intermediate plasmid load for *N. apis* and *N. bombi* methods, but this was improved by bias correction (Fig. 3). The accuracy domain of corrected loads ranged from 5.3 $\log_{10}$ to 10.3 $\log_{10}$ copies/bee for the three methods in this bee species. By contrast, plasmid quantification in mason bee homogenate yielded systematic large and negative biases of about −1 $\log_{10}$ copies/bee, with tolerance limits falling outside the acceptable limits in most cases (*N. apis* method: −1.06 $\log_{10}$ copies/bee, *N. ceranae* method: −1.04 $\log_{10}$ copies/bee, *N. bombi* method: −0.86 $\log_{10}$ copies/bee). As previously, method accuracy (trueness and precision) was considerably improved by bias correction (Fig. 4). After bias correction, the accuracy domains of the three methods ranged from 5.0 $\log_{10}$ to 10.0 $\log_{10}$ copies/bee.

Discussion

This article describes the development and validation of the first probe-based method of real-time simplex qPCRs to accurately quantify three *Nosema* species: *N. apis*, *N. ceranae* and *N. bombi* in three bee species (*A. mellifera*, *B. terrestris* and *O. bicornis*). This method provides an additional relevant tool to the existing molecular methods for the epidemiological studies of these parasites, which may spread within and between bee species. Molecular methods, in contrast with spore counting by microscopy, ease the distinction between microsporidia species and possible environmental contamination by other spore-shaped microorganisms (e.g. yeasts). To overcome the issue of multi-copy rRNA genes for which the variable and unknown copy numbers impair quantification trueness, our method development focused on the single-copy housekeeping gene *RPB1*, which codes for the DNA-dependent RNA polymerase II largest subunit. This locus harbours variability in *N. ceranae* (Gomez-Moracho et al., 2015; Hatjina et al., 2011; Roudel et al., 2013), and in *N. apis* (Maside et al., 2015). Nevertheless, the gene sequence is highly conserved, with only a few SNPs (<4% SNPs in our consensus sequences) mostly at the third position of codons, hence with little or no consequences on protein function (Ironside and Corradi, 2013). The design of our specific primers and probes took into account the existing polymorphism in the *N. apis* and *N. ceranae* sequences retrieved from databases since it targeted conserved sections of the built consensus sequences. For *N. bombi*, the specific primers and probe were designed on the single *RPB1* sequence that was available in databases. Although this might have excluded natural variability at this locus, we could expect that this natural variability would likely be limited from the observation of *N. apis* and *N. ceranae* *RPB1* homologue sequences.

In addition, while excluding the detection of other bee pathogens (evaluated *in silico*), our primers and probes

Table 2. qPCR parameters for the three targets, including the parameters of the standard curves (mean intercept, slope and R^2), mean PCR efficiency E_{PCR} and linear uncertainty U_{LIN}, and the minimum and maximum coefficient of variation of Cq values (CV) of the replicate dilutions of the standard.

Target	E_{PCR} (%)	Slope	Intercept	R^2	CV on replicate Cq (%)	U_{LIN} (log_{10})
N. apis	93.09	−3.50	45.27	>0.99	0.03 – 2.74	0.10
N. ceranae	92.11	−3.53	43.23	>0.99	0 – 4.79	0.18
N. bombi	99.84	−3.33	42.56	>0.99	0.04 – 4.45	0.12

Table 3. Experimental assessment of the qPCR specificities on *Nosema* samples. Expected loads for naturally infected bee samples are microscopy estimates in log_{10} copies/mL. Loads are expressed in log_{10} copies/PCR for recombinant plasmids. Hyphens stand for the absence of signal detection in the exclusivity assessment.

