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Polymorphism of *16s rRNA* Gene: Any Effect on the Biomolecular Quantitation of the Honey Bee (*Apis mellifera* L., 1758) Pathogen *Nosema ceranae*?

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Abstract: The microsporidian *Nosema ceranae* is a severe threat to the western honey bee *Apis mellifera*, as it is responsible for nosemosis type C, which leads the colonies to dwindle and collapse. Infection quantification is essential to clinical and research aims. Assessment is made often with molecular assays based on *rRNA* genes, which are present in the *N. ceranae* genome as multiple and polymorphic copies. This study aims to compare two different methods of Real-Time PCR (qPCR), respectively relying on the *16S rRNA* and *Hsp70* genes, the first of which is described as a multiple and polymorphic gene. Young worker bees, hatched in the laboratory and artificially inoculated with *N. ceranae* spores, were incubated at 33 °C and subject to different treatment regimens. Samples were taken post-infection and analyzed with both qPCR methods. Compared to *Hsp70*, the *16S rRNA* method systematically detected higher abundance. Straightforward conversion between the two methods is made impossible by erratic *16s rRNA*/*Hsp70* ratios. The *16s rRNA* polymorphism showed an increase around the inoculated dose, where a higher prevalence of ungerminated spores was expected due to the treatment effects. The possible genetic background of that irregular distribution is discussed in detail. The polymorphic nature of *16S rRNA* showed to be a limit in the infection quantification. More reliably, the *N. ceranae* abundance can be assessed in honey bee samples with methods based on the single-copy gene *Hsp70*.

Keywords: *Nosema ceranae*; nosemosis; polymorphic gene; heat shock protein; *Hsp70* gene; ribosomal RNA; microsporidia; multicopy gene

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1. Introduction

The nosemosis type C is a disease of honey bees caused by the microsporidium *Nosema ceranae* [1,2]. The parasite, native of the Asian honey bee *Apis cerana* [3], spread rapidly around the world becoming one of the diseases causing colony decline and collapse in the European honey bee *Apis mellifera*, often replacing *Nosema apis* [4–8]. The microsporidium is an intracellular obligate parasite with high tropism for the honey bee ventriculum [3,9,10], infecting its epithelial cells to replicate [11]. Usually, the *N. ceranae* infection tends to be asymptomatic, but in symptomatic individuals [5,12], the disease may be shortening the lifespan of workers, inducing lethargic behavior and reducing the foraging activity [9,13,14]. *N. ceranae* represents an important threat for pollinators and hymenopterans, other than honey bees [15–21]. Although the transmission routes remain unknown [15,22], *N. ceranae* spores were found in the hive pest *Aethina tumida* and the regurgitated pellets of the bee-eater *Merops apiaster* [23,24]

All microsporidia have very small genomes, usually less than 20 million megabases (Mb) [25]. The *N. ceranae* genome appears to be approximately 7.86 Mb [26], similarly to *N. apis* (8.50 Mb) [27].

The ribosomal DNA (rDNA) represents the portion of DNA coding for the RNA that makes up the ribosomes of a living organism (rRNA) [28]. Typically, rDNA is organized within the genome in repeated copies in tandem. Each copy represents a well-defined unit, which is made up of two main components: Small Sub Unit rDNA (SSU-rDNA) and Large Sub Unit rDNA (LSU-rDNA), which represent the coding sequences for the rRNAs that will, respectively, constitute the minor and major subunits of the ribosome [29]. The rRNAs, depending on their sedimentation coefficient, take on different names in various living organisms. In the case of *N. ceranae* the SSU-rRNA is represented as *16S*, while the LSU-rRNA as *18S*. The two coding sequences are separated from each other by a non-coding one called Internal Transcribed Spacer (ITS), while between each repeated unit of rDNA there is another non-coding sequence called Intergenic Spacer (IGS) [29]. These DNA sequences are frequently used for phylogenetic studies of eukaryotic organisms [1], and the same approach was used to study *N. ceranae* also [3]. Therefore, rDNA has also been used to distinguish the different species belonging to the genus *Nosema*, especially *N. ceranae* from *N. apis*, both capable of infecting *A. mellifera* [30–33], as well as to evaluate the degree of intraspecific variability of those two microsporidia [31,34,35].In general, the quantification of *N. ceranae* abundance relies on primer pairs designed on different sequences of the same SSU-rRNA (*16S rRNA*) [36].

