Experimental evidence and meta-analysis indicate the negative effect of nosemosis on the survivorship of honeybees

Ostap-Chec, M.[1,2], Cait, J.[3], Scott, R.W.[4], Arct, A.[5], Moroń, D.[5], Rapacz, M.[6], Miler, K.[5*]

[1] Doctoral School of Exact and Natural Sciences, Jagiellonian University, Kraków, Poland

[2] Institute of Environmental Sciences, Faculty of Biology, Jagiellonian University, Kraków, Poland

[3] Department of Integrative Biology, College of Biological Science, University of Guelph, Guelph, Ontario, Canada

[4] Biological Sciences Platform, Holland Bone and Joint Program, Sunnybrook Research Institute; Department of Laboratory Medicine & Pathobiology, University of Toronto, Ontario, Canada

[5] Institute of Systematics and Evolution of Animals of the Polish Academy of Sciences, Kraków, Poland

[6] Department of Plant Physiology, Breeding and Seed Science, Faculty of Agriculture and Economics, University of Agriculture in Krakow, Poland

*****Correspondence:

Monika Ostap-Chec, Institute of Environmental Sciences, Faculty of Biology, Jagiellonian University, Gronostajowa 7 St., 30-387 Kraków, Poland. E-mail: ostap.monika@gmail.com

Krzysztof Miler, Institute of Systematics and Evolution of Animals of the Polish Academy of Sciences, Sławkowska 17 St., 31-016 Kraków, Poland. E-mail: miler@isez.pan.krakow.pl

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Abstract

Nosemosis, caused by microsporidian parasites of the genus *Nosema*, is considered a significant health concern for insect pollinators, including the most economically significant, the honeybee (*Apis mellifera* L.). Despite its acknowledged importance, the impact of this disease on honeybee survivorship remains unclear. In this study, we used a standard laboratory cage trial to compare mortality rates between healthy honeybees and those infected with *Nosema*. We also conducted a systematic review and meta-analysis of existing literature to explore how nosemosis contributes to increased mortality in honeybees tested in such widely used standard trials. Our review and meta-analysis included 50 studies that reported relevant experiments involving healthy and *Nosema*-infected individuals. We excluded studies that failed to report survivorship curves or information on potential moderators affecting the impact of nosemosis, such as spore inoculation dose, the age of the inoculated honeybees, or factors that may impact energy expenditure. Both our experimental results and meta-analysis revealed a consistent, robust effect of infection, indicating a threefold increase in mortality among the infected group of honeybee workers (hazard ratio for infected individuals = 3.16 [1.97, 5.07] and 2.99 [2.36, 3.79] in the experiment and meta-analysis, respectively). However, the meta-analysis also indicated high heterogeneity in the effect magnitude, which was not explained by our moderators. Furthermore, there was a serious risk of bias within studies and potential publication bias across studies. Our findings underscore knowledge gaps in the existing literature. We stress that laboratory cage trials should be viewed as an initial step in evaluating the impact of *Nosema* infection on mortality. Complementary field and apiary studies are imperative in search of effective treatment measures to preserve honeybee populations.

Keywords: *Apis mellifera*, bees, honeybee, meta-analysis, mortality, *Nosema apis*, *Nosema ceranae*, nosemosis, pollinators, *Vairimorpha*

1. Introduction

Numerous insects in most climatic zones play important roles in pollination (Klein et al. 2006, Garibaldi et al. 2013). Among these, the honeybee (*Apis mellifera* L.) can be considered a key pollinator for many crops and wild plants due to the high efficiency of its workers in collecting and transferring pollen between flowers, which is essential for fertilization and subsequent fruit and seed production (Abrol 2012). The significance of honeybees is also underscored by their large colonies that enable the recruitment of high numbers of workers for pollination (Abrol 2012). This makes honeybees particularly valuable for commercial agriculture, where large-scale pollination is often key to ensuring high yields and the quality of crops (Aizen and Harder 2009, Morse and Calderone 2000). However, within the agricultural context, honeybees, and consequently beekeepers, face a wide range of significant issues (Genersch 2010). These encompass challenges such as exposure to pesticides and chemicals, along with susceptibility to diseases and parasites (Potts et al. 2010, Goulson et al. 2015).

Nosemosis is viewed as one of the most significant health concerns for the honeybee (Moritz et al. 2010, Hristov et al. 2020). This disease, caused by microsporidian parasites of the genus *Nosema* (currently reclassified as *Vairimorpha* (Tokarev et al. 2020), but hereinafter referred to as *Nosema* for consistency with existing literature), encompasses two species that infect honeybees: *N. apis* and *N. ceranae*. Both parasites complete their life cycle within the honeybee midgut cells. Upon ingestion, spores reach the midgut, germinate, and inject their contents into host cells. Following phagocytosis, the cells are destroyed to release new spores (Gisder et al. 2011, Huang and Solter 2013). Consequently, typical signs of nosemosis include gut lesions and dysentery (reviewed in Goblirsch 2017). These symptoms result in weakened and lethargic hosts, ultimately compromising the colony's foraging capacity (Koch et al. 2017). The spores can infect other digestive tract cells or be excreted,

contaminating nesting environments and floral resources. The fecal-oral transmission facilitates the easy and rapid spread of these parasites to new habitats and hosts (Fürst et al. 2014). Therefore, while *N. apis* was originally confined to Europe and North America, and *N. ceranae* to South East Asia, both species are now distributed worldwide (Paxton et al. 2007, Chen et al. 2008, Grupe and Quandt 2020). The exacerbation of this spread can be at least in part attributed to the global trade of honeybee colonies and related products (Higes et al. 2008a, Mutinelli 2011).

