Nosema ceranae infection in honeybee samples from Tuscanian Archipelago (Central Italy) investigated by two qPCR methods

Giovanni Cilia a,b,1, Simona Sagona b,1, Matteo Giusti b, Pedro Emanuel Jarmela dos Santos a, Antonio Nanetti a, Antonio Felicioli b,∗

a CREA Research Centre for Agriculture and Environment, Via di Saliceto 80, 40128 Bologna, Italy
b Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, 20159 Pisa, Italy

1. Introduction

Microsporidia are unicellular eukaryotes infecting vertebrate and invertebrate animal hosts. So far, three microsporidia species have been described associated to the honey bees, all belonging to the Nosema genus. The infections are known as nosemosis irrespective they are caused by Nosema apis Zander (Fries, 1993), Nosema ceranae (Fries et al., 1996) or Nosema neumannii (Chemurot et al., 2017). The first two species are spread worldwide and responsible for known symptoms (Fries et al., 2006; Higes et al., 2007; Klee et al., 2007; Martin-Hernandez et al., 2007). However, N. neumanni was recently detected only in Ugandan bees and its consequences for the host bees have not been described yet.

N. apis and N. ceranae are generally considered original parasites of European and Asian honey bees, respectively (Botías et al., 2012). However, the second showed capable to effectively infect Apis mellifera individuals also, which allowed its spread far beyond the Asian continent (Fries et al., 2006).

It was suggested that N. ceranae may have replaced N. apis in Italy (Ferroglio et al., 2013) and in other countries (Ansari et al., 2017; Calderon et al., 2008; Fries et al., 2006; Giersch et al., 2009; Higes et al., 2009b; Invernizzi et al., 2009; Klee et al., 2007; Pacini et al., 2016; Tapasztí et al., 2009), although the possibility of a direct competition between the two species is still debated.

In Italy, N. ceranae was found in honey bee colonies reared in all regions (Ferroglio et al., 2013; Klee et al., 2007; Maiolino et al., 2014; Mutinelli et al., 2010; Porrini et al., 2016), but small islands have never been investigated in this respect. Thus, no data are available for the islands of Tuscanian Archipelago: Giannutri (area: 26 ha), Montecristo (103 ha), Pianosa (104 ha), Capraia (193 ha), Giglio (204 ha), Gorgona (223 ha) and Elba (22.350 ha).

Both Nosema species do infect the epithelial cells of the honey bee ventriculum (Fries et al., 1996; Higes et al., 2007) with effects recognizable both at individual and colony level, like reduced lifespan, lethargic behaviour and poor honey and pollen harvest (Eiri et al., 2015; Higes et al., 2009c, 2008).

Diarrhoeic faeces, high mortality at the hive entrance and swollen bee abdomens are commonly seen in N. apis infected colonies (Bourgeois et al., 2010), but N. ceranae infections tend to produce subtle symptoms that are difficult to spot in the field. Nevertheless,
damage may be severe and result into adult population decline until colony collapse (Fries et al., 1996; Giersch et al., 2009; Higes et al., 2006).

*Nosema* spp. spores can be detected by light microscopy in the honey bee ventriculum, but reliable species determination requires advanced techniques. Although individual spore morphology may be examined by transmission electron microscope with this purpose (Chen et al., 2009; Forsgren and Fries, 2010; Fries et al., 1996), biomolecular bioassays are generally preferred for practical reasons and because they allow accurate quantification by the Real-Time PCR (qPCR) approach.

The 16S rRNA gene is commonly used in qPCR assays where species discrimination and quantification are required (Chen et al., 2008; Forsgren and Fries, 2010; Higes et al., 2006; Klee et al., 2007; Martin-Hernandez et al., 2007). However, this is a multi-copy gene characterized by a variable number of sequences in the *N. ceranae* genome. This suggested to develop a new qPCR method, which is based on the single-copy *N. ceranae* gene *Hsp70* (Cilia et al., 2018).

The aim of this investigation was to seek the presence of *Nosema* spp. in managed colonies from the Tuscanian Archipelago and, concurrently, compare the results of the qPCR methods based on the 16S rRNA and *Hsp70* genes.

## 2. Materials and methods

In July and August of 2017, honey bee workers were sampled on the islands of Tuscanian Archipelago in order to determine the *Nosema* infection.

