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Vairimorpha infections in endemic Moroccan honeybees*:* A cross-sectional study

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Abstract

Biodiversity and food security on Earth would be impossible without the contribution of animal and insect pollinators, with the honeybee (*Apis mellifera*) being the primary agent. However, the distribution and diversity of these insect populations, particularly native species, are increasingly threatened by various biotic and abiotic stressors, which contribute to Colony Collapse Disorder (CCD) in apiaries worldwide. A microsporidian fungus of the genus *Vairimorpha* (formerly *Nosema*)) that infects the insect gut causes the disease known as nosemosis, which is among the biotic factors potentially associated with CCD. In this study, we examined 206 samples from various apiaries in the Souss-Massa region of Morocco to assess the epidemiological status of this parasitosis in Moroccan honeybees. Spore detection and quantification of *Vairimorpha* spp. were performed using microscopy, while molecular techniques were employed to differentiate *Vairimorpha apis* from *Vairimorpha ceranae*. The microscopic analysis revealed a prevalence rate of 78.6%, a mean abundance of 7×10^5 spores/bee/apiary, and a mean infection intensity of 9×10^5 spores/bee/apiary. Molecular analysis indicated that *V. ceranae* is more prevalent than *V. apis*. Consequently, as observed in many other regions worldwide, *V. ceranae* has become invasive in native Moroccan honeybees populations, highlighting the need to consider this new ecological status in future control and conservation programs.

Key words: *Apis mellifera intermissa*, *Apis mellifera sahariensis*, Moroccan honeybee, Nosema spores counting, *Vairimorpha apis*, *Vairimorpha ceranae*.

Infecciones por *Vairimorpha* en abejas endémicas de Marruecos: un estudio transversal

Resumen. La biodiversidad y la seguridad alimentaria en la Tierra serían imposibles sin la contribución de los animales e insectos polinizadores, como la abeja melífera (*Apis mellifera*), el actor principal. Sin embargo, la distribución y diversidad de esas poblaciones de insectos, especialmente las especies nativas, se ven amenazadas por muchos factores estresantes bióticos y abióticos que causan el trastorno de colapso de colonias (CCD) en colmenares de todo el mundo. Un hongo (*Vairimorpha*) que infecta el intestino del insecto causa la enfermedad (nosemosis), que se eleva entre los factores bióticos que podrían estar relacionados con la aparición del CCD. En el estudio actual, hemos examinado 206 muestras de diferentes colmenares en la región de Souss-Massa (Marruecos), para analizar la situación epidemiológica relacionada con la parasitosis en las abejas melíferas marroquíes. La herramienta de microscopio se utilizó para detectar y contar las esporas de *Vairimorpha* spp., y la herramienta molecular se utilizó para discriminar *Vairimorpha apis* de *Vairimorpha ceranae.* Los resultados del análisis microscópico mostraron una tasa de prevalencia del 78,6%, una abundancia media de 7×10^5 esporas/abeja/colmenar y una intensidad de infección media de 9×10^5 esporas/abeja/colmenar. La herramienta molecular mostró que *V. ceranae* es más prevalente que *V. apis*. Por lo tanto, como en muchos otros lugares del planeta, *V. ceranae* también se ha vuelto invasiva en las abejas melíferas nativas de Marruecos, lo que nos impulsa a considerar este nuevo estado ecológico en futuros programas de control y conservación.

Palabras clave: *Apis mellifera intertermissa*, *Apis mellifera sahariensis*, abeja marroquí, recuento de esporas de Nosema, *Vairimorpha apis*, *Vairimorpha ceranae*.

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INTRODUCTION

Among principal pollinator insects, the honeybee (*Apis mellifera,* Linnaeus 1758) plays an essential role in our ecosystem biodiversity, agricultural production, and food production systems (Kevan and Phillips 2001, Klein et al. 2003). Many subspecies (~30) of *Apis mellifera* are recorded across different biotopes worldwide, and eleven of them are endemic in Africa (Pirk et al. 2016). In Morocco, two African honeybee subspecies namely *Apis mellifera sahariensis* (Baldensperger 1932) and *Apis mellifera intermissa* (Maa 1953), are breeding across the country, essentially in traditional apiaries (Aglagane et al. 2023). Nevertheless, this biological diversity is susceptible to many biotic and abiotic stressful factors causing the referred Colony Collapse Disorder (CCD) that threatens beekeeping worldwide (VanEngelsdorp et al. 2009, Goulson et al. 2015). Furthermore, the intensive use of some pesticides (e.g. Neonicotinoids), climate change, and some pathological issues (e.g. *Varroa* mite, foulbrood, nosemosis) could contribute to such disorder occurrence (Neumann and Carreck 2010, Williams et al. 2010, van der Sluijs and Vaage 2016).

