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Insect pollinators in the Anthropocene: How multiple environmental stressors are shaping pollinator health

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Insect pollinators in the Anthropocene: How multiple environmental stressors are shaping pollinator health

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Editorial: Insect pollinators in the Anthropocene: how multiple environmental stressors are shaping pollinator health

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Editorial on the Research Topic

Insect pollinators in the Anthropocene: how multiple environmental stressors are shaping pollinator health

Loss of biodiversity, particularly concerning insect pollinators, is a defining feature of the Anthropocene, and may have potentially severe consequences for ecosystem function and food security (Potts et al., 2010). A range of abiotic and biotic stressors, such as habitat destruction and fragmentation, pests and pathogens, climate change, intensified agriculture, poor nutrition, and pollution, are likely responsible for the observed insect declines, including bees and other insect pollinators (Figure 1) (Sánchez-Bayo and Wyckhuys, 2019). These environmental stressors most certainly interact with one another and generate complex effects that amplify individual stressors. There are still knowledge gaps concerning how even the most important stressors may interact to affect insect pollinators. In this Research Topic, we highlight research focusing on how environmental stressors shape pollinator health.

The effects of climate change on insect pollinator health remain poorly understood, but will most likely result in changes to their behavior, physiology, phenology, and distribution (Harvey et al., 2023). Unlike honey bees that can regulate temperature at the colony level, solitary bees are subjected to ambient temperatures in the environment, possibly leaving them more susceptible to the changing climate and extreme weather. Scalici et al. explored how the interaction between temperature and geographical origin affect the fitness of blue orchard bees, *Osmia lignaria*. Developing bees that were reared on temperatures warmer than their native ranges had reduced fitness. Furthermore, developmental differences were observed between populations, suggesting a possible interaction between genetics and environment which may explain how certain populations were better adapted to temperature and environmental change. Such findings are crucial to predict potential

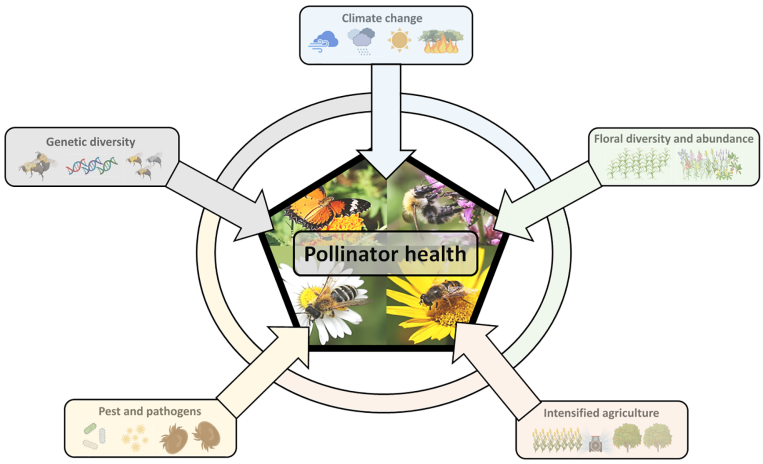

FIGURE 1
Key factors affecting pollinator health in the Anthropocene. Research in this Research Topic addresses how multiple environmental stressors, including climate change, pest and pathogens, genetic diversity, intensified agriculture, and floral diversity and abundance, are shaping pollinator health. Figure created with BioRender.com.

impacts of climate change on insect pollinators, yet they also highlight the urgent need for additional research to improve our understanding.

Many insect populations are especially susceptible to habitat loss and fragmentation (Foley et al., 2005; Potts et al., 2010), as they are dependent on abundant and diverse floral resources (Baude et al., 2016). Agricultural intensification is a key driver in the loss of natural habitats, causing reduced floral diversity and abundance as well as decreasing natural nesting sites (Kremen et al., 2002). Levenson and Tarpy studied conservation habitat in agroecosystems and showed that even small acreages (i.e., <1 acre) of flower cover positively supported bee diversity and abundance in agroecosystem. Bottero et al. further highlighted the important role of habitat heterogeneity when they sampled pollinator taxa across eight European countries and determined that pollinators responded to landscape and climate parameters in taxon- and crop-specific ways. Bee abundance was positively correlated with landscape diversity in oilseed rape fields. Less-intensively managed habitats also positively influenced pollinator abundance. Both studies emphasize habitat restoration to improve resource availability to support pollinator populations. Additional tools to assess bee nutritional health will be needed to identify bee nutritional status. For example, pollen diets can increase biomarkers of oxidative stress in honey bees and thus act as a possible novel tool to assess nutritional deficiencies in bees as shown in Yazlovytska et al.

Pests and pathogens are believed to be a key factor influencing population dynamics of wild and managed bee species (Cameron et al., 2011; Neumann et al., 2012). The ectoparasitic mite, *Varroa destructor*, and its associated viruses remain the greatest threat to apiculture globally (Rosenkranz et al., 2010). To mitigate the negative effects of *V. destructor* parasitism and potential viral spread, effective treatments at the appropriate times are required to prevent further unsustainable losses of managed honey bee colonies (Steinhauer and Saegerman, 2021). Jack et al. revealed that temporal efficacy of mite treatments can vary across seasons, with treatments in winter and spring being more effective at reducing mite populations in colonies compared to treatments in summer and fall. These findings are key in aiding beekeepers to control *V. destructor*, and lay the foundation for future treatments and spatial distribution models. Sobkowich et al. explored the spatial distribution of *V. destructor* infestations in honey bee colonies using a population-level epidemiological approach over a five-year period. They identified a stable cluster of mite infestations with other individual clusters occurring sporadically throughout their study site in Southern Ontario; no link between mite infestation and environmental factors was detected. Mitigating mite populations in managed honey bee colonies will have positive downstream effects for colony health and may also reduce the potential virus spillover to wild bee species (Nanetti et al., 2021). Schauer et al. studied the potential impact of viral spillover from managed honey bees to wild bees and demonstrated that Deformed Wing Virus A (DWV-A) does not replicate in the mason bee, *Osmia bicornis*, and is thus unlikely infectious. Nevertheless, there is a potential that *O. bicornis* may act as a host, as DWV-A recovered 16 days post-microinjection was infectious to honey bees. Besides potential spillovers, pathogens can also interact with environmental pollutants such as agrochemicals. Thebeau et al. showed that concurrent exposure to four fungicide products commonly used in blueberry production may increase honey bee larvae susceptibly to *Melissococcus plutonius*, the causative agent of European Foulbrood. Such findings are essential to improving our understanding of the mechanistic pathways and consequences underlying pesticide and pathogen interactions.

Sustaining insect pollinators in the Anthropocene will require mitigating the multitude of stressors insect pollinators simultaneously encounter. There is still a need to understand how many different environmental factors affect insect pollinators on their own, or in

concert. However, it is becoming increasingly clear that providing flower-rich habitats can mitigate the effects of environmental stressors. Altogether, this information will contribute towards decision making processes for environmental management. A framework will need to be developed for pesticide applications in different cropping systems, as microclimate and presence of different bee species in an area needs to be considered prior to pesticide application decisions as discussed in Decourtye et al. Further, Barrett et al. highlight the relevance of keeping a balance between conservation and animal welfare goals when establishing insect pollinator monitoring programs that are key to improving our understanding population dynamics of pollinators.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Spatial analysis of *Varroa destructor* and the relationship with surrounding landscape types in Southern Ontario

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Elevated colony losses have continued to be an issue for Canadian beekeepers for more than a decade. Numerous studies have identified unmanaged *Apis mellifera* colony infestation by the *Varroa destructor* mite as a main cause of the problem. *V. destructor* spread externally of the hive through a phoretic stage in their life cycle. Consequently, their movement outside the hive is influenced by honey bee flight behaviours, which can range to multiple kilometers from the originating hive in any direction. *V. destructor* are therefore of regional concern as neighboring colonies and yards share nearby forage which can serve as fomites. Additionally, mites can be transmitted through bee behaviours such as robbing and drifting, thus impacting surrounding colonies. Understanding the distribution of mites across a population is key for surveillance and equitable allocation of resources. Spatial patterns of *V. destructor* infestations in Southern Ontario, Canada, were investigated using a combination of cluster analysis, scan statistics, and geostatistical modelling, using 5 years of provincial apiary inspection data, from 2015 to 2019. A collection of disease clusters of *V. destructor* infestations was identified and found to be stable over multiple years with several other individual clusters occurring sporadically throughout Southern Ontario during the same study period. Universal kriging was applied to the *V. destructor* data in combination with regional colony density, and land use data as covariates, producing an isopleth map of the prevalence risk for *V. destructor* infestation. No substantial link between *V. destructor* infestation and environmental factors was found. This study highlights the need for more data and investigation to determine the cause of the identified clusters and areas of elevated risk. These results are hypothesis-generating but simultaneously provide information for government agencies, industry organizations, and beekeepers into the spatial distribution of *V. destructor* at a macro scale.

KEYWORDS

Varroa destructor*, spatial scan analysis, disease cluster detection, universal kriging, epidemiology, geostatistical modelling, *Apis mellifera

Introduction

Between 2015 and 2019, Ontario beekeepers reported an average overwinter colony loss of 30.5% (Canadian Association of Professional Apiculturalists, n.d.). Other Canadian provinces reported similarly high losses, with an average of 25.7% overwinter colony loss in 2019 across all 10 provinces. This amount of loss is beyond the accepted level of 5–15% (Vidal-Naquet, 2018). Elevated levels of colony loss have been experienced consistently since the Canadian Association of Professional Apiculturists (CAPA) began reporting on the issue of "colony collapse disorder" in 2007 (Canadian Association of Professional Apiculturalists, n.d.). Despite the high percentage of colony loss, beekeepers in Canada have managed to maintain a consistent population of colonies in the past 5 years (Agriculture and Agri-Food Canada, 2019). This paradox demonstrates the effectiveness of modern advancements in beekeeping, allowing for beekeepers to compensate continuing large losses through techniques such as colony splitting, and commercialization of queen and nucleus colonies, but the issue of long-term colony health still remains.

First reported in Canada in 1989 (McElheran, 1990), the parasitic mite, *Varroa destructor*, has continued to be one of the greatest threats to beekeeping in Canada and has spread to most beekeeping regions across the country (Currie et al., 2015). *V. destructor* (commonly, and henceforth, referred to as *Varroa* or *Varroa* mites) is a phoretic mite, which feeds on adult honey bees for survival, and acts as a parasite to honey bee larva during developmental stages (Rosenkranz et al., 2010). *Varroa* mites also serve as a vector for several viruses, including deformed wing virus, and black queen cell virus (Tentcheva et al., 2004). Clinically, the infestation of a honey bee colony by *Varroa* mites, and the associated symptoms, is referred to as varroosis. Varroosis has been found to be most detrimental when co-prevalent with other parasites and abiotic stressors (Roberts et al., 2017), but left untreated, is capable of decimating entire honey bee colonies. *Varroa* mites have been considered by numerous researchers as the greatest contributor to weakened colonies and overwinter colony losses (Guzmán-Novoa et al., 2010; Van Der Zee et al., 2015; Barroso-Arévalo, et al., 2019).

Varroa mites are an endemic and treatable issue in beekeeping in Canada and around the majority of the world. Therefore, the effects of an infestation can usually be mitigated when detected early. However, the presence of *Varroa* may go undetected due to sampling error or an absence of testing. If detected, chemical and non-chemical treatment options are available. Some chemical treatment regimens for *Varroa* may be detrimental to the colony's health if administered incorrectly, though not all have been shown to have negative effects (Giovenazzo and Dubreuil, 2011). *Varroa* mites have also demonstrated resistance to various chemical treatment options due to improper administration or rotation (Rawn et al., 2019). Non-chemical treatment options against *Varroa* infestations exist but have been shown to be less effective at reducing *Varroa* load (Haber et al., 2019). Flaws in both *Varroa* detection and *Varroa* treatment could influence the regional *Varroa* abundance, as neighboring yards may contract *Varroa* as a result of bees robbing from a weakened colony possessing a high *Varroa* load (Peck and Seeley, 2019), or other means of transmission. Because no treatment is 100% effective, and the eradication of mites is not possible, integrated pest management (IPM) strategies are important to keep mite levels below critical thresholds. Adequate knowledge on the pest of interest and their distributions across the population is key for effective surveillance and IPM.