Samples were taken on asymptomatic apiaries from the island of Pianosa (42°35’05.2”N 10°05’22.3”E), Capraia (43°01’54.4”N 9°29’59.8”E), Gorgona (43°26’00.1”N 9°54’18.0”E), and Elba (42°51’43.3”N 10°25’03.1”E) islands (Fig. 1). On Giannutri and Montecristo, no managed apiaries are known. On Giglio, the contacted beekeepers refused to take samples for this research.

On each island, two colonies from the same apiary were sampled. Each sample consisted on 50 forager bees, which were refrigerated until analysis. The samples were divided in two subsamples of 25 bees, which were extracted separately. For extraction, the gastrum (later referred to as abdomen as of common use) was carefully dissected with tweezers. The abdomens of each subsample were pooled and homogenized (in 1 ml of DNA free water) with a Tissue Lyser II (Qiagen, Hilden, Germany) operated for 3 min at 30 Hz.

Total DNA was extracted with High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) following the manufacturer’s instructions.

Two aliquots from each DNA extract were taken and analysed by different qPCR methods. In one case, primers and probes for *N. apis* and *N. ceranae* designed on a 16S rRNA gene sequence were used as described by Forsgren and Fries (2010). The optimized amplification and quantitation protocol was: 15 min at 98°C followed by 40 cycles of denaturation for 5 s at 98°C, annealing/extension for 10 s at 63°C and melt curve analysis from 65 to 95°C (in 0.5°C increments) 10 sec/step. The other aliquot served to quantify *N. ceranae* copies in each sample using the *Hsp70* gene-based primers and probes described by Cilia et al. (2018). In this case, amplification and quantitation were performed according to the following cycling conditions: initial activation step 95°C for 10 min, PCR cycling (40 cycles of 95°C for 15 s, 56°C for 60 s).

All the analyses were conducted in duplicate and the final infection data reported as average of *Nosema* number copies per honey bee.

Data obtained were statistically analysed through a three-way ANOVA. Number of *N. ceranae* copies were tested against colony, qPCR method and replicate. Statistical significance threshold was set at P = 0.05. Where applicable, multiple comparison of means was made by using Newman-Keuls test.

Regression was calculated on the averages of the replicate analyses from each colony to compare the two qPCR methods.

All the statistics were calculated with the package STATISTICA (Sistema software di analisi dei dati), ver. 7.1, StatSoft Italia srl (2005).

![Fig. 1. Map of Tuscanian Archipelago (Central Italy) with the distances in kilometres (km) between each island and the nearest land.](image-url)
3. Results

All of the samples resulted in DNA amplification below the detection limit for *N. apis*. Table 1 reports the number of *N. ceranae* copies resulting from each replicate analysis performed with the two methods. No detectable *N. ceranae* infections were found in the samples from Elba Island, whereas the samples from Gorgona, Capraia and Pianosa samples were positive.

Samples positive for *N. ceranae* contained an average of 13.99 ±10.34 s.e.; s.d. = 25.34) * 10^6 copies per bee with the 16S rRNA-based method, but only 1.33 (±0.71 s.e.; s.d. = 1.74) * 10^6 by using the Hsp70 sequences.

A principal effects ANOVA conducted on the data of Table 1 resulted in a significant effect of colony (F(5, 16) = 4.020, p = 0.015) and analytical method (F(1, 16) = 5.380, p = 0.034) but not of replicate (F(1, 16) = 0.004, p = 0.950).

On average, the 16S rRNA-based technique detected 8.59 (±2.91 s.e.) times the *N. ceranae* copies found with the Hsp70 method, and the ratio between the copies detected with the two methods (16S rRNA/Hsp70) was in the range 1.35–16.53.

Results show both high between-sample variation of this ratio (s.d. = 7.12) and insufficient correlation between the paired replicate averages to make direct conversion between the two methods possible (Pearson r = 0.832 (F(1, 4) = 9.020, p = 0.040)).

In a regression analysis of data (y = b0 + b1 * x), the number of copies detected with 16S rRNA and Hsp70 methods were respectively considered dependent and independent variables, with good fit to the linear model (F(1,4) = 9.02p < 0.040), b0 = 0.004, p = 0.950).

