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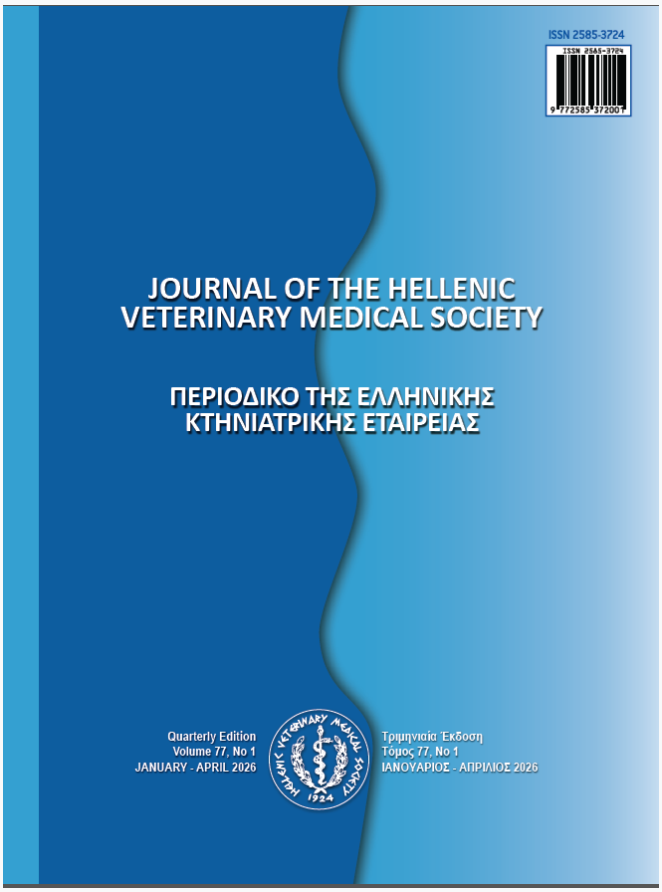

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Research article
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Epidemiology of *Nosema* spp. in *Apis mellifera* Colonies in Attica, Greece: Molecular Survey and Risk Factor Analysis

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ABSTRACT: Nosema disease, caused by microsporidian fungi of the genus *Nosema*, poses a significant threat to honey bee (*Apis mellifera*) populations worldwide. This study calculated the prevalence of *Nosema* species in honey bee colonies in the Attica region of Greece and assessed the impact of various management practices on infection rates. A survey was conducted in 33 apiaries in the Attica region during the spring of 2022. A total of 130 colonies were sampled and analyzed using species-specific multiplex Polymerase Chain Reaction targeting the 16S rRNA gene to detect *Nosema* species. A questionnaire was administered to beekeepers to gather data covering apiary characteristics, hive type, management practices, and clinical observations of nosemosis symptoms. The results revealed a high prevalence (69.2%) of *Nosema ceranae* in the sampled colonies, while *Nosema apis* or co-infection with both species was not detected. The absence of *N. apis* aligns with similar findings across Southern Europe, where *N.* ceranae has largely displaced the formerly dominant *N.apis.* Statistical analysis identified significant associations between *N. ceranae* prevalence and certain management factors. Colonies in migratory apiaries had significantly higher infection rates compared to stationary ones. Additionally, hives with screened bottom boards were less frequently infected than those with solid boards, suggesting a potential protective effect through enhanced ventilation and hygiene. Beekeeper occupation was also linked to infection risk: professional beekeepers' colonies exhibited a significantly higher prevalence than those of sideline and hobbyist beekeepers. Notably, the study found poor agreement between beekeeper-reported symptoms and laboratory-confirmed infections (Kappa = 0.046), indicating that reliance on visual signs alone is insufficient for accurate diagnosis of *N. ceranae*. This highlights the need for routine molecular diagnostics in colony health management. Overall, this study provides data on *N. ceranae* prevalence in Attica, Greece, for the first time and underscores the role of management practices in disease transmission. The findings emphasize the need for improved diagnostic awareness and targeted health strategies to mitigate the spread and impact of *N. ceranae* in Greek apiculture.