The phoretic nature of *Varroa* mites and the flight behaviour of honey bees, implies that the presence of *Varroa* is a landscape-wide issue and is not localized to single bee yard outbreaks unless geographically isolated. Increased *Varroa* load in a single yard may result in subsequent transmission to nearby colonies as *Varroa* is transmitted by means of robbing, drifting (if colonies within yards are not adequately spaced), or through fomites in the environment (Peck et al., 2016; Peck and Seeley, 2019). Therefore, regional population levels of *Varroa* should be considered when making management decisions. Insufficient *Varroa* detection and treatment suggest a need to switch from reactive to proactive population medicine for *Varroa* management, for which enhanced surveillance is necessary.

Geospatial epidemiological studies can address all aspects of the epidemiologic triad: agent, host, and environmental risk factors of disease (Berke, 2005). Clustering tendencies can indicate if the disease agent's prevalence is spatially related, while the detection of clusters can give an indication of whether the host's susceptibility and behaviours are influencing the distribution of disease. Furthermore, spatial regression and trend analysis can help identify which environmental risk factors may be contributing to the prevalence of the disease. This approach can therefore provide insight into all major aspects of *Varroa* distribution and spread mechanics at a population level. To date, few spatial epidemiological studies have investigated the prevalence of *Varroa*, and none have been identified in the literature for Ontario or Canada. In one geospatial study of varroosis in New Zealand, Stevenson et al. (2005) identified clusters of *Varroa* infestations, as well as a spatial dependence structure that decays over distance from an infected yard. Similar patterns may exist in Ontario and should be investigated.

Previous studies have explored the impacts of surrounding landscape on the health of managed honey bees, but found no association with *Varroa* (Dolezal et al., 2016). However, this study by Dolezal et al. (2016) investigated only two landscape categories: high cultivation and low/no cultivation. Further investigation into more landscape classifications is therefore warranted to confirm that this choice of binary classification is not suppressing a true association. Surrounding land-use may influence mite loads due to variations in diversity and quantity of available forage, as well as potential for mite transfer from feral bee colonies in natural landscapes or managed bees in higher colony density areas. The diet of honey bees has previously been linked to health issues such as immunocompetence (Alaux et al., 2010) and surrounding land-use type has been shown to impact the quantity and quality

of forage and food accumulation, and bee health in general (Sponsler and Johnson, 2015; Dolezal et al., 2016). Landscapes with higher colony densities, could possess greater mite prevalence because of the increase in density of susceptible colonies for mite transfer to occur, a theory accepted in human epidemiology where population density is related to disease transmission (Tarwater and Martin, 2001), but not yet accepted in bee research.

Honey bee colonies surrounded by natural landscapes are more likely to forage on a more diverse diet and have access to ample food sources but simultaneously may interact more with feral colonies, potentially spreading and contracting mites more frequently. Bees located in heavily cultivated landscapes are less likely to interact with untreated feral colonies but have access to a less diverse diet and may spread mites between other managed colonies because of the increased density observed in areas of farmland in Ontario (Sobkowich et al., 2021). Various environmental stressors (e.g., pesticides found in areas of cultivated land) may also play a role in the susceptibility of a colony towards *Varroa* mites (Morfin et al., 2019). In locations where colonies exist beside a large body of water, the immediate foraging landscape is effectively reduced and may lead to less available forage resulting in a greater competition for nectar sources, which may contribute to mite spread due to shared forage or increased robbing (Peck et al., 2016; Peck and Seeley, 2019). In contrast, bees in an urban setting may experience similar issues of reduced forage quantity but may face less competition because of decreased colony density (Sobkowich et al., 2021). These scenarios are the justification for a five-category landscape classification system to be evaluated for an association with *Varroa* prevalence. These five landscape categories are: natural land, primary agricultural forage, secondary agricultural forage, urban/developed land, and water bodies (Table 1).

Geostatistical kriging allows for spatial prediction of prevalence even in locations or areas where the sample size is otherwise too small. Kriging can be used to predict the prevalence over the entire study area which can inform policy decisions, aid in the efficient allocation of resources, and provide a basis for a risk-based sampling model for future inspections (Carrat and Valleron, 1992; Berke, 2004). Universal kriging is based on a spatial general linear model (GLM) to study the impact of potential risk factors, such as land-use types derived from satellite imagery (as applied in this study) in the presence of spatial dependence.

The goal of this study is to explore the spatial distribution of *Varroa* infestations in managed honey bee colonies of Southern Ontario, using a population-level epidemiologic approach, over a 5-year study period. This study has three main objectives to achieve this goal: (1) explore the spatial distribution of *Varroa* and the tendency for spatial clustering of varroosis cases; (2) locate high-risk clusters of varroosis; and (3) use geostatistical modelling to determine the effects of the five various land-use types on *Varroa* infestation to estimate and map the prevalence-risk.

TABLE 1 Definitions and examples of the five land-usage categories considered.

Land-usage category	Definition	Examples
Primary agricultural forage	Human cultivated land possessing crops commonly visited by honey bees for pollination and forage.	Apples, blueberries, canola, stone fruits, etc. A complete list can be found in Pollinator Partnership Canada (2017).
Secondary agricultural forage	Human cultivated land possessing crops that are foraged by honey bees in times of necessity.	Barley, oats, sod, winter wheat, etc.
Natural land	Land existing in its natural state without, or with minimal, human interference.	Conservation areas, grasslands forests, shrublands, etc.
Developed land	Land where substantial human interference has taken place to alter the state.	Exposed land, greenhouses, residential areas, urban centers, etc.
Water	Areas covered by a body of water.	Lakes, rivers, etc.

Materials and methods

Data on *Varroa destructor* were received from the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA). The data were collected by trained inspectors. Inspections occur mainly for three reasons: regulatory inspections, confirmation of *Varroa* status of commercialized queens or nucleus colonies, or to address beekeeper concern of poor colony health. *Varroa* inspection data are based on the standard alcohol wash method (Dietemann et al., 2013) and reported as a total count per 300 bees. These counts were converted to a value of mites per 100 bees, referred to subsequently as the "*Varroa* rate." Inspected colonies were recorded with their GPS location, date of inspection, and the observed *Varroa* rate. Geographic coordinates of yard locations were truncated down to two decimal places (approximately 1.11 km spatial resolution) to maintain the privacy of exact yard locations. The cleaned dataset contained 3,786 colony-level observations in Southern Ontario between 2015 and 2019. Regional colony density values were derived from OMAFRA registry data from the 2018 beekeeping season, aggregated by census consolidated subdivision (CCS; Statistics Canada, 2018) to maintain beekeeper privacy. The 2018 registry dataset was the most recent and complete at the time of analysis. Each inspection location was assigned a colony density value based on the CCS region that the inspection occurred within.

Land usage data were acquired from the Government of Canada, and the Agriculture and Agri-Food division, through their Annual Crop Inventory program (Government of

Canada, 2020). The data were in raster file format, produced by optical and radar-based satellites. The raster file contained land use information at a spatial resolution of 30 m with a reported accuracy of 85%. Seventy-two distinct land-use types were used to define the provincial landscape of Ontario using optical and radar-based satellite imagery. These 72 categories were then aggregated into the 5 categories of interest for this study: natural land, developed land, primary agricultural forage, secondary agricultural forage, and water. Agricultural land was deemed as primary forage if included in the Pollinator Partnership Canada Guide for Planting Forage for Honeybees (Pollinator Partnership Canada, 2017). All other cultivated land types were deemed as secondary agricultural forage. A more detailed definition for each of the 5 land-use categories is presented in Table 1.

Spatial distribution of *Varroa* in Southern Ontario and determination of *Varroa* mite clustering

A sampled *Varroa* rate of greater than or equal to 3 mites per 100 bees was considered a case colony, as outlined by the OMAFRA treatment threshold guidelines (Kozak et al., 2021). Further reference to cases in this study is with respect to a colony found to have a *Varroa* rate at or above the threshold of 3 mites per 100 bees. Locations of cases and controls were plotted to a map of the province for data exploration.

As proposed by Diggle and Chetwynd (1991), the D-function was applied to assess spatial clustering of cases. Estimation of the D-function further provides an approximation for the spatial range at which clustering may be occurring. A confidence band derived from the standard errors was used to determine the presence of spatial clustering.

Detection of high-risk clusters of *Varroa* mite prevalence

Clusters of *Varroa* case locations were detected using the spatial scan statistic implemented in the SaTScan software (Kulldorff and Information Managemnet Services Inc., 2009). The scan statistic was applied for each of the 5 study years individually, and the results were then overlayed onto a map to check for temporal stability of *Varroa* case clusters across beekeeping seasons. The spatial scan analysis used the Bernoulli model (Kulldorff, 1997), with a purely spatial method to detect regions of high rates. A circular scanning window was used, with a maximum cluster size of 20% of the population at risk. A maximum of 20% was used in place of the standard 50% maximum to uphold biological relevance, owing to the scale of the study area (the distribution of colonies) in relation to the typical movement and flight ranges of bees. This reduced maximum cluster size has been used previously by researchers looking to account for low levels of data, spatial discontinuity or to look specifically for smaller clusters (Ma et al., 2016). The standard Monte-Carlo method, with 999 replications, was used as a means to estimate the value of p for detected clusters. All non-overlapping clusters identified at a 5% significance level were highlighted on a map of Southern Ontario. The 95% confidence intervals of the standardized morbidity ratios were estimated using the Vandenbroucke method (Vandenbroucke, 1982).

Spatial regression modelling of *Varroa* prevalence using environmental covariates

For spatial regression modelling, the Ontario land use data were merged with the *Varroa* rate data using a buffer analysis in QGIS software (QGIS Development Team, 2020). A Lambert azimuthal equal-area projection was applied to preserve the study's area size and minimize distance distortions. The locations of bee yards inspected for *Varroa* from all five study years were used as centroids for a buffer analysis. Buffers with a 3 km radius approximating the average foraging range of honey bees around their hives (Visscher and Seeley, 1982; Pollinator Partnership Canada, 2017) were used to link the land-use raster data to the inspection data. A summary of the percentage of each of the five land use categories within each buffer was calculated and merged with the *Varroa* inspection data. The five land use categories and regional colony density values were considered as covariates in the model building process. For spatial modelling, counts of the number of mites for each inspection were used (mites per 100 bees sampled). Repeat inspection observations at the same geographical location were addressed by averaging the counts.

The generalized linear model component of regression-kriging was fit by comparing the results of regression models for each covariate. A Gaussian family GLM model was used to model the continuous *Varroa* rate, and an iteratively reweighted least squares approach was used to fit the GLM model. The Akaike information criterium (AIC) from each regression model was used as an indication of greatest model fit. Simple and multiple regression models were considered using the land-use types, and colony density as covariates. The covariate(s) with the lowest AIC value was selected. This model would then be put forward in the regression-kriging model building process. Because this study is hypothesis generating, *p*-values were considered as exploratory metrics only (Matthews et al., 2017). Estimated regression coefficients (β) for the simple regression models with their 95% Wald confidence intervals were presented in a forest plot to visualize the magnitude and direction of their potential effect on *Varroa* prevalence.

Universal (regression) kriging is a two-part process which combines a generalized regression model of the dependent variable with kriging interpolation of the residuals over a geographic area. The regression model, to estimate the influence of an independent variable(s), is fit first using ordinary least squares, then the covariance function of the residuals is used to

TABLE 2 Descriptive statistics of the dependent and independent variables used.

Variable	Obs.[i]	Mean	Std. Dev.	Min.	Max.
Dependent					
Varroa destructor Rate	1,082	0.863	2.65	0	51
Independent					
Regional Colony Density	1,370,880	2.17	2.82	0.01	14.7
Developed Land (%)	1,370,880	0.09	0.12	0.01	0.95
Forageable Land (%)	1,370,880	0.35	0.13	0.002	0.76
Non-Forageable Land (%)	1,370,880	0.23	0.17	0	0.84
Natural Land (%)	1,370,880	0.27	0.18	0.01	0.98
Water Coverage (%)	1,370,880	0.02	0.07	0	0.69

[i]independent variable observation counts represent the grid resolution of the raster.

derive generalized least squares coefficients from which the residuals can be re-estimated iteratively (Hengl et al., 2007). The variogram is then modelled for the residuals and kriging is performed to predict the regression model residuals over the study area. The predicted residuals are then combined with the regression output, using a spatially continuous raster of the independent variable(s), to produce a continuous prediction of the *Varroa* rate. Residuals from the final selected GLM model were obtained and the corresponding variogram of residuals was estimated through weighted least squares estimation (WLSE) using initial nugget, sill, and range parameters from visual inspection of the empirical variogram. A spherical variogram model was used to represent the GLM residual variogram. Following the fit of the regression model, and variogram model, universal kriging was applied to predict the prevalence of *Varroa* mites onto a grid covering the entire study area for mapping.