Target	Sample type	Origin / ID	Expected load (log_{10} spores/mL or copies/PCR)	Experimental load (log_{10} copies/mL or /PCR)		
				N. apis	*N. ceranae*	*N. bombi*
N. apis	Honey bee (n = 1)	Aland		5.96	–	–
	Recombinant plasmids (n = 4)	pNa	5.00	5.01	–	–
		pNa_DQ996230	5.00	5.42	–	–
		pNa_i382-c8	5.00	5.61	–	–
		pNa_i1074-c9	5.00	5.59	–	–
N. ceranae	Honey bee (n = 9)	R08/2019 (1)	6.77	–	7.38	–
		R08/2019 (2)	6.83	–	4.78	–
		0126	4.30	–	6.70	–
		3398	6.52	–	5.65	–
		0153	6.29	–	7.97	–
		6530	<6	–	2.67	–
		6531	<6	–	6.02	–
		6534	6.40	–	7.58	–
		6538	<6	–	5.40	–
	Recombinant plasmids (n = 2)	pNc	5.00	–	5.01	–
		pNc_i1994-c9	5.00	–	5.16	–
N. bombi	Bumble bee (n = 2)	S1075	6.92	–	–	9.07
		S1076	7.16	–	–	9.10
	Recombinant plasmid (n = 1)	pNb	5.00	–	–	5.00

enabled the sensitive discrimination of the three *Nosema* species in an easy to set up, simplex PCR design that yields good performances (PCR efficiencies were between 95 % and 110 %). The LOQs of our qPCRs allow for the sensitive detection of the three *Nosema* species. Our LOD_{PCR} and low minimum LOQ_{PCR} for *N. ceranae* and *N. bombi* qPCRs (2 log_{10} plasmid copies/PCR corresponding to 100 DNA copies/PCR) is consistent with the LOQ of 188 DNA copies/PCR recently reported for the *N. ceranae* qPCR targeting the *hsp70* gene (Cilia et al., 2018a). For *N. apis* qPCR, the LOD_{PCR} was estimated at a higher, but still low, plasmid load, 3 log_{10} plasmid copies/PCR (i.e. 1,000 copies/PCR), meaning that the real LOD_{PCR} is between 100 and 1,000 plasmid copies/PCR. The higher intercept values of the *N. apis* standard curves were consistent with this slightly higher LOD_{PCR}. These performance differences between *N. apis* qPCR and the two other qPCRs could be explained by the larger *N. apis* amplicon. Note that the three qPCRs showed low coefficients of variation of Cq values, revealing their precision and reproducibility. Furthermore, the three qPCRs yielded satisfactory trueness results with nearly unbiased plasmid quantifications, far below the recommendations of French standard U47-600 (±0.25 log_{10}; AFNOR, 2015). Note also that Cq precocity in our three methods, indicated by the intercepts of standard curves, eases the transfer of these classical qPCR methods to high-throughput qPCR methods based on very small PCR reaction volumes, which requires the earliest Cq values.

Using spiked bee homogenates, the three methods systematically overestimated *Nosema* loads (within acceptable limits) in honey bee and bumble bee homogenates, while they systematically underestimated *Nosema* loads in mason bee homogenate by about 1 log_{10} copies/bee. Correction of these systematic biases increased result accuracy and facilitated comparisons of *Nosema* loads between bee species. In honey bee homogenate, the three *Nosema* methods enabled

