The first detection of *Nosema ceranae* (Microsporidia) in the small hive beetle, *Aethina tumida* Murray (Coleoptera: Nitidulidae)

Giovanni CILIA¹, Ilaria CARDAIO¹, Pedro Emanuel Jarmela dos SANTOS¹,
James D. ELLIS², Antonio NANETTI¹

¹CREA-AA, Centro di Ricerca Agricoltura e Ambiente, Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Via di Saliceto 80, 40128, Bologna, Italy

²Entomology and Nematology Department, University of Florida, Bldg. 970 Natural Area Dr., P.O. Box 110620, Gainesville, FL 32607-0620, USA

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Abstract – In order to investigate the possible infection of *Nosema ceranae* in small hive beetle (SHB), *Aethina tumida*, in 2017, beetle specimens were sampled in Gainesville (Florida). By Real-Time PCR (qPCR), using previously developed primers based on the 16S rRNA gene, *N. ceranae* was detected in 7 out of 10 SHB specimens, proving that the microsporidia can be transmitted by the alien beetle. This is the first report of *N. ceranae* infection in *A. tumida*, although evaluated on a few specimens.

Nosema ceranae / small hive beetle / honey bee / qPCR / transmission / *Aethina tumida*

1. INTRODUCTION

Nosemosis of western honey bees (*Apis mellifera*) is an infection caused by microsporidians belonging to the *Nosema* genus. It occurs in individual bees and can impact overall colony health (Fries et al. 2013). For decades, nosemosis was attributed to *Nosema apis* (Zander 1909). More recently, two additional species were found to be associated with honey bees: *Nosema ceranae* (Fries et al. 1996) and *Nosema neumannii* (Chemurot et al. 2017), the latter being detected recently and only in Ugandan bees, with still unknown distribution and undescribed effects on *A. mellifera*.

N. ceranae was first identified infecting the ventricular epithelial cells of the Asian honey bee *Apis cerana* (Fries et al. 1996), a honey bee

species generally accepted as the original host (Botías et al. 2012). However, the microsporidium was later found in *A. mellifera* colonies living in several areas of the world (Fries et al. 2006; Higes et al. 2006; Klee et al. 2007; Martin-Hernandez et al. 2007). Examination of preserved samples indicates that contact with *A. mellifera* must have occurred long before *N. ceranae* was recognised as a species (Chen et al. 2008; Ferroglio et al. 2013; Teixeira et al. 2013). The infections may be detrimental to the colonies and lead to their decline and collapse (Cox-Foster et al. 2007; Higes et al. 2008a), which makes *N. ceranae* a major threat to honey bees. Transmission occurs by incompletely elucidated routes; however, it is known that other Hymenoptera and insectivorous birds may be implicated in the dissemination of spores in the environment (Plischuk et al. 2009; Valera et al. 2011, 2017).

The small hive beetle (SHB), *Aethina tumida* (Murray 1867), is an invasive pest of *A. mellifera*. Native to Sub-Saharan Africa (Lundie 1940;

Corresponding author: G. Cilia,

giovanni.cilia@crea.gov.it

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Schmoke 1974), it has been detected in several countries worldwide since 1996 and presently, it is distributed on all continents except Antarctica (Al Toufailia et al. 2017; Lee et al. 2017; Neumann et al. 2016). It is widely distributed in North America and parts of Australia (Neumann et al. 2016), but has recently been found in Europe (da Silva 2014; Granato et al. 2017; Murilhas 2004; Palmeri et al. 2015). This species is an ecological generalist (Ellis and Hepburn 2006) that is capable of creating persistent populations with low density of honey bees (Arbogast et al. 2009).

Adult SHBs live in honey bee colonies, making them likely to encounter honey bee pathogens that they may be capable of spreading between bees or colonies (Eyer et al. 2009; Schäfer et al. 2010), thus increasing concerns related to SHB infestations. Herein, the aim of this investigation was the evaluation of *Nosema* spp. spores in SHB adults, possibly contributing to the transmission of nosemosis.