All analyses, unless otherwise stated, were performed using the open-source software R (R Core Team, 2020). The package "gstat: Spatial and Spatio-Temporal Geostatistical Modelling, Prediction and Simulation" was used to perform kriging (Pebesma, 2004; Gräler and Pebesma, 2016).

Results

A total of 3,786 observations were collected over the 5-year study period from 2015 to 2019 at 1,082 unique locations. The annual number of observations declined from 1,030 in 2015, to a total of 939, 757, 551, and 509 inspections conducted in 2016 to 2019, respectively. The observed annual prevalence of *V. destructor* cases (≥ 3 mites per 100 bees) varied during the 5-year study period around an average of 13.6% of colonies sampled. From 2015 to 2019, the prevalence estimates of *Varroa* cases in Ontario were 21.1% (95% CI [18.6, 23.7%]), 8.9% (95% CI [7.2, 11.0%]), 16.3% (95% CI [13.7, 19.1%]), 4.2% (95% CI [2.7, 6.2%]) and 15.2% (95% CI [12.1, 18.5%]) respectively. 95% confidence intervals were derived using Z-scores and the observed mean and standard deviation. Complete descriptive statistics of the dependent and independent variables used in regression modelling is presented in Table 2.

Spatial distribution of *Varroa* in Southern Ontario and determination of *Varroa* mite clustering

Producing a point map of the locations of cases (colonies infected by ≥3 mites per 100 bees) and controls illustrates that the locations of inspections in Southern Ontario during the study period are geographically diverse. Furthermore, sample sites are representative of the provincial colony density, with a greater apparent number of inspections in the Niagara Peninsula and fewer observations in the northeast. Cases appear to be present across the entire study area (Figure 1).

Plots of the D-function for each of the study years, and the entire study period combined, indicate the presence of spatial clustering of cases, as illustrated by crossing the 95% confidence limit (Figure 2). The distance at which spatial clustering was detected is not consistent over the study period, with 2015 demonstrating the largest range of clustering at approximately 100 km. Subsequent years to 2015 demonstrated noticeably lesser degrees of spatial clustering with 2018 indicating negligible amounts of clustering present. The 2016, 2017, and 2019 years of data all showed relatively equal results of clustering at an approximate range of 10 km. When the data from the 5-year study period were combined, spatial clustering of *Varroa* cases was detected by the D-function at a range of approximately 25 km (Figure 2).

Detection of high-risk clusters of *Varroa* mite prevalence

At least one and up to three spatial clusters of *Varroa* cases were detected for each year in the study period (Table 3).

Figure 3 shows the locations of high-risk clusters detected in each year of the study period combined to a single map of the province. The map gives an indication of a temporal stability of clusters in the northwestern quadrant of Southern Ontario, with some satellite clusters occurring sporadically elsewhere throughout the study area. All observed clusters presented a standardized morbidity ratio (SMR) of greater than 2 with a maximum observed SMR of 12.19 (95% CI: 3.85, 25.23; Table 3).

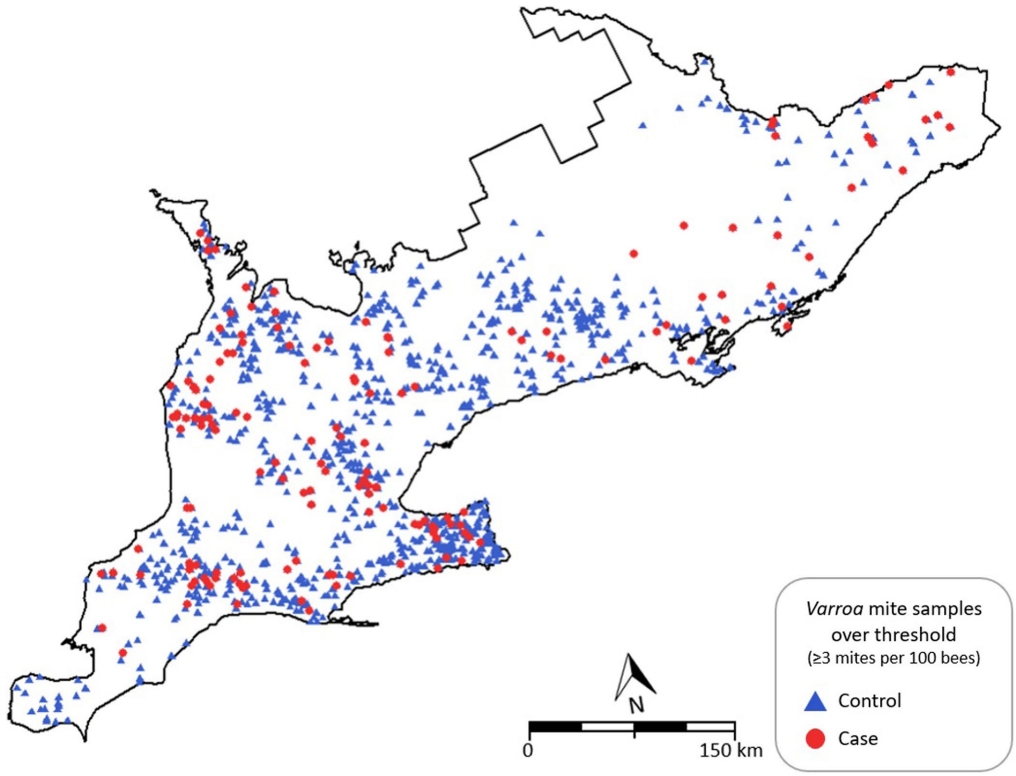

FIGURE 1
Point map of Southern Ontario indicating *Varroa destructor* counts above threshold (≥3 mites per 100 bees) as cases in red circles and controls in blue triangles, 2015–2019.

Spatial regression modelling of *Varroa* prevalence using environmental covariates

For regression modelling, the annual data were aggregated over time. Preliminary simple GLMs indicated no evidence of an association between *Varroa* rate and any of the five land-use types. The estimated regression coefficients (β) for the 5 land-use variables all possessed large confidence intervals at the 95% level, and *p*-values larger than 0.5. Regional colony density provided minimal evidence for a small negative association, with an estimated regression coefficient of $\beta = -0.05$ (95% CI: −0.11, 0.01, $p = 0.09$), indicating a decrease in *Varroa* rate by 0.05 for an increase of 1 colony per square kilometer. The degree of northing also indicated some evidence for a positive association with *Varroa* rate [$\beta = 1.5$; 95% CI: (−0.23, 3.23); $p = 0.09$] but sufficient evidence of an association was not found for easting, indicating little evidence of a large-scale spatial trend across the study area. A forest plot of the results from the preliminary simple regression models is presented in Figure 4. The model with regional colony density as the sole independent covariate produced the lowest AIC and was put forward in the regression kriging process. Multiple regression modelling, by backwards model selection, did not result in a better fitting model.

The variogram estimated from the final GLM model residuals is presented in Figure 5. A spherical variogram model with parameters: nugget = 2.98, partial sill = 4.17, and range = 27.58 km sufficiently represents the spatial correlation structure of the residuals of the GLM model (Figure 5).

The predicted values of the *Varroa* rate derived from the spatial regression model ranged from 0 to 15.9 (μ: 0.11), compared to the observed *Varroa* prevalence range of 0 to 51 (μ: 0.86). Model fit was assessed using leave-one-out cross-validation; no evidence for lack of fit was indicated by the histogram of residuals or map of residuals. The RMSE = 2.7 appears large compared to the *Varroa* prevalence but this is an effect of a few outliers (MAE = 0.007). Predicted values from the model for the whole study area are presented as an isopleth map in Figure 6. The map indicates a heterogenous spread of *Varroa* across the study area with several areas of increased risk. The locations with the greatest estimated risk both reside in the mid-north-east region of Southern Ontario near the municipalities of Peterborough and Bancroft. Several other areas across the study area showed high *Varroa* rates compared to their surroundings. Most of Southern Ontario was predicted to have an overall low rate of *Varroa* mites (Figure 6).

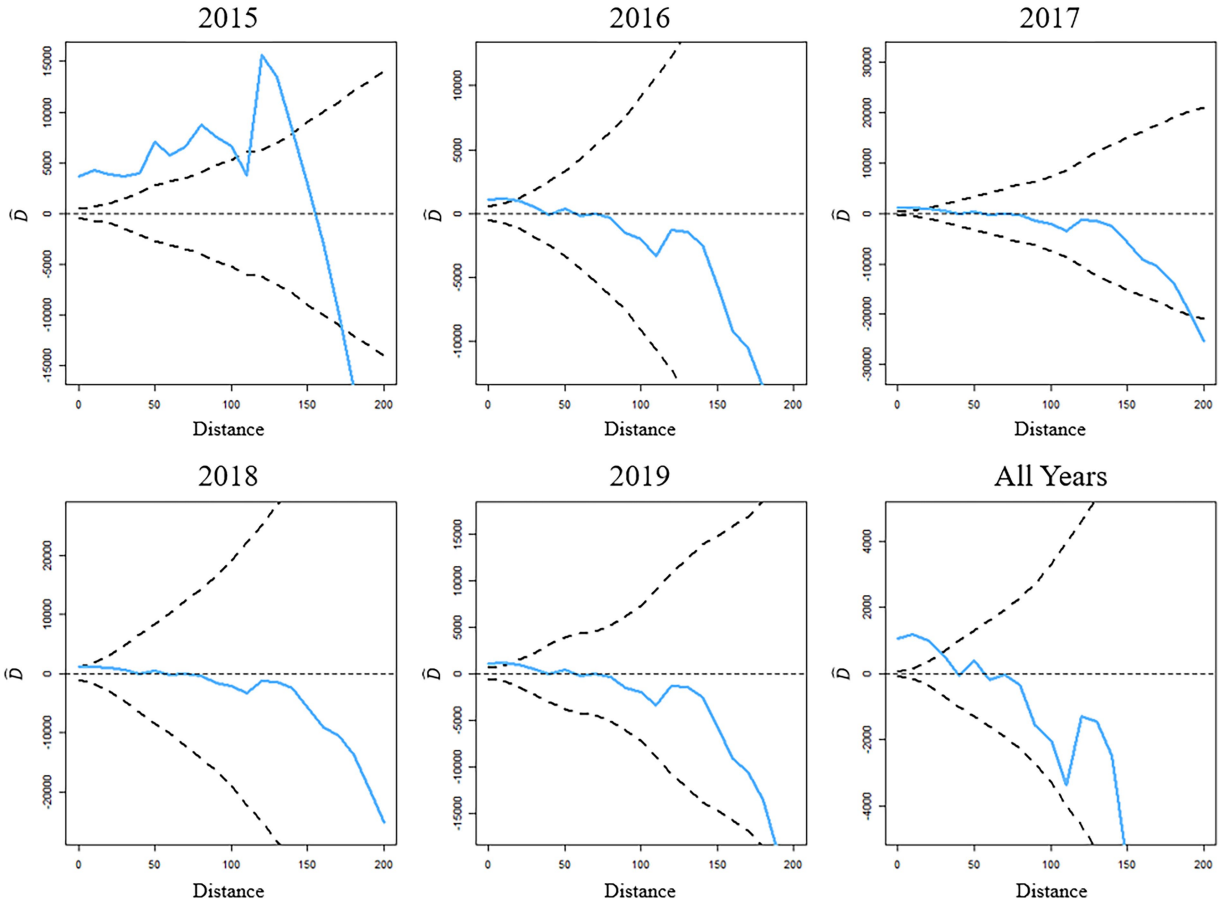

FIGURE 2
D-Functions for inspected colony locations in Southern Ontario with *V. destructor* counts equaling or exceeding 3 mites per 100 bees for each of the study years and the 5-year study period combined. Upper and lower 95% confidence limits derived from MCMC are indicated by the dotted lines.

TABLE 3 Results from spatial scan analysis for clusters of high-risk of *Varroa destructor* cases in Southern Ontario (2015–2019).