Among the two agents responsible for nosemosis, *N. ceranae* has garnered significantly more attention compared to *N. apis*. Originally identified solely in *Apis cerana* (Fries et al. 1996), three decades ago *N. ceranae* expanded its range to *Apis mellifera* (Klee et al. 2007) and other bee species (Martín-Hernández et al. 2018, Grupe and Quandt 2020), demonstrating varying infectivity based on geographical origin and host species (Chaimanee et al. 2013, Porrini et al. 2020). *N. ceranae* displays less host-specificity than *N. apis* and exhibits less seasonality, resulting in year-round infection cycles (Higes et al. 2010). Moreover, *N. ceranae* appears to have higher biotic potential at different temperatures compared with *N. apis* (Martín-Hernández et al. 2009). The spread of *N. ceranae* is often associated with worker and colony mortality, including colony collapse syndrome (e.g. in Spain, Higes et al. 2008b, 2009, 2010, Botías et al. 2013), but conflicting research suggests that nosemosis may not significantly contribute to beekeepers' losses (e.g. Cox-Foster et al. 2007, Gisder et al. 2010, Fernández et al. 2012, Pohorecka et al. 2014, Schüler et al. 2023). Thus, the precise impact of *N. ceranae* infection on honeybee mortality remains unclear and requires further investigation.

Laboratory cage trials, a widely used standard method in *Nosema* research, involve the confinement of control and *Nosema*-infected honeybees in cages and strictly controlled conditions (Fries et al. 2012). This method is frequently used, primarily in search of an

effective treatment against the parasite (e.g. van den Heever et al. 2016, Borges et al. 2020, Naree et al. 2021a, Chaimanee et al. 2021). Unfortunately, the method has never been formally validated in the context of *Nosema* infection. The trials are often poorly standardized across studies, leading to highly varied infection effects. For example, concerning mortality, some studies show a powerful effect of *N. ceranae* infection (e.g. Van der Zee et al. 2014, Naree et al. 2021b), while others indicate no discernible effect (e.g. Retschnig et al. 2014, Huang et al. 2015, Hosaka et al. 2021, Duguet et al. 2022). Such discrepancies may stem from different experimental setups that involve, for example, various levels of artificial inoculation or ages of the hosts, both of which can affect nosemosis development (e.g. Berbeć et al. 2022, Jabal-Uriel et al. 2022). Additionally, considering that nosemosis operates through energy-related processes, such as hosts' starvation or failed thermoregulation due to impaired digestive system function (Mayack and Naug 2009, Martín-Hernández et al. 2011, Vidau et al. 2014), assessing infection effects in honeybees maintained in laboratory cages with minimal energy expenditure, stable temperature, and *ad libitum* access to food might be suboptimal methodologically. Hence it is not clear how justified the use of this method is in *Nosema*-related research.

In this study, we conducted an experiment to compare mortality rates between healthy and *Nosema*-infected honeybees, using a typical laboratory cage trial setup. We focused on *N. ceranae* infection due to its likely more invasive and dangerous character than *N. apis*. Furthermore, we conducted a systematic review and meta-analysis of existing literature to explore the impact of *N. ceranae* infection on increased mortality among honeybee workers in similar experimental setups. Our primary objective was to check the effect of nosemosis on honeybee survivorship to assess the suitability of mortality evaluation in caged honeybees as a method. We hypothesized that, generally, *N. ceranae* infection would lead to a significant decrease in honeybee survivorship. However, we also hypothesized that the magnitude of this

effect would depend on mediating factors that often vary between studies, such as the spore dosage used for inoculation or the age of the experimentally inoculated honeybees. We also included incubation temperature and food supplementation, factors that may impact energy expenditure, as potential mediators of the effect of infection on mortality. All these factors are likely to influence the development and the course of nosemosis (Goblirsch 2017).

2. Material and methods

2.1. Survivorship experiment

2.1.1. Preparation of spores for infection and their genetic identification

The spore suspension for experimental infection was freshly prepared. Utilizing a stock population of infected honeybees, the digestive tract of several individuals was homogenized with a micro-pestle and suspended in distilled water. To isolate spores, the suspension underwent centrifugation (Frontier 5306, Ohaus, Switzerland) for 5 minutes at 2,000 G, repeated three times, with each round involving the replacement of the supernatant with fresh distilled water. Subsequently, the supernatant was substituted with a 1M sucrose solution, and the spore concentration was assessed using a Bürker hemocytometer under a Leica DMLB light microscope equipped with phase contrast (PCM) and a digital camera. Achieving a final concentration of 100,000 spores per 10 µL involved appropriate dilution of the infection solution with 1M sucrose solution. This dose was subsequently used for individual infection in the experiment (a typical dose used to ensure infection, Fries et al 2012). To verify the identity of *N. ceranae* spores, PCR was employed following the protocol outlined by Berbeć et al. (2022). Briefly, 50 µL of the spore suspension was incubated in TNES buffer (100 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.3% SDS, 200 mM NaCl) with 8 µL of proteinase K (10 mg/ml) for 2 hours at 56°C with shaking. Following centrifugation, DNA from the supernatant was precipitated by adding an equal volume of 100% isopropanol, washed twice

in 70% ethanol, and resuspended in 50 µL of nuclease-free water. PCR amplification, using species-specific primers complementary to the rRNA genes of *Nosema* species and a PCR Mix Plus kit containing PCR anti-inhibitors (A&A Biotechnology), was carried out under the following conditions: 94°C for 3 minutes for initial denaturation and 35 cycles (94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds). Primers were employed following Chen et al. (2008). The resulting amplification products were analyzed through gel electrophoresis to confirm the exclusive presence of *N. ceranae*.