Among those parasitic pathologies, *Nosema* infection (disease: nosemosis) occurs in honeybees, it is caused by an intracellular microsporidian that targets the ventricular epithelium tissue (midgut) of the adult insect (Higes et al. 2020). This fungus genus is recently redefined as *Vairimorpha* (Tokarev et al. 2020). It is essentially represented by two species *Vairimorpha (Nosema) apis* and *Vairimorpha (Nosema) ceranae*. Morphologically identical, the two pathogens have long been confused because their spores are closely identical (Ptaszyńska et al. 2014). However, the epidemiology of these two species is very different: *V. apis* causes diarrhea that beekeepers recognize and know how to control; on the other hand, *V. ceranae* does not cause symptoms (dry form) allowing by that a distinguishing element for a differential diagnosis besides the molecular tool (Martín-Hernández et al. 2007).

Nosemosis disease could affect the three bee castes (queen, worker, male) and appears as dysenteric syndrome when it is caused by *V. apis*, while it is characterized by a dry pattern when it is caused by *V. ceranae*, in addition to other common signs essentially related to behavioral disorder (dragging bees, weakness, flightless, spatial disorientation, etc.) (Fries 1993, Fries et al. 2006). Like any host-parasite relationship, an equilibrium is established between the bee and *Vairimorpha*, and the disease rises only up when that balance is broken due to many stressful conditions (Martín-Hernández et al. 2011, Chen et al. 2013, Kurze et al. 2018).

Therefore, monitoring *Vairimorpha* infection should combine the clinical condition of the hive and the measurement of the bee parasite burden. Before any health management strategy in apiaries, a preliminary scan should be done to determine the *Vairimorpha* prevalence, abundance, and infection intensity. That was the aim of the current study; it concerned the study of *Vairimorpha* infection across the Sous-Massa apiaries (Morocco) to set up a preliminary dashboard, dressing up the related health status, and guiding bee industry managers in any further intervention against that pathogen.

MATERIALS AND METHODS

Sampling Methodology. The current study concerned a total of 206 individual samples, composed of a pool of 80 to 100 adult bee workers from hives at the same spot, representing 206 different apiaries scattered around the Souss-Massa geographical area (Figure 1). They were collected and conserved in 70% ethanol flasks before being sent to the laboratory for analysis.

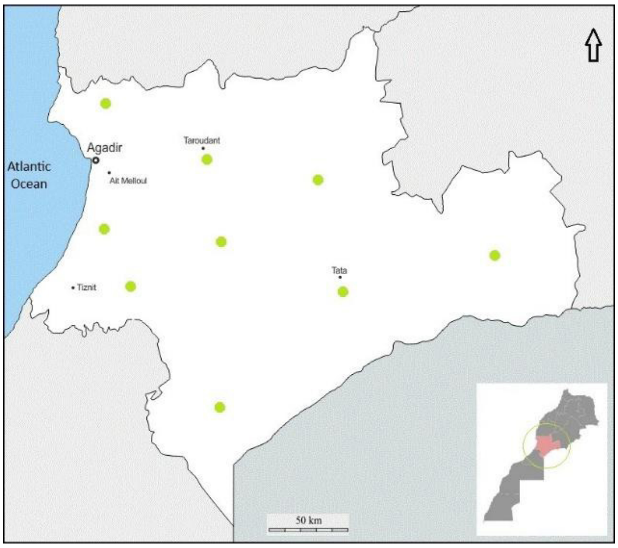

Figure 1. The delimitation of the Souss-Massa region within the Moroccan territory, the green dots showed the localization of the studied apiaries.

Microscopic and Molecular analyses. Briefly, for each individual sample, 60 abdomens of worker honeybees were macerated in 60 ml of water, filtered to extract and count *Vairimorpha* spores using a haemocytometer under a phase-optic microscope and the calculating formula to estimate the bee burden as described in chapter 3.2.4 of the World Organization of Animal Health terrestrial manual protocol (WOAH 2018): $Z = \alpha/\beta \, . \, \delta \, . \, 250{,}000$. Where Z = spore numbers per bee, α = total number of spores counted, β = number of squares counted, and δ = dilution factor.

DNA was extracted from the filtrates used for the microscopic analysis using the Macherey-Nagel™ NucleoSpin™ Mini kit for DNA (MN GmbH, Düren, Germany). The detection of *Vairimorpha* specific genes was done using a triplex quantitative real time PCR kit (MycoReal Kit Nosema apis & ceranae®: ingenetix GmbH, Vienna, Austria) distinguishing and targeting both *Vairimorpha (Nosema) apis* and *Vairimorpha (Nosema) ceranae* species.

Terminology and Statistical analysis. The employed terminology in this study (Prevalence, Abundance, Intensity) agrees with the standard ecological terms in parasitology (Margolis et al. 1982, Bush et al. 1997). Their formulas are:

$$\text{Prevalence (\%)} = \frac{\textit{Number of positive samples}}{\textit{Total sample size}} . 100$$

$$\text{Abundance} = \frac{\textit{Spore number per bee}}{\textit{Total sample size}}$$

$$\text{Intensity} = \frac{\textit{Spore number per bee}}{\textit{Total sample size}}$$

Using GraphPad PRISM® v5.00 software for data analysis. Outputs are presented as percentages for prevalence and means ± standard deviation for parasite abundance and intensity.