		High-risk clusters				
Year	**Cluster**	**Coordinates**	**Radius (km)**	**Standardized morbidity ratio**	**SMR (95% CI)**	**P-Value**
2015	1	(773.9, −587.9)	71.80	2.25	(1.75, 2.81)	0.001
	2	(338.1, −806.7)	28.80	3.95	(2.41, 5.86)	0.001
	3	(402.1, −918.5)	2.20	4.58	(1.95, 8.29)	0.018
2016	1	(370.1, −852.1)	16.89	12.19	(3.85, 25.23)	0.002
	2	(730.0, −698.7)	33.09	4.33	(2.15, 7.27)	0.019
2017	1	(326.3, −771.3)	13.61	7.14	(3.67, 11.76)	0.001
	2	(401.0, −757.1)	50.50	2.57	(1.52, 3.90)	0.046
2018	1	(429.3, −792.9)	48.96	9.58	(5.07, 15.46)	0.001
2019	1	(315.8, −920.2)	11.27	6.28	(3.77, 9.41)	0.001
	2	(675.7, −775.1)	48.19	4.38	(2.45, 6.89)	0.001

Discussion

This is the first study to comprehensively assess the spatial distribution of *Varroa destructor* in managed Ontario bee colonies at a population level. This study provides insight into all three aspects of the epidemiological triad: host, agent, and environmental risk factors for *Varroa* prevalence.

Spatial clustering of *Varroa* infestations were detected using the D-function (case–control data) and similarly through the estimation of the variogram (*Varroa* count data). Both methods

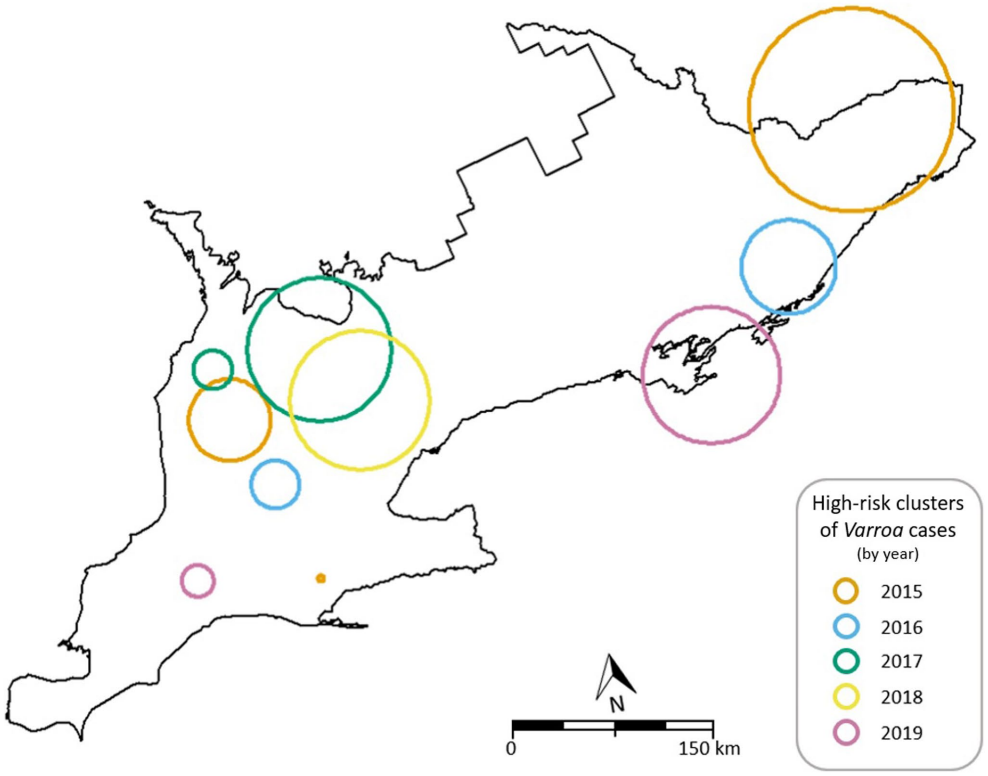

FIGURE 3
Map of the detected high-risk clusters of *V. destructor* cases by year in Southern Ontario using the spatial scan statistic.

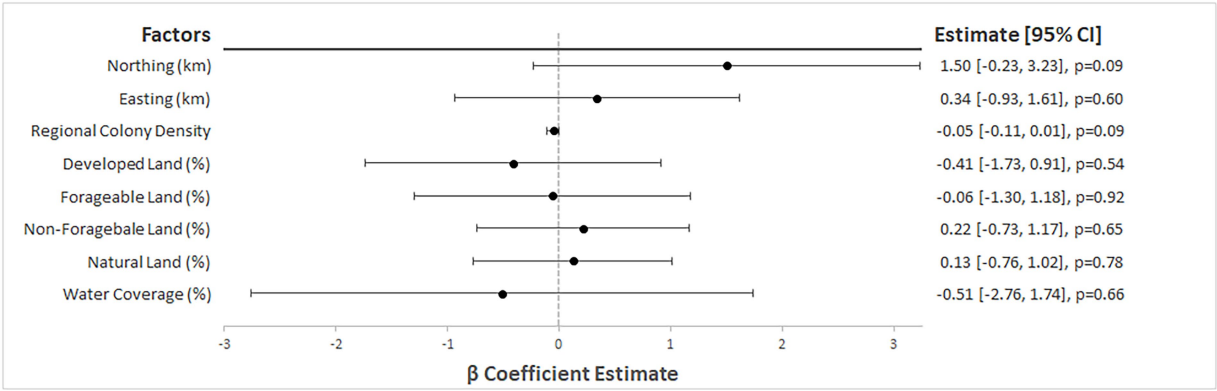

FIGURE 4
Forest plot of estimated beta coefficients from preliminary simple linear modelling of *V. destructor* rate in Southern Ontario managed honey bee colonies (2015–2019).

presented results of clustering occurring up to a range of around 25 km. In this context, clustering is indicative of the geographic extent to which *Varroa* mites are communicated between colonies of bees, be it through natural contact and exchange during foraging or through the relocation of colonies throughout the season. The nature of mite exchange was not identified in this study.

The distance at which clustering was observed in individual years varied noticeably, ranging from 100 km in 2015 to 10 km in 2016, 2017, and 2019. Only in 2018 was no spatial clustering

observed. This discrepancy might be attributed to small sample sizes, or low *Varroa* case prevalence observed in 2018 (4.2%) compared to the 5-year average case prevalence (13.6%). Without an adequate representation of both cases and controls, in terms of numbers and spatial sampling intensity, there may be a lack of power to detect spatial clustering. When all inspection data were aggregated over the five-year study, effectively increasing the sample size and spatial representation of cases and controls, the D-function provided evidence of spatial clustering upwards of 25 km (Figure 2). According to the geospatial epidemiologic triad, clustering can be thought of as a representation of agent factors, and the agent's tendency to spread within localized areas, which is common for infectious diseases. *Varroa* mites are communicable between bees through the environment and within yards (Rosenkranz et al., 2010). This passing of mites between colonies is limited by the foraging range of the honey bees, and the number of contacts (with other bees, or colonies) in the vicinity of an infested colony (Rosenkranz et al., 2010), and thus spatial clustering might occur in a semi-localized range, as observed of around 25 km. While a single bee may have a limited flight radius of up to 10 km (Beekman and Ratnieks, 2000), mites may be passed along a chain of colonies to reach further distances during the year. Furthermore, colonies and equipment may be moved even further distances during the beekeeping season, extending the possible range of transmission.

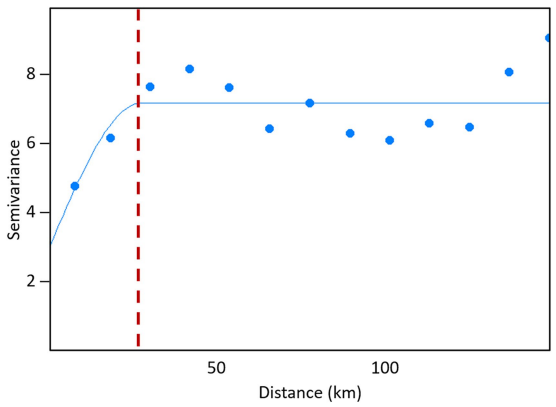

FIGURE 5
Variogram of generalized linear model residuals (points), with spherical variogram model (solid blue line) and spatial correlation range (red dashed line) for sampled rates of *V. destructor* mites in managed honey bee colonies in Southern Ontario (2015–2019).

This finding of spatial clustering of *Varroa* mite infestations offers the basis for a *Varroa* notification system where beekeepers could be notified if elevated mite levels are detected in the immediate vicinity of their colonies (25 km). Thus, allowing for

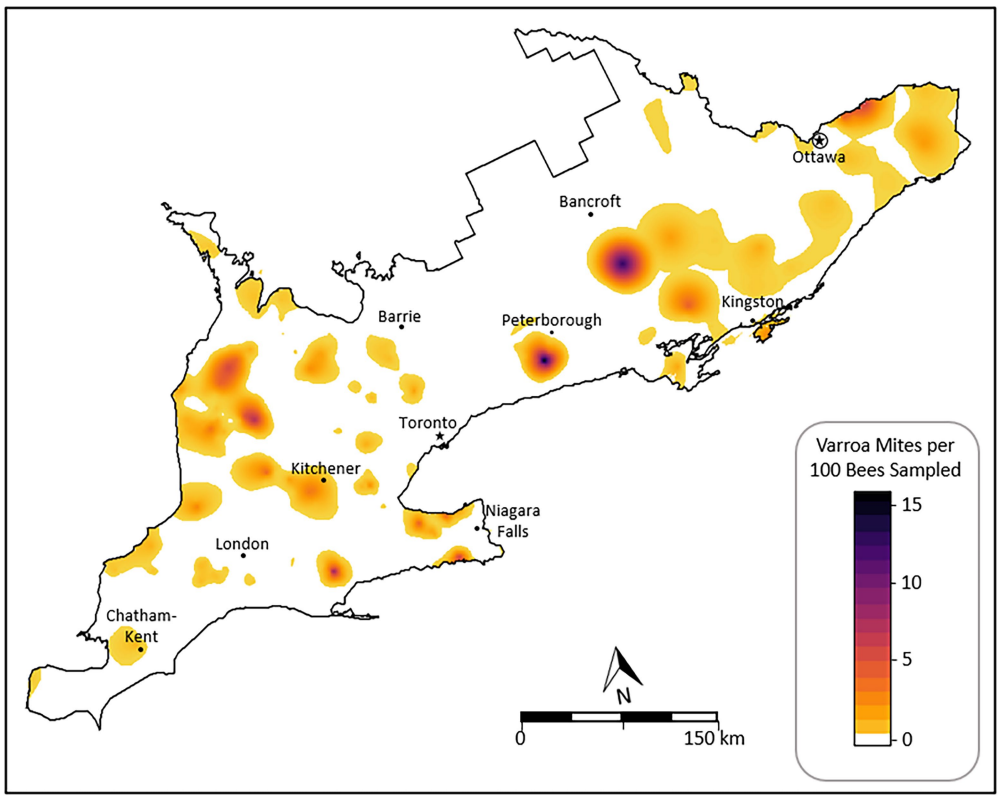

FIGURE 6
Isopleth map of predicted *V. destructor* rates for Southern Ontario derived from spatial regression modelling.

more intensive monitoring of their colonies to detect an increase in mite load early, and allow for risks to be mitigated to prevent further spread and colony weakening.

Several high-risk clusters were identified in this study for all years studied. All but 3 of the 10 observed clusters were found to have a SMR with a lower 95% confidence limit of greater than 2, indicating at least a doubling of the rate of *Varroa* cases than expected. The specific locations of these clusters varied from year to year, but recurring patterns were seen as well. Most notable was the reoccurrence of clusters of cases in the northwestern quadrant of Southern Ontario for 4 of the 5 years studied (Figure 3). This grouping of observed clusters covers a large area but provides evidence to suggest that there is temporal stability of *Varroa* clusters in this region. This region possesses one of the higher honey bee colony densities in Ontario (Sobkowich et al., 2021) which could explain the higher-than-expected rates of *Varroa*, as population density has been suggested to play a role in *Varroa* transmission (Rosenkranz et al., 2010). An increase in regional colony density would inherently result in an increase of susceptible colonies and an increased occurrence of robbing, drifting, and other intra-colony bee interaction events which have all been suggested as viable means of mite transmission (Peck et al., 2016; Peck and Seeley, 2019; Kulhanek et al., 2021). However, conflicting to this is the lack of observed clusters in the Niagara peninsula (southeastern most region of Southern Ontario), which possesses the highest colony density in the province (Sobkowich et al., 2021).

No clusters of *Varroa* infested colonies were identified in the Niagara region in the 5 study years, which could provide evidence against the hypothesis linking population density to *Varroa* prevalence. Similarly, the regression analysis showed a mild negative correlation between *Varroa* rate and colony density, which is contrary to what would be expected in support of this hypothesis. However, the colony density values used are based upon self-reports from colony registration and therefore may be representative of stationary colonies or overwinter locations but not the locations in which colonies spend the majority of the season.