2.1.2. Experimental procedure

We obtained newly emerged worker honeybees from two unrelated and queenright colonies. The selected colonies were in overall good condition, having undergone treatment with oxalic acid against *Varroa destructor* in early spring. Additionally, before the experiment, they were confirmed to be *Nosema*-free through spore count assessment in several randomly collected foragers using hemocytometry. For the experiment, we obtained workers by selecting a single bee-free frame with a capped brood from each colony and placing it in an incubator (KB53, Binder, Germany) at 32 °C overnight. Subsequently, we created two groups per colony, each consisting of 20 individuals randomly selected from the available bees. These individuals were then transported to the laboratory for feeding, which occurred individually on Petri dishes. In the experiment, individuals in the infected groups (one from each colony) received a 10 µl drop of 1M sucrose solution with 100,000 spores of *N. ceranae*. Individuals in the control groups (another from each colony) received a 10 µl drop of 1M sucrose solution each. After the complete consumption, each individual was transferred to its designated cage. The cages were provided with *ad libitum* water and gravity feeders with 1M sucrose solution and were placed in an incubator (KB400, Binder, Germany) at 34 °C. Water and food were renewed every morning in each cage. The mortality assessment lasted for 38 days, continuing until the death of all individuals, and was performed blind (cages were coded). We refrained

from counting weakened or lethargic individuals as dead, leaving them in their cages. All dead individuals were frozen and later analyzed to confirm their infection status (control vs. infected).

2.1.3. Analysis of experimental samples

The level of infection in honeybees after the survival experiment was examined using hemocytometry. The digestive tract of each frozen honeybee was homogenized using micro-pestle in 300 μL distilled water. The spores were counted in a hemocytometer, analogically to the preparation of spores for infection. The contents of 5 small squares (volume: 0.00125 μL) were counted. If the number of spores counted per sample was less than 10, the contents of 5 large squares (volume: 0.02 μL) were counted. To determine the total number of spores per individual we used the following formula: number of spores per individual = number of spores per sample × 300 μL / total solution volume of sample. We analyzed 40 control and 39 infected honeybees in total (1 infected sample was misplaced).

2.1.4. Statistical analysis

To analyze the survivorship data, we used the Cox mixed-effect regression with a fixed factor of the group and random factor of the colony in R (coxme, survival, and survminer packages) and visualized the results using Kaplan-Meier curves (Kassambara et al. 2021, Therneau 2022, 2023).

2.2. Systematic review and meta-analysis

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al. 2015). The review and its protocol have not been pre-registered.

2.2.1. Eligibility criteria

We first prepared a list of eligibility criteria for studies to be included in the systematic review and meta-analysis: (i) in English, (ii) with the use of workers of the honeybee (*Apis mellifera*),

(iii) comprising a laboratory experiment with a treatment group, i.e. workers exposed to *N. ceranae* spores, and a control group, i.e. workers exposed to no *Nosema* treatment, and (iv) reporting a measure of mortality in the form of a survivorship curve.

2.2.2. Data sources and search

We completed an electronic search of documents on October 31, 2022. For this, we used Web of Science™ and the Scopus databases. Our search used the phrase "nosema mortality apis" in the topic, as well as a forward search (i.e. documents citing one or more works on the list) refined by the same phrase in the topic. References were de-duplicated using Mendeley. The screening was conducted in two phases. First, we screened the documents for potential inclusion based on their titles and abstracts. Second, we retrieved the full texts of potentially eligible documents for further reading. This action was done independently by two investigators and the full texts retrieved by just one or both were used.

2.2.3. Extraction of study details and study exclusion

From the selected studies, we extracted the following types of data: (i) inoculation dose (spores per individual), (ii) age of the inoculated workers, (iii) food provided to the workers (plain sucrose or with supplements), (iv) the number of individuals per cage as well as the number of replicates and the resulting group size (N), (v) days of mortality assessment (i.e. duration of the survivorship analysis), and (vi) incubation temperature. Further, we noted (vii) the inoculation method (individual or collective) as a factor likely to compromise the accuracy of the above-mentioned data type (i) and, as such, a potential methodological mediator of the effect of infection on mortality. This action was completed by one investigator. Studies were excluded if (i) survivorship figures were unreadable, (ii) infection dose or method was not specified, (iii) group size was not reported, (iv) *Nosema* spores used were of mixed species, (v) worker age was unknown. There was also one case in which the survivorship was reported for only 24 hours and another case in which the results were already reported in a different

publication (duplicate results). Both these works were excluded. For the full list of references containing the studies excluded during the second phase of screening see Appendix A.

Among the included studies, some did not specify the number of replicates (4 studies) or incubation temperature (1 study). In these cases, we noted the number of replicates as 1 and the temperature as 33 °C (the median from the other studies). Further, some works used multiple *Nosema* populations with different geographic origins (1 study) and multiple inoculation doses (3 studies), but with a single control group of workers. Thus, a single infected group of workers had to be chosen. In the case of multiple *Nosema* populations, we selected the group infected by spores from a population corresponding geographically to the honeybee population. In the case of multiple inoculation doses, we selected the group with the lowest inoculation dose as studies that utilize low doses are rare.

