Journal of Microbiological Methods 244 (2026) 107451

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Molecular detection of honeybee pathogens in honey from a UK citizen science program

Michael J.R. Bennett [a,b,*], Lindsay K. Newbold [a,*], Susheel Bhanu Busi [a], Richard Pywell [a], Holly Tipper [a], Joanna Savage [a], Jennifer Shelton [a], Manasa Suresh [a], Ellie Grove [a], Hyun S. Gweon [b], Ben A. Woodcock [a,*]

[a] UK Centre for Ecology & Hydrology (UKCEH), MacLean Building, Benson Lane, Crowmarsh Gifford, Wallingford OX10 8BB, United Kingdom
[b] University of Reading, Whiteknights Campus, Reading RG6 6UR, United Kingdom

ARTICLE INFO

Keywords:
Apis mellifera
Honey
Environmental DNA
Disease and pathogens

ABSTRACT

Fruits, legumes, oilseeds and nuts have a high dependency on insect pollination, of which the honey bee (*Apis mellifera* L.) is a critical contributor. However, declines in wild insect pollinators and managed honey bees have been widely reported. An increase in the prevalence of diseases is likely to be directly or indirectly contributing to these declines. In the United Kingdom, the fungal chalkbrood, the microsporidian *Nosema*, and the bacterial European Foulbrood (EFB), represent three major non-viral diseases of honey bees. Many of these diseases are economically important to honey bees and have the potential to cross over to wild bee species. Rapid large-scale monitoring of these pathogens provides advantages in controlling these diseases through pre-emptive mitigation measures. The UK National Honey Monitoring Scheme provides this kind of monitoring, and this study assesses the efficacy of molecular methods to detect these diseases within honey samples obtained through this citizen science scheme. The study used quantitative polymerase chain reaction (qPCR) from DNA extracted from 150 honey samples, which revealed chalkbrood (13%) and *Nosema* (11%) as the most abundant pathogens, whereas the legally notifiable EFB was discovered in less than 2% of colonies. Asymptomatic infections, including that of the high-risk EFB, were common. There is evidence to suggest that both asymptomatic and symptomatic disease expression become more prevalent throughout the year. Monitoring for these diseases is recognised as being vital in the control and containment of these pathogens. The application of low-cost, non-invasive methods with a large-scale monitoring scheme, could support mitigation as well as identify currently asymptomatic but highly contagious or notifiable diseases prior to outbreaks.

1. Introduction

Insect pollination is critical to around 90% of all flowering plants (Tong et al., 2023), and approximately one-third of world crops (Klein et al., 2007). Of these, the managed western honey bee (*Apis mellifera)* is the most abundant and widespread pollinator in the world (Visick and Ratnieks, 2023). In addition to its role as a pollinator of crops and wild plants, this species contributes around USD 200 billion annually to the global economy through honey production (Requier et al., 2019; Vidal-Naquet, 2015). Despite being a managed organism, declines of 30% have been observed in the past few decades (Nikita Grover et al., 2022). The majority of these losses are linked to colony collapse disorder (CCD), where there is a major reduction in adult workers in the hive, leading to colony death (Vanengelsdorp et al., 2009). Annually, colony losses regularly occur at a rate of between 5 and 15%, usually during winter (Vidal-Naquet, 2015), however since 2006 this percentage has doubled in some areas (Nikita Grover et al., 2022). This mirrors the wider global decline in wild pollinators, putting this vital ecosystem service at risk (Potts et al., 2010a; Powney et al., 2019).

Pollinator declines have been attributed to habitat loss and fragmentation, intensive agriculture, including pesticide use, invasive species and climate change (Potts et al., 2010a; Vasiliev and Greenwood, 2021). In addition, increased disease prevalence (often linked to CCD) has been promoted by the globalisation and transport of bees across national borders as well as changes in the climate (Boncristiani et al., 2020; Neov et al., 2019). While impacts of diseases are not unique to

* Corresponding authors at: UK Centre for Ecology & Hydrology (UKCEH), MacLean Building, Benson Lane, Crowmarsh Gifford, Wallingford OX10 8BB, United Kingdom.
E-mail addresses: mbennett@ceh.ac.uk (M.J.R. Bennett), lise@ceh.ac.uk (L.K. Newbold), bawood@ceh.ac.uk (B.A. Woodcock).

https://doi.org/10.1016/j.mimet.2026.107451
Received 3 February 2026; Accepted 2 March 2026
Available online 3 March 2026
0167-7012/© 2026 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

honey bees, the managed status of this species means the prevalence of diseases are more obvious (Potts et al., 2010b; Higes et al., 2008).

In the UK, the rare bacterial disease, European Foulbrood (EFB, caused by *Melissococcus plutonius*) is sufficiently virulent and contagious to be government notifiable diseases whose detection leads to the colonies' destruction (Bees Act, 1980). Whilst not as virulent, diseases caused by fungal (*Ascosphaera apis* - chalkbrood) and microsporidian (*Nosema apis* and *Nosema ceranae*) parasites are more widely distributed across the UK and may reflect the impacts of chronic infection upon honey bee populations (Rowland et al., 2021; Fries, 2010).

Detection of these diseases is critical to ensuring healthy hives and preventing widespread outbreaks, as such, accurate methodologies for pathogen detection, quantification, and monitoring are essential in understanding the drivers of honey bee disease and preservation of honey bee populations. In the UK, reporting and subsequent inspection is done by both individual beekeepers, and through government agencies, such as the National Bee Unit (NBU) (Rowland et al., 2021). However, this relies largely on visual inspection of symptomatic bees, and so in many cases disease diagnosis occurs at a stage where treatment options are limited, leading to colony collapse or even spread of more virulent diseases, like EFB to surrounding hives (Milbrath, 2021). Compounding this, visual disease detection at a symptomatic stage requires expertise and so its accuracy may vary considerably depending on individual beekeeper experience. Therefore, the key to the prevention of such outbreaks is regular monitoring which is accurate and timely as well as sensitive to pre-symptomatic infection.

Quantitative and highly sensitive molecular methods, such as quantitative PCR (qPCR), provide opportunities to deliver such detection including that of asymptomatic diseases (Smith and Osborn, 2009; Shahrajabian and Sun, 2024). This method and other PCR based approaches, has been successfully applied in the identification of the major honey bee diseases over the past three decades. Govan et al. (1998) was able to detect EFB, using PCR on DNA isolated from infected larvae. More recently, Mackay et al. (2024) demonstrated successful identification of honey diseases from hive swab-extracted DNA, while Revainera et al. (2020) utilised honey-derived DNA to detect *A. apis* and *N. ceranae*.