Potential bias might exist since a large proportion of honey bee colonies in Niagara belong to large-scale commercial operations, offering mobile pollination services to other provinces throughout the beekeeping season. Colonies are screened for *Varroa* before being moved for pollination services, and therefore there may be an inflation of low *Varroa* count observations, and a simultaneous overestimation of colony density, as commercial operations treat their colonies before the inspection to ensure a satisfactory result for travel. This is largely but not always the case. Additionally, blueberry pollination in Eastern Canada, occurring each spring is a large draw for commercial beekeepers in Ontario offering pollination services. Given the known population dynamics of *Varroa* mites, screening for mites in the spring is likely to produce a bias towards low counts (Fanelli and Tizzani, 2020). Many beekeepers intending to mobilize their bees in the spring may choose to operate in Niagara due to the more southern location to build colony strength earlier in the season compared to cooler climates elsewhere. Therefore, the hypothesis of a relationship between *Varroa* prevalence and colony density cannot be rejected considering the nature of the current data (i.e., based on registration locations rather than foraging locations of colonies).

Natural land had been hypothesized to increase the odds of varroosis due to transmission of mites from feral colonies (Peck et al., 2016), but was not found to be associated with *Varroa* rate in the regression analysis. Chemurot et al. (2016), in Uganda, proposed a relationship between colony placement in farmland and *Varroa* prevalence which was also not observed in the current study. None of the land-use covariates examined in this study showed sufficient evidence of an association with *Varroa* rate, suggesting that other factors have stronger effects on *Varroa* prevalence such as beekeeping management practices, including control measures, abiotic factors that fluctuate over time such as weather, or biotic factors such as mite and bee behaviours. Time-dependent factors, such as temperature or precipitation, were not accounted for in this analysis but may lend themselves well to time-series modelling approaches.

The isopleth map of *Varroa* rates (Figure 6) illustrates an overall low rate for Southern Ontario with sporadic high-rate areas throughout the province. Notably, the high-rate area south of Bancroft (Figure 6) exists in an area of low sampling as seen in Figure 1 and therefore may be an overprediction of the true rate. The North-western quadrant of the study area exhibited several clusters over the 5-year study period (Figure 2) when using varroosis case locations based on the 3-mite threshold. This pattern is similarly illustrated by the kriged map where the *Varroa* rate is shown to be greater overall compared to the rest of the study area (Figure 6). In the region south of Peterborough, a high rate was estimated by spatial modelling, which contrasts to the findings seen through cluster detection as no cluster of varroosis cases were observed in this area. This contradiction is likely the result of a repeat of high *Varroa* count samples each year, but not multiple high *Varroa* count samples in a single given season. Furthermore, the difference in the data structure used in this study (i.e., binary case and control data used for cluster detection and *Varroa* rates for spatial modelling) could have led to differences in data analysis results. This problem has been termed the modifiable areal unit problem and is a common source of bias in geostatistical studies such as the present work (Waller and Gotway, 2004).

Studies such as this are reliant on large sample sizes, accurately recorded, and serving as a representative sample for the target population. Inspections are not truly random samples and may be biased in some cases towards beekeepers with higher *Varroa* loads or beekeepers better skilled at treating for pests and diseases (i.e., commercial operations). In cases where an inspection is requested by the beekeeper to address recent issues with their colonies, there is a greater likelihood that *Varroa* may be present, as *Varroa* is recognized as a common pest and is responsible for colony weakening and reduced hive activity (Barroso-Arévalo et al., 2019). On the other hand, in cases that an inspection was

requested for the purpose of verifying the disease-free status of colonies to be sold as queens and nuclei, there may be a bias towards lower levels of *Varroa*. Routine apiary inspections also occur and are expected to be more representative of the true population but are still prone to sampling and measurement bias. The reason for colony inspection was not explored in the present work but should be explored in future studies.

A limitation of this study is inconsistent sampling locations from year to year. In order to sample Ontario beekeepers representatively, inspections cannot be guaranteed to occur in the same geographical locations every year. For this reason, one region may be over-sampled one year and under-sampled in the next as inspectors may choose to group inspections by proximity. This limitation was the primary reason for the decision to treat the annual data as a whole during geospatial modelling rather than 5 distinct years. This also suggests that there may be years where a high-risk cluster exists but is not detected, since sufficient repeat sampling did not occur in that region over the year. This could explain the absence of clusters in the Peterborough region despite a high predicted risk through modelling. The supposed grouping of inspections may also explain some of the clusters observed in this study, but the consistency and significance of clusters observed over 5 years suggest that a true effect may be in place. Further studies could address this limitation through the use of a continuous cohort of colonies spread across the study area, rather than the repeated cross-sectional sampling approach used in this study. Furthermore, there are various predispositions and factors related to mite infestations that were not able to be assessed, including: biological controls (e.g., brood removal to limit opportunities for mite reproduction), cultural controls (e.g., beekeeper selection towards hygienic queens and stocks), and chemical controls (e.g., administration of *Varroa* control treatments). Data regarding these various factors was not available for this study but all would be expected to influence mite counts (Harbo and Harris, 2009; Rosenkranz et al., 2010; Vidal-Naquet, 2018). However, this study focused on population level and environmental factors and therefore the omission of these factors is deemed acceptable, under the assumption that they are spatially independent. Future studies may want to consider these factors if collecting primary data.

With advancements in communications and the low cost and absence of necessary technical tools to sample bees for *Varroa* mites, the collection of these data lends itself well to a citizen science approach (Thomas-Bachli et al., 2020; Khayli et al., 2021). With the implementation of citizen science and self-reported *Varroa* mite counts by beekeepers, agencies can achieve a greater number of observations per year, and cover a greater spatial area, without the need to increase inspector resources. Furthermore, this approach frees up inspectors to allow for more strategic sampling and respond to inspection requests from operations experiencing difficulties. Skepticism exists around the quality of self-reported data, but evidence exists to suggest that citizen science approaches can produce data that are equal to or greater than the quality obtained by professionals (dependent on the difficulty of data collection, upon other factors; Kosmala et al., 2016).

This study provides evidence for temporally stable clusters of varroosis throughout Southern Ontario, which were not explained sufficiently by the environmental factors considered in this study but suggest that there are environmental (i.e., meteorological) and management influences at play. A spatial clustering effect was also observed, suggestive of the transmission patterns of *Varroa* mites and the influence that neighbouring yards have on each other's mite counts. The results of this study provide a launch point to further assess the spatial patterns of *Varroa* identified. Intervention efforts should focus on areas of Southern Ontario exhibiting clusters of excess *Varroa*, and especially the regions in the northwest, where clusters appear over multiple years. The predicted risk map identifies areas where *Varroa* is likely to exist at elevated levels and therefore highlights the need for more data and investigation to identify the cause of these increased *Varroa* rates. It is recommended that inspections and intervention programs focus their efforts on these areas, while citizen science efforts could provide data elsewhere in the province, resulting in an enhanced province wide *Varroa* surveillance system.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Data sharing agreement does not permit the sharing of these data. Requests to access these datasets should be directed to PK, paul.kozak@ontario.ca.

Author contributions

KS and OB: study conception and design, analysis and interpretation of results. PK: data collection. KS, OB, TB, DP, and PK: draft manuscript preparation. All authors reviewed the results, contributed to the article, and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Informing policy and practice on insect pollinator declines: Tensions between conservation and animal welfare

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Climate change, agricultural intensification, and other anthropogenic ecosystem challenges have caused declines in the diversity and abundance of insect pollinators. In response to these declines, entomologists have called for greater attention to insect pollinator conservation. Conservation primarily aims to protect groups of non-human animals—populations or species—with only secondary concern for the welfare of individual animals. While conservation and animal welfare goals are sometimes aligned, they often are not. And because animal welfare comes second, it tends to be sacrificed when in tension with conversation priorities. Consider, for example, lethal sampling to monitor many pollinator populations. Growing evidence suggests that the welfare of individual insect pollinators may be morally significant, particularly in the Hymenoptera and Diptera. Considering insect welfare in conservation practices and policies presents many challenges as, in the face of rapid, anthropogenic change, it may be impossible to avoid harming individual animals while promoting diverse populations. We suggest some practical, implementable strategies that can allow for more robust integration of animal welfare goals into insect pollinator conservation. By following these strategies, entomologists may be able to find policies and practices that promote the health of ecosystems and the individual animals within them.

KEYWORDS

ethics, pollinator conservation, insects, animal welfare, monitoring programs, green infrastructure, policy

Introduction

Insect pollinators are in peril: anthropogenic climate and habitat changes have caused abundance, diversity, and body size declines, as well as range, phenology, and ecological relationship shifts (Cane et al., 2006; Bartomeus et al., 2011; Kuhlmann et al., 2012; Burkle et al., 2013; Barrett and Johnson, 2022; Turley et al., 2022). In

the face of this rapid and unprecedented biodiversity crisis (Schachat and Labandeira, 2021), entomologists have called for greater attention to pollinators in conservation and policymaking.

Conservationists aim to maintain biological diversity, ecological health, and ecosystem integrity (Trombulak et al., 2004). These goals require focusing on populations and species—groups of non-human animals—with secondary concern for the welfare of the individual members of those groups. Although conservation goals are sometimes aligned with individual welfare—that is, how a single organism is faring from its own subjective perspective—they can conflict too. For instance, population control measures may enhance ecosystem integrity while causing harm to the individual animals being controlled. While such measures may be necessary, conservationists are increasingly concerned with minimizing such harms (Dubois et al., 2017; Sekar and Shiller, 2020). Our aim here is to consider the prospects for harm minimization in the context of conserving insect pollinators.

The welfare of sentient organisms—i.e., organisms with the capacity to feel pain (Singer, 2002)—matters morally. There is currently no scientific consensus on insect sentience (Adamo, 2016; Klein and Barron, 2016; Birch, 2020; Chittka, 2022; Gibbons et al., 2022). However, two important groups of insect pollinators– the Hymenoptera (including wasps and bees), and the Diptera (including flies) –meet many of the criteria in the Birch et al. (2021) framework for assessing animal sentience. Using this framework to review over 300 scientific studies of insect neurobiology and behavior, Gibbons et al. (2022) found "substantial evidence for sentience" in Hymenoptera and "strong evidence for sentience" in Diptera. There is also "strong evidence for sentience" in decapod crustaceans, which guaranteed their protection by the UK government in the 2022 Animal Welfare (Sentience) Act (Birch et al., 2021). Though not decisive, there is reason to take the possibility of insect sentience, and thus welfare, seriously. A precautionary approach could involve making efforts to minimize possible harm by treating insects as though they are sentient while collecting additional data (Fischer, 2016, 2019; Birch, 2017). Since most insect conservation efforts are not structured around a precautionary approach, adopting such a stance could have significant implications for the design and implementation of interventions, programs, and policies—as we will demonstrate in the following section.

Potential conservation-welfare conflicts for insect pollinators

Coordinated action to improve wild pollinator conservation first became highly publicized in the United States through "The Forgotten Pollinators" book and Arizona-Sonora Desert Museum pollinators' campaign (Buchmann and Nabhan, 1996). Since the 1990s, numerous pollinator conservation actions and policies have been implemented or proposed (Williams, 2003; Byrne and Fitzpatrick, 2009; Hall and Steiner, 2019; Marselle et al., 2020; Stout and Dicks, 2022). In the next two subsections, we briefly review two examples that highlight tensions between conservation goals and individual insects' welfare. We also suggest some ways that those tensions might be reduced *via* adjustments to standard practices or policies.

Monitoring programs that include lethal sampling

Most proposals for conserving insect pollinators include the need to increase community-level monitoring efforts (Dicks et al., 2016; Woodard et al., 2020; but see Tepedino and Portman, 2021) to inform interventions and assess their impact. Monitoring programs often include passive lethal sampling methods, such as pan traps, that keep field labor, expenses, and expertise relatively low while allowing for subsequent species-level identifications. Biweekly tests of pan traps, combined with sweep-netting, suggest that they do not affect long-term trends in bee abundance or diversity (Gezon et al., 2015; but see vane traps, Gibbs et al., 2017).

However, lethal monitoring programs present obvious welfare problems (Fischer and Larson, 2019). Insects drown, starve/desiccate, or die *via* poisoning, all of which may induce pain and stress. This is a special problem for "bycatch" insects, which comprised nearly 63% of individual captured arthropods in pan traps in Gonzalez et al. (2020). These insects are rarely used to generate data and are often discarded, offering no clear conservation benefit and constituting a negative welfare impact. Additionally, conservationists are increasingly concerned about the impact of lethal monitoring on target species (Tepedino and Portman, 2021; Montero-Castaño et al., 2022), particularly those that are vulnerable or threatened: a case study of North American bumble bees showed an increase in the number of lethal collections since the 1990s, even though data demonstrating taxonomic resilience of many recently imperiled bumble bee species are sparse (Miller et al., 2022).