Keyword: *Nosema ceranae*; honey bee; prevalence; epidemiology; Greece

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INTRODUCTION

Bees have provided humans with products such as honey, wax, propolis, royal jelly, and venom since prehistoric times (Harissis, 2009). More importantly, their role in pollination makes them essential to both global economies and ecological balance. (Klein et al., 2007). The Food and Agriculture Organization of the United Nations (FAO) estimates that 87 of the most important crops, such as fruits and vegetables for human consumption, depend on pollination (FAO,2018).

However, over the past 20 years, substantial losses in honey bee populations—particularly in the Northern Hemisphere—have been recorded, raising concerns and prompting research into the causes of these losses (vanEngelsdorp et al., 2008, 2009, 2010). This phenomenon has garnered significant global attention and stimulated scientific investigation into its potential causes (Neumann and Carreck , 2010). Although no single factor has been conclusively identified as the primary driver of bee population decline, a multitude of stressors, including intensive agriculture, pesticide use, environmental changes, management practices, poor nutrition, and pathogens, have been implicated in honey bee decline (Soroker et al.,2010; Goulson et al., 2015; Hristov et al., 2020).

The contribution of pathogens such as microsporidia *Nosema* spp., to colony mortality, as well as the interactions between their prevalence, remains insufficiently understood and warrants further investigation (Bordin et al., 2022).

The reclassification of *Nosema* within the genus *Vairimorpha* has been proposed in recent literature (Tokarev et al., 2020), however, it continues to spark considerable discussion and differing viewpoints among researchers (Bartolomé et al., 2024). Due to this ongoing debate, the present study employs the historically and widely accepted designation of the genus *Nosema*.

Nosemosis is a prevalent and impactful disease of adult honey bees, caused by intracellular microsporidian parasites of the genus *Nosema*, which are highly specialized fungi (Gill and Fast, 2006; Ren et al., 2016). These pathogens invade the epithelial cells of the bee's midgut, disrupting digestive processes, weakening the immune system, and ultimately compromising the overall health and productivity of the colony. While *Nosema apis* was historically considered the primary causative agent (Fries,1988, 2010), in European honey bees *(Apis mellifera), Nosema. ceranae,* initially identified in the Asian honey bee *(Apis cerana),* (Fries et al., 1996), has more recently emerged as a dominant and potentially more virulent species in global honey bee populations. *N. ceranae* is known to infect not only managed honey bee species (*A. mellifera* and *A. cerana*) (Higes et al., 2007) but also a broad range of other insects, including wild bees (Purkiss and Lach, 2019), bumblebees (*Bombus* spp.) (Li et al., 2011), wasps (Porrini et al., 2017), butterflies, and the small hive beetle *Aethina tumida* (Nanetti et al., 2021). This expanded host range contributes to the pathogen's potential for widespread dissemination, both geographically and across different pollinator communities, including transmission from wild populations to managed colonies (Nanetti et al., 2021). Given its ecological versatility and ability to infect multiple pollinator taxa, *N. ceranae* is suspected to play a role in the global decline of pollinators (Klee et al., 2007; Giersch et al., 2009; Higes et al., 2013), either as a primary pathogenic agent or synergistically with other environmental stressors (Doublet et al., 2014). Other *Nosema* species, such as *N. neumanni* (Chemurot et al.,2017) and *N. bombi,* (Vavilova, 2017), have been identified in honey bees, but their distribution and pathogenicity are not fully described.

Nosema infection can have a significant negative impact on honey bee colonies, affecting individual bee physiology, behaviour, and colony health (Paris et al., 2018). Infected bees may exhibit reduced lifespan, impaired flight ability, and altered foraging behavior (Higes et al., 2013), which can lead to colony weakening and increased mortality (Fries, 2010). Infections may be chronic and asymptomatic in the early stages. A combination of various stress factors appears to influence, to a lesser or greater extent, the health of honey bee colonies and their ability to survive following infection by *N. ceranae*. Viral pathogens such as the chronic bee paralysis virus (Toplak et al.,2013) and black queen cell virus, along with exposure to neonicotinoid pesticides like imidacloprid, which is commonly used in agriculture, have been implicated in increasing mortality among *N. ceranae*-infected bees (Alaux et al., 2010; Doublet et al., 2015). These stressors may act synergistically with *N. ceranae*, exacerbating its effects and compromising the immune response and resilience of infected bees. Given its insidious progression and high transmission potential, elucidating the epidemiology of Nosemosis remains a research priority.