The first contribution of *N. ceranae* genome highlighted the possible presence of multiple copies of rDNA [26]. Various subsequent studies demonstrated their existence, and revealed a high degree of polymorphism between them [6,34,37]. Additionally, in other microsporidia, e.g. *Encephalitozoon cuniculi*, multiple copies of ribosomal genes are present, albeit they are homogeneous within the same genome [38]. The point that ribosomal genes are repeated within the genome is considered normal in eukaryotes. However, the repetitions are usually homogeneous or denote minimal variations in their sequences [39]. That occurs thanks to the phenomenon of concerted evolution, normally defined as the process by which the paralogous genes (genes repeated within the genome) present in a given species show greater sequence similarity compared to ortholog genes (genes with different origins but with similar functions) present in other related species [40]. Concerted evolution, therefore, provides for molecular mechanisms that prevent the independent evolution of repeated sequences, keeping them homogeneous within the genome. Ribosomal genes are among the best known examples of concerted evolution [39]. In the literature, however, there are other cases of eukaryotes in which rDNA was found to violate this mechanism; polymorphic copies of rDNA in the same genome were found both in eukaryotic microorganisms, *Cryptosporidium parvum* [41], *Plasmodium berghei* [42], and *Prototheca wickerhamii* [43], and in more complex eukaryotes, as grasshoppers [44] and flatworms [45]. The fact that rDNA variants are present in genome-wide does not necessarily mean that they are expressed in different ribosomes. However, in *N. ceranae,* the genetic variants of rDNA are transcribed and translated into functional proteins, suggesting changeable roles during the development life stages or under different environmental conditions [37].. Various subsequent studies have then demonstrated their actual existence, also and revealing revealed a high degree of polymorphism between them [6,34,37] [6,36,38]. Additionally, in other microsporidia, such ase.g. *Encephalitozoon cuniculi*, multiple copies of ribosomal genes are present, however, thesealbeit they are homogeneous within the same genome [38]. The point that ribosomal genes are repeated within the genome is considered normal in eukaryotes. However, the repetitions are usually homogeneous with each other, withor denote minimal variations in their sequences [39]. This That occurs thanks to the phenomenon of concerted evolution, normally defined as the process by which the paralogous genes (genes repeated within the genome) present in a given species, show greater sequence similarity to each other, rather thancompared to ortholog genes (genes with different origins but with similar functions) are present in other related species [40]. Concerted evolution, therefore, provides for molecular mechanisms that prevent the independent evolution of repeated sequences, keeping them homogeneous within the genome. Ribosomal genes are among the best known examples one of the greatest examples of concerted evolution [39].

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On the other side, the *Hsp70* gene codifies for one of the highest conserved proteins throughout the evolution [46]. That gene was found in a single copy in all organisms, including archaeobacteria, plants, fungi, and animals, as the coded protein is strictly related to species-specific functions [47,48]. In the case of *Nosema* spp., that protein protects the spores against overheating, therefore contributing to the microsporidium survival in hostile environments [49,50]. The literature shows that the highly conserved single-copy gene *Hsp70* represents a better alternative to rDNA genes in the molecular approach to species-specific identification, quantification, and genotyping [51–60].

Several theories have been envisaged to explain the origin of the genetic variability observed in the *N. ceranae* rDNA. One of the most frequently advocated is the possibility that single colonies of *A. mellifera* become infected by multiple genetic variants of the microsporidium, thereafter multiplying asexually [7,34,61]. However, this hypothesis alone does not account for the variability observed within the different isolates, either considered as an entire colony or individual hosts [6,34]. This statement arises from the fact that the high levels of polymorphism reported for the various sequences within the different isolates (colonies or single bees), were found to be shared with other isolates from different geographical origins. In addition, subsequent attempts aimed at differentiating populations based on that polymorphism did not produce statistically valid results [7,34,61]. In other words, the different isolates are highly polymorphic within themselves, but they do not appear different from each other, suggesting a certain degree of gene flow [7,49]. A further source of genetic variability could be due to recombination, that may occur between the different genetic variants of *N. ceranae* infecting the same colony of *A. mellifera* [6,34]. Evidence of recombination between genetic sequences of *N. ceranae* was brought to light both for SSU-rDNA [34,37] and for several genes encoding structural proteins, such as *DNA-directed RNA polymerase II subunit RPB1*, *Protein-tyrosine-phosphatase* 1 (*PTP1)*, *PTP2,* and *PTP3* [34,37] and for several genes encoding structural proteins, such as *DNA-directed RNA polymerase II subunit RPB1*, *Protein-tyrosine-phosphatase* 1 (*PTP1)*, *PTP2,* and *PTP3* [6,7,49,61–63]. Such recombination events were reported in some cases as frequent [34,37,49,63], while in other cases as rare [6,61]. In addition to these results on *N. ceranae*, there are others on *N. apis*, reporting the observation of recombination phenomena for the RPB1 and PTP2 genes for this species [62].. Such recombination events would bewere reported considered in some cases as frequent [34,37,49,63], while in other cases as rare [6,61]. In addition to these results on *N. ceranae*, there are others on *N. apis*, which also reporting the observation of recombination phenomena for the RPB1 and PTP2 genes for this species [62].

Recombination in *N. ceranae* may result from a meiosis process, which occurs during the sexual phases of the life cycle, or parasexual phenomena, such as mitotic crossing-over, non-homologous recombination, and gene conversion [62].Recombination in *N. ceranae* cmay result from a ould occur through the meiosis process, which occurs during the sexual phases of the life cycle, or they could result from parasexual phenomena, such as mitotic crossing-over, non-homologous recombination, and gene conversion [62].