There are several ways to reduce the welfare costs of monitoring initiatives. First, researchers could focus on developing protocols that minimize bycatch. For example, smaller pan traps reduce bycatch without changing bee monitoring efficacy (Gonzalez et al., 2020). Second, making bycatch (and target; Montero-Castaño et al., 2022) specimens/data more accessible could reduce the necessity for other lethal sampling studies (Spears and Ramirez, 2015;

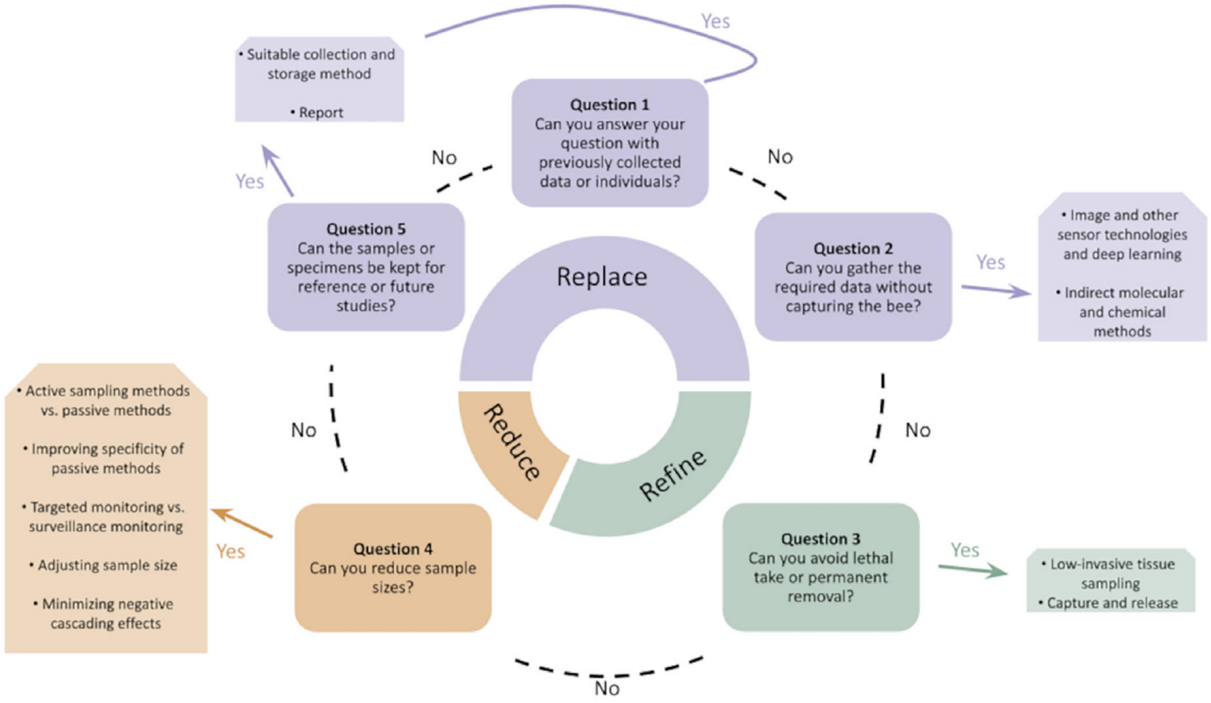

FIGURE 1
Decision-making framework for replacing, refining, and reducing lethal bee captures in monitoring programs, reproduced from Montero-Castaño et al. (2022) (CC BY 4.0).

Fischer and Larson, 2019). Third, scientists could reduce suffering in lethal monitoring programs by hastening insect time-to-death *via* different (or increased concentrations of) lethal agents.

There is little guidance available about the appropriate level of temporal or spatial sampling effort for many monitoring initiatives (but see: Lebuhn et al., 2013). Likewise, there is little guidance about how to handle biases and deficiencies in particular methodologies (Cane et al., 2000; Baum and Wallen, 2011; Didham et al., 2020), which may lead to "more is better" or "all of the above" approaches (Rhoades et al., 2017; Portman et al., 2020). However, sampling that does not provide additional, action-relevant information to support conservation goals should be avoided for welfare, conservation, and cost/storage/effort reasons (Droege et al., 2016; Tepedino and Portman, 2021). Consider the thousands of *Dialictus* (Halictidae) that are collected in pan traps and often go unidentified to species due to the lack of available taxonomic expertise. Most of these individual bees offer little value to monitoring and conservation efforts (Portman et al., 2020), yet represent a significant negative welfare impact. To avoid over collection, models built from meta-analyses of capture data in different habitats with different methods could be used to estimate the actual sampling effort (temporal frequency, sites, methods) required to answer specific questions of interest before establishing a sampling protocol.

Additionally, scientists could switch wholly or partially to non-lethal sampling methods depending on research needs; when new methods are non-invasive, this may support both welfare and conservation goals (Montero-Castaño et al., 2022; **Figure 1**). Expert transects, where taxonomic experts go into the field to collect data on insect diversity using transects, can produce similar species accumulation curves as pan traps for hoverflies and bees in some habitats while collecting far fewer individuals (O'Connor et al., 2019; but see Rhoades et al., 2017). Conservation and insect welfare goals are thus also aligned in the need to train additional taxonomic experts (Hopkins and Freckleton, 2006) that could support less-lethal monitoring programs. Developing/validating new, non-invasive methods (like eDNA; Thomsen and Sigsgaard, 2019) or using a community- (e.g., "citizen")-science-driven photographic BioBlitz (Bickerman-Martens et al., 2017; or iNaturalist-style databases, Gazdic and Groom, 2019) could also support conservation-relevant data collection. Barlow and O'Neill (2020) and Miller et al. (2022) review other non-lethal techniques not yet widely employed for pollinator monitoring, including: telemetry/radar, automated visual monitoring, machine-learning identification, molecular analyses, acoustic monitoring, and fecal sampling.

Finally, some scientists have suggested that large-scale, community-level monitoring may be overemphasized for obtaining conservation-relevant data on pollinator ecology (Tepedino and Portman, 2021). Population-level studies of a few, carefully chosen pollinator species that are field- or photograph-identifiable could serve just as well for answering *many* action-relevant questions (Portman et al., 2020; Tepedino and Portman, 2021; Dorian and Crone, 2022). Visual monitoring is already used for some large and easily identifiable groups such as bumble bees and certain butterflies (Montgomery et al., 2021), alongside netting in areas where lethal sampling might harm endangered species (Portman et al., 2020; and see non-lethal protein mark-recapture for vulnerable species, Boyle et al., 2018).

Creating diverse habitats in agricultural areas

Habitat fragmentation/simplification caused by agricultural intensification can negatively impact pollinator foraging activity and movement, thereby reducing abundance and diversity. This has led to calls for increasing "green infrastructure" for biodiversity maintenance in agricultural areas (Brown and Paxton, 2009; Dicks et al., 2016), which may include native plants alongside agricultural fields (Williams et al., 2015) or creating habitat corridors to allow for increased movement across resource-poor areas (Blüthgen et al., 2022). Green infrastructure may provide welfare benefits to wild pollinators by increasing resource availability and diversity, with positive impacts on health (St. Clair et al., 2020). However, increasing the proximity of wild pollinators to agricultural areas also harms the many animals newly inhabiting these spaces through increased exposure to agrochemicals with lethal or sublethal welfare effects (Susan et al., 2019) and other anthropogenic welfare challenges (e.g., exposure to light pollution and vehicle strikes near road verges; Phillips et al., 2019; Owens et al., 2020).

Some of these welfare effects could be mitigated by incentivizing simultaneous reductions in agrochemical usage, alongside the diversification of agricultural systems and creation of pollinator protection zones in areas where green infrastructure will be created (which may also support honey bee welfare; St. Clair et al., 2020). While this additional incentive structure may reduce the total amount of green infrastructure that can be created, each incentive is both a conservation and welfare benefit to the pollinators in that area. This holistic approach to improving pollinator conservation *via* multiple means demonstrates one of the ways that policy might be re-structured (and re-budgeted) if welfare and conservation were considered simultaneously. Notably, some of these same issues (and urban heat island effects) will also affect wild bee populations in urbanized areas with greenspace development (Baldrock, 2020), but different incentive structures will be needed in these spaces.

Discussion

There is significant value in conserving species, populations, and ecosystems. It is also morally important to consider the welfare of non-human animals (Fischer, 2021), including many invertebrates (Koperski, 2022). We have demonstrated that conservation and welfare goals may sometimes conflict, as in the expansion of lethal pollinator monitoring programs and the creation of green infrastructure near some agricultural habitats. In many cases, it is not possible to achieve conservation goals without some harm to non-human animals. However, there appear to be ways for researchers, conservationists, farmers, and policymakers to reduce harms to non-human animals while pursuing their conservation goals. So, a precautionary approach to insect welfare is compatible with their aims (and, in some cases, may even help them achieve their aims: Capozzelli et al., 2020).

One way to promote harm reduction is to encourage welfare-oriented cost-benefit analyses in grant applications and conservation management plans—a practice that is familiar from environmental cost-benefit analysis (Atkinson and Mourato, 2008). In some cases, making the costs explicit may be sufficient to show that they are negligible relative to the potential welfare benefits.

In other cases, of course, it will be less clear what to prioritize. Eventually, then, it will be important to develop frameworks for comparing the relative importance of various costs (financial, temporal, etc.), specific conservation goals, and welfare impacts (e.g., more resource-intensive monitoring methods and the particular welfare impacts of those methods). One path forward involves developing tools that allow stakeholders to express the value they assign to avoiding negative welfare impacts in monetary terms, which could then be aggregated to determine how much stakeholders ought to be willing to pay to avoid causing those impacts (Lusk and Norwood, 2011). While economists, animal welfare scientists, and philosophers are in the early days of creating such tools—for insects and non-insects alike—entomologists can contribute to these efforts by studying insect welfare and quantifying the insect welfare impacts of different conservation practices.

In the interim, it is important simply to make welfare impacts on insects salient in discussions of conservation practices and policies. Insects warrant some consideration and

we can reduce many harms to them without compromising conservation goals.

Author contributions

MB: conceptualization. MB, BF, and SB: writing/editing. All authors contributed to the article and approved the submitted version.

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Are fungicides a driver of European foulbrood disease in honey bee colonies pollinating blueberries?

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Introduction: Blueberry producers in Canada depend heavily on pollination services provided by honey bees (*Apis mellifera* L.). Anecdotal reports indicate an increased incidence of European foulbrood (EFB), a bacterial disease caused by *Melissococcus plutonius,* is compromising pollination services and colony health. Fungicidal products are commonly used in blueberry production to prevent fungal diseases such as anthracnose and botrytis fruit rot. Pesticide exposure has been implicated in honey bee immunosuppression; however, the effects of commercial fungicidal products, commonly used during blueberry pollination, on honey bee larval susceptibility to EFB have not been investigated.

Methods: Using an *in vitro* infection model of EFB, we infected first instar honey bee larvae with *M. plutonius* 2019BC1, a strain isolated from an EFB outbreak in British Columbia, Canada, and chronically exposed larvae to environmentally relevant concentrations of fungicide products over 6 days. Survival was monitored until pupation or eclosion.

Results: We found that larvae chronically exposed to one, two, or three fungicidal products [Supra® Captan 80WDG (Captan), low concentration of Kenja™ 400SC (Kenja), Luna® Tranquility (Luna), and/or Switch® 62.5 WG (Switch)], did not significantly reduce survival from EFB relative to infected controls. When larvae were exposed to four fungicide products concurrently, we observed a significant 24.2% decrease in survival from *M. plutonius* infection (p=0.0038). Similarly, higher concentrations of Kenja significantly reduced larval survival by 24.7–33.0% from EFB (p<0.0001).

Discussion: These *in vitro* results suggest that fungicides may contribute to larval susceptibility and response to *M. plutonius* infections. Further testing of other pesticide combinations is warranted as well as continued surveillance of pesticide residues in blueberry-pollinating colonies.