This study aimed to investigate the epidemiology

of *Nosema* species in honey bee colonies in the Attica region of Greece and to identify potential risk factors associated with the disease. To date, information regarding the occurrence and distribution of Nosemosis in the Attica peninsula remains limited.

MATERIALS AND METHODS

Study Area and Sample Collection

The study was conducted in the Attica peninsula of Greece during the spring of 2022. Thirty-three apiaries, both stationary and migratory, were included in the study. Four honey bee colonies were randomly selected and sampled from each apiary, except for two apiaries from which three hives were sampled due to weather constraints. In total, 130 samples were collected from the entrance flying boards of the selected colonies. Care was taken to avoid collecting young bees engaged in orientation flights (Fries et al.,2013). To collect forager bees, hive entrances were closed for 15 minutes (Meana et al., 2010; Evans et al. 2013; Csaki et al., 2015; Pacini et al., 2021). Each sample consisted of 60 forager bees. This sample size was selected based on the recommendation by Pirk et al. (2013), who suggest that a minimum of 59 bees per colony is required to detect a Nosema infection prevalence of 5% or higher with 95% confidence. The samples were stored at –20 °C until further processing and analysis. During field visits, participating beekeepers were requested to complete a questionnaire.

Questionnaire

A questionnaire was designed to gather information on beekeeping practices and the characteristics of apiaries. The questionnaire included both closed-ended and open-ended questions covering beekeeper demographics, beekeeping experience, apiary type (stationary or migratory), hive type, bottom board type, and preventive treatments. The questionnaire also solicited the beekeeper's subjective assessment of the colony's health status, with response options including 'healthy and asymptomatic' or indicating the presence of symptoms such as diarrheal feces, fecal staining on the flight board, reduced brood area, diminished colony population, or decreased honey production

Sample Processing and DNA Extraction

The abdomens of 60 forager honey bees from each sample were removed using a sterile disposable scalpel, and they were macerated in 10 ml of distilled water. A further 3 ml was added, and the suspension was filtered through a two-layer gauze and then centrifuged at 2000 rpm for 10 minutes (MULTIMAGE – Model 0412-1, HIGH HOPE INT'L GROUP JIANGSU MEDICINES & HEALTH PRODUCTS IMP. & EXP. CORP. Ltd., China). The supernatant was discarded, and the pellet was resuspended in 1 ml of distilled water. After that, DNA extraction was performed using the NucleoSpin® DNA Insect Isolation Kit (MACHEREY–NAGEL, Germany; Cat. No. 740470.50), following the manufacturer's protocol.

PCR Analysis

Duplex Polymerase chain reaction (PCR) was then performed to detect and identify Nosema species. Specific primers were used to differentiate between *N. apis* and *N. ceranae*. Two small-subunit 16S rRNA gene regions were simultaneously amplified using two primer sets: 218 MITOC FOR/REV amplifying a 218 bp fragment for *N. ceranae* and 321 APIS FOR/REV amplifying a 321 bp fragment for *N. apis*(Martín-Hernández et al., 2007). Each PCR reaction was prepared in a total volume of 20 μL, containing 2 μL of dNTPs, 0.2 μL of Taq DNA polymerase, 2 μL of 10× buffer, 2.4 μL of $MgCl_2$, 1 μL of primer mix for each primer pair (0.4 μM per primer), 6.4 μL of dH_2O, and 5 μL of template DNA. The amplification was performed using a TECHNETC-312 thermal cycler (Techne Inc., UK). The thermal cycler conditions were 5 min at 94 °C for initial double-strand denaturation followed by 35 cycles of DNA denaturation at 94 °C for 45 s, primer annealing at 62 °C for 45s, and extension at 72 °C for 45 s, with a final 7-min extension at 72 °C. Following PCR amplification, the samples were analyzed by electrophoresis on a 1.5% (w/v) agarose gel using a 100 bp molecular weight DNA ladder (FastGene 100 bp DNA Ladder HH3 RTU, NIPPON Genetics EUROPE GmbH). Electrophoresis was performed in 1× TAE (Tris-acetate ethylene diamine tetra-acetic acid) buffer containing ethidium bromide for DNA visualization.

Statistical Analysis

The qualitative variables were presented using frequencies (n) and percentages (%).