The use of honey as a source of environmental DNA (eDNA) to assess disease occurrence has advantages and appeal for many beekeepers as it is non-invasive and can be collected as part of standard beekeeping operations (Ribani et al., 2020). Furthermore, antibacterial properties of honey improve DNA preservation, making this stored hive product particularly suitable for detecting diseases (Nolan et al., 2019; Cirtwill and Wirta, 2025). To this effect, a recent review suggested the need for more in-depth studies using honey in the detection of bee pathogens as a priority (Soares et al., 2023). Building on this, Revainera et al. (2020) and Ribani et al. (2020) have used commercial honey samples (i.e. extracted for human consumption) to establish the prevalence of the major honey bee pathogens at national and international scales, clearly demonstrating the methods potential for national monitoring. Yet, these studies used commercially harvested honey samples, often taken at the end of the honey production season. Therefore, these samples represent an amalgam of annual hive production, limiting temporally linked data on disease prevalence. This limits the understanding of the effect of pathogens during incubation and asymptomatic periods (Kunat-Budzyńska et al., 2025). Further, recognition of temporal patterns of honey bee disease is essential in determining when potential treatments, for example probiotics, should be applied (Nekoei et al., 2023; Abdi et al., 2023). To counteract this, accurate monitoring models would require large scale collection of temporally linked honey samples directly removed from recently laid down honey storage cells in hive combs to allow for a more precise timing of infection. However, the scale of such a study is outside the limits of most research projects. Citizen science promises to bridge this gap, using an experienced and invested network of national beekeepers to collect and monitor their own hives.

This project tests the viability of using molecular methods within the framework of UK based citizen science derived honey samples, to detect symptomatic and asymptomatic disease expression within honey bee hives. We assess how qPCR can be used to reliably detect the globally important honey bee pathogens associated with the diseases of chalkbrood, *Nosema* infection and EFB. Furthermore, we compared the efficacy of the molecular approach in disease detection with conventional visual symptom-based assessment made by beekeepers. Finally, through the project's unique citizen science derived sampling methodology, we report spatial and temporal patterns in disease prevalence in the UK. The findings are discussed within the context of low-cost national scale monitoring to support the disease mitigation and management strategies.

2. Materials and methods

2.1. Sampling protocol and selection

In this project we used 150 Honey samples that were obtained through the National Honey Monitoring Scheme (NHMS) archive (https://honey-monitoring.ac.uk/). This national citizen science scheme works with approximately 3500 beekeepers participating across the UK, both amateur and professional, that directly sample honey from the most recently constructed comb within hives providing scientific data from the past few weeks. These samples are returned to the national repository housed at the UK Centre for Ecology & Hydrology (UKCEH) that contain over 6000 of these samples. The wider purpose of this is to monitor national patterns of pollinator foraging preferences through plant pollen DNA suspended within the honey and to support post regulation pesticide monitoring (Woodcock et al., 2022; Oliver et al., 2021). A subset of 90 archived honey samples from England were selected from 2022 (Fig. 1a). The samples were collected along a gradient of low (0% within 2 km of hives) to high (95%) arable agricultural land use and temporally distributed to represent the early (late April–June) and late (August), peaks in honey bee activity. To contrast samples originating from arable landscapes, an additional 60 samples from high urban and sub-urban land uses (30–96%) were also assessed. Ten urban / sub-urban samples were collected each month from an even distribution from May to October. Associated with these samples was meta-data provided by beekeepers that included information on disease prevalence as identified by the beekeeper at the point of collection. Overall, the honey samples were collected within all nine ITL-1 (International Territorial Level) regions within England (North East, North West, Yorkshire and the Humber, East Midlands, West Midlands, East of England, London, South East, and South West).

2.2. DNA extraction and quantification

To capture the eDNA from each honey sample, approximately 15 g of honey was weighed, diluted to 50 mL and incubated at 56 °C. Excess wax was removed using stomacher strainer bags (Seward Ltd), prior to the filtration as per the procedure detailed in Oliver et al., 2021. Resultant filters were shredded and placed in a 5 mL lysis tube with 0.8 g of lysing matrix A (MP biomedical) and stored at −80 °C. Extraction of DNA was conducted using the procedures outlined in the Qiagen DNeasy 96 plant kit with amendments to account for sample type (see Supplementary Materials section).

To verify extraction success and quantify DNA yield, concentration was measured on a Qubit 4 fluorometer using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). The 150 honey samples were obtained from the NHMS archive and so a comparison was made between these concentrations and those obtained when the samples were previously extracted, 2 years prior, to determine DNA quality and to ensure there was no degradation (see Supplementary Materials for statistical analysis protocol).