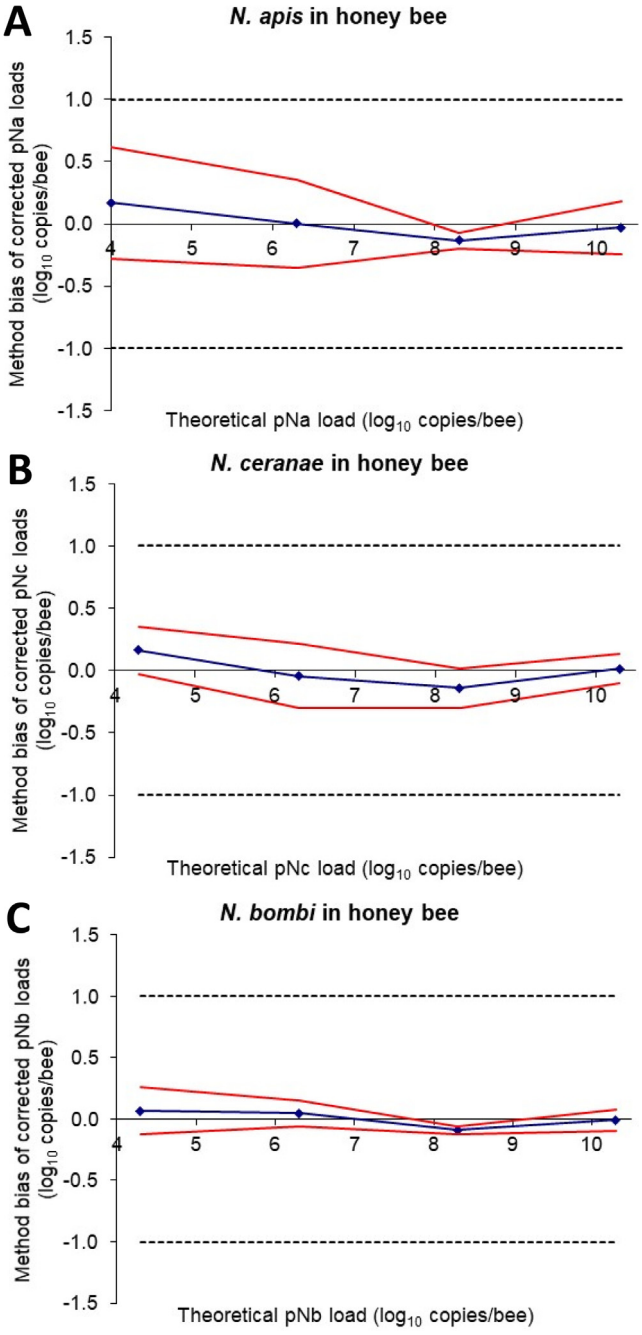

Fig. 2. Method accuracy of the plasmid load quantifications in honey bee homogenate for the three *Nosema* qPCR methods (blue line) calculated for each theoretical plasmid load, with the associated lower and upper tolerance limits (95 % CI, red lines) and acceptable accuracy limits set at ±1 $\log_{10}$ (blue dotted lines). (A) *N. apis* target, (B) *N. ceranae* target, (C) *N. bombi* target. Plasmid loads were corrected for the systematic bias: 0.63 $\log_{10}$ copies/bee for *N. apis* target, 0.16 $\log_{10}$ copies/bee for *N. ceranae* target, 0.37 $\log_{10}$ copies/bee for *N. bombi* target. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