2. MATERIALS AND METHODS

2.1. Sample collection

One hive of European-derived honey bees with overt SHB infestation was selected in summer 2017 from an experimental apiary managed at the University of Florida, Gainesville, Florida, USA. No evident signs of nosemosis were present in the colony. A sample of 40 SHB adults was randomly collected from combs and the hive floor and further split into two subsamples, one of 30 and one of 10 individuals.

2.2. DNA extraction and qPCR analysis

Prior to analysis, the SHBs were washed with 95% ethanol to remove any external *Nosema* spores that may have been present. Following this, the abdomens were dissected, crushed, and DNA extracted with a DNeasy Blood & Tissue Kit (Qiagen S.p.a., Milan, Italy) following the manufacturer's instructions. The 30 abdomens of the first subsample were pooled and processed together to determine the average *Nosema* spp. abundance. The second subsample was used for individual SHB examinations and to estimate the proportion of positive individuals.

Multiplex Real-Time PCR (qPCR) was performed in duplicate from the same DNA extracts using primers and probes for *N. apis* and *N. ceranae* as reported by Fries et al. 2013.

The optimised amplification and quantitation protocol for Multiplex qPCR was as follows: 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 s/step.

Light microscopy was used to evaluate the stage of *Nosema* spores in SHBs. Spore counting was estimated by haemocytometer as described by Fries et al. (2013).

3. RESULTS

The qPCR analysis performed on the pooled sample resulted in 15,646 *N. ceranae* copies being found (Table I), corresponding to an average of 521.5 copies per individual SHB. The number of copies found in the individual SHBs averaged 691.3 with an s.d. of 1,544.1 (Table I). However, *N. ceranae* was not found in three of those samples, which indicates a 70% prevalence of positive individuals. Considering all the analysed samples ($N = 40$), the average number of detected *N. ceranae* copies per adult SHB was 564.0. *N. apis* was not found in any of the samples.

4. DISCUSSION AND CONCLUSION

Data show for the first time that SHB abdomens are associated with *N. ceranae*. This does not imply that SHBs are vectors or mechanical transmitters of the pathogen, but it does suggest either is possible. More work needs to be done to clarify this.

N. ceranae shows reproductive capability in different Hymenopteran hosts but there is no present evidence that it can multiply in Coleopteran species. In fact, it is possible that the SHBs in this investigation did not contain vegetative stages of *N. ceranae* but rather spores, as was observed under the light microscope. Histological investigations should be made to assess *N. ceranae* ability to complete its lifecycle in the SHB. This could revise the views of the transmission of nosemosis.

Table 1. *Nosema ceranae* copies detected by RT-PCR in small hive beetle abdomens and relevant Ct data (asterisks denote the same Ct as the negative control)

Sample	Mean <i>N. ceranae</i> copies $\pm$ s.d.	Mean Ct $\pm$ s.d.
Pooled sample (30 individuals)	15,646 $\pm$ 6.82	32 $\pm$ 2
SHB 1	< LOD	40*
SHB 2	420 $\pm$ 1.32	34 $\pm$ 6
SHB 3	969 $\pm$ 2.13	34 $\pm$ 1
SHB 4	< LOD	40*
SHB 5	4.3 $\pm$ 0.05	36 $\pm$ 4
SHB 6	< LOD	40*
SHB 7	120 $\pm$ 3.58	32 $\pm$ 6
SHB 8	320 $\pm$ 1.01	34 $\pm$ 2
SHB 9	5000 $\pm$ 0.98	35 $\pm$ 2
SHB 10	80 $\pm$ 1.09	33 $\pm$ 4

Several bee species may be infected by *N. ceranae*. These include some stingless bees (Meliponini) (Porrini et al. 2017), the solitary bees *Osmia cornuta*, *Osmia bicornis*, *Heriades truncorum*, and *Andrena ventralis* (Ravoet et al. 2014), and the bumblebee species *Bombus brasiliensis* (Plischuk and Lange 2016), *Bombus atratus*, *Bombus morio*, and *Bombus bellicosus* (Plischuk et al. 2009). The microsporidium also was detected in the social wasp *Polybia scutellaris* (Vespidae) (Porrini et al. 2017). Also, in regurgitated pellet of *Merops apiaster*, bee-eater bird, vital *N. ceranae* spores was detected (Higes et al. 2008b). All of these species potentially share the same environment with *A. mellifera* and *Aethina tumida*.