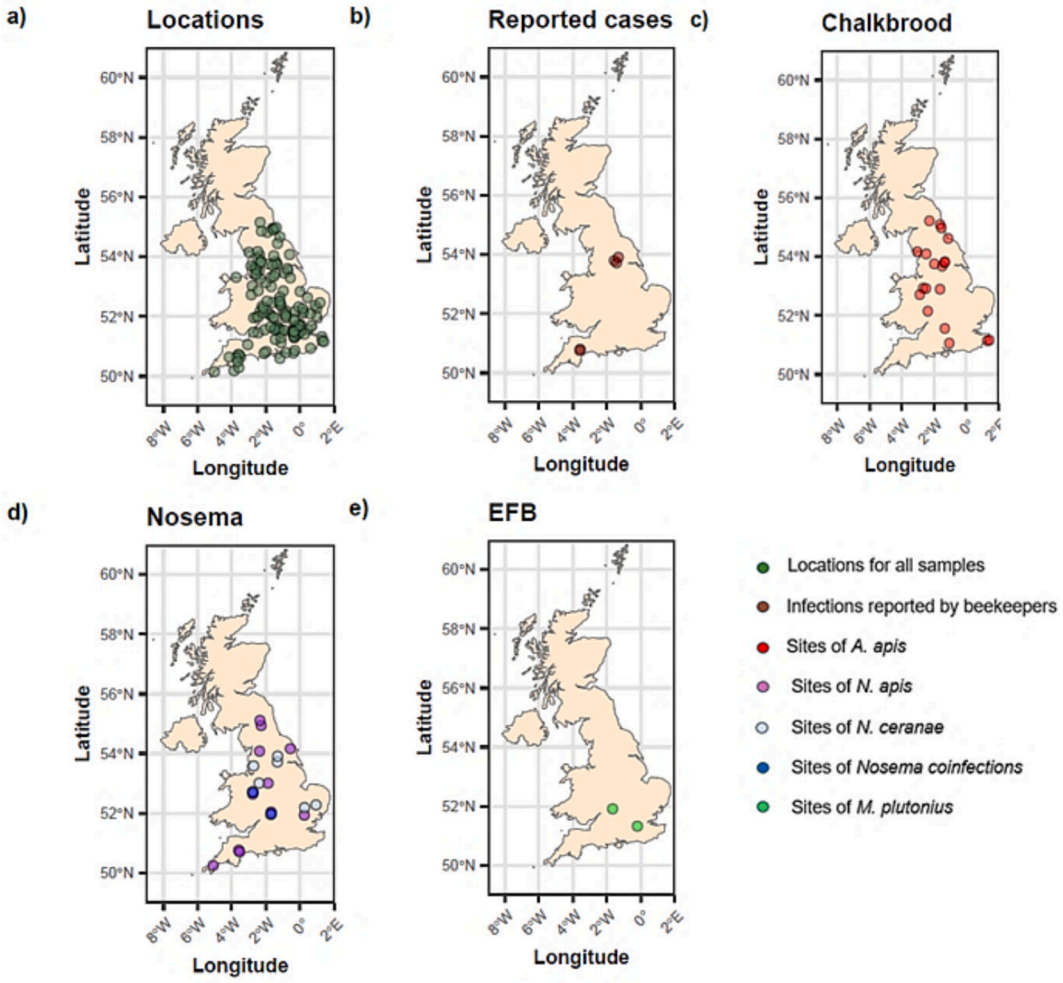

Fig. 1. Distribution of honey bee diseases in the UK. a) Locations from where all 150 samples were collected from. b) Sites where beekeepers had reported symptoms of disease within hives. c) The hives that were discovered to have been infected by *A. apis* (chalkbrood) through qPCR detection. d) Geographical points of hives where *N. apis*, *N. ceranae* and *Nosema* coinfections were found together via qPCR. e) Locations of hives where *M. plutonius* (EFB) was detected utilising qPCR.

2.3. Pathogen abundance via qPCR

Reactions were run on a Roche LightCycler 480 using the systems LightCycler Software release 1.5. The abundance of *A. apis* (chalkbrood) was quantified using qPCR through the following conditions;

To 5 μL of Power Up SYBR Green master mix, 0.2 μL of 20 μM of both forward and reverse primers (Table 1), 3.6 μL of Molecular Grade water, and 2.5 μL of (0.05 ng - 2.88 ng) DNA template was added to make up a 10 μL reaction volume. For this assay, thermocycling conditions commenced with an initial denaturation stage at 95 °C for 3 mins, proceeded by 45 cycles of amplification with 95 °C for 15 s, 56 °C for 15 s and 72 °C for 15 s.

The abundance of *N. ceranae* and *N. apis* (*Nosema* infection) and *M. plutonius* (EFB) was quantified through the following conditions;

To 5 μL solution of Thermo-Scientific Fast Advanced Taq Master Mix, 1 μL of primer-probe mix, with final concentrations of 0.3 μM forward and reverse primers with 0.15 μM of probe solution (Table 1), 1.5 μL of molecular grade water, and 2.5 μL of (0.05 ng - 2.88 ng) DNA template was added to make up a 10 μL reaction volume. For the *N. apis, N. ceranae* and *M. plutonius* assays, thermocycling conditions first imposed an initial denaturation stage at 95 °C for 5 mins, proceeded by 45 cycles of amplification with 95 °C for 10 s and 65 °C for 30 s. All primers and probes for all assays were selected from previous honey bee pathogenic studies outlined in Table 1.

Synthetic oligonucleotide DNA standards (gBlocks) were designed from published sequences and used to produce a standard curve from which pathogenic gene copies could be calculated (see Supplementary Materials section for source sequence and accession number). Only runs

Table 1
Primer and probe sequences used in qPCR assays on honey samples involved in the project. Reference dictates projects that originally developed sequences.

Assay	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Probe (5′-3′)	Reference
Nosema ceranae	CGGATAAAAGAGTCCGTTACC	TGAGCAGGGTTCTAGGGAT	6FAM_5'-CGTTACCCTTCGGGGAATCTTC	(Chen et al., 2009)
Nosema Apis	CCATTGCCGGATAAGAGAGT	CCACCAAAAACTCCCAAGAG	5HEX_5' ATAGTGAGGCTCTATCACTCCGCTG	(Chen et al., 2009)
Melissococcus plutonius	GACCTGTTTAGCTATTATCACTA	CACCTACAATGAATGATTCATTC	6FAM_5'-TCCGCCTAAGCTACCACCTAAGAA	(Grossar, 2023)
Aschophaera Apis	GCACTCCCACCCTTGTCTA	CAGGCTCGCGAGAACCC	No probe required	(James and Skinner, 2005)

where standard curves had efficiency values ranging between >90–110%, and r^2 values of at least 0.99 were included in analysis (Bustin et al., 2009). To maximise this calculation, at least four of the eight dilutions were used to improve standard curve efficiency (Ruijter et al., 2021).

Absolute quantification was used to generate quantification cycle (Cq) values calculated from the amplification plots produced on the LightCycler software. Samples were considered positive for disease when both replicates had a Cq value <35. Cq values above the threshold of 35, were considered as indicative of non-specific amplification and variability, and were therefore regarded as false positives (Bustin et al., 2025; Ruiz-Villalba et al., 2021). Samples and standards had two replicates per plate, and if one sample replicate was reported as having a difference greater than 1 Cq from the other, these reactions were repeated. Further, to account for potential contamination negative controls and extraction blanks were included in the analysis, none of which amplified. To validate target specificity, a subset of positive sample sequences was verified through Sanger sequencing and GenBank (Source Bioscience).

Gene copy number was calculated utilising the efficiency of the standard curve and the resultant value was converted into gene copy per microlitre (μL) and then normalised to gene copy number per nanogram (ng) of DNA for each of the samples, using individual DNA concentrations (see Supplementary Materials for details and gene copy numbers, Appendix A2).

2.4. Statistical analysis

All statistical analyses and plots were conducted using R 4.4.0 statistical environment (R Core Team, 2024). All statistical calculations were done using the base functions via the *stats* package in R.

2.4.1. Disease prevalence

The number of positive cases for each pathogen was analysed to determine which diseases were significantly more prevalent. The presence/absence format of the data required a Chi-squared test of independence to be implemented, to reveal significant differences in number of positive cases between each disease.

2.4.2. Asymptomatic infection prevalence

Infections by beekeepers were considered symptomatic and cases of disease that were detected by qPCR, but not by beekeepers, were asymptomatic infections. A non-parametric Wilcoxon test was used to determine whether the number of cases detected by qPCR (symptomatic and asymptomatic) was significantly higher than that reported by beekeepers (symptomatic only). The choice to use the Wilcoxon test was based on a poor fit to parametric distributions for this data.

2.4.3. Interspecific variation

Pathogen gene copies obtained through qPCR were used to reveal whether there were significant differences between diseases. The diseases were the explanatory variable and gene copy number was the response. To compare gene copy number between infections a non-parametric Kruskal-Wallis test was utilised as the datasets did not meet the assumptions required for parametric testing.

2.4.4. Seasonal variation in disease detection

Pathogen gene copy data from the qPCR was analysed to determine whether there was a seasonal pattern in honey bee infections in response to the time of year that the samples were collected. Disease copy number, that was calculated from the qPCR, was used as the response, and sample collection time period as the explanatory covariate. Samples were split into two time periods based on what month the samples were collected in. Early samples were those obtained between April and the end of July, and late samples were honey extracts attained between August and the end of October. Where a sample contained multiple diseases, the summed gene copy across those diseases for a sample was used as the response. For samples, where no disease was identified, the copy number was set to zero. A non-parametric Kruskal-Wallis test was used to analyse the gene copy differences between different time periods for each pathogen. This comparative analysis was selected due to the disease datasets not conforming to parametric criteria.