KEYWORDS

pesticides, fungicides, European foulbrood, honey bees (*Apis mellifera*), blueberries

1. Introduction

Honey bee pollination is crucial to blueberry production in North America, contributing 90% of the value of Canada's blueberry crops each year (Government of Canada, 2018). Unfortunately, blueberry growers face a shortage of pollination services, in part due to a reported increased incidence of European foulbrood (EFB) disease in blueberry pollinating honey bee colonies

(Wardell, 1982; Guarna et al., 2019; Olmstead et al., 2019; Thebeau et al., 2022). The negative economic consequences of EFB outbreaks include lost honey, reduced pollination service revenue, and increased treatment and colony replacement costs (Laate et al., 2020), thereby threatening the continued profitability of the blueberry and beekeeping industries and calling for scientific investigation of the predisposing factors for this disease.

European foulbrood occurs when the Gram-positive bacterium *Melissococcus plutonius* colonizes the midgut of honey bee larvae and outcompetes the larvae for nutrition (Forsgren, 2010; Laate et al., 2020). Clinical signs of EFB include yellow to brown, twisted and/or deflated larvae; dead larvae that dry to form a rubbery scale on the back of the brood cell; and a sour odor from affected brood due to secondary bacterial infection (Cheshire and Cheyne, 1885). EFB often emerges in honey bee colonies when under stress. For example, in the early spring when nursing bee populations are low and pollen and nectar resources are scarce in the environment, the colony's brood may suffer from inadequate care and feeding, predisposing them to EFB (Forsgren, 2010; Kane and Faux, 2021).

Previously, colonies with EFB were observed to spontaneously recover when stressors such as inadequate food and water resources were alleviated (Forsgren, 2010); however, recent outbreaks of EFB associated with blueberry pollination have been described as refractory to traditional management practices (Olmstead et al., 2019; Laate et al., 2020; Thebeau et al., 2022). Proposed causes of the increased clinical severity of EFB include highly virulent (Djukic et al., 2018; Grossar et al., 2020; Thebeau et al., 2022) or antimicrobial-resistant strains of *M. plutonius* (Masood et al., 2022), and environmental factors such as the poor nutritional quality of blueberry pollen (Wardell, 1982; Olmstead et al., 2019); however, the role of pesticide exposure as a predisposing factor for EFB during blueberry pollination has been incompletely explored. For example, several studies have investigated the risk of pesticides on survival of honey bee brood through larval exposure to unrealistically high concentration of active ingredients (Mussen et al., 2004; Wade et al., 2019). Moreover, researchers studying synergistic effects of pesticides on larval survival have focused on combinations that also include insecticides with known negative effects (Prado et al., 2019; Wade et al., 2019; Wood et al., 2020). Nonetheless, the investigation of field-relevant concentrations of fungicide products containing proprietary ingredients has never been explored as a potential predisposing factor for EFB.

Fungicides are widely used in Canadian highbush blueberry production to prevent anthracnose and botrytis fruit rot (Everich et al., 2009; Province of British Columbia, 2022; Mussen et al., 2004). Frequently used fungicidal products include Supra® Captan 80WDG (Captan; active ingredient captan [*N*-Trichloromethylthio-4-cyclohexane-1,2-dicarboximide]), Kenja™ 400SC (Kenja; active ingredient isofetamid), Luna® Tranquility (Luna; active ingredients fluopyram and pyrimethanil) and Switch® 62.5 WG (Switch; active ingredients cyprodinil and fludioxonil) (Province of British Columbia, 2022). Captan is a broad-spectrum, dicarboximide fungicide (Mussen et al., 2004) that is commonly combined with Kenja, Luna, and Switch to prevent the development of resistance (Province of British Columbia, 2022). Modes of action of the active ingredients in these commonly used fungicidal products include succinate dehydrogenase inhibitors (isofetamid and fluopyram) which inhibit the mitochondrial electron transport chain (Umetsu and Shirai, 2020); anilinopyrimidines (pyrimethanil and cyprodinil) which inhibit methionine and protein biosynthesis (Fritz et al., 2003); and phenylpyrroles (fludioxonil) which disrupt cellular signal transduction (Bersching and Jacob, 2021).

Chronic exposure of honey bees to multiple fungicides during blueberry pollination is common (Graham et al., 2021; Guarna, 2021; Rondeau and Raine, 2022). A review of fungicide risk to bees identified a total of 90 different fungicides within North American and European honey bee colony derivatives, with the greatest number of fungicides present in pollen samples (Rondeau and Raine, 2022). Moreover, residue analysis of pooled honey and pollen samples from 3 to 5 colonies after blueberry pollination has confirmed concurrent detection of 4–5 fungicide residues within these colonies (Guarna, 2021), with fluopyram, pyrimethanil, cyprodinil, and fludioxonil detected in bee bread at concentrations up to 572 ng/g for fludioxonil. Fluopyram, pyrimethanil, cyprodinil, and fludioxonil have also been identified throughout Europe, North America, and Africa, with concentrations up to 16,400 ng/g fludioxonil reported in pollen (Rondeau and Raine, 2022). These four fungicides were also detected in blueberry pollinating colonies in the United States (Graham et al., 2021). Furthermore, residues of Captan have been found in concentrations as high as 18,970 ng/g in pollen collected from honey bee colonies pollinating crops, including blueberries, in the United States (Johnson et al., 2010; Mullin et al., 2010; Rondeau and Raine, 2022).

Chronic exposure to multiple fungicides may increase the susceptibility of honey bee colonies to EFB, considering that the exposure to combinations of agrochemicals has been found to elicit synergistic negative effects on honey bee adults and larvae (Johnson et al., 2013; Wade et al., 2019). For example, Wood et al. (2020) demonstrated chronic exposure to a fungicide and an insecticide decreased larval survival from EFB *in vitro.* Similarly, Bartling et al. (2021) showed that individual fungicide exposure decreased survival of adult bees infected with *Pseudomonas.* However, to our knowledge, there has been no investigation of potential synergistic effects of exposure to multiple fungicides on honey bee immunity and susceptibility to infectious disease.

Considering the chronic fungicide exposure of colonies pollinating blueberries and the previously reported negative effects of fungicides and adjuvants on honey bees, we urgently need to determine whether formulated fungicide exposure can explain EFB outbreaks during blueberry pollination in North America. Therefore, in this study, we used an *in vitro* larval infection model of EFB to investigate the effects of chronic exposure to four formulated fungicidal products commonly used in blueberry production on honey bee survival from EFB. Specifically, we sought to (1) determine the effects of field-relevant concentrations of individual fungicidal products on honey bee larval survival, (2) determine if individual fungicidal products increases mortality from EFB infection, and (3) determine if larvae co-exposed to combinations of two, three, or four fungicide products are more susceptible to EFB.

2. Materials and methods

2.1. Fungicide preparation

Four water soluble formulated fungicidal products were tested in this study: Supra® Captan 80WDG (Captan; Product 33,641, Lot BO9044965060, Terralink Horticulture, Abbotsford, BC, Canada), Kenja™ 400SC (Kenja; Product 31,758, Lot V31758-170324, Terralink

Horticulture), Luna® Tranquility (Luna; Product 30,510, Lot NK43HX1965, Terralink Horticulture), and Switch® 62.5 WG (Switch; Product 28,189, Lot YGM9C28004, Terralink Horticulture). Products were stored in their concentrated form (wettable granules or liquid concentrate) in opaque containers at room temperature until use. Stock solutions of diluted fungicidal products were prepared in sterile water and stored at 4°C for up to 1 week until incorporation in the larval diet.

2.2. Fungicidal product concentration range determination

To determine a field-relevant concentration range for the four fungicidal products used in the experiment, we used BeeRex (United States Environmental Protection Agency, 2015), a United States Environmental Protection Agency tool for terrestrial pesticide risk assessment for honey bees (Table 1). Using BeeRex (United States Environmental Protection Agency, 2015), the total exposure to the active ingredients of the four fungicidal products for 6-day-old worker honey bee larvae was determined in two ways; (1) by input of the foliar application rate (kg/hectare) for the preventative control against botrytis fruit rot in highbush blueberries (Province of British Columbia, 2022), and (2) through input of the maximum residue concentrations in pollen/bee bread and honey of the active fungicide ingredients (Mullin et al., 2010; Guarna, 2021). For products with two active ingredients (Luna [125 g/l fluopyram and 375 g/l pyrimethanil] and Switch [37.5% cyprodinil and 25.0% fludioxonil]), we used residue information for the active ingredients in lowest concentration in the fungicidal product (i.e., fluopyram and fludioxonil) to calculate the exposure. The residue concentration for Switch (fludioxonil) was not available for honey and was replaced with the maximum bee bread residue concentration in the BeeRex model. The residue concentrations for isofetamid in pollen/bee bread and honey were not available.

To determine the fungicide product concentrations to be tested *in vitro* (Table 1), we considered the field application rate of the fungicide product, as well as residue data where available (Table 1). For Captan and Kenja, the calculated fungicide product exposure based on the application rate (19,000 and 6,000 ng/bee, respectively) caused a significant reduction in larval survival (Supplementary Figure S1); therefore, 10-, 100-, and 1,000-fold dilutions were performed to obtain high, medium, and low concentrations, respectively, for *in vitro* testing (Table 1). For Luna (i.e., fluopyram) and Switch (i.e., fludioxonil), the 'high' concentration tested *in vitro* corresponded to the BeeRex-calculated concentration based on the application rates (1,800 ng/bee and 3,000 ng/bee, respectively), while the 'low' concentration tested *in vitro* corresponded to the BeeRex-calculated concentration based on maximum reported residues quantified in pollen/bee bread and honey sampled from blueberry-pollinating colonies (Table 1). Medium concentrations were not calculated for Luna and Switch as both the high and low concentrations did not significantly decrease larval survival from control. The final concentrations in the larval diet were calculated based on the concentration of the active substance consumed over the 6-day larval period (160 µl, Schmehl et al., 2016).

2.3. Preparation of *Melissococcus plutonius* for *in vitro* larval infection

Melissococcus plutonius isolate 2019BC1 (Wood et al., 2020; Masood et al., 2022) was used to infect honey bee larvae *in vitro*. To prepare this isolate for larval infection, 100 µl of thawed liquid culture of 2019BC1, previously stored at −80°C in 20% glycerol, was inoculated into 100 ml of KSBHI liquid media (brain heart infusion, supplemented with 0.15 M KH_2PO_4, and 1% soluble starch) and incubated at 37°C under microaerophilic conditions (Arai et al., 2012) for 48 h, shaking at 200 rpm. The liquid culture was then stored in 1 ml aliquots with 20% glycerol at −80°C. The CFU/mL of the culture was determined by using a thawed culture aliquot and plating serial dilutions on KSBHI agar with 3 µg/ml nalidixic acid (Arai et al., 2012). On the day of larval infection, an aliquot of liquid culture was warmed to 37°C and diluted in PBS to a concentration of 1.0×10^5 CFU/ml based on the previously determined CFU/ml. Post larval infection, the CFU/ml of the thawed aliquot was intermittently re-determined for accuracy. While the re-evaluation of CFUs post larval infections would have ideally been performed for each *M. plutonius* infection day, we found minimal variability among aliquots when we confirmed *M. plutonius* CFUs post-infection.

2.4. *In vitro* larval rearing, fungicide exposure, and *Melissococcus plutonius* infection

Larval infection with *M. plutonius* and concurrent dietary exposure to fungicide products was adapted from the protocols of Schmehl et al. (2016) and Wood et al. (2020). Briefly, using recipes outlined by Schmehl et al. (2016), we prepared three diets, labeled 'A,' 'B,' and 'C,' using sterile royal jelly (Stakich Inc., Troy, MI, United States), glucose (Fisher Chemicals, Fair Lawn, NJ, United States), fructose (Fisher Chemicals), yeast extract (Becton, Dickson and Company), and sterile distilled water. Diets increased in sugar and protein content from 'A' to 'C.' For larval diets containing fungicidal products, diluted formulated fungicidal products replaced the distilled water fraction in all three diets. For all fungicidal products, the concentration remained constant within diets 'A' – 'C' (Table 1). Diets were stored at −20°C until use.

From mid-May until mid-August in 2020 and 2021, we produced age-synchronized frames of honey bee worker brood, by inserting an empty wax-drawn brood frame into a cage containing the queen in one or more of 15 honey bee colonies. After 24 h, frames with eggs were removed from the queen cage and incubated in the adjacent brood chamber for 3 days until hatching. Frames of first instar larvae were transported back to the laboratory for grafting using a portable incubator at 35°C.

In the laboratory, within a biological safety cabinet, first instar larvae were individually transferred (grafted) from the brood frame into 48-well sterile tissue culture plates (STCP; Figure 1). The day of grafting was considered day 0 (D0). Each well of the STCP contained a sterile, 1 cm in diameter plastic cup, each with 10 µl of control diet 'A' pre-warmed to 35°C. STCPs remained on an electric heating pad at 35°C during grafting.