As resulted by the three-way ANOVA test, colony was a significant factor of variability, suggesting heterogeneous levels of infection in the considered hives. Conversely, the replicate did not result into significant effect. This is compatible with high repeatability of both qPCR methods (Cilia et al., 2018; Martin-Hernandez et al., 2007). However, Hsp70 qPCR assay systematically detected less *N. ceranae* copies than the alternate method, whose results were one or two orders of magnitude higher. This and the unsteady 16S rRNA/Hsp70 ratio calculated at sample level agrees with the variable number of sequences of the 16S rRNA gene of *N. ceranae* (Sagastume et al., 2016). Molecular diagnostics may take advantage of multi-copy genes in that they allow designing high sensitivity tests (more possibility to detect the target gene), but the fluctuations in the number of 16S rRNA sequences in the *N. ceranae* genome may ultimately affect the quantification reliability (Sagastume et al., 2011). Conversely, the Hsp70 method bases on a highly-conserved region of the *N. ceranae* genome (Gomez-Moracho et al., 2014; Wang et al., 2017), making it suitable to accurately quantification.

The results of the 16S rRNA amplification may be correlated to the highly variable number of copies of multi-copy genes. The PCR primers may affect their capability to anneal to the template influencing the assay sensitivity. On the other hand, the results of the Hsp70 gene amplification, detecting a lower number of copies, may be correlated to its highly conserved sequence. The designed PCR primers anneal to the template once, with high sensitivity and specificity.

In conclusion, the results of this investigation provide evidence that, as well known, *N. ceranae* may successfully proliferate in asymptomatic honey bee colonies living in small Mediterranean islands. In this respect, samples collected from the comparatively larger Elba island Elba should be considered more occasional finding than a realistic representation of the overall situation.

**Table 1**

Replicates of *Nosema ceranae* gene copies detected with 16S rRNA and Hsp70 qPCR assay in honey bee samples from Tuscanian Archipelago.

| Island | Colony | Replicate | 16S gene copies/bee | Hsp70 gene copies/bee |
|--------|--------|-----------|---------------------|-----------------------|
| Capraia | C1     | 1         | 3.16E+05            | 2.28E+04              |
|        |        | 2         | 3.28E+05            | 2.44E+04              |
|        | C2     | 1         | 1.16E+07            | 3.12E+06              |
|        |        | 2         | 1.28E+07            | 3.24E+06              |
| Gorgona | G1    | 1         | 4.52E+04            | 3.36E+04              |
|         |        | 2         | 4.96E+04            | 3.64E+04              |
|         | G2    | 1         | 5.88E+06            | 3.96E+05              |
|         |        | 2         | 6.12E+06            | 4.16E+05              |
| Pianosa | P1    | 1         | 5.52E+05            | 3.80E+05              |
|         |        | 2         | 5.80E+05            | 4.28E+05              |
|         | P2    | 1         | 6.36E+07            | 3.88E+06              |
|         |        | 2         | 6.60E+07            | 3.96E+06              |
| Elba    | E1    | 1         | <LOD               | <LOD                  |
|         |        | 2         | <LOD               | <LOD                  |
|         | E2    | 1         | <LOD               | <LOD                  |
|         |        | 2         | <LOD               | <LOD                  |
environments characterized by genetic isolation. A comparison between different q-PCR assays showed profound differences in the results when the primers are designed on single-copy or multi-copy sequences. The choice of analytical method may then be critical when reliable quantification is required.

References

Ansari, M.J., Al-Ghamdi, A., Nuru, A., Khan, K.A., Alatal, Y., 2017. Geographical distribution and molecular detection of Nosema ceranae from indigenous honey bees of Saudi Arabia. Saudi J. Biol. Sci. 24, 583–591. https://doi.org/10.1016/j.sjbs.2017.01.054.

Botías, A., Anderson, D.L., Meana, A., Garrido-Bailón, E., Martín-Hernández, R., Higes, M., 2012. Further evidence of an oriental origin for Nosema ceranae (Microsporidia: Nosematidae). J. Invertebr. Pathol. 100, 110–113. https://doi.org/10.1016/j.jip.2012.02.014.

Bourgeois, A.L., Rinderer, T.E., Beaman, L.D., Danka, R.G., 2010. Genetic detection and quantification of Nosema apis and N. ceranae in the honey bee. J. Invertebr. Pathol. 103, 53–58. https://doi.org/10.1016/j.jip.2009.10.005.