3. Results

3.1. Prevalence of pathogens

Preceding pathogen analyses, comparative tests revealed that the measurements of archived DNA concentrations were not significantly different to those extracted 2 years prior ($W = 19{,}973$, $p > 0.05$). Of the 150 samples tested, the most prevalent infections were the fungal pathogens, *A. apis* (19 samples, 13%), and *Nosema* spp. (17 samples, 11%). Far less prevalent was the bacterial *M. plutonius* (3 samples, 2%, 2 were from the same location but different time of year), by using the thresholds outlined in the methods section (< 35 CT). Testing between prevalences of different diseases revealed a significant difference between *A. apis* abundance and *M. plutonius* ($x^2 = 11.04$, $P < 0.001$), and between the combined *Nosema* prevalence and *M. plutonius* ($x^2 = 9.05$, $P < 0.005$).

3.2. Geographical distribution of pathogens

Chalkbrood (*A. apis* infection) was the sole disease reported by beekeepers. Symptomatic infections were reported by beekeepers in the South West of England and Yorkshire (Fig. 1b). The South West cases of chalkbrood were not detected via qPCR, nor was there any detection in the East of England, but evidence of this pathogen's presence was discovered in every other ITL-1 region of this country (Fig. 1c). Generally, positive qPCR signal for *Nosema* was discovered in every ITS-1 region, excluding the South East, East Midlands and North West. The parasite, *N. apis*, which is more commonly associated with the western honey bee, was found to be more dispersed across the country than *N. ceranae*, which was mainly concentrated towards the Midlands, where coinfections of these *Nosema* species occurred (Fig. 1d). Positive qPCR detection of *M. plutonius* occurred in a small area from the South East to the West Midlands, with the two sites separated by less than 80 miles (Fig. 1e).

3.3. Molecular and reported data

When comparing beekeeper reported disease cases to those identified by qPCR, there were significantly more cases detected through qPCR (number of samples with positive PCR result) ($W = 9075$ and $P < 0.001$). Furthermore, qPCR detected a greater range of pathogens (*Nosema*, chalkbrood and EFB), than those reported by beekeepers (chalkbrood).

3.4. Interspecific pathogen gene copy variation

There was no significant differences in gene copy numbers among the pathogens (Kruskal Wallis $H(2) = 6.60$, $df = 3$, $P > 0.05$). There were high levels of intraspecific variation of gene copy within the samples that detected *A. apis* and *M. plutonius* producing the highest average gene copies (Fig. 2, Appendix A3).

3.5. Within year transitions in disease occurrence

The gene copy numbers reported for both *A. apis* (Kruskal-Wallis $H(2) = 4.96$, $df = 1$, p-value <0.05) and *N. apis* (Kruskal-Wallis $H(2) = 11.77$, $df = 1$, p-value <0.001) were higher in the late (August–October) season than early (April–July) (Fig. 3a & b). In the case of *N. apis* no cases were discovered early in the season. While there was no significant

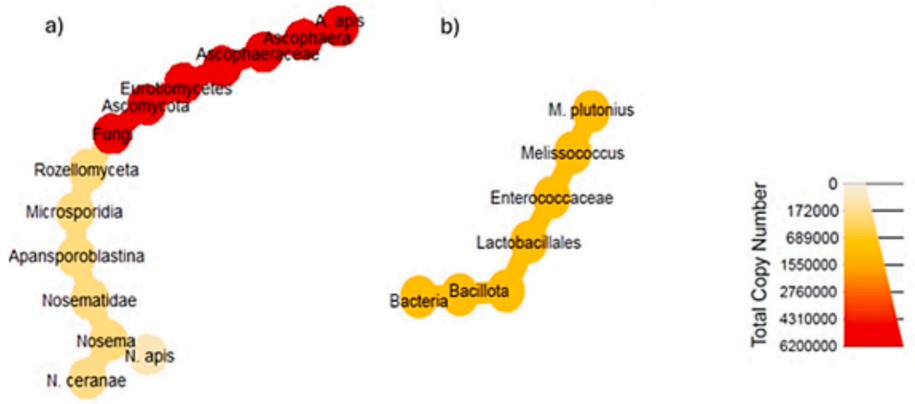

Fig. 2. Heat tree map showing total gene copy numbers for each pathogen as discovered via qPCR. Nodes depict taxonomic ranks, starting in the centre going from Domain and finishing with the Species on the tips. The colour legend describes the number of total gene copies discovered for each species. a) Heat tree map displaying the gene copies for fungal pathogens. b) Bacterial pathogen gene copies mapped onto a heat tree.

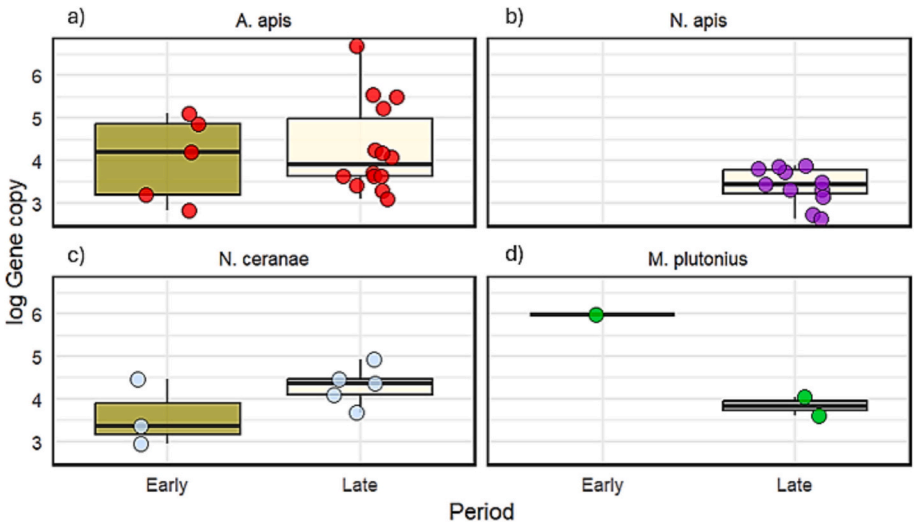

Fig. 3. Box plots and jitter points depicting the log pathogen gene copies dependent on time of the year. a) Boxplots and red jitter points for the gene copy data of *A. apis*. b) Orange jitter points and boxplots exhibiting gene copy for *N. apis*. c) Gene copy data for *N. ceranae* in box plots and represented with blue jitter points. d) *M. plutonius* displayed in green jitter points with boxplots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

change in gene copy number for *N. ceranae* between the early and late season ($p > 0.05$), there was a non-significant trend of the disease being more frequently detected later in the year (Fig. 3c). As *M. plutonius* was infrequently detected we did not formally test the significance of temporal trends (Fig. 3d).