2.2.4. Risk of bias

The risk of bias was assessed by two investigators independently, with disagreements resolved by consensus, using a modified SYRCLE tool (Hooijmans et al. 2014) and visualized with a summary plot in R using the robvis package (McGuinness and Kothe 2019). We used the following assessment categories: (1) whether baseline characteristics of animals allocated to the control and infected groups were likely similar (low bias if were similar, high bias if were not similar, unclear if there were differentiating factors of unknown effect), (2) whether housing conditions in the incubator were randomized (low if cage positions were switched, high if cages were stationary, unclear if randomization was not mentioned), (3) whether investigators assessing mortality were blind to the group identity of animals (low bias if were blind, high bias if were not blind, unclear if blinding was not mentioned), (4) whether the contamination was measured and reported (low bias if was measured and reported, high bias if was not measured or not reported, unclear if was not mentioned), (5) whether there was any commercial funding for the study (low bias if none, high bias if funded by industry, unclear if

was not mentioned). These assessment categories had the highest relevance in the context of our systematic review and meta-analysis.

2.2.5. Data extraction for survivorship

To assess worker mortality in the selected studies, we computed hazard ratios (HRs) by reconstructing Kaplan-Meier curves from figures included in the papers using a Web Plot Digitizer (Rohatgi 2022). This action was performed first by one investigator, and then checked for completeness and accuracy by another investigator, with corrections applied if needed by consensus. Curve construction, HRs, and confidence interval calculations were performed via methods and R script from Guyot et al. (2012).

2.2.6. Meta-analysis

Analyses were conducted in R using the random-effects meta-analysis function (rma) in the metafor package (Viechtbauer 2010). Data are reported such that increased death in infected groups resulted in HR > 1.

2.2.7. Publication bias

Publication bias, the failure to publish null results from small studies, was assessed visually by examining the degree of effect asymmetry in the funnel plot (standard error plotted against HRs, Appendix B) and was evaluated statistically via the rank correlation test.

2.2.8. Exploration of data heterogeneity

Heterogeneity was assessed using the Q and I^2 statistics. To explore potential causes of data heterogeneity, we conducted meta-regressions. These regressions included inoculation dose, inoculation age of workers, and incubation temperature as continuous moderators, and food supplementation (sucrose or supplement) and inoculation method (individual or collective) as categorical moderators in the random-effects model.

2.2.9. Quality of evidence

The strength of the body of evidence was assessed by one investigator using the Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) guidelines (Guyatt et al. 2011). The quality of evidence was based on the study design and decreased for risk of bias, publication bias, inconsistencies, indirectness, or imprecision in the included studies as well as increased for the size of the effect or dose-response relationship.

3. Results

3.1. Survivorship experiment

Cox regression showed a significant effect of the infection (control vs. infected group of workers, $z = 4.77$, $p < 0.001$). The hazard ratio for the group infected by *N. ceranae* spores was 3.16 [1.97, 5.07] (Fig. 1). The infected group had high infection levels (average number of spores per individual: 18,536,634) with 8 individuals that had no detectable spores. In the control group, we found 5 *Nosema*-contaminated samples (range: 30,000-165,000 spores per individual).

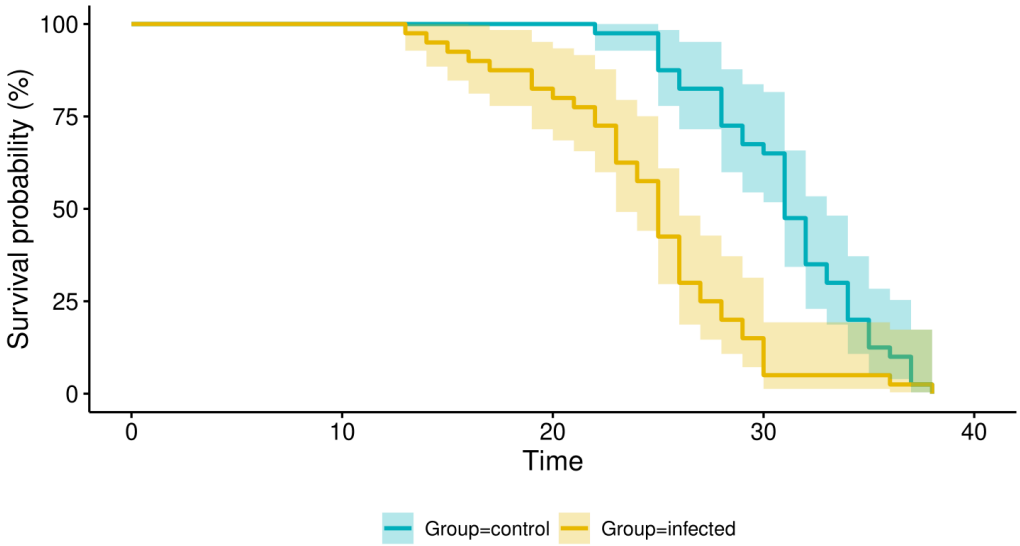

Fig. 1. Kaplan-Meier survival curves for the control and infected groups of workers. The two groups differ significantly in survival. Shading indicates 95 % confidence intervals.