The maintenance of these genes in a reduced *N. ceranae* genome suggests their possible expression within a process of sexual reproduction [64]. Inferences about the presence of cryptic sexual reproduction have also been suggested for another species belonging to the *Nosema* genus, *Nosema granulosis* [65]. In this regard, it has been hypothesized

that the common ancestor of the genus *Nosema* was endowed with sexual reproduction, which was lost repeatedly and independently in the different evolutionary lines [66].In this regard, it has been hypothesized that the common ancestor of the genus *Nosema* was endowed with sexual reproduction, which was lost repeatedly and and that this was then lost several times independently in the different evolutionary lines [66]. Despite that opens to the sexuality of *N. ceranae*, to date cytological evidence of the entire process or any of its phases has never been achieved [49] and which makes this microsporidium still considered asexual. A further hypothesis involves the clonal reproduction of *N. ceranae*, which would involve a high genetic variability in relation to polyploidy [6]. That stems from the possibility hypothesis that the microsporidia possess at least a tetraploid genome or two heterogeneous diploid nuclei [67].

Therefore, although clonal reproduction is the norm and the observed recombination events are considered ancestral [67], the repeatedly found heterogeneity of *N. ceranae* rDNA could be due to the presence of either multiple genetic variants that reproduce clonally or a single variant that is polyploid and therefore heterozygous for some markers [64]. The cause of these recombinations would be meiosis, which requires the fusion of two nuclei [64]. That process could take place between two heterogeneous nuclei present within the same spore or between nuclei belonging to genetically different spores during the multiplication of *N. ceranae* within the intestine *of A. mellifera* [64].

This investigation aimed to evaluate two different qPCR assays for quantifying the number of copies of *N. ceranae* present within individuals of *A. mellifera*. The two assays were based on sequences belonging to different target genes: *16S rRNA* and *Hsp70* which, due to their intrinsic characteristics, may affect the quantification differently.

2. Materials and Methods

2.1. Experimental Design

This study is a part of a laboratory treatment trial encompassing treated groups and untreated controls. The results on the effects against the *N. ceranae* infection have been reported previously [68]. In this paper, we concentrate on methodological aspects.

Briefly, in summer 2018, 450 newborn *A. mellifera ligustica* workers reared in an incubator from the brood of colonies selected from the apiary of CREA-AA (Bologna, Italy, 44°31′27.1″ N 11°21′03.6″ E) were inoculated with 187,500 purified *N. ceranae* spores/bee. The bees were reared in hoarding cages in groups of 25 each, fed with the test diets and kept at 33 °C throughout the experiment [68]. Whenever possible, five living individuals were sampled per cage, albeit bee mortality did not allow to complete the bee sampling in all groups. For this reason, 306 bees could be collected in total, which were stored at −20 °C until the molecular analysis.

2.2. DNA Extraction and qPCR

The sampled bees were analyzed individually. Once dissected, each digestive tract from the ventriculum to the rectum was collected in a microtube and homogenized in 1 mL nuclease-free water using Tissue Lyser II (Qiagen, Hilden, Germany) for 3 min at 30 Hz, as previously reported [69].

From each homogenate, the total DNA was extracted using a Quick DNA Microprep Plus Kit (Zymo Research, Irvine, CA, USA) following the procedure indicated for the processing of solid tissue. The DNA obtained was split into two aliquots, which were analyzed separately using two different primer pairs and probes designed on the sequence of the *16s rRNA* [70] and *Hsp70* [50] genes of *N. ceranae* (Table 1). For each specific target gene, 15 µL total reaction volume was prepared using 2xQuantiTect Probe PCR Master Mix (Qiagen), 2 µM forward and reverse primers, 500 nM probe, and 3 µL DNA extract.

Table 1. Primer sequences used for the qPCR analysis with the *Hsp70* and *16S rRNA* genes and TaqMan Probes.

Gene	Primer	Sequence (5′-3′)	Reference
16S rRNA	Forward	AAGAGTGAGACCTATCAGCTAGTTG	[70]
	Reverse	CCGTCTCTCAGGCTCCTTCTC	
	Taqman Probe	ACCGTTACCCGTCACAGCCTTGTT	
Hsp70	Forward	GGGATTACAAGTGCTTAGAGTGATT	[50]
	Reverse	TGTCAAGCCCATAAGCAAGTG	
	Taqman Probe	TGAGCCTACTGCGGC	

The standard curve for each target gene was generated by the amplification of recombinant plasmids cloned with CloneJET PCR Cloning Kit (ThermoFisher Scientific, Waltham, MA, USA) containing the *N. ceranae*-specific DNA fragment diluted from 10^0 to 10^9 copies, as previously reported [69].

All the analyses were performed with two technical replicates.

2.3. Statistical Analysis

The *N. ceranae* abundance was determined at the individual level (N = 306) by averaging each of the two replicates for the two methods of qPCR. The number of *N. ceranae* copies detected in the same bee by the two methods was compared using a two-tailed T-test for paired samples. Due to a large number of samples, the control of the assumption of normality was not considered as stringent. The association between continuous variables was evaluated through the two-tailed Pearson correlation index. In addition, the abundance values obtained with both methods were used for the construction of a linear regression model, using the abundance values measured with *16S rRNA* as the dependent variable and those obtained with *Hsp70* as an explanatory independent variable. A further linear regression model was developed using as dependent variable the ratio between the abundance values obtained with both methods for each sample (*16S rRNA/Hsp70)* and as independent variable the abundance values measured with *Hsp70*.