4. Discussion

This study assessed the viability of molecular methods for monitoring honey bee pathogens within honey collected as part of a national-scale citizen science beekeeper monitoring program. The scale of this citizen science scheme provides opportunities for relatively low-cost, rapid mapping of diseases, improving understanding of both disease prevalence in managed honey bees as well as potential risks of spill-over effects into wild bee populations. Comparative analysis between DNA concentrations of differently aged samples revealed the preservation qualities of honey (Appendix A1). This emphasises using a long-term citizen science scheme that possess samples which allow for the tracking of historical trends in current disease prevalence, as well as potentially new emerging diseases yet to be identified. The project demonstrates the potential of the approach for assessing the prevalence, geographical and seasonal distribution of diseases at national scales. This includes the role of this approach in detection of asymptomatic infections in comparison to visual pathogen detection, practiced by beekeepers and the national inspection service run by the government.

4.1. Prevalence and geographical distribution

This project found that fungal pathogens were significantly more prevalent than bacterial, with *A. apis* detected in 13% of honey samples. This finding supports that of the NBU inspectors who rely on traditional visual monitoring of hives, who consistently find significantly more cases of chalkbrood than the foulbrood diseases in the UK (Rowland et al., 2021). Further comparison of the current analysis to Rowland et al.'s study shows similarities in underlying geographical patterns for diseases, the most notable being the absence and low risk of chalkbrood in the South West of England, despite its high prevalence elsewhere in the country (Fig. 1c). It is likely that the detected distribution of *A. apis*, the causative agent of chalkbrood, is linked to the low virulence of the pathogen allowing for it to spread across most of the country (Vojvodic et al., 2011). This low virulence results in a lower rate of mortality, which provides the opportunity for the spores to disperse within and out

of the hive (Kun et al., 2023). Furthermore, the cellular structure of fungal spores enables the pathogenic cells to persist in the natural environment potentially facilitating rapid dispersal through hives (Seekles, 2023).

In Europe, the case abundance of *A. apis* is usually lower than that of *Nosema,* which is predominantly caused by the species *N. ceranae* (Ribani et al., 2020). However, this investigation discovered that *Nosema* was the second most prevalent disease, detected in approximately 11% of hives, with a widespread distribution being detected in 6 NTL-1 regions of England (Fig. 1c). This difference may reflect the contrasting pollen isolation methods used, as this study employed vacuum filtration whereas Ribani et al. (2020) used centrifugation. Consequently, due to differing spore size, (*A. apis* spores persist in ascus formations which are larger at >36 μm in width) (Evison and Jensen, 2018; Anderson et al., 1998), it is feasible that *Nosema* spores, although around 2.8 μm in width (Galajda et al., 2021), may still have passed through the 1.2 μm filters (Spurgeon, 2006; Sivasubramani et al., 2004), whereas this is less likely with *A. apis*. Although there is a potential reduction in the sensitivity of *Nosema* detection, a comparison between both microsporidian parasites of this genus can still be made.

There were differences in the number of cases and geographical spread between the two *Nosema* species. The original cause of the infection in the UK, *N. apis*, was detected in 2% more hives and found across a wider area in comparison to *N. ceranae* (Fig. 1d). Approximately 60 years ago, *N. ceranae* began to infect western honey bees after this species' boundary overlapped with that of Asian honey bees (*Apis cerana*), the original host for this microsporidian parasite (Fries, 2010). Research in the last decade has revealed that *N. ceranae* is increasing in prevalence and potentially outcompeting *N. apis*, especially in Europe (Rubanov et al., 2019). As England is separated from mainland Europe, it is plausible that there has been a lag in transmission of *N. ceranae* to this country, which could explain the relatively fewer cases of this disease in comparison to *N. apis* that this project reports. Long-term monitoring, such as that suggested here, may play a key role in tracking the emergence of *N. ceranae* as well as providing insights into its interaction with *N. apis*. Moreover, it could aid in determining whether restriction measures of hives infected with this type of *Nosema* are required, similar to the notifiable disease, EFB.

The Bacterial disease, EFB, has been discovered at low numbers within the UK, reported both here and through governmental monitoring programs (Rowland et al., 2021). Transmission routes of the pathogen that causes EFB, *M. plutonius*, are believed to occur from foragers carrying these pathogens from infected colonies encountering other bees from neighbouring hives (Forsgren et al., 2018). Consequently, the dispersal of the cells of the disease may occur at a relatively slow rate, while the restriction measures intended to isolate and destroy infected hives would also directly reduce risks of transmission (Bees Act, 1980). As a result this was consistent with expectations that *M. plutonius* was detected at low prevalence among sampled hives. However, two colonies involved in this project detected the presence of EFB (Fig. 1e), both within 10 miles from known infected hives (NBU, 2022). Therefore because of flight ranges of foraging bees (Beekman and Ratnieks, 2001), it is probable that honey bees from the apiaries involved in this project, encountered individuals originating from the infected hives. An important application of this eDNA approach is to undertake early identification of the notifiable EFB before symptomatic infection occurs, which could lead to a path of treatment or early containment to reduce the spread of this disease.

4.2. Molecular and reported data

Asymptomatic disease presence as identified by qPCR was significantly more prevalent than that reported by the participating beekeepers who provided the honey samples. This disparity is not surprising given that many infections are asymptomatic until the colony experiences stress (Van Haga et al., 2012). Stress can be a result of numerous factors that cause change to the status of the colony, such as low nutrition, extreme climatic changes, and pesticide exposure (Aronstein and Murray, 2010; Woodcock et al., 2022). A further consideration is that these pathogens may experience a long asymptomatic incubation period, this has already been observed in *Nosema* and although EFB is more virulent than the fungal pathogens, there have been EFB asymptomatic colonies detected (Higes et al., 2008; Erban et al., 2017). Therefore, the high rate of asymptomatic infection observed in this study is not unexpected.

Incorrect identification may lead to not just misreporting, but potentially inappropriate treatment and in extreme cases risks linked to notifiable diseases like EFB not being quarantined and destroyed. Only three substantiated cases of chalkbrood were identified by the beekeepers, with two other cases reported that were not detected by qPCR (Fig. 1b). It is highly probable that these cases were misidentifications of a similar disease called Stonebrood, caused by *Aspergillus* spp., the symptoms of which can be mistaken for that of chalkbrood (Foley et al., 2014). Indeed, this highlights one of the risks associated with self-reporting of diseases as significant expertise may be required to separate symptoms alone.