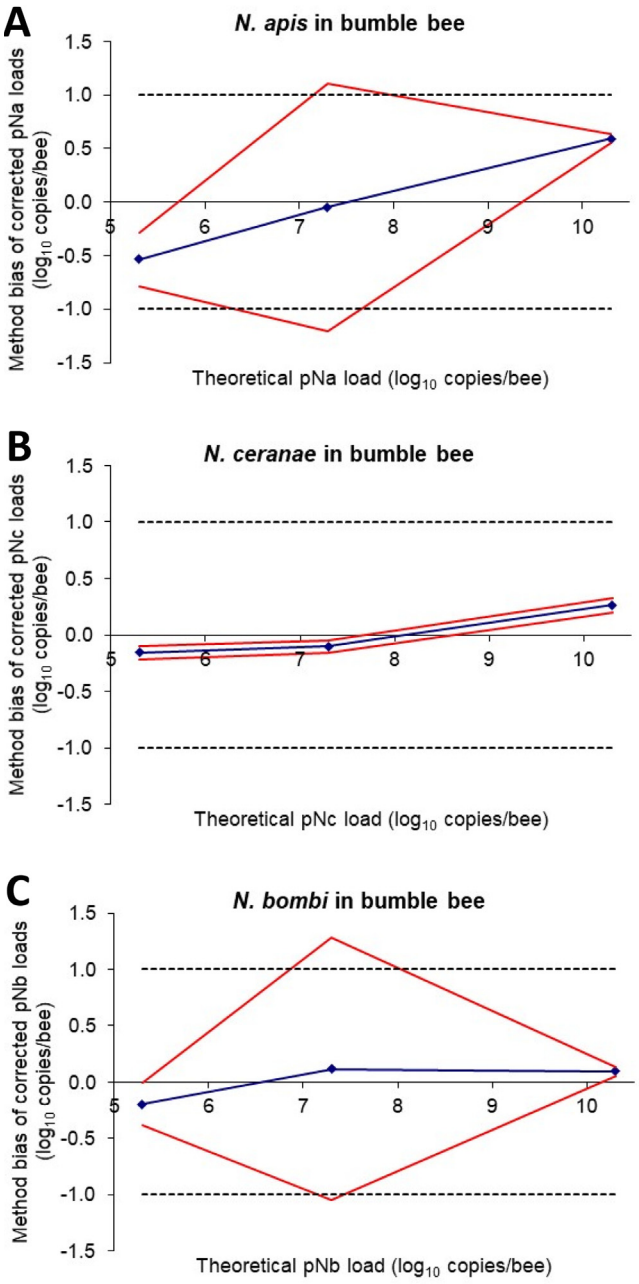

Fig. 3. Method accuracy of the plasmid load quantifications in bumble bee homogenate for the three *Nosema* qPCR methods (blue line) calculated for each theoretical plasmid load, with the associated lower and upper tolerance limits (95 % CI, red lines) and acceptable accuracy limits set at ±1 $\log_{10}$ (blue dotted lines). (A) *N. apis* target, (B) *N. ceranae* target, (C) *N. bombi* target. Plasmid loads were corrected for the systematic bias: 0.54 $\log_{10}$ copies/bee for *N. apis* target, 0.11 $\log_{10}$ copies/bee for *N. ceranae* target, 0.49 $\log_{10}$ copies/bee for *N. bombi* target. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