The agreement between beekeeper's assessment of Nosemosis symptoms and PCR results was evaluated using the Kappa agreement index.

Unifactorial analyses were made using the Chi-square test or Fisher exact test to analyse the relation between the qualitative outcome variable (presence of *N. ceranae*) and qualitative variables of characteristics of beekeeping operations and hives.

All assumptions of logistic regression analysis, e.g., homoscedasticity, multicollinearity of independent variables, were examined.
All tests were two-sided, and statistical significance was set at $p < 0,05$. All analyses were carried out using the statistical package SPSS vr 21.00 (IBM Corporation, Somers, NY, USA).

RESULTS

Of the 130 colony samples tested with multiplex PCR, no sample tested positive for *N. apis* or *N. apis* and *N. ceranae* co-infection. Ninety colony samples tested positive for *N. ceranae* (Table 1).

The prevalence of *N.ceranae* in Attica, Greece was 69.2% with 95% CI (60.5%-77.0%) (Table 2) (Chart 1)

Evaluation of beekeepers' questionnaire data shows that 23% of the colonies were in a stationary apiary and 77% in a migratory one. Furthermore, 57% of the hives had a solid bottom board, while 43% had a screened bottom board. What's more, 89.2% of the hives were wooden and 10.8% of the hives were plastic. Regarding the beekeepers' primary occupation, 27.7% were professional, 46.2% were sideline beekeepers, and 26.2% were hobbyists. These data are presented in Table 3.

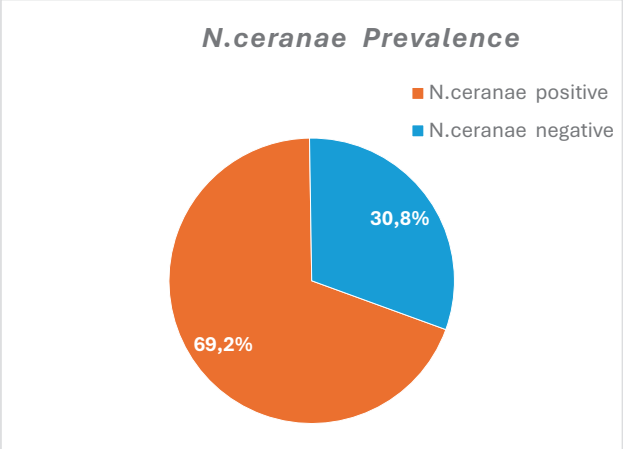

Figure 1. Prevalence of *Nosema ceranae* in honey bee samples collected from colonies in the Attica region during spring 2022.

Table 1. Results of molecular detection (mPCR) of the microsporidia *Nosema apis* and *Nosema ceranae*, in honey bee samples collected from colonies in Attica region during spring 2022.

Infective agent	Positive samples (n)	Negative samples (n)	Total number of samples tested
N. apis	0	130	130
N. ceranae	90	40	130

Table 2. Prevalence of *Nosema. ceranae* in honey bee samples collected from colonies in the Attica region during spring 2022.

Infective agent	Prevalence	95% CI
N. ceranae	69.2%	(60.5-77.0)

CI: Confidence Interval

Table 3. Distribution of surveyed honey bee colonies according to key operational characteristics, including the type of apiary (stationary or migratory), bottom board style (solid or screened), hive material (wooden or plastic), and the primary occupation of the beekeeper (professional, sideline, or hobbyist). Data are expressed as absolute numbers with corresponding percentages (%). The information was obtained from questionnaires completed by beekeepers during hive sampling conducted in the Attica region in Spring 2022.

Characteristics	N(%)
Type of beehive: stationary /migratory	30(23)/100(77)
Type of bottom board: solid /screened	74(57)/56(43)
Type of hive: wooden /plastic	116(89.2)/14(10.8)
Occupation of beekeeper: professional/sideline/hobbyist	36(27.7)/60(46.2)/34(26.2)

An unifactorial analysis of the presence of *N. ceranae* in honey bee colonies revealed several significant associations with apiary and beekeeper characteristics. Colonies of mobile apiaries exhibited a significantly higher prevalence of *N. ceranae* infection compared to those in stationary apiaries (74% vs. 53.3%, p = 0.042). Similarly, hives with screened bottom boards showed a lower prevalence of infection than those with solid bottom boards (59% vs. 77%, p = 0.035). No significant difference in infection rates was noted between wooden and plastic hives (70% vs. 64.3%, p = 0.761). Beekeeper occupation was also significantly associated with *N. ceranae* presence, with infection rates differing between professional (89%), sideline (55%), and hobbyist beekeepers (73.5%) (p = 0.002). Further pairwise analysis indicated a statistically significant difference between professional and sideline beekeepers (p = 0.003) (Table 4).