Calderón, R.A., Sanchez, L.A., Yahé, O., Fallas, N., 2008. Presence of Nosema ceranae in Africanized honey bee colonies in Costa Rica. J. Apic. Res. 47, 328–329. https://doi.org/10.3896/00218839.2008.11101485.

Chernourot, M., De Smet, L., Brunain, M., De Rycke, R., de Graaf, D.C., 2017. Nosema neumannii n. sp. (Microsporidia, Nosematidae), a new microsporidian parasite of honeybees, Apis mellifera in Uganda. Eur. J. Protistol. 61, 1–10. https://doi.org/10.1016/j.ejop.2017.07.002.

Chen, Y., Evans, J.D., Smith, L.B., Petris, J.S., 2008. Nosema ceranae is a long-present and wide-spread microsporidian infection of the European honey bee (Apis mellifera) in the United States. J. Invertebr. Pathol. 97, 186–188. https://doi.org/10.1016/j.jip.2007.07.010.

Chen, Y.P., Morgan, J.D., Murphy, C., Gutell, R., Zunker, M., Gundens-Rindal, D., Petris, J.S., 2009. Morphological, molecular, and phylogenetic characterization of Nosema ceranae, a microsporidian parasite isolated from the European honey bee, Apis mellifera. J. Eukaryot. Microbiol. 56, 142–147. https://doi.org/10.1111/j.1550-7408.2008.00374.x.

Chen, Y.W., Chung, W.P., Wang, C.H., Solter, L.F., Huang, W.F., 2012. Nosema ceranae infection intensity highly correlates with temperature. J. Invertebr. Pathol. 111, 264–267. https://doi.org/10.1016/j.jip.2012.08.014.

Cilia, G., Cabrini, R., Maiorana, G., Cardiao, L., Dall’Olio, R., Nannetti, A., 2018. A novel TaqMan® assay for Nosema ceranae quantification in honey bee, based on the protein coding gene Hsp70. Eur. J. Protistol. 63, 44–50. https://doi.org/10.1016/j.ejop.2018.01.007.

Eiri, D.M., Suwanapong, G., Endler, M., Nieh, J.C., 2015. Nosema ceranae can infect honey bee larvae and reduces subsequent adult longevity. PLoS One 10, https://doi.org/10.1371/journal.pone.0126340.

Ferroglio, E., Zanet, S., Peraldo, N., Tachis, E., Triscuglio, A., Laurino, D., Porporato, M., 2013. Nosema ceranae has been infecting honey bee Apis mellifera in Italy since at least 1993. J. Apic. Res. 52, 60–61. https://doi.org/10.3896/00218839.2013.0005772X.2017.1382041.

Forsgren, E., Fries, L., 2010. Comparative virulence of Nosema ceranae and Nosema apis in individual European honey bees. Vet. Parasitol. 170, 212–217. https://doi.org/10.1016/j.vetpar.2010.02.010.

Fries, I., 1993. Nosema Apis - a parasite in the honey bee colony. Bee World 74, 5–19. https://doi.org/10.1080/00057722.1993.11991495.

Fries, I., Feng, F., da Silva, A., Sлемenda, S.B., Pieniazek, N.J., 1996. Nosema ceranae n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee Apis ceranae (Hymenoptera, Apidae). Eur. J. Protistol. 32, 356–365. https://doi.org/10.1016/S0953-4739(96)80059-9.

Fries, I., Martin, R., Meana, A., García-Palencia, P., Higes, M., 2006. Natural infections of Nosema ceranae in European honey bees. J. Apic. Res. 45, 230–233. https://doi.org/10.1080/00100347.2006.1101355.

Giersch, T., Berg, T., Galea, F., Hornitzky, M., 2009. Nosema ceranae infects honey bees (Apis mellifera) and contaminates honey in Australia. Apidologie 40, 117–122. https://doi.org/10.1051/apido:2007050.

Gomez-Moracho, T., Maside, X., Martin-Hernández, R., Higes, M., Bartolomé, C., 2014. High levels of genetic diversity in Nosema ceranae within Apis mellifera colonies. Parasitology 141, 475–481. https://doi.org/10.1017/S0031182013001790.

Higes, M., García-Palencia, P., Martín-Hernández, R., Meana, A., 2007. Experimental infection of Apis mellifera honeybees with Nosema ceranae (Microsporidia). J. Invertebr. Pathol. 94, 211–217. https://doi.org/10.1016/j.jip.2006.11.001.