3.2. Systematic review and meta-analysis

3.2.1. Study characteristics

Our search yielded 1599 documents in total (273 from Web of Science™ and 1326 from Scopus). After de-duplication (n = 314 duplicates) and removal of patents (n = 1) 1284 documents were screened for potential inclusion based on their titles and abstracts. This action yielded a total of 123 publications. Upon reading the full texts of the selected 123 publications, we were able to identify 52 eligible studies. During data extraction for survivorship, we excluded 1 additional article (containing 2 studies that used separate groups of animals) because no mortality occurred in the control groups for over 2 weeks, which made resulting hazard ratios improbably large and thus unusable. By this action, the number of studies included in the meta-analysis decreased to 50 from 44 publications (Fig . 2).

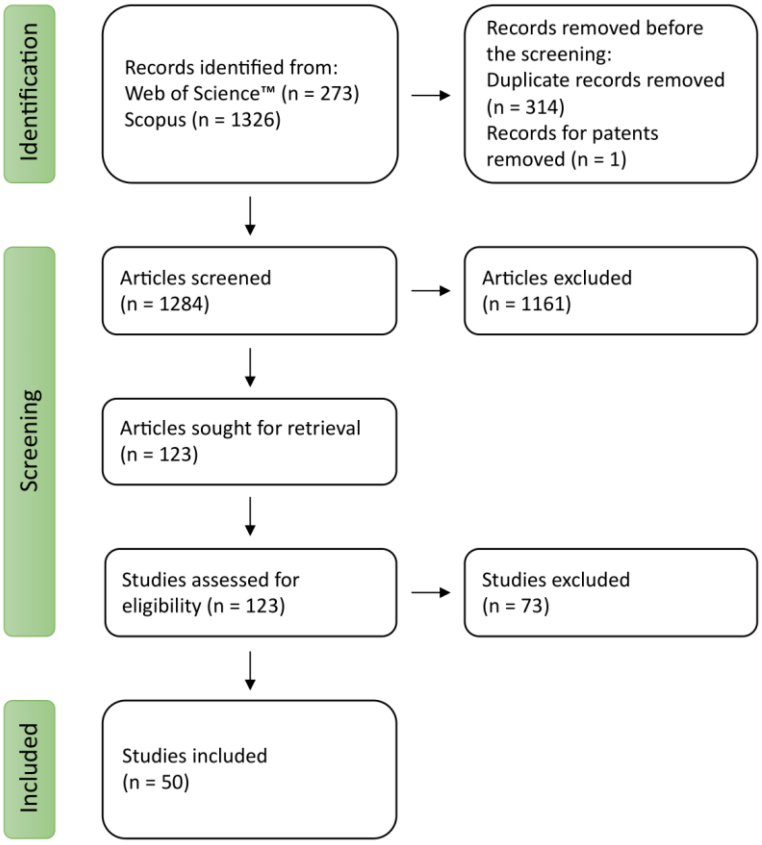

Fig. 2. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram.

Among the included studies (Table 1), most used a plain sucrose diet (70 % of studies) as opposed to any kind of diet supplementation (30 % of studies). Similarly, most studies implemented individual (82 %), not collective spore feeding method (18 %). Freshly emerged, one-day-old workers were most often used (in 38 % of studies) and an inoculation dose of 100,000 spores per individual was most often implemented (in 42 % of studies). In the majority of works included in the meta-analysis, the mortality assessment lasted for longer than 2 weeks (70 %), and the workers undergoing assessment were most frequently kept at 33 or 34 °C (in 58 % of studies).

3.2.2. Risk of bias

Overall, the risk of bias was serious (Fig. 3). Baseline characteristics of animals participating in the studies were mostly similar between groups, except for a few studies in which the control and infected groups were kept in separate housing incubators. Randomization of the position of cages within the housing incubator was applied in only one study, thanks to which it avoided possible effects related to the position of a cage in the incubator. Notably, not a single study mentioned any form of blinding applied during the mortality assessment. Considering that only one study listed specific criteria for counting an individual as dead, this made the omission of blinding quite conspicuous and created space for investigator bias during mortality assessment. Several studies failed to control the contamination of the control groups and infection levels in the artificially infected groups. There was no commercial funding in any of the studies.

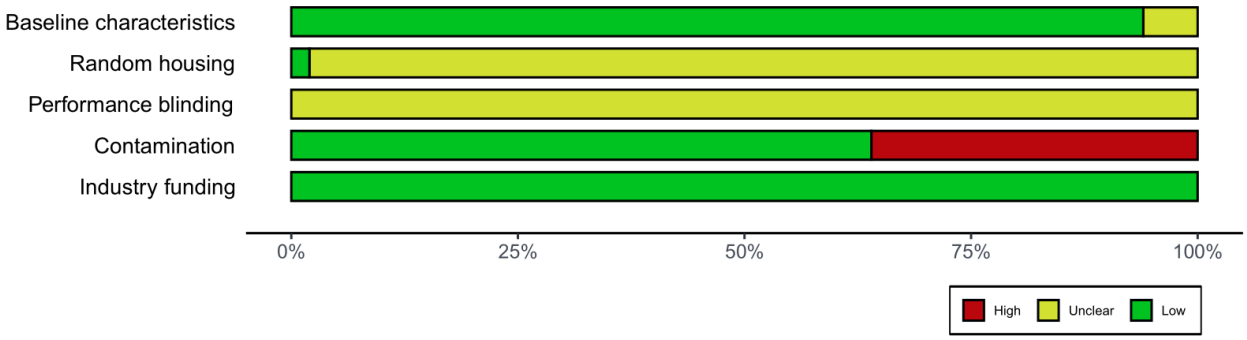

Fig. 3. Summary plot of the results from the SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE) risk of bias tool. Green indicates a low risk of bias, yellow is unclear, and red indicates a high risk of bias.