An evaluation of the agreement between beekeepers' reported presence or absence of symptoms of nosemosis in the colony and the laboratory-confirmed presence of *N. ceranae* revealed an inferior level of concordance. The Kappa agreement index was 0.046 (95% CI: 0–0.12), indicating minimal agreement beyond chance (Table 5).

It has to be noted that molecular detection of *N. ceranae* does not necessarily indicate clinical nosemosis or colony-level disease, as PCR may detect very low spore levels or even vegetative forms of the microsporidia (Fries et al. 2013). A quantitative assessment of infection intensity, such as microscopic examination of the samples (Cantwell,1970), would provide a reflective indication of colony health status and potential risk for disease.

DISCUSSION

This study revealed that 69.2% of the examined colonies were infected with *N. ceranae*, while *N. apis* was not detected in any of the samples. This result indicates a clear dominance of *N. ceranae* within the honey bee populations of the Attica region, consistent with the global trend of *N. ceranae* progressively replacing *N. apis* as the predominant microsporidian species.

The widespread prevalence of *N. ceranae* has been similarly documented across several Mediterranean countries. In a comparative study involving Spain, Portugal, France, and Israel, high infection rates of *N. ceranae* were observed across most surveyed areas, with *N. apis* being detected only sporadically. Notably, in the CIAPA apiary in Spain, all

Table 4. Unifactorial analysis of the presence of *Nosema ceranae* and potential associations between hive-related variables and the presence of *N. ceranae* in honey bee colonies. The variables analysed include the type of beehive (stationary or migratory), type of bottom board (screened or solid), hive material (wooden or plastic), and the primary occupation of the beekeeper (professional, sideline, or hobbyist). Data are expressed as number of colonies (n) with corresponding percentages (%). The p-values indicate the statistical significance of differences between *N. ceranae*-negative (n = 40) and *N. ceranae*-positive (n = 90) groups. Asterisks (*) denote statistically significant differences (p < 0.05) in comparison to the primary category within each variable.

Variable		*N. ceranae* Presence-Negative (n=40)	*N. ceranae* Presence-Positive (n=90)	p-value
Type of beehive	stationary	14(46.7)	16(**53.3**)	0,042*
	migratory	26(26.0)	74(**74.0**)	
Type of bottom board	screened	17(23.0)	57(**77.0**)	0,035*
	solid	23(41,1)	33(**58.9**)	
Hive material	wooden	35(30.2)	81(**69.8**)	0,761
	plastic	5(35.7)	9(**64.3**)	
Occupation	professional	4(11,1)	32(**88.9**)	0,002*
	sideline	27(45.0)	33(**55.0**)*	
	hobbyist	9(26.5)	25(**73.5**)	

All variables were presented as N(%), * p<0.05 vs main

Table 5. Agreement between laboratory results and the beekeeper's reported absence (negative) or presence (positive) of symptoms of Nosemosis in the colony.
Cohen's Kappa coefficient was calculated to assess the level of agreement beyond chance. A Kappa value of 0.046 (95% Confidence Interval: 0–0.12) indicates minimal agreement. The distribution of observations includes the number and percentage of cases classified as *Nosema ceranae* positive or negative by both methods. These results highlight the limited reliability of field-based symptom recognition for suspicion of *N. ceranae* infection.

				Kappa (95% CI)	
Agreement of laboratory results and beekeeper evaluation				**0.046 (0-0.12)**	
			Symptoms of Nosemosis - beekeeper		Total
			negative	positive	
Nosema ceranae laboratory result	negative	n	37	3	40
		%	28.5%	2.3%	30.8%
	positive	n	77	13	90
		%	59.2%	10.0%	69.2%
Total		n	114	16	130
		%	87.7%	12.3%	100.0%

colonies tested positive for *N. ceranae* throughout the study period, whereas *N. apis* was identified in only a single colony in April 2018 (Jabal-Uriel et al., 2022).