3. Results

The averages of the values obtained with the two different methods are reported in Figure 1. The t-test performed on the samples indicated a higher number of copies detected with the *16S rRNA* method (1,568,482.9 ± 53,725.1 se, SD = 939,805.8) compared to the *Hsp70* ones (152,579.8 ± 5,853.1 se, SD = 102,388.2) (t (305) = + 28.991, $p = 0.000$).

A positive correlation was calculated between the two analytical data series (r (306) = 0.851, $p = 0.000$). In a linear regression model (Table 2), the abundance obtained with *16S rRNA* was considered as the dependent variable versus the abundance obtained with Hsp70 considered as the independent predictor variable (Figure 2).

Table 2. Characterization of the regression model relating the *16S rRNA* abundance (dependent variable) to the *Hsp70* abundance (independent variable).

Model	F (1.304) = 800.403, $p = 0.000$, Adj. $R^2 = 0.724$
Intercept	376,209.4 ± 50,726.0 s.e. (95% CI = 276,390.9, 476,028.0), t (304) = 7.417, $p = 0.000$
Slope	7.8 ± 0.3 s.e. (95% CI = 7.3, 8.4), t (304) = 28.291, $p = 0.000$

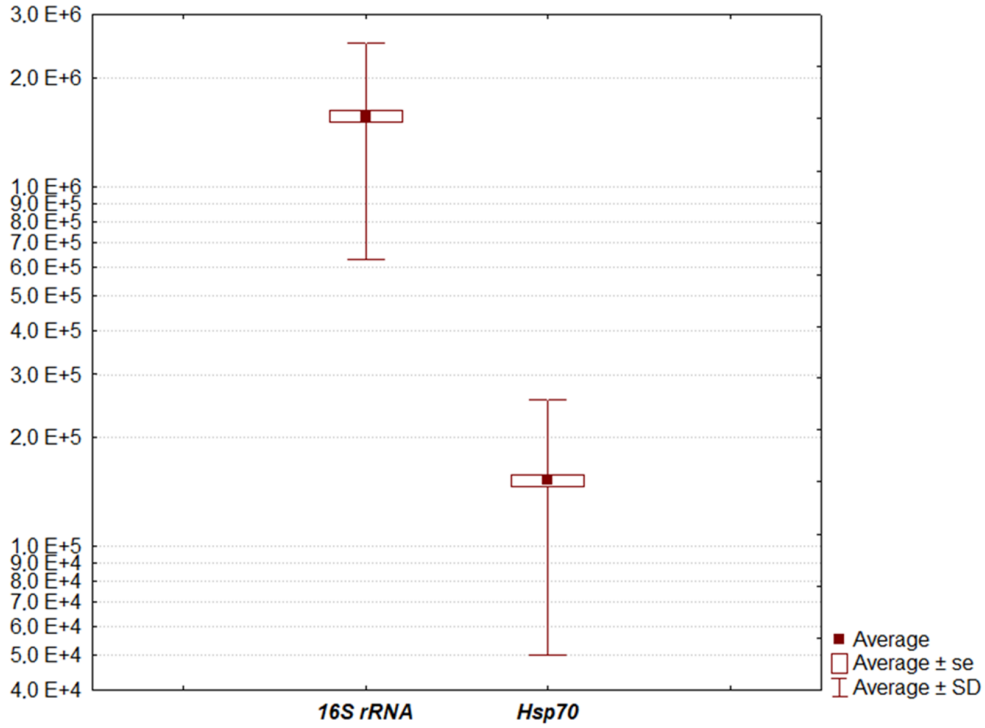

Figure 1. Boxplot showing average, standard error and standard deviation of the individual *N. ceranae* abundance values measured with the two methods examined here (*16S rRNA* and *Hsp70*). The number of copies is reported against a logarithmic vertical axis.