3.2.3. Meta-analysis

A random-effects meta-analysis of the hazard ratios (HRs) showed a significant effect of infection with *N. ceranae* spores (HR = 2.99 [2.36, 3.79]) (Fig. 4). There was high heterogeneity (Q = 647.32, df = 49, p < 0.0001; I^2 = 94.44 %).

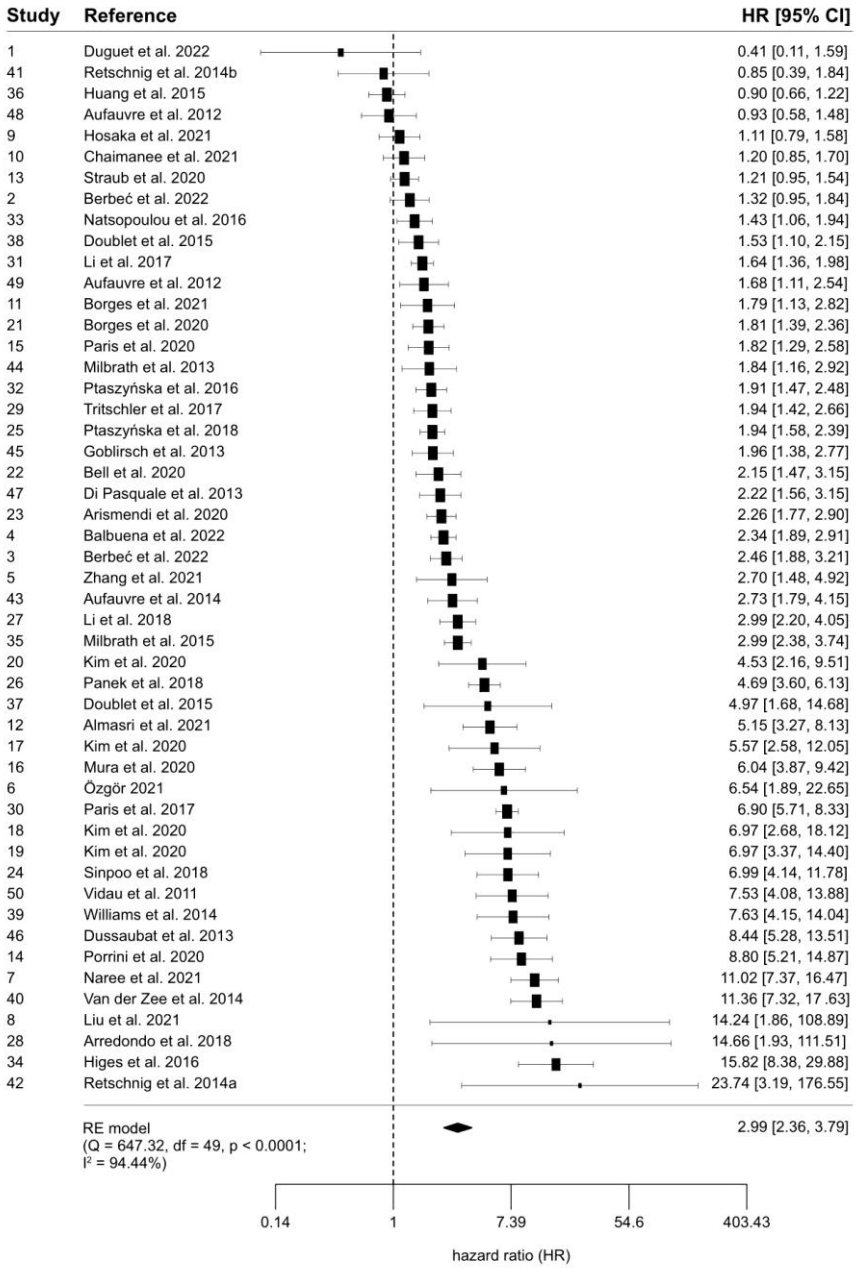

Fig. 4. Forest plot of the random-effects meta-analysis of hazard ratios (HRs) and 95 % confidence intervals. Diamond demonstrates the overall estimate (with the width reflecting the 95 % CI). Black squares indicate the study HRs and their sizes indicate the weight of the study in the meta-analysis. The dashed line indicates no difference between groups (HR = 1). The Q and I^2 statistics are tests of heterogeneity.

3.2.4. Publication bias

Visual inspection of the funnel plot and the rank correlation test indicated the presence of publication bias (tau = 0.223, p = 0.022) (Appendix B).

3.2.5. Exploration of data heterogeneity

Included moderators did not significantly explain HR magnitudes ($F_{5,44}$ = 1.812, p = 0.130, R^2 = 10.92 %). A trend emerged demonstrating that HRs increased with increasing inoculation doses ($F_{1,44}$ = 3.650, p = 0.063). Another trend indicated a positive relationship between the increased age of workers at inoculation and larger HRs ($F_{1,44}$ = 3.800, p = 0.058).