RESULTS AND DISCUSSION

For the microscopic results, the qualitative screening allowed us to note the presence of *Vairimorpha* spp. spores (Figure 2) in most analyzed samples with an overall prevalence rate of 78.6% of *Vairimorpha* spp. spore detection by apiary was registered. Furthermore, the quantitative assessment of the infection levels, by counting the number of spores by bee, revealed an infection abundance rate of 709 573 spores/bee/apiary, and an intensity infection of 902 296 spores/bee/apiary (Table 1). In addition, the abundance dispersion analysis shows that 72.8% of apiaries are below 10^6 spores/bees, 17.5% between 10 x 10^6 and 20 x 10^6, and only 9.7% of apiaries exceed 20 x 10^6 spores/bee.

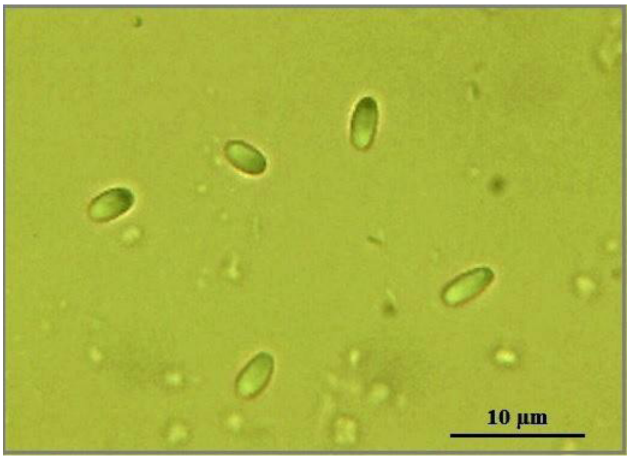

Figure 2. Original image showing *Vairimorpha* spp. spores.

Across a subsample of 84 microscopic positive samples, the molecular analysis showed that the dominant species is *V. ceranae* detected in 100% (84/84) of positive samples. In contrast, *V. apis* was only detected in bi-infected apiaries beside *V. ceranae* at a rate of 6% (5/84) in positive samples.

Table 1. Intensity and Abundance scores for *Vairimorpha* spp. infection at the apiary scale.

	Intensity	**Abundance**
Mean	902 296.30	709 572.82
SD	980 366.23	944 588.61
Min	10 000	0.00
Max	7 325 000	7 325 000

Our study showed that *Vairimorpha.* spp. is very prevalent with a noticeable dominance of *V. ceranae* species. This finding is in concordance with the latest multiple studies conducted in variable climatic and geographical zones around the globe (González et al. 2020, Ostroverkhova 2020, Ostroverkhova et al. 2020, Jabal-Uriel et al. 2022, Lage et al. 2022, Lopes et al. 2022).

V. ceranae was first reported in the Asian honeybee (*Apis cerana*) before adopting the European honeybee (*Apis mellifera*) as a new host and spreading on the five continents (Fries et al. 1996). *V. ceranae* seems to be more virulent than *V. apis* and is capable of causing massive mortality in the hive (Williams et al. 2014). Nevertheless, the detection of the disease due to *V. ceranae,* without diarrheic symptoms, is essentially based on excreted spore counting that could inform us of infection levels. Hence, the quantitative parameters based on the excreted spores such as the infection intensity are depending on intrinsic factors, especially the insect immune status, and the extrinsic factors, particularly the bee diet composition and the pollen abundance (Zheng et al. 2014, Li et al. 2017).

However, the correlation between the infection intensity and the adult insect morbidity doesn't always mean causality; hence, the disease outbreaks could be predicted under certain climatic conditions, especially by monitoring the annual temperatures allowing us to expect high infection levels in low-temperature periods (Meana et al. 2010, Chen et al. 2012).

In addition to that, the ecological health status of hives is influenced by other environment-related factors; particularly the poor nutrition conditions caused by the scarcity of flowering during long periods of drought increasing the vulnerability of the bees to parasitic diseases, and the climate changing effects that encourage health troubles to rise especially when summers are longer than usual (Dolezal and Toth 2018, Flores et al. 2019).

Confirming the same trend globally, *V. ceran*ae has become invasive and replaced *V. apis* in the Moroccan honeybees. This status changes the epidemiological data and should be considered by the technical managers in the diagnosis, prevention, and disease control strategies especially in the threatened endemic species: nosemosis may occur without showing any dysenteric symptom; thus, the excretions of parasite forms should be monitored at the level of the hive and apiary, particularly in low-temperature periods, to take adequate control measures.

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