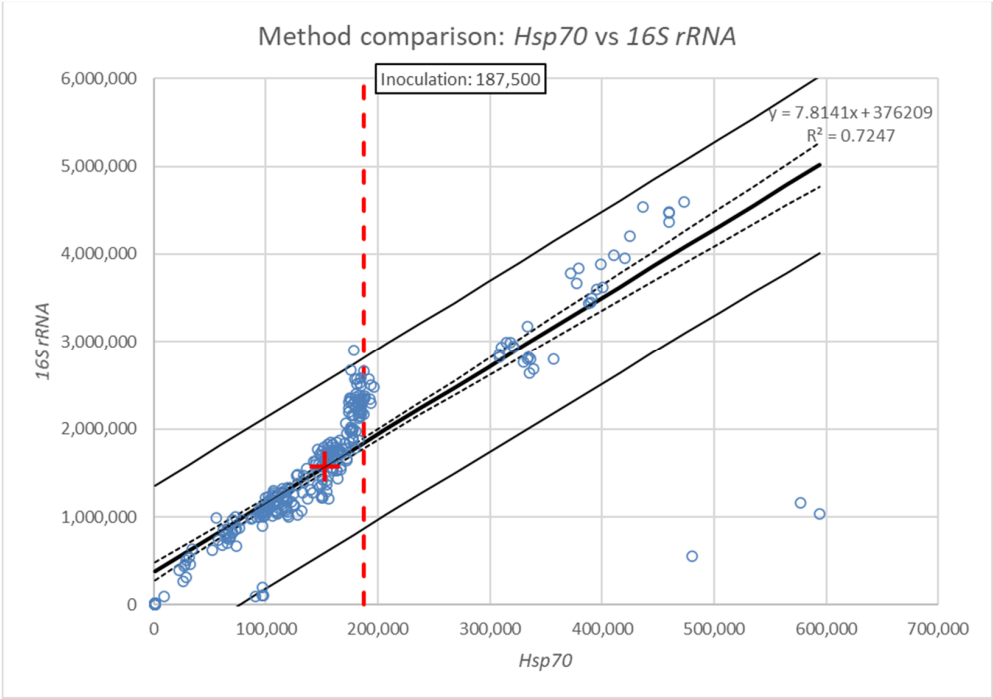

Figure 2. Linear regression analysis between the abundance measured with *16S rRNA* (dependent variable) and with *Hsp70* (independent variable) methods. Legend-blue circles: experimental data, red cross: average values, red dashed line: inoculated dose, thick solid line: regression line, dashed thin lines: confidence interval of regression, solid thin lines: prediction interval.

The regression model is significant (p = 0.000), indicating the existence of a relationship between the two variables. Similarly, the intercept and slope values are also significant

(p = 0.000). The obtained regression index (R^2) is high (0.724), therefore in this model, the independent variable (*Hsp70*) has a strong influence in the determination of the dependent one (*16S rRNA*). However, the forecast interval is very wide, and this is not compatible with an effective forecast of the value obtained with *16S rRNA*, starting from that obtained with *Hsp70* and vice versa. In addition, the graph highlights an anomaly in the distribution of the values, which tends to concentrate around the number of copies relating to the dose of inoculum (187500). This is related to the fact that most of the bees analyzed were subjected to treatments with substances that produced efficacy against *N. ceranae*.

Concerning the *16S rRNA/Hsp70* ratio, the minimum and maximum values were 1.01 and 18.68, respectively. The distribution of values reached maximum frequency in the range 10.00 to 12.00 (50.3% of all values), with most of the remaining ones positioned in the ranges 8.00 to 10.00 and 12.00 to 14.00 (total 41.2% of all values. Only a few other observations fell outside the ranges mentioned (8.5% of all values).

In a further linear regression model (Table 3), the *16S rRNA/Hsp70* ratio was considered as the dependent variable against the abundance obtained with *Hsp70* considered as the independent predictor variable (Figure 3).

Table 3. Characterization of the regression model relating the abundance ratio *16S rRNA/Hsp70* (dependent variable) to the *Hsp70* abundance (independent variable).

Model	F (1.304) = 38.740, p = 0.000, Adj. R^2 = 0.110
Intercept	11.9 ± 0.2 s.e. (95% CI = 11.5, 12.4), t (304) = 53.551, p = 0.000
Slope	$-7.6 \times 10^{-6} \pm 1.2 \times 10^{-6}$ s.e. (95% CI = -10×10^{-6}, -5.2×10^{-6}), t (304) = −6.224, p = 0.000

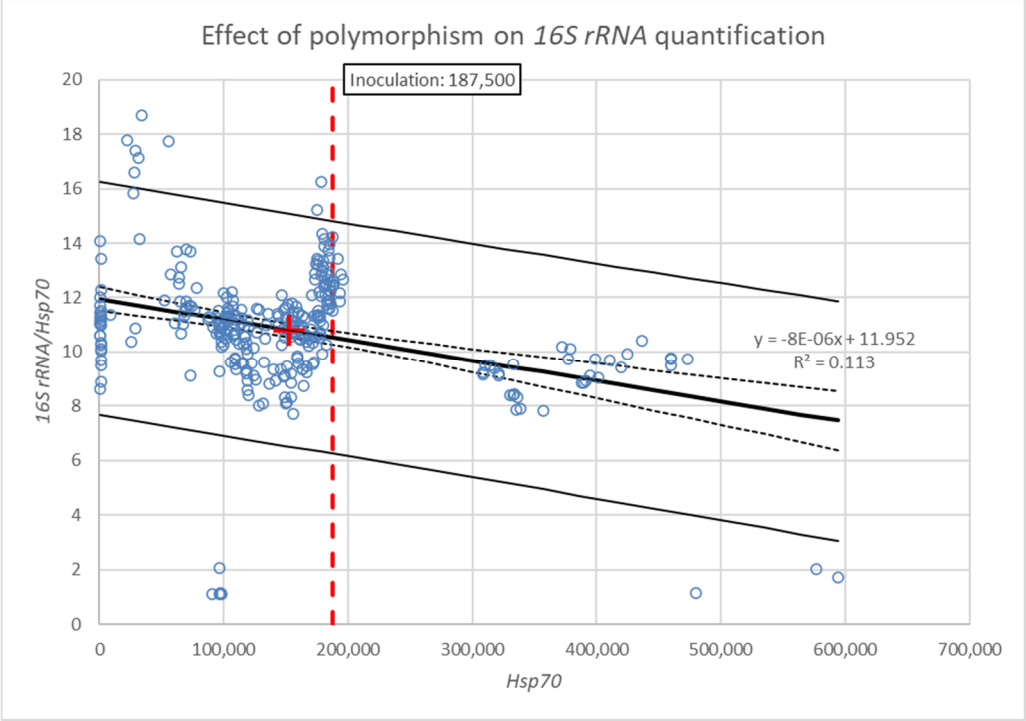

Figure 3. Figure 3. Linear regression analysis between the 16S rRNA/Hsp70 ratio (dependent variable) and the abundance measured with the Hsp70 method (independent variable). Legend-blue circles: experimental data, red cross: average values, red dashed line: inoculated dose, thick solid line: regression line, dashed thin lines: confidence interval of regression, solid thin lines: prediction interval.