3.2.6. Quality of evidence

All studies included in the systematic review and meta-analysis were considered randomized trials. The overall quality of evidence was high (Table 2). Even with a serious risk of bias, likely publication bias, and serious inconsistencies indicated by high heterogeneity, the included studies had no serious indirectness or imprecision. Moreover, the detected effect was large and there was a trend towards dose response.

4. Discussion

Both our experiment and meta-analysis indicated a consistent and strong impact of infection, with mortality increasing about threefold in the group of honeybees infected with *N. ceranae* compared to uninfected individuals. Importantly, we found no evidence of the incubation temperature, food supplementation, or inoculation method influencing the magnitude of the mortality effect. While trends suggested higher infection effects in studies where honeybees

were infected with larger doses of spores or in studies involving older hosts, the moderators collectively accounted for only 10.92 % of the large heterogeneity in HRs (I^2 = 94.44 %). The results from our experiment closely aligned with the findings of the meta-analysis. Thus, our experiment can serve as a valuable reference for standardizing research methodologies in laboratory cage trials.

The lack of strong evidence regarding the significance of any of the included moderators was surprising. Future studies must account for high heterogeneity in the effect of *Nosema* infection on honeybees. Given the type of studies incorporated into our systematic review and meta-analysis (Table 1), we strongly recommend future research to address the impact of infection on mortality in older individuals, including those considerably older than a few days. Considering that older bees are the ones leaving the hive to forage and are more likely to encounter *N. ceranae* in the environment than in-hive bees (Li et al. 2017, Lourenço et al. 2021), assessing the effects of infection on aged individuals would provide a more accurate representation of real-life scenarios. This is particularly relevant since the oldest foragers typically exhibit the highest infection levels that can be counted in millions of spores (Smart and Sheppard 2012, Li et al. 2017, Jabal-Uriel et al. 2022). Investigating the impact of large spore doses on young honeybees can obscure the estimation of *N. ceranae* true effect.

Our findings indicate that *N. ceranae* is a serious threat to honeybees in terms of mortality. This study strongly supports the idea that nosemosis can significantly contribute to honeybee colony losses. The implications are particularly concerning given the ease with which *N. ceranae* infects various species, including bumblebees and solitary bees (Martín-Hernández et al. 2018, Grupe and Quandt 2020). However, it’s crucial to treat cage trials as an initial step in assessing infection effects and possible treatment measures. The subsequent step should involve conducting similar research under natural conditions within colonies, where bees experience not only individual immunity but also full social immunity. The value

of this approach is confirmed when we compare the results of studies such as those by Li et al. (2018) and Lourenço et al. (2021). In the former, artificially infected bees were examined in cages, while in the latter, artificially infected bees were introduced into colonies. The results showed a significantly lower spore load in bees living in colonies compared to those maintained in cages 12 days post-infection. This demonstrates the significant role that the colony environment and its components, including substances with high antibiotic activity like propolis (Simone-Finstrom et al. 2017, Mura et al. 2020) or secondary plant metabolites in pollen and honey (Erler and Moritz 2015), can play. Equally important are the behavioural defence mechanisms of bees, such as avoiding trophallaxis among sick bees (Naug and Gibbs 2009) or consumption of honey with higher antibiotic activity by diseased bees (Gherman et al, 2014). In natural colony conditions, however, honeybees face various challenges, including exposure to pesticides and other chemical substances. The synergistic impact of these challenges, combined with *Nosema* infection, surely increases the mortality rate among bees and affects queen survival (Dussaubat et al. 2016, Aufauvre et al. 2012, 2014, Paris et al. 2018). Additionally, *Nosema*-infected bees experience higher levels of hunger, leading to energetic stress (Mayack and Naug 2008, Alaux et al. 2010). The expenditure of energy in the colony environment is considerably higher than in laboratory cages due to task performance. Therefore, not only should experimental methodologies be critically evaluated for ecological accuracy, but outcomes obtained in controlled settings need validation within the context of natural environments. This approach is essential to ensure that the results and conclusions capture the complexity of real-world effects.

Our study indicates that *N. ceranae* significantly influences the mortality of worker bees and, as such, likely poses a serious survival threat to the honeybee and the ecosystem services it provides (Papa et al. 2022). The search for the treatment of nosemosis is ongoing (Iorizzo et al. 2022). Significant attention is paid to the application of Good Beekeeping

Practices as a form of prevention (Formato et al. 2022). However, while these methods yield some results, they still prove insufficient (Huang et al. 2013, Holt and Grozinger 2016, Garrido et al. 2023, Lang et al. 2023, Prouty et al. 2023). There is a pressing need for further research on *N. ceranae*, especially in the context of its effective control strategies that consider the microbiological safety of the hive products (i.e. pollen and honey).

