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NOTES AND COMMENTS

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First detection of *Nosema ceranae* in honey bees (*Apis mellifera* L.) of the Macaronesian archipelago of Madeira

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ABSTRACT

The Microsporidia *Nosema ceranae* is an invasive pathogen affecting honey bee health, particularly in warm climates. In this study, *N. ceranae* was detected for the first time in honey bees (*Apis mellifera* L.) of the Madeira archipelago, indicating that this pathogen is now spreading across the entire Macaronesia. *Nosema apis* was not detected, and the high prevalence (67.7%) of *N. ceranae* indicates its dominance over *N. apis*.

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The archipelago of Madeira, together with that of the Azores and the Canaries, form the biogeographical region of Macaronesia (*sensu* Beyhl et al. (1995)). These volcanic archipelagos share a mild climate and a highly diverse flora, thereby offering ideal conditions for apiculture. In addition, they share honey bee populations that are genetically close to the Iberian honey bee *Apis mellifera iberiensis*, as revealed by wing morphometry and mitochondrial DNA (Álvarez et al., 1997, 2001; Ferreira et al., 2020; Miguel et al., 2015; Muñoz et al., 2013). These findings support historical introductions of *A. m. iberiensis* into Macaronesia during colonization by the Portuguese and Spanish settlers (Ferreira et al., 2020), although natural expansion out of the north African coast has also been proposed (de la Rúa et al., 1998, 2006).

Over recent decades, beekeeper-mediated introductions of honey bees or hive products brought along *Varroa destructor* into the three archipelagos and *N. ceranae* (recently renamed by Tokarev et al. (2020) as *Vairimorpha ceranae*) into the Canaries and the Azores (Lopes et al., 2022; Muñoz et al., 2014). However, in the Azores, there are still six islands that are free of *V. destructor* and two that are free of *N. ceranae* (Lopes et al., 2022). While *V. destructor* was also introduced on Madeira in 2001 (Silva, 2016), the question remains whether this archipelago is free of *N. ceranae*, such as in the case of the Azorean island of Flores, which, in spite of being *V. destructor*-colonized, is still *naïve* to this pathogen.

To gain a fuller picture of the *N. ceranae* epidemiological status in Macaronesia, in 2014, we conducted a survey on Madeira, the largest island of the Madeira archipelago. On the other inhabited island of the archipelago, Porto Santo, honey bee colonies were re-introduced in 2014 from the Azorean island of Terceira, after the die-off of the managed population due to the arrival of *V. destructor* in 2003 (Silva, 2016). Given that *N. ceranae* is present on Terceira (Lopes et al., 2022), it is possible that the honey bee population of Porto Santo is also infected.

Prior to the *N. ceranae* jump from *Apis cerana* to *Apis mellifera*, *Nosema apis* was thought to be the single etiological cause of nosemosis in the world (Higes et al., 2006; Huang et al., 2007). While the role of *N. ceranae* in colony losses is a contentious matter (reviewed by Martín-Hernández et al. (2018)), there is evidence that it has higher biological potential in warmer climates than *N. apis* (Martín-Hernández et al., 2009). In this context, it has been claimed that *N. ceranae* is an important stressor in the Mediterranean countries (Hges et al., 2010) and likely in Madeira because of the year-round mild climate.

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MAP and RMH designed the experiment. SKS performed the multiplex PCR and the related DNA extractions under supervision of RMH and MH. All data curation and graphic representation were performed by ARL with assistance of DH. ARL and MAP wrote the manuscript. All the authors critically reviewed the manuscript for important intellectual content.

The goal of this study was to survey the honey bee population of Madeira for the presence of *N. ceranae* and *N. apis* and evaluate their prevalence and distribution. To achieve this goal, between February and March of 2014, three colonies from 31 apiaries were sampled along the coast where, due to the island's rough orography, most apiaries are located (Figure 1A). Total DNA was extracted from a pool of 120 workers for each of the 93 samples, following the protocol described by Martín-Hernández et al. (2012). The 93 DNA extracts were simultaneously screened for *N. ceranae*, *N. apis*, and an internal control of *A. mellifera* (COI gene) using the multiplex-PCR assay developed by Martín-Hernández et al. (2012). The assay comprised a negative control of extraction, a non-template control (NTC), a positive control for *N. apis*, and another for *N. ceranae*. The QIAxcel Advanced System (Qiagen®) was used to analyse the PCR products. The amplification of the internal *A. mellifera* control in all 93 samples confirmed the success of the DNA extraction. No amplicons were observed in the extraction controls or NTCs, indicating that there was no cross-contamination during sample processing.