Additionally, in this case, the regression model, the intercept values, and the slope values are significant ($p = 0.000$), however, the coefficient of determination (R^2) is low (0.113), indicating the presence of other factors capable of influencing the dependent variable, not considered in the model. The graph highlights the already observed accumulation of data around the inoculation zone, but furthermore, it indicates that near this threshold the *16S rRNA/Hsp70* ratio tends to assume greater variability, signaling an increase in the polymorphism of the *16S rRNA* gene in correspondence with these values.

The analysis of the standardized residues of the *16S rRNA/Hsp70* ratio revealed a data distribution compatible with normality, as only few values fell outside the 95% boundaries of the normally distributed data (Figure 4).

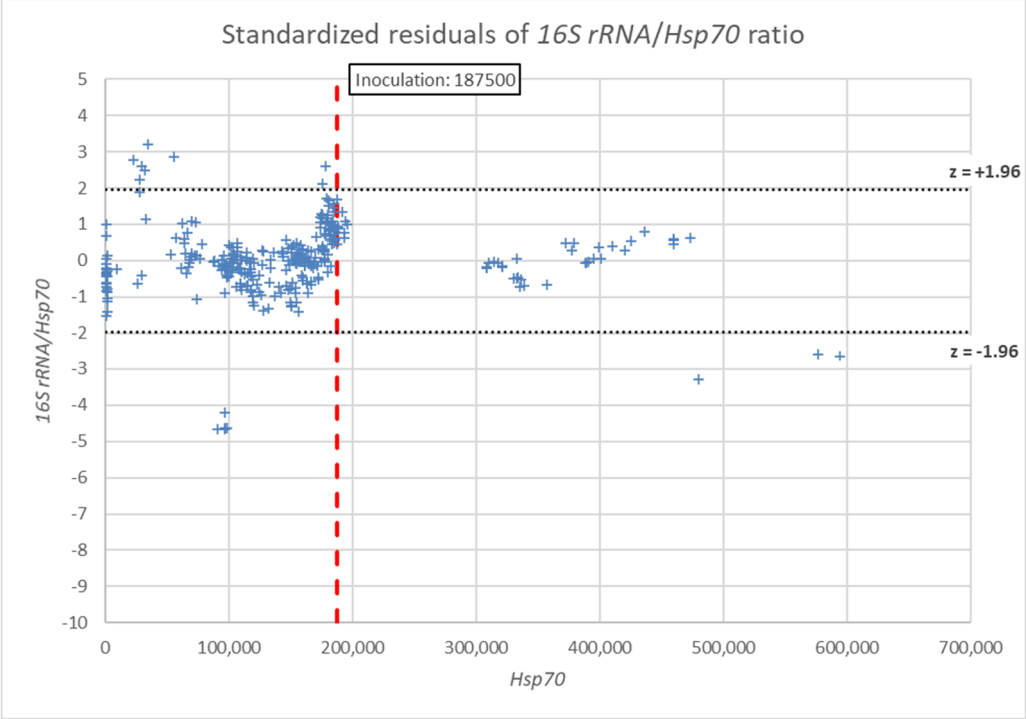

Figure 4. Scatterplot showing the relationship between the error variance of the 16S rRNA/Hsp70 ratio, and the abundance measured with the Hsp70 method (N = 306). Legend-blue crosses: individual standardized residuals, red dashed line: inoculated dose, dotted black lines: 95% interval of the normally distributed data (z = ±1.96).

4. Discussion

In this investigation we wanted to compare two different methods of qPCR, in use to quantify the number of *N. ceranae* copies present in infected *A. mellifera* samples. Those methods differ mainly for the target sequence in the microsporidian DNA, in that they respectively amplify sequences belonging to the genes coding for *16S rRNA* (SSU-rDNA) and *Hsp70* (Heat Shock Protein).