The use of eDNA plays a particularly important role in diagnosis for some honey bee pathogens whose symptoms are not easily recognised (Tiedge et al., 2025), e.g. *N. ceranae* (Soares et al., 2023). When honey bees are infected with *N. ceranae*, the bees display fewer notable symptoms than those infected with *N. apis* (Vidal-Naquet, 2015). Sluggishness of adults infected with *N. ceranae*, for example, can go unnoticed by beekeepers or could be misconstrued with other diseases that cause this behaviour (Rubanov et al., 2019), such as Deformed Wing Virus (DWV) (Schroeder and Martin, 2012).

4.3. Interspecific and seasonal variation in pathogen load

Whilst we did identify differing abundances of pathogen detection across our samples, direct interspecies load comparisons should be taken with caution (Fig. 2a & b). Quantitative PCR detection is not an exact representation of pathogen load within hives as there are many molecular factors obscuring direct comparison between spore count and gene copy number (Traver and Fell, 2011; Thirugnanasambandam et al., 2024). As alluded to earlier, there can be a difference in detection thresholds that would lead to differences in gene copy number (Guimarães-Cestaro et al., 2016). In addition, the differences in qPCR conditions utilised between *A. apis* and the other pathogens, would result in differences in amplification rates and therefore gene copy number (Morinha et al., 2020). Consequently, comparative conclusions cannot be definitively made between the gene copies of different pathogens that have been produced in this study (Fig. 2a & b). However, there is still a positive correlation between gene copies and pathogen load (Traver and Fell, 2011), therefore the gene copies obtained in this study can still be used as evidence of within hive prevalence to allow for comparison of seasonal patterns of these diseases (Fig. 3a-d).

A known factor affecting disease distribution and infection rate is the behavioural patterns of host honey bees tied to seasonality, along with the associated climatic conditions (Higes et al., 2008). The majority of symptomatic and asymptomatic infections were discovered later in the year, specifically August (Fig. 3a-d). In addition, the later time period was significantly higher in terms of pathogen gene copy, though specifically for *A. apis* and *N. apis*, not *N. ceranae* nor *M. plutonius*. Growth assessments, within growth media and bees, have demonstrated that *A. apis* and *N. apis* maximal rate of spore production is around 30 °C (Mraz et al., 2021; Martin-Hernandez et al., 2009), a temperature which was regularly reached at the end of July and August of 2022 (Burt, 2025). Furthermore, with the increase of temperature in August, foragers and workers travel further distances and more often for water (Couvillon et al., 2014; Schmaranzer, 2000). Consequently, the increase in activity leads to greater exposure risk through airborne spores or directly through foraged pollen (Sulborska et al., 2019; Pereira et al.,

2019). Therefore, high abundance and pathogen gene copy number in mid-summer, as identified here, may be expected. However, these observations could differ from year to year due to the extreme climatic events becoming more frequent and the weather patterns less predictable. Adopting a quantitative and eDNA-based approach to analyse samples in an ongoing monitoring campaign, such as the NHMS, can provide insight into the causes of disease prevalence, especially under uncertain future climate conditions.

5. Conclusion

Citizen science schemes that collect biological samples at relatively low cost have an important societal role in encouraging the public to get involved in the sciences. Such schemes often provide important archives of spatially and temporally referenced samples that can be used to understand wider environmental, management or policy questions relevant to the protection of pollination ecosystem services. This project clearly demonstrates how honey bee disease prevalence information can be gained at minimal expense and disturbance directly from their hives, at a national scale citizen science model. From a disease mitigation perspective, the project highlights the value of these molecular procedures in discovering honey bee infections at early stages of asymptomatic infection. Through the use of preventative/prophylactic measures being implemented at these points to avoid colony collapse at a later stage, as well as providing insights into wider risks for interspecific transmission to wild species. In the meantime, this monitoring could be utilised to understand the drivers of symptomatic infection, such as increased pesticides exposure and changes in climate, as well as how diseases interact with the gut microbiome and individual immunity of honey bees.

CRediT authorship contribution statement

Michael J.R. Bennett: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing, Conceptualization, Data curation. **Lindsay K. Newbold:** Conceptualization, Resources, Supervision, Writing – review & editing, Funding acquisition. **Susheel Bhanu Busi:** Formal analysis, Visualization, Writing – review & editing. **Richard Pywell:** Conceptualization, Funding acquisition, Writing – review & editing. **Holly Tipper:** Methodology, Writing – review & editing. **Joanna Savage:** Data curation, Funding acquisition, Writing – review & editing, Resources. **Jennifer Shelton:** Conceptualization, Methodology, Resources, Writing – review & editing. **Manasa Suresh:** Methodology, Writing – review & editing. **Ellie Grove:** Methodology, Writing – review & editing. **Hyun S. Gweon:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Ben A. Woodcock:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

To the best of our knowledge, the named authors of this work have no conflicts of interest.

Acknowledgements

Thanks to the beekeepers who supported the work by providing honey to the National Honey Monitoring Scheme. This NHMS is funded by the Natural Environment Research Council (NERC) and the Biotechnology and Biological Sciences Research Council (BBSRC) joint research programme NE/W005050/1 AgZero+: Towards sustainable, climate-neutral farming. MB is funded through a NERC SCENARIO doctoral training partnership with additional financial support from the British Beekeepers Association (BBKA). SBB is supported by the Biotechnology and Biological Sciences Research Council (BBSRC), part of the UK Research and Innovation (UKRI), the Earlham Institute Strategic Programme Grant Decoding Biodiversity BBX011089/1, and its constituent work packages, BBS/E/ER/230002C (Decode WP3 Linking Fine-Scale Microbial Diversity to Ecosystem Functions).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2026.107451.

Data availability

The gene copy data can be found in Appendix A2 within the Supplementary Materials section. All locations of hives that participated in this project cannot be disclosed due to General Data Protection Regulations.