Likewise, SHBs were detected in the nests of several bee species, nests in which they showed the ability to reproduce the bumblebee *Bombus impatiens* (Spiewok and Neumann 2006) and the stingless bees *Austroplebeia australis* (Halcroft et al. 2011), *Dactylurina staudingeri* (Mutsaers 2006), *Melipona beecheii* (Lóriga Peña et al. 2014), and *Trigona carbonaria* (Greco et al. 2010).

The knowledge about the effects of *N. ceranae* infections and SHB infestations on the host species above is limited. However, considering the low specificity of both pests and the high

prevalence of *Nosema* positive SHBs in limited samples of this investigation, the possible role of SHB in the transmission of *Nosema* spp. within the members of the reared and wild community of pollinators should be considered further as a matter of ecological concern. The infection of *Nosema ceranae* in SHBs adults may confirm the transmission of pathology from original host to other species, like Deformed Wing Virus (DWV) replicative form funded in *Vespa crabro* (Forzan et al. 2017a; Forzan et al. 2017b).

The presence of spores in the SHB abdomens suggests that they may have been ingested by the beetles. This is compatible with the fact that SHB adults feed on colony stores (Buchholz et al. 2008; Neumann et al. 2016; Pirk and Neumann 2013) and with the description of bee-collected pollen as a possible *N. ceranae* spore reservoir (Higes et al. 2008c). Another possible contamination route may be related to the SHB ability to interact with the workers to elicit a trophallactic food transfer via antennal stimulation (Ellis 2005; Ellis et al. 2002).

The average number of spores detected in the SHB abdomens is 6.6 times the median infective dose (ID₅₀ = 85 spores) for the honey bee (Forsgren and Fries 2010). This investigation does not elucidate the mode of transmission to the bees but, in case those spores are shared between a limited number of individuals and are fully viable, the possibility to generate SHB-mediated *Nosema* transmission becomes realistic.

The transmission routes of *N. ceranae* between bees and SHBs need further investigation. In bees, *N. ceranae* is transmitted via the oral-oral and faecal-oral route (Higes et al. 2009; Smith 2012). Both routes may be viable for the interaction between honey bees and SHBs given that the beetles are fed by honey bee workers. This could be a route of transmission in both directions. In addition, adult and larval SHBs defecate inside the hive with the larval faeces suspected of causing honey to ferment (Cuthbertson et al. 2013). This, too, could be a transmission route. The influence of different SHB life-history stages on *N. ceranae* infections should be studied to evaluate their potential epidemiological and ecological impact.

The no detection of *N. apis* may be a consequence of the climate where the samples were taken (warm/temperate with mild winters and

hot summers), season (summer), or of the still controversial possible competition between the two *Nosema* species (Chen et al. 2012; Forsgren and Fries 2010; Martín-Hernández et al. 2012).

The detection of *N. ceranae* spores in the SHB abdomens at levels quantitatively sufficient to produce an outbreak suggests a possible role of *Aethina tumida* in nosemosis infection, though that careful investigation of this topic is needed to confirm this hypothesis. The low host specificity of SHB and its increasing spread may represent a further risk factor, ultimately jeopardising pollinator diversity. Further research efforts are needed to elucidate whether *Aethina tumida* adults act as a dead-end host, mechanical transmitter, or vector of *N. ceranae*.

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Contributions Conceived and designed the experiments: GC. Performed the experiments: IC, GC, and PJ. Analysed the data: IC. Wrote the original draft: AN, GC, PJ, IC, and JE. Review and editing: JE, AN, and GC. Supervision: AN and JE. All authors read and approved the final manuscript.

La première détection de *Nosema ceranae* (Microsporidia) dans le petit coléoptère des ruches, *Aethina tumida* Murray (Coleoptera: Nitidulidae)

Nosema ceranae / petit coléoptère de la ruche / abeille / qPCR / transmission / *Aethina tumida*

Erster Nachweis von *Nosema ceranae* (Microsporidia) in dem Kleinen Beutenkäfer, *Aethina tumida* Murray (Coleoptera: Nitidulidae)

Nosema ceranae / Kleiner Beutenkäfer / Honigbiene / qPCR / Transmission / *Aethina tumida*

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