The comparison indicates that the abundance obtained with the qPCR technique based on *16S rRNA* is systematically higher. This observation is in line with what reported in other comparative studies considering the same methods [8,69], where the authors detected instable ratios between the data obtained on different samples. This variability was also observed in the present study, with values of the *16S rRNA/Hsp70* ratio oscillating between 1.01 and 18.68. Those values are in line with previous observations [8], where the ratio varied within a range of 1.35 and 16.53. Besides, the distribution of the *16S rRNA/Hsp70* ratio did not appear evenly distributed, as the frequency peaked between 10.00 and 12.00, with more than 50% of all observed values included in that range and 91.5% of all values in the range 8.00–13.00. This indicates that the observed large variability was concentrated

within a specific range. The average ratio was 10.80, which is higher but still in line with the previously observed value of 8.59 [8]. Since in the *N. ceranae* genome the *Hsp70* gene is present as a single copy [49,52], whereas the *16S rRNA* gene is polymorphic, with high variability, and presenting an undefined number of copies in its genome [34,37], we can assume that the *16S rRNA/Hsp70* ratio reflects the number of *16S rRNA* copies present in the *N.* [34,37], it we can be assumed that the *16S rRNA/Hsp70* ratio reflects the copy number of *16S rRNA* copies present in the *N. ceranae* genome of the considered samples.

Based on both observed data and literature, it is possible to hypothesize that *16S rRNA* is generally present in *N. ceranae* in an average number of copies oscillating between 8 and 14. This hypothesis is in agreement with what reported by Bourgeois et al. (2010), who suggest that approximately 10 copies of the gene are present [70]. However, it is necessary to consider that the qPCR analysis was performed on the total DNA extracted from a single individual of *A. mellifera*, which can contain millions of *N. ceranae* copies [71]. As all the data obtained, including the *16S rRNA/Hsp70* ratio, refer to a given infecting *N. ceranae* population, the number of *16S rRNA* copies represent the average for the population found within the host bees in the sample. As indicated, the calculated ratio largely falls within a specific range. However, the lowest (e.g., 1.12; 1.73; 2.01) and highest (e.g., 16.61; 17.78; 18.68) values (Supplementary Table S1) account for high genetic variability in *N. ceranae* [6,49,64,67]. To explain the genetic variability observed in this microsporidium, two main explanations were proposed. The first hypothesis suggests the presence of a single clonal *N. ceranae* population, with dikaryotic and polyploid spores [6,67]. Although this hypothesis partly clarifies the extensive genetic variability in *N. ceranae*, it does not appear explanatory of the observed values. Considering, as suggested, a tetraploid genome, it would be possible to have four variants of ribosomal genes in each spore [67]. These variants could differ from each other both in terms of repeated number of copies and nucleotide sequences which, taken together, would determine the average copy number of *16S rRNA* for a given *N. ceranae* spore. However, assuming clonal reproduction, all spores would maintain a high degree of genetic similarity and, therefore, the values observed in different isolates should be characterized by constancy or little variability, making it unlikely to observe the ratios mentioned above. The second hypothesis includes the possibility that every single infected individual of *A. mellifera* contains a heterogeneous population made of a number of *N. ceranae* genetic variants [7,34,49,61,64]. Those variants, would be polymorphic for a certain number of sequences in their genome, including the *16S rRNA* gene, that would be present in multiple copies. Therefore, the average number of *16S rRNA* copies for a given *N. ceranae* population infecting an individual *A. mellifera*, would depend on the specific genetic variants present within the host. This second hypothesis appears to account for a larger variability of the *16S rRNA/Hsp70* ratio [7,34,49,61,64]. Theose genetic variants, would in addition to being polymorphic for a certain number of sequences placed alongin their genome, including would also be polymorphic forthe *16S rRNA* gene, as well asthat would be presenting a different number of repetitions for this genein multiple copies. Therefore, the average number of copies of *16S rRNA* copies for a given *N. ceranae* population of *N. ceranae* infecting an individual of *A. mellifera*, wouldill depend on the specific genetic variants present within the host. TThrough this second hypothesis, therefore, appears to account for a larger it is possible to include greater variability of the *16S rRNA/Hsp70* ratio.

However, the aspects above provide only a limited perspective. Indeed, there is clear evidence of recombination in *N. ceranae*, both in sequences coding for specific proteins [7,49,61–63] and in ribosomal genes [6,34,37]. Recombination requires fusion of two nuclei followed by meiosis, which allows to obtain new variants. This could happen both between two nuclei present within the same spore and between nuclei coming from different spores, admitting, in this case, the presence of a cryptic sexual phase in an organism that has been so far considered asexual [64]. The occurrence of recombinant events could therefore lead to the generation of variants with a new number of copies of the *16S rRNA* gene. Recombination events could also generate new nucleotide sequences