In Italy, surveys conducted from April to September 2014 and from May to September 2015 found a *N. ceranae* infection rate of 63%, with no detection of *N. apis* during either period. These findings reinforce the notion that *N. ceranae* has effectively become the dominant Nosema species in Italian honey bee populations (Papini et al., 2017).

This pattern extends to the Balkan Peninsula as well. In Bulgaria, a study conducted across three regions showed that 52.8% of samples were positive for *N. ceranae*, while *N. apis* were absent (Shumkova et al., 2018).

Our study also revealed an apparent discrepancy between beekeepers' assessments of Nosema-related disease and laboratory confirmation of *N. ceranae* infection. Claing et al. (2024) reported a similar lack of correlation between *Nosema* spp. detection and clinical symptoms in honey bee colonies in southwestern Quebec, Canada. Notably, *N. ceranae* often infects colonies without causing obvious clinical signs, complicating field diagnosis (Martín-Hernández et al., 2018). This variability likely arises from factors such as host genetics, nutrition, climate, and synergistic effects with environmental pollutants and co-pathogens (Paris et al., 2018). Moreover, sensitive molecular diagnostics such as PCR can detect even low pathogen levels before they cause symptoms. In many cases, robust bee populations with strong immune systems may suppress the microsporidian's pathogenic effects (Li et al., 2017). Therefore, microscopic spore counting using a hemocytometer (Cantwell, 1970) remains essential for the quantitative assessment of infection intensity. This approach provides a reliable indication of colony health status and the associated risk of overt clinical nosemosis, thereby supporting evidence-based management decisions. Future studies aiming to link pathogen presence with colony health outcomes would benefit from incorporating quantitative methods, such as the Cantwell spore count, to evaluate infection intensity and disease risk.

The results of this study demonstrate that *N. ceranae* infection rates are significantly higher in colonies managed under migratory beekeeping practices compared to those maintained in stationary apiaries. This observation aligns with the findings of Jara et al. (2021), who also reported elevated levels of *N. ceranae* in migratory colonies in Spain, suggesting that the increased stress and environmental variability associated with frequent relocation may contribute to heightened susceptibility to infection. However, our findings contrast with those of Cestaro et al. (2017), who did not detect statistically significant differences

in *N. ceranae* prevalence between migratory and stationary operations in Brazil. This discrepancy may be attributed to regional environmental conditions, methodological differences, or other management practices that could modulate pathogen transmission and host response.

An additional factor of notable importance revealed by our study is the type of bottom board used in hives. Colonies housed in hives with solid bottom boards had a significantly higher likelihood of *N. ceranae* infection compared to those with screened bottom boards. Screened bottom boards are known to enhance ventilation and facilitate the removal of debris and pathogens from the hive interior, which may contribute to a less favorable environment for Nosema proliferation and transmission. This finding highlights the potential of hive design modifications as a practical and non-chemical approach to mitigating disease risk in apiculture.

In this study, professional beekeeping was identified as a significant risk factor for *N. ceranae* prevalence, indicating a statistically significant relationship between professional management and infection. Further research needs to be done to elucidate the specific mechanisms behind this association

In contrast, no statistically significant association was observed between the construction material of the hive, wooden or plastic, and *N. ceranae* infection. This suggests that while material properties may affect hive insulation or durability, they do not appear to play a direct role in the risk of infection with this microsporidian pathogen.

Together, these results highlight the multifactorial nature of *N. ceranae* epidemiology and suggest that both management practices and hive type features can significantly influence disease dynamics within colonies. Further research is needed to investigate the underlying mechanisms and to evaluate the long-term effects of these factors on colony health and productivity.

CONCLUSION

This study provides valuable insights into the epidemiology of Nosema disease in the Attica region of Greece. The findings confirm the predominance of *N. ceranae in* honey bee colonies and the absence of *N. apis*. Several management-related factors, including apiary mobility, hive type, and beekeeper occupation, were significantly associated with infection risk. The poor correlation between beekeeper symptom assessments and molecular diagnoses highlights the need for improved awareness and routine diagnostic testing. Continued surveillance and integrated health management strategies are crucial for mitigating the impact of *N. ceranae* on managed and wild pollinator populations in Greece and beyond.

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Competing Interests

The authors declare that they have no competing interests.

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