References

Abdi, K., Ben Said, M., Crotti, E., Masmoudi, A.S., Cherif, A., 2023. The promise of probiotics in honey bee health and disease management. Arch. Microbiol. 205, 73.
Bees Act, 1980. https://www.legislation.gov.uk/ukpga/1980/12.
Anderson, D.L., Gibbs, A.J., Gibson, N.L., 1998. Identification and phylogeny of sporecyst fungi (*Ascosphaera spp.*) using ribosomal DNA sequences. Mycol. Res. 102, 541–547.
Aronstein, K.A., Murray, K.D., 2010. Chalkbrood disease in honey bees. J. Invertebr. Pathol. 103, S20–S29.
Beekman, M., Ratnieks, F.L.W., 2001. Long-range foraging by the honey-bee, *Apis mellifera L.* Funct. Ecol. 14, 490–496.
Boncristiani, H., Ellis, J.D., Bustamante, T., Graham, J., Jack, C., Kimmel, C.B., Mortensen, A., Schmehl, D.R., 2020. World honey bee health: the global distribution of western honey bee (*Apis mellifera L.*) pests and pathogens. Bee World 98, 2–6.
Burt, S., 2025. Evaluation and verification of new UK air temperature extremes during the July 2022 heatwave. Part 1: maximum temperatures. Weather 80, 220–228.
Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.
Bustin, S.A., Ruijter, J.M., Van Den Hoff, M.J.B., Kubista, M., Pfaffl, M.W., Shipley, G.L., Tran, N., Rodiger, S., Untergasser, A., Mueller, R., Nolan, T., Milavec, M., Burns, M. J., Huggett, J.F., Vandesompele, J., Wittwer, C.T., 2025. Miqe 2.0: revision of the minimum information for publication of quantitative real-time PCR experiments guidelines. Clin. Chem. 71, 634–651.
Chen, Y., Evans, J.D., Zhou, L., Boncristiani, H., Kimura, K., Xiao, T., Litkowski, A., Pettis, J.S., 2009. Asymmetrical coexistence of *Nosema ceranae* and *Nosema apis* in honey bees. J. Invertebr. Pathol. 101, 204–209.
Cirtwill, A.R., Wirta, H., 2025. DNA in honey could describe the changes in flower visits and microbe encounters of honey bees over decades. Sci. Rep. 15, 8807.
Couvillon, M.J., Riddell Pearce, F.C., Accleton, C., Fensome, K.A., Quah, S.K.L., Taylor, E. L., Ratnieks, F.L.W., 2014. Honey bee foraging distance depends on month and forage type. Apidologie 46, 61–70.
Erban, T., Ledvinka, O., Kamler, M., Hortova, B., Nesvorna, M., Tyl, J., Titera, D., Markovic, M., Hubert, J., 2017. Bacterial community associated with worker honey bees (*Apis mellifera*) affected by European foulbrood. PeerJ 5, e3816.
Evison, S.E., Jensen, A.B., 2018. The biology and prevalence of fungal diseases in managed and wild bees. Curr. Opin. Insect Sci. 26, 105–113.
Foley, K., Fazio, G., Jensen, A.B., Hughes, W.O., 2014. The distribution of *Aspergillus spp.* opportunistic parasites in hives and their pathogenicity to honey bees. Vet. Microbiol. 169, 203–210.
Forsgren, E., Locke, B., Sircoulomb, F., Schäfer, M.O., 2018. Bacterial diseases in honey bees. Curr. Clin. Microbiol. Rep. 5, 18–25.
Fries, I., 2010. *Nosema ceranae* in European honey bees (*Apis mellifera*). J. Invertebr. Pathol. 103, S73–S79.
Galajda, R., Valencakova, A., Sucik, M., Kandracova, P., 2021. *Nosema* Disease of European Honey Bees. J. Fungi (Basel) 7.
Govan, V., Brözel, V., Allsopp, M., Davison, S., 1998. A PCR detection method for rapid identification of *Melissococcus plutonius* in honey bee larvae. Appl. Environ. Microbiol. 64, 1983–1985.
Grossar, D., 2023. Genetic diversity and effects of the bacterial pathogen *Melissococcus plutonius* in Swiss honey bee populations *(Apis mellifera)*. Univerity de Lausanne.
Guimarães-Cestaro, L., Serrão, J.E., Message, D., Martins, M.F., Teixeira, É.W., 2016. Simultaneous detection of *Nosema spp.*, *Ascosphaera apis* and *Paenibacillus larvae* in honey bee products. J. Hymenopt. Res. 49, 43–50.
Higes, M., Martin-Hernandez, R., Botias, C., Bailon, E.G., Gonzalez-Porto, A.V., Barrios, L., Del Nozal, M.J., Bernal, J.L., Jimenez, J.J., Palencia, P.G., Meana, A., 2008. How natural infection by *Nosema ceranae* causes honey bee colony collapse. Environ. Microbiol. 10, 2659–2669.
James, R.R., Skinner, J.S., 2005. PCR diagnostic methods for *Ascosphaera* infections in bees. J. Invertebr. Pathol. 90, 98–103.