on that gene. If the new sequences were formed at the pairing sites of the primers used in the qPCR assay, the primers would not pair, preventing amplification and leading to a low *16S rRNA/Hsp70* ratio value. It is then necessary to consider that in the specific case of rDNA further mechanisms, in addition to meiosis, could lead to recombination. One of them is the Sister Chromatid Exchange (SCE) implying that, during DNA replication, two identical chromosomes pair up and exchange portions [72]. Considering that the ribosomal genes of *N. ceranae* are present at least in tandem and as variably repeated number of copies within the same genome [26,34], new sequences of this gene could be generated through the recombination of the different repetitions [34], without the need of a fusion between two nuclei. However, this mechanism would not be effective in the case of the *Hsp70* gene, as it represents a highly conserved portion present in a single copy in the *N. ceranae* genome [49,52]. For this reason, the *16S rRNA* gene can be considered much more prone to recombination than *Hsp70*. The picture may be completed considering that a number of microsporidian genes derives from the horizontal genetic transfer (HGT), to which other organisms, mainly bacteria, concur [73]. In *N. ceranae*, the 0.44 to 1.31% of the genes result from HGT events [73]. The occurrence of such events between prokaryotes and eukaryotes are not rare and, in most cases, they end in the functional loss of the newly acquired gene or in its more or less rapid erosion [74]. Erosion is the phenomenon by which a given species loses races and populations, but in this case, the term refers to the loss of *N. ceranae* alleles [75,76]. Factors promoting HGT events include an organism's ability to acquire exogenous DNA, accessibility to its "germline" and the frequency of exogenous DNA "donors" in the environment [74]. From this point of view, *N. ceranae* and other microsporidia, as a unicellular eukaryotic parasite, are candidates particularly prone to acquire exogenous DNA. Each *N. ceranae* spore represents a single germline and the organ in which it stabilizes in *A. mellifera*, the ventricle, as a part of the digestive system is colonized by an extensive bacterial flora, consisting of at least 9 different species [77]. To this adds the environmental microbial contamination. Therefore, microsporidia like *N. ceranae*, when penetrating the host, may come into contact with exogenous bacterial DNA dispersed in the environment, that may subsequently acquired [73]. Such a mechanism might have occurred with *16s rRNA* bacterial genes, which may have been repeatedly incorporated independently into the different genetic variants of *N. ceranae*, further expanding the observed variability of the number of *16S rRNA* copies. It is, therefore, clear that qPCR methods based on the amplification of *16S rRNA* are not accurate in precisely defining the extent of the infection. Doubts about the use of this gene in diagnostics have also been previously raised. In a comparative study, it has been indicated that primer-based PCR assays capable of amplifying a sequence belonging to *16S rRNA* can produce false negatives or, in the case of mixed infections of *N. apis* and *N. ceranae*, overestimate the infection of the latter pathogen [78]. Further evidence is provided reporting false negative results using assays based on *16S rRNA* [79], and indicating that assays based on this gene can amplify sequences belonging to *N. apis*, so altering the analytical result [80]. It is true that both studies base on End Point PCR assays; however, those primers can be used in Real Time PCR assays also and, as the low reliability has to be ascribed to the molecular characteristics of *16S rRNA,* those statements can also be extended to the latter technique. The other assay considered in the study is based on the amplification of a highly conserved sequence belonging to the *Hsp70 gene*. This gene is present in a single copy within the *N. ceranae* genome [49,52] and no evidence of intraspecific genetic variability has been reported so far [50]. Furthermore, it was shown to be specific for *N. ceranae*, generating no reaction with DNA from *N. apis* [50]. For these characteristics, this assay represents a valid and reliable alternative in the quantification of the number of *N. ceranae* copies in a honey bee sample.

The regression analysis highlighted an anomaly in the distribution corresponding to a number of copies close to the inoculated dose (187,500 spores/bee), indicating an increased *16S rRNA* polymorphism. Given the specific experimental conditions, the bees were unable to defecate, so unloading the intestinal spores. Likely, in that area of the distribution concentrated spores that were killed or made quiescent by the experimental treatments

in progress, whereas vegetative forms of the pathogen were prevailing elsewhere. The coincidence of the high concentration of spores in an area of high polymorphism of *16S rRNA* would therefore be compatible with a potential accentuation of this phenomenon in the forms of resistance compared to the vegetative ones. Alternatively, the dead or quiescent spores could undergo degradation within the infected tissues, thus releasing nucleic acids in the cellular matrix. Fragments of dispersed *16S rRNA* could therefore be acquired from viable spores or vegetative forms of *N. ceranae* in a similar way to what happens with other microorganisms [73], thus altering the quantification and thus also increasing the eventual *16S rRNA/Hsp70* ratio.

5. Conclusions

The experiment showed that the use of qPCR assays based on the *16S rRNA* gene results in the systematic detection of a higher number of copies than the assay based on the *Hsp70* gene, thus overestimating the quantitative infection. This is certainly related to the multi-copy nature of *16S rRNA*, however, the ratio between the two measurements is not constant, making it impossible to obtain a quick conversion factor between the two values. Considering that *Hsp70* is present in a single copy, the cause of this variability is to be found in the nature of *16S rRNA*, which is subject to a more frequent recombination. Therefore, the qPCR assay based on *Hsp70* appears to be a valid and reliable alternative for the precise quantification of the number of copies of *N. ceranae*. Using diagnostic tools with these characteristics it will be possible to develop effective treatments against this pathogen, thus reducing the pollinator decline due to this pathogen. In addition, the experimental conditions characterized by artificial infections with *N. ceranae* spores, and the impossibility of defecation seem capable of influencing the polymorphism of the *16S rRNA* gene through an increase in dead spores within the intestine of *A. mellifera*. These would inherently possess a greater polymorphism than the vegetative forms or could constitute a source of exogenous *16S rRNA* for the viable forms of *N. ceranae*, representing a further negative aspect to consider if a qPCR assay based on this gene is to be used.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app12010422/s1, Table S1. Detailed information for all samples in this study: honey bee ID, number of copies detected in each replicate for each target gene (*Hsp70* and *16srRNA*) and their ratio.

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