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5. References

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Study	Reference	Food	Inoculation age (days)	Inoculation method	Inoculation dose	Group size	Number of replicates	N	Mortality assessment (days)	Temperature
1	Duguet et al. 2022	Plain sucrose	3	Individual	120,000	20	4	80	14	34
2	Berbeć et al. 2022	Plain sucrose	2	Collective	10,000	150	4	600	7	30
3		Plain sucrose	10	Collective	10,000	150	4	600	7	30
4	Balbuena et al. 2022	Plain sucrose	2	Collective	100,000	70	3	210	30	30
5	Zhang et al. 2021	Plain sucrose	5	Individual	100,000	45	3	135	20	34
6	Özgör 2021	Plain sucrose	1	Individual	1,000,000	30	1	30	12	34
7	Naree et al. 2021	Supplement	1	Individual	100,000	50	3	150	30	34
8	Liu et al. 2021	Plain sucrose	10	Individual	100,000	20	3	60	10	35
9	Hosaka et al. 2021	Plain sucrose	1	Individual	140	30	3	90	21	33
10	Chaimanee et al. 2021	Plain sucrose	3	Individual	100,000	30	3	90	10	34
11	Borges et al. 2021	Plain sucrose	1	Individual	50,000	40	1	40	54	30
12	Almasri et al. 2021	Plain sucrose	1	Individual	100,000	30	7	210	21	33
13	Straub et al. 2020	Supplement	1	Collective	10,000	20	22	440	14	34
14	Porrini et al. 2020	Supplement	2	Individual	1,000,000	50	5	250	13	28
15	Paris et al. 2020	Plain sucrose	2	Collective	100,000	50	3	150	22	33
16	Mura et al. 2020	Plain sucrose	1	Individual	100,000	21	3	63	30	31
17	Kim et al. 2020	Plain sucrose	4	Individual	50,000	20	3	60	15	34
18		Plain sucrose	4	Individual	50,000	20	3	60	15	34
19		Plain sucrose	4	Individual	50,000	20	3	60	15	34
20		Plain sucrose	4	Individual	50,000	20	3	60	15	34
21	Borges et al. 2020	Plain sucrose	1	Individual	50,000	40	3	120	54	33
22	Bell et al. 2020	Plain sucrose	1	Individual	40,000	123	1	123	9	36
23	Arismendi et al. 2020	Supplement	2	Individual	100,000	75	4	300	20	30
24	Sinpoo et al. 2018	Plain sucrose	5	Individual	100,000	30	3	90	14	34
25	Ptaszyńska et al. 2018	Plain sucrose	3	Collective	125,000	40	10	400	16	35
26	Panek et al. 2018	Supplement	1	Individual	50,000	130	4	520	21	33
27	Li et al. 2018	Supplement	1	Individual	100,000	40	4	160	21	34
28	Arredondo et al. 2018	Plain sucrose	3	Collective	500,000	50	3	150	7	35
29	Tritschler et al. 2017	Plain sucrose	1	Collective	100,000	50	6	300	14	35
30	Paris et al. 2017	Supplement	6	Individual	100,000	45	24	1080	22	33
31	Li et al. 2017	Plain sucrose	2	Individual	100,000	40	6	240	26	34
32	Ptaszyńska et al. 2016	Plain sucrose	3	Individual	32,000	40	6	240	21	30
33	Natsopoulou et al. 2016	Plain sucrose	4	Individual	100,000	18	5	90	42	30
34	Higes et al. 2016	Plain sucrose	5	Individual	50,000	25	3	75	30	27

35	Milbrath et al. 2015	Plain sucrose	1	Individual	30,000	100	3	300	30	30
36	Huang et al. 2015	Supplement	5	Individual	100,000	30	3	90	50	30
37	Doublet et al. 2015	Plain sucrose	2	Individual	100,000	30	3	90	13	30
38		Plain sucrose	2	Individual	100,000	30	4	120	25	30
39	Williams et al. 2014	Plain sucrose	2	Individual	35,000	20	3	60	30	33
40	Van der Zee et al. 2014	Plain sucrose	5	Individual	50,000	25	4	100	25	34
41	Retschnig et al. 2014b	Supplement	2	Individual	100,000	20	5	100	14	34
42	Retschnig et al. 2014a	Plain sucrose	1	Collective	100,000	20	4	80	14	30
43	Aufauvre et al. 2014	Supplement	1	Individual	125,000	165	1	165	25	33
44	Milbrath et al. 2013	Plain sucrose	5	Individual	125,000	20	2	40	32	33
45	Goblirsch et al. 2013	Supplement	1	Individual	10,000	30	3	90	28	28
46	Dussaubat et al. 2013	Plain sucrose	5	Individual	40,000	30	3	90	19	33
47	Di Pasquale et al. 2013	Supplement	1	Individual	100,000	70	1	70	50	34
48	Aufauvre et al. 2012	Supplement	1	Individual	125,000	50	3	150	22	33
49		Supplement	1	Individual	125,000	50	3	150	22	33
50	Vidau et al. 2011	Supplement	5	Individual	125,000	50	3	150	20	35

Table 1. Overview of studies included in the systematic review and meta-analysis. The table presents information on studies investigating worker honeybee mortality in control and *Nosema*-infected individuals. Each study is catalogued with details including reference, the type of food provided (plain sucrose or supplemented), worker age at spore inoculation, method of inoculation (individual or collective feeding), estimated spore dose per individual, group sizes with replicates, total number of tested individuals (N), duration of mortality assessment, and incubation temperatures.

Number of studies	Overall risk of bias	Inconsistencies	Indirectness	Imprecision	Publication bias	Effect	Dose response	Quality
50	Serious	Serious	Not serious	Not serious	Likely	HR = 2.99 [2.36, 3.79] (large)	$F_{1,44} = 3.650$, $p = 0.063$	High

Table 2. Grading of Recommendations, Assessment, Development, and Evaluations (GRADE) assessment of confidence of cumulative evidence.

Appendices

Appendix A. List of references excluded at the second phase of screening.

Appendix B. Funnel plot (standard error plotted against hazard ratios) for the studies included in the meta-analysis.