Of the 93 colony samples, 30 (32.3%) were negative for both *Nosema* species (Figure 1B). *N. apis* was not detected, whereas *N. ceranae* was found in 63 colonies (67.7%). This finding suggests the dominance of *N. ceranae* over *N. apis* and it is unaffected by sampling time because the colonies were sampled in February and March, therefore matching the seasonal peak of *N. apis* infection (Fries, 1993). Interestingly, the prevalence of *N. ceranae* on Madeira lies between that reported for the Canaries (75%; Muñoz et al. (2014)) and for the Azores (50.7%; Lopes et al. (2022)). This south-north gradient might be explained by *N. ceranae* arriving at the Canaries first and at the Azores last, which is supported by the observation that there are two Azorean islands still free of *N. ceranae* (Lopes et al., 2022). Alternatively, but not mutually exclusive, this pattern is shaped by the climate in which the warmer and dryer Canaries and Madeira are more favourable to *N. ceranae* (Martín-Hernández et al., 2009).

The analysis by apiary revealed that in 16 of the 31 (51.6%) surveyed apiaries, all the colonies tested positive for *N. ceranae*, six (19.4%) had two-thirds of positive colonies, three had one-third (9.7%), and six (19.4%) had no positive samples (Figure 1B). This observation suggests that once *N. ceranae* enters an apiary, it rapidly spreads across all the colonies. Interestingly, most of the *N. ceranae*-negative colonies are clustered on the southern side of the island, and, except for one single apiary, which only had two positive colonies, all northern apiaries had the three sampled colonies positive for *N. ceranae* (Figure 1B). This pattern suggests that *N. ceranae* first entered Madeira from the north, and, at the moment of this study, it was spreading to the south. Alternatively, *N. ceranae* entered Madeira elsewhere and a more favourable environment for the development of *N. ceranae* infection in the north explains

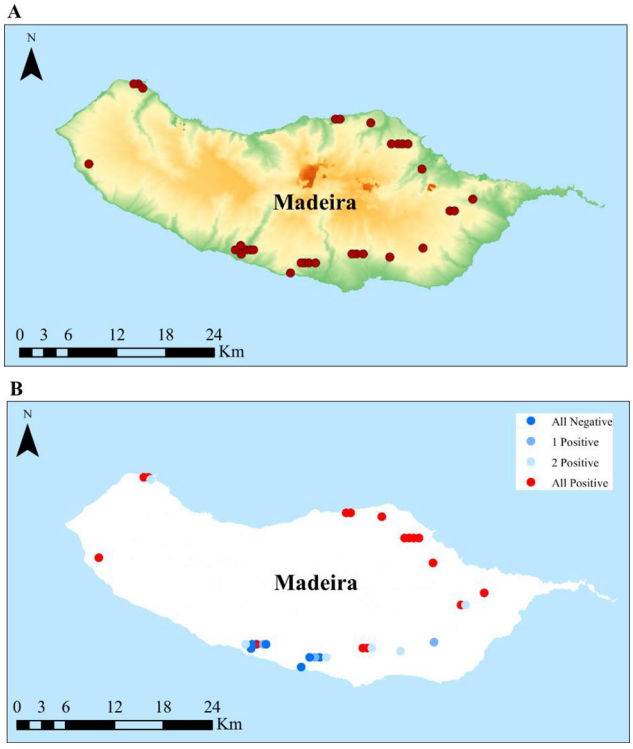

Figure 1. Map of Madeira showing (A) the orography of the island, with the location of the sampled apiaries, and (B) the location of the apiaries with the three sampled colonies *N. ceranae*-positive (red dots), two colonies *N. ceranae*-positive (light blue dots), one colony *N. ceranae-positive* (blue dots), and no *N. ceranae*-positive colonies (dark blue dots).

the higher prevalence. The northern side of Madeira is more humid and colder than the southern side. But more importantly, because it is dominated by the densely-wooded primeval Laurissilva (laurel) forest, the northern side offers poor pollen sources for honey bees, and there is mounting evidence that nutrition has a pivotal role in the development of *N. ceranae* infection (Castelli et al., 2020).

This study constitutes the first report of *N. ceranae* on Madeira. The lack of detection of *N. apis* suggests that *N. ceranae* is possibly the sole species causing nosemosis on Madeira, as reported elsewhere (Cilia et al., 2019; Gajger et al., 2010; Stevanovic et al., 2011). The distributional pattern of the pathogen suggests that it entered Madeira not long before the sampling in 2014 and it is possibly still expanding. Another cross-sectional survey is warranted to assess whether *N. ceranae* prevalence in the south has reached the levels of the north, which would help understand the dynamics of the dispersion.

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