Klein, A.M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Tscharntke, T., 2007. Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. B 274, 303–313.
Kun, A., Hubai, A.G., Kral, A., Mokos, J., Mikulecz, B.A., Radvanyi, A., 2023. Do pathogens always evolve to be less virulent? The virulence-transmission trade-off in light of the COVID-19 pandemic. Biologia Futura 74, 69–80.
Kunat-Budzyńska, M., Łabuć, E., Ptaszyńska, A.A., 2025. Seasonal detection of pathogens in honey bees kept in natural and laboratory conditions. Parasitol. Int. 104, 102978.
Mackay, J.F., Hewett, R.E., Smith, N.T., Waters, T.L., Scandrett, J.S., 2024. The Foster method: rapid and non-invasive detection of clinically significant American Foulbrood disease levels using eDNA sampling and a dual-target qPCR assay, with its potential for other hive pathogens. J. Apic. Res. 64 (2), 693–700.
Martin-Hernandez, R., Meana, A., Garcia-Palencia, P., Marin, P., Botias, C., Garrido-Bailon, E., Barrios, L., Higes, M., 2009. Effect of temperature on the biotic potential of honey bee microsporidia. Appl. Environ. Microbiol. 75, 2554–2557.
Milbrath, M., 2021. Honey bee bacterial diseases. Honey Bee Med. Vet. Pract. 277–293.
Morinha, F., Magalhães, P., Blanco, G., 2020. Different qPCR master mixes influence telomere primer binding within and between bird species. J. Avian Biol. 51.
Mraz, P., Hybl, M., Kopecky, M., Bohata, A., Konopicka, J., Hostickova, I., Konvalina, P., Sipos, J., Rost, M., Curn, V., 2021. The effect of artificial media and temperature on the growth and development of the honey bee brood pathogen *Ascosphaera apis*. Biol.-Basel 10.
NBU, 2022. Disease Incidence. https://www.nationalbeeunit.com/diseases-and-pests/reports-charts-and-maps/disease-incidence.
Nekoei, S., Rezvan, M., Khamesipour, F., Mayack, C., Molento, M.B., Revainera, P.D., 2023. A systematic review of honey bee (*Apis mellifera*, Linnaeus, 1758) infections and available treatment options. Vet. Med. Sci. 9, 1848–1860.
Neov, B., Georgieva, A., Shumkova, R., Radoslavov, G., Hristov, P., 2019. Biotic and abiotic factors associated with colonies mortalities of managed honey bee (*Apis mellifera*). Diversity 11.
Nikita Grover, A., Kalia, P., Sinha, R., Garg, P., 2022. Colony collapse disorder: a peril to apiculture. J. Appl. Nat. Sci. 14, 729–739.
Nolan, V.C., Harrison, J., Cox, J.A.G., 2019. Dissecting the antimicrobial composition of honey. Antibiotics (Basel) 8.
Oliver, A.E., Newbold, L.K., Gweon, H.S., Read, D.S., Woodcock, B.A., Pywell, R.F., 2021. Integration of DNA extraction, metabarcoding and an informatics pipeline to underpin a national citizen science honey monitoring scheme. MethodsX 8, 101303.
Pereira, K.S., Meeus, I., Smagghe, G., 2019. Honey bee-collected pollen is a potential source of *Ascosphaera apis* infection in managed bumble bees. Sci. Rep. 9, 4241.
Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010a. Global pollinator declines: trends, impacts and drivers. Trends Ecol. Evol. 25, 345–353.
Potts, S.G., Roberts, S.P., Dean, R., Marris, G., Brown, M.A., Jones, R., Neumann, P., Settele, J., 2010b. Declines of managed honey bees and beekeepers in Europe. J. Apic. Res. 49, 15–22.
Powney, G.D., Carvell, C., Edwards, M., Morris, R.K.A., Roy, H.E., Woodcock, B.A., Isaac, N.J.B., 2019. Widespread losses of pollinating insects in Britain. Nat. Commun. 10, 1018.
R Core Team, 2024. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Url: https://www.R-project.org/.
Requier, F., Garnery, L., Kohl, P.L., Njovu, H.K., Pirk, C.W.W., Crewe, R.M., Steffan-Dewenter, I., 2019. The conservation of native honey bees is crucial. Trends Ecol. Evol. 34, 789–798.
Revainera, P.D., Quintana, S., De Landa, G.F., Iza, C.G., Olivera, E., Fuentes, G., Plischuk, S., Medici, S., Ruffinengo, S., Marcangelli, J., Fuselli, S., 2020. Molecular detection of bee pathogens in honey. J. Insects Food Feed 6, 467–474.
Ribani, A., Utzeri, V.J., Taurisano, V., Fontanesi, L., 2020. Honey as a source of environmental DNA for the detection and monitoring of honey bee pathogens and parasites. Vet. Sci. 7.
Rowland, B.W., Rushton, S.P., Shirley, M.D.F., Brown, M.A., Budge, G.E., 2021. Identifying the climatic drivers of honey bee disease in England and Wales. Sci. Rep. 11, 21953.
Rubanov, A., Russell, K.A., Rothman, J.A., Nieh, J.C., Mcfrederick, Q.S., 2019. Intensity of *Nosema ceranae* infection is associated with specific honey bee gut bacteria and weakly associated with gut microbiome structure. Sci. Rep. 9, 3820.
Ruijter, J.M., Barnewall, R.J., Marsh, I.B., Szentirmay, A.N., Quinn, J.C., Van Houdt, R., Gunst, Q.D., Van Den Hoff, M.J.B., 2021. Efficiency correction is required for accurate quantitative PCR analysis and reporting. Clin. Chem. 67, 829–842.
Ruiz-Villalba, A., Ruijter, J.M., Van Den Hoff, M.J.B., 2021. Use and misuse of C(q) in qPCR data analysis and reporting. Life (Basel) 11.
Schmaranzer, S., 2000. Thermoregulation of water collecting honey bees (Apis mellifera). J. Insect Physiol. 46, 1187–1194.
Schroeder, D.C., Martin, S.J., 2012. Deformed wing virus: the main suspect in unexplained honey bee deaths worldwide. Virulence 3, 589–591.
Seekles, S.J., 2023. The breaking of fungal spore dormancy: A coordinated transition. PLoS Biol. 21, e3002077.
Shahrajabian, M.H., Sun, W., 2024. The significance and importance of dPCR, qPCR, and SYBR green PCR kit in the detection of numerous diseases. Curr. Pharm. Des. 30, 169–179.
Sivasubramani, S.K., Niemeier, R.T., Reponen, T., Grinshpun, S.A., 2004. Fungal spore source strength tester: laboratory evaluation of a new concept. Sci. Total Environ. 329, 75–86.
Smith, C.J., Osborn, A.M., 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol. Ecol. 67, 6–20.
Soares, S., Rodrigues, F., Delerue-Matos, C., 2023. Towards DNA-based methods analysis for honey: an update. Molecules 28, 2106.
Spurgeon, J., 2006. A new filter cassette for the direct microscopic examination of airborne fungal spores. Aerosol Sci. Technol. 40, 1025–1033.
Sulborska, A., Horecka, B., Cebrat, M., Kowalczyk, M., Skrzypek, T.H., Kazimierczak, W., Trytek, M., Borsuk, G., 2019. Microsporidia *Nosema spp*. - obligate bee parasites are transmitted by air. Sci. Rep. 9, 14376.
Thirugnanasambandam, I., Vucurevich, T., Shelton, T., Kav, N.N., Sheedy, C., Laroche, A., Challis, J.K., 2024. Assessment of real-time PCR techniques for the detection of airborne fungal pathogens of wheat: role of DNA extraction on spore quantification. Acs Agricult. Sci. Technol. 4, 1063–1073.
Tiedge, T.M., Love, K.R., Meiklejohn, K.A., 2025. Utilizing eDNA from bacteria, fungi, plants, and arthropods associated with mock geologic evidence for sample-to-sample comparisons and study site separation: A feasibility study. Foren. Sci. Int. Genet. 78, 103284.
Tong, Z.Y., Wu, L.Y., Feng, H.H., Zhang, M., Armbruster, W.S., Renner, S.S., Huang, S.Q., 2023. New calculations indicate that 90% of flowering plant species are animal-pollinated. Natl. Sci. Rev. 10, nwad219.
Traver, B.E., Fell, R.D., 2011. Prevalence and infection intensity of *Nosema* in honey bee (Apis mellifera L.) colonies in Virginia. J. Invertebr. Pathol. 107, 43–49.
Van Haga, A., Keddie, B., Pernal, S., 2012. Evaluation of lysozyme-HCl for the treatment of chalkbrood disease in honey bee colonies. J. Econ. Entomol. 105, 1878–1889.
Vanengelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B.K., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y., Underwood, R., Tarpy, D.R., Pettis, J.S., 2009. Colony collapse disorder: a descriptive study. PLoS One 4, e6481.
Vasiliev, D., Greenwood, S., 2021. The role of climate change in pollinator decline across the Northern Hemisphere is underestimated. Sci. Total Environ. 775, 145788.
Vidal-Naquet, N., 2015. Honey Bee Veterinary Medicine: *Apis mellifera L*. 5m Books (UK).
Visick, O.D., Ratnieks, F.L.W., 2023. Density of wild honey bee, *Apis mellifera*, colonies worldwide. Ecol. Evol. 13, e10609.
Vojvodic, S., Jensen, A., James, R., Boomsma, J., Eilenberg, J., 2011. Temperature dependent virulence of obligate and facultative fungal pathogens of honey bee brood. Vet. Microbiol. 149, 200–205.
Woodcock, B.A., Oliver, A.E., Newbold, L.K., Soon Gweon, H., Read, D.S., Sayed, U., Savage, J., Bacon, J., Upcott, E., Howell, K., Turvey, K., Roy, D.B., Gloria Pereira, M., Sleep, D., Greenop, A., Pywell, R.F., 2022. Citizen science monitoring reveals links between honey bee health, pesticide exposure and seasonal availability of floral resources. Sci. Rep. 12, 14331.