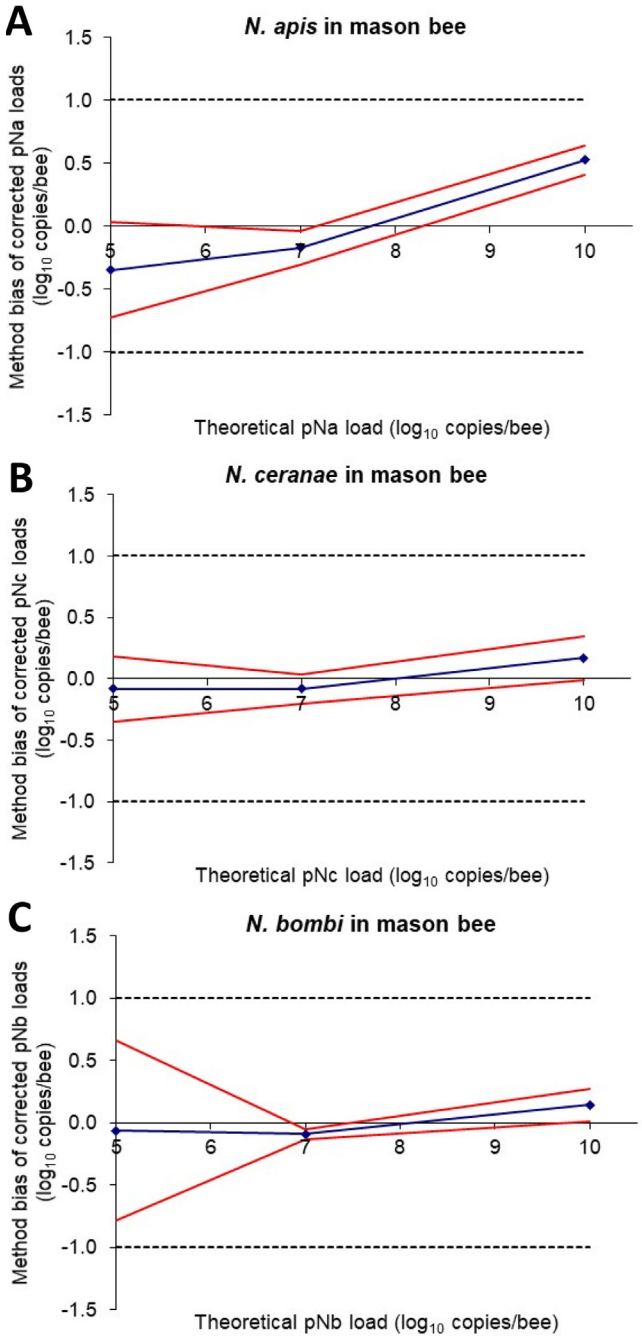

Fig. 4. Method accuracy of the plasmid load quantifications in mason bee homogenate for the three *Nosema* qPCR methods (blue line) calculated for each theoretical plasmid load, with the associated lower and upper tolerance limits (95 % CI, red lines) and acceptable accuracy limits set at ±1 $\log_{10}$ (blue dotted lines). (A) *N. apis* target, (B) *N. ceranae* target, (C) *N. bombi* target. Plasmid loads were corrected for the systematic bias: −1.06 $\log_{10}$ copies/bee for *N. apis* target, −1.04 $\log_{10}$ copies/bee for *N. ceranae* target, −0.86 $\log_{10}$ copies/bee for *N. bombi* target. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

accurate plasmid quantifications from 4 $\log_{10}$ to 10.3 $\log_{10}$ copies/bee. The accuracy domain of the three methods was narrower in bumble bee and mason bee homogenates, ranging from 5 $\log_{10}$ to 10.3 $\log_{10}$ copies/bee. Nevertheless, the accuracy ranges in the three bee species framed the empirical diagnosis threshold established by microscopy at 6 $\log_{10}$ *N. ceranae* spores/honey bee, associated with declining populations and reduced food stores in honey bee colonies (Emsen et al., 2020).

Our harmonised simplex PCR conditions make it possible to assess the prevalence and loads of the three *Nosema* species simultaneously on the same PCR run. The simplex PCR design prevents loss of sensitivity due to multiplexing. Our qPCR parameters open perspectives in the transfer of these qPCRs to high-throughput methods. These three qPCR methods open further exciting perspectives in the discriminative and quantitative assessment of nosemosis in bee pollinators, especially of infections by *N. ceranae* and *N. bombi*, which are often associated with unclear, chronic and variable symptoms. Our methods provide specific and efficient tools for studying the spread of the two honey bee parasites *N. apis* and *N. ceranae*, and of the bumble bee parasite *N. bombi*, both within and between three bee species (*A. mellifera*, *B. terrestris* and *O. bicornis*). Moreover, these quantitative methods could be implemented to study coinfections and the synergy between *Nosema* species (e.g. *N. apis* and *N. ceranae*; Ozkirim et al., 2019), and between *Nosema* parasites and viruses (e.g. *Black queen cell virus*, Chronic bee paralysis virus, and *Deformed wing virus*; Doublet et al., 2015a, Doublet et al., 2015b; Toplak et al., 2013) or trypanosomatids (e.g. *Lotmaria passim*; Stevanoniv et al., 2016; Vejnovic et al., 2018; Williams et al., 2021). Importantly, accurate *Nosema* quantifications would make it possible to determine the coinfection dynamics precisely, for instance at what point one parasite takes over from the other. Finally, accurate parasite loads combined with data on the bee environment from holistic studies (meteorological data, agricultural and apicultural practices) could fuel risk assessments of managed and wild bee health.

CRediT authorship contribution statement

Aurélie Babin: Conceptualization, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Frank Schurr:** Data curation. **Marie-Pierre Rivière:** Resources, Writing – review & editing. **Marie-Pierre Chauzat:** Resources, Writing – review & editing. **Eric Dubois:** Conceptualization, Formal analysis, Data curation, Resources, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejop.2022.125935.

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