Each STCP was divided into four groups of 12 larvae, including one negative control group (grafting control; GC) per plate to ensure adequate grafting and rearing techniques, and intermittent (once every 1–4 weeks) infection control groups (IC) to confirm successful *M. plutonius* infection. STCPs with <75% survival in the GC at D6 were removed from the study (Wood et al., 2020). Each fungicidal product was included in the larval diet at two to three different incremental concentrations (Table 1), with or without *M. plutonius* infection (Figure 1). Next, using the same experimental design, larvae were exposed to combinations of two, three, or four fungicide

products (which corresponds to two to six active ingredients) alone or in combination with *M. plutonius* (Figure 2). For combination of two or three fungicide products, we selected the highest concentrations that did not significantly reduce survival (high concentration of Captan, Luna, and Switch, and the low concentration of Kenja); for larvae exposed to four fungicide products, we selected the low concentrations of Captan, Kenja, Luna, and Switch to approximate the exposure based on residues (Table 1). A minimum of three technical replicates ($n = 36$ larvae) and two to six biological replicates (different queens corresponding to different genetic lineages) were used for each treatment group (Supplementary Table S1).

After grafting, each larva received an additional 9.5 µl of control diet A (GC, IC), or fungicide product-containing diet A, combined with either 0.5 µl of *M. plutonius* inoculum [~50 CFU (mean = 61.8 CFU, SD = 24.0); Supplementary Table S1, or 0.5 µl of PBS (Figure 1)]. STCP with larvae were incubated at 35°C (mean = 34.69°C, SD = 0.24) within a desiccator containing approximately 400 ml of supersaturated potassium sulfate solution to maintain the relative humidity at approximately 94% (mean = 98.25%, SD = 5.25) (Schmehl et al., 2016). Temperature and relative humidity were recorded hourly in the desiccator using a thermometer hygrometer probe.

Larvae were fed according to the schedule described by Schmehl et al. (2016) and adapted by Wood et al. (2020). On day 1 (D1) of *in vitro* rearing, the larvae were not fed. From D2 to D5, larvae received 20, 30, 40, and 50 µl of either control (GC, IC) or fungicide product-containing diet B (D2) or diet C (D3-D5; Schmehl et al., 2016). Larval survival was monitored daily using a dissecting microscope. Dead larvae, characterized by darkened coloration, lack of mobility, and arrest of spiracle movement, were removed daily (Schmehl et al., 2016).

Survival data is presented until D6 for all treatment groups exposed to one, two, or three fungicide products. Experiments were limited to six days because larval survival from D0 to D6 was shown to be reflective of larval survival until adulthood (18 days; Supplementary Figure S2). Only the treatment groups exposed to four fungicide products, and corresponding controls, were reared to adulthood, as outlined below.

On D6 of *in vitro* rearing, to prepare for pupation, honey bee larvae that consumed all larval diet were individually transferred to a new 'pupal' STCP containing a 1 cm in diameter circular Kimwipe™ tissue in each well and incubated at 35°C (mean = 34.50°C, SD = 0.31) within a desiccator containing approximately 400 ml of supersaturated sodium chloride to maintain the relative humidity at 75% (mean = 75.55%, SD = 6.16) (Schmehl et al., 2016). Temperature and relative humidity were recorded hourly. Larvae that did not consume all diet by D6 were kept in the larval desiccator at 94% humidity until death or until all diet was consumed, at which point surviving larvae were transferred to a pupal STCP. Pupal survival was monitored daily by visual inspection until honey bees emerged as adults at 15–18 days after grafting. Dead pupae, characterized by deflation or brown discoloration, were removed daily.

2.5. Statistical analysis

Stata 16 (StataCorp LLC, College Station, TX, United States) was used for analyses. Data are reported as the median and interquartile range. A Shapiro–Wilk test was used to assess normality. Pearson's chi-squared test was used to compare percent survival among groups. Survival analysis was performed with a Mantel-Cox log-rank test. Level

TABLE 1 Fungicidal product information and concentrations tested during *in vitro* larval exposure.

Fungicidal product[1]	Active ingredients	Application rate (kg/ha)[2,3]	Total larval exposure based on application rate (ng/bee)[4]	Maximum residue concentrations (mg/kg)[5,6,7]		Total larval exposure based on residues (ng/bee)[8]	Concentrations tested *in vitro* (ng/bee)			Final concentrations in larval diet (ng/µl)[9]		
				Pollen/ bee bread	Honey		Low	Medium	High	Low	Medium	High
Supra® Captan 80WDG (Captan)	80% captan	2	19,000	10	0.019	38	19	190	1,900	0.12	1.19	11.9
Kenja™ 400SC (Kenja)	400 g/l isofetamid	0.496	6,000	—	—	—	6	60	600	0.04	0.38	3.57
Luna® Tranquility (Luna)	125 g/l fluopyram 375 g/l pyrimethanil	0.6	1800	0.026	0.016	2	2	—	1,800	0.01	—	11.25
Switch® 62.5 WG (Switch)	37.5% cyprodinil 25.0% fludioxonil	0.975	3,000	0.57	—	70	70	—	3,000	0.44	—	21.89

For each fungicidal product, the table describes the active ingredients, the application rates, maximum residue concentrations in pollen/bee bread and honey, total larval exposure based on application and residue concentrations using BeeRex (United States Environmental Protection Agency, 2015), concentrations tested *in vitro*, and final concentrations in larval diet. [1]Captan active ingredient: *N*-Trichloromethylthio-4-cyclohexane-1,2-dicarboximide. [2]Province of British Columbia (2022). [3]For fungicidal products with two active ingredients (Luna and Switch), the values in the table represent the active ingredient in lowest concentration in the fungicidal product (fluopyram and fludioxonil, respectively). [4]Total larval exposure was rounded to the nearest thousand for Captan and Kenja. [5]Guarna (2021). [6]Mullin et al. (2010). [7]Residue data for Kenja, and residue concentrations for fludioxonil in honey were not available. [8]To calculate total larval exposure of Switch (fludioxonil), the maximum bee bread residue was used in place of the honey residue concentration in the BeeRex model (United States Environmental Protection Agency, 2015). [9]1 mg/kg is proportionate to 1 ng/µl.

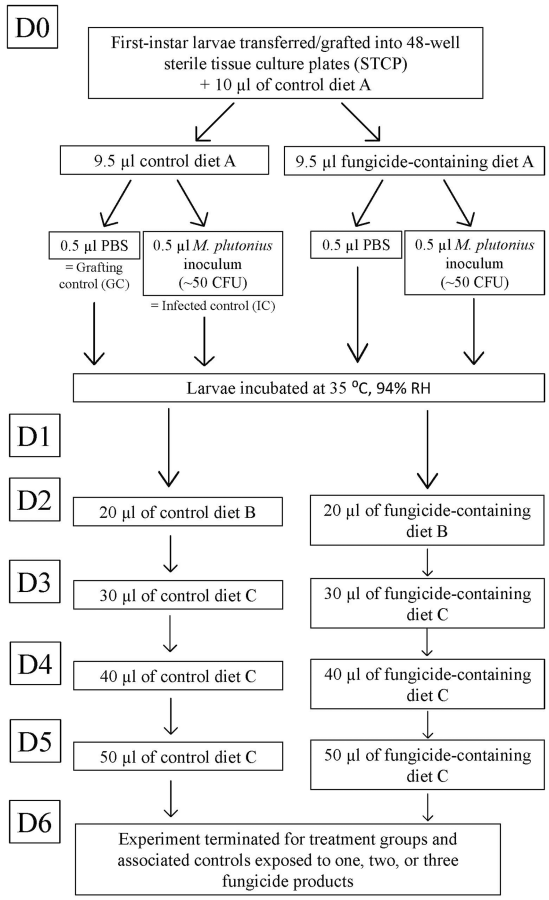

FIGURE 1
Diagram of experimental fungicide exposure and larval infection for larvae reared until day 6 (D6). Larvae exposed to one, two, or three fungicide products were reared for 6 days *in vitro*. The flow chart outlines timeline of infection and chronic fungicidal exposure. Larvae were monitored daily for survival.

A

	Kenja (isofetamid)	**Luna** (fluopyram & pyrimethanil)	**Switch** (cyprodinil & fludioxonil)
Captan (captan)	$p = 0.4629$	***$p = 0.0003$	$p = 0.0967$
	***$p = 0.0001$		-

B

	Kenja (isofetamid)	**Luna** (fluopyram & pyrimethanil)	**Switch** (cyprodinil & fludioxonil)
Captan (captan)	**$p = 0.0038$		

FIGURE 2
Summary of combination fungicide exposure groups and their effects on larval survival with *M. plutonius* infection. Honey bee larvae were exposed to combination of two, three **(A)**, or four **(B)** fungicide products [i.e., two, three, four **(A)**, or six **(B)** active ingredients]. Survival was monitored for 6 days **(A)** or 18 days **(B)**. ** and *** indicate significant effects on survival with $p<0.01$ and $p<0.001$, respectively by a Mantel–Cox log rank test (green boxes indicate significant increases in survival and red boxes indicate significant decreases in survival). Combinations not tested are indicated by "-."

of significance was $p<0.05$. A Bonferroni correction was used for multiple comparisons using the level of significance $p<0.016$ with the Mantel–Cox log-rank test.

3. Results

Larval survival was not negatively affected by exposure to maximum field-relevant concentrations of Captan, Luna, and Switch compared to grafting control (GC) larvae (Figure 3A).

Surprisingly, larvae infected with *M. plutonius* and exposed to the medium and high concentrations of Captan, the high concentration of Luna, and the low and high concentrations of Switch, experienced significant 16.7–58.4% increases in survival compared to infected control (IC) larvae [Figure 3B, $x^2(1)=31.70$, $p<0.0001$; $x^2(1)=17.05$, $p<0.0001$; $x^2(1)=55.46$, $p<0.0001$; $x^2(1)=7.564$, $p=0.006$; $x^2(1)=19.74$, $p<0.0001$; Supplementary Table S1].

Larval exposure to medium and high concentrations of Kenja, without *M. plutonius* infection, significantly decreased larval survival compared to GC by 16.7 and 83.4%, respectively [Figure 3A, $x^2(1)=12.13$, $p=0.0005$; $x^2(1)=169.4$, $p<0.0001$]. Similarly, when larvae were infected with *M. plutonius* and exposed to the medium or high concentrations of Kenja, larval survival significantly decreased by 33 and 24.7%, respectively, compared to IC larvae [Figure 3B, $x^2(1)=46.96$, $p<0.0001$; $x^2(1)=22.17$, $p<0.0001$]. Larval survival was not negatively affected by exposure to the low concentration of Kenja compared to GC (Figure 3A), whereas larvae infected with *M. plutonius* and exposed to the low concentration of Kenja experienced a significant 50.0% increase in survival compared to IC larvae [Figure 3B, $x^2(1)=32.39$, $p<0.0001$].

Larval exposure to combinations of two or three fungicides did not negatively affect survival relative to GC (Figure 4), whereas larvae infected with *M. plutonius* and exposed to combinations of either Captan and Luna, or Captan, Kenja, and Luna, had significant 25.1 and 26.3% increases in larval survival compared to IC larvae, respectively [Figure 4, $x^2(1)=13.15$, $p=0.0003$; $x^2(1)=11.62$, $p=0.0007$].

Larval survival was not significantly decreased by concurrent exposure to low concentrations of four fungicidal products (Figure 5); however, when combined with *M. plutonius* infection, exposure to four fungicidal products resulted in a significant 24.2% decrease in larval survival compared to IC larvae [Figure 5, $x^2(1)=8.398$, $p=0.0038$].

4. Discussion

In this investigation, we tested the effects of four formulated fungicide products used in highbush blueberry production on honey bee larval survival from EFB using an *in vitro* larval infection model (Schmehl et al., 2016; Wood et al., 2020). Previous investigators have found that at least four fungicide residues were detectable in pollen from blueberry pollinating honey bee colonies (Graham et al., 2021; Guarna, 2021; Rondeau and Raine, 2022), supporting the relevancy of our study.

Chronic oral larval exposure to the fungicide products Supra® Captan 80WDG (Captan), Luna® Tranquility (Luna), and Switch® 62.5 WG (Switch), when applied individually, did not negatively impact larval survival compared to grafting controls (GCs), nor did they have any significant negative effect on larval survival from EFB. The lack of significant negative effects following fungicide product exposure with and without *M. plutonius* infection are not surprising, as other researchers also have not found significant negative effects on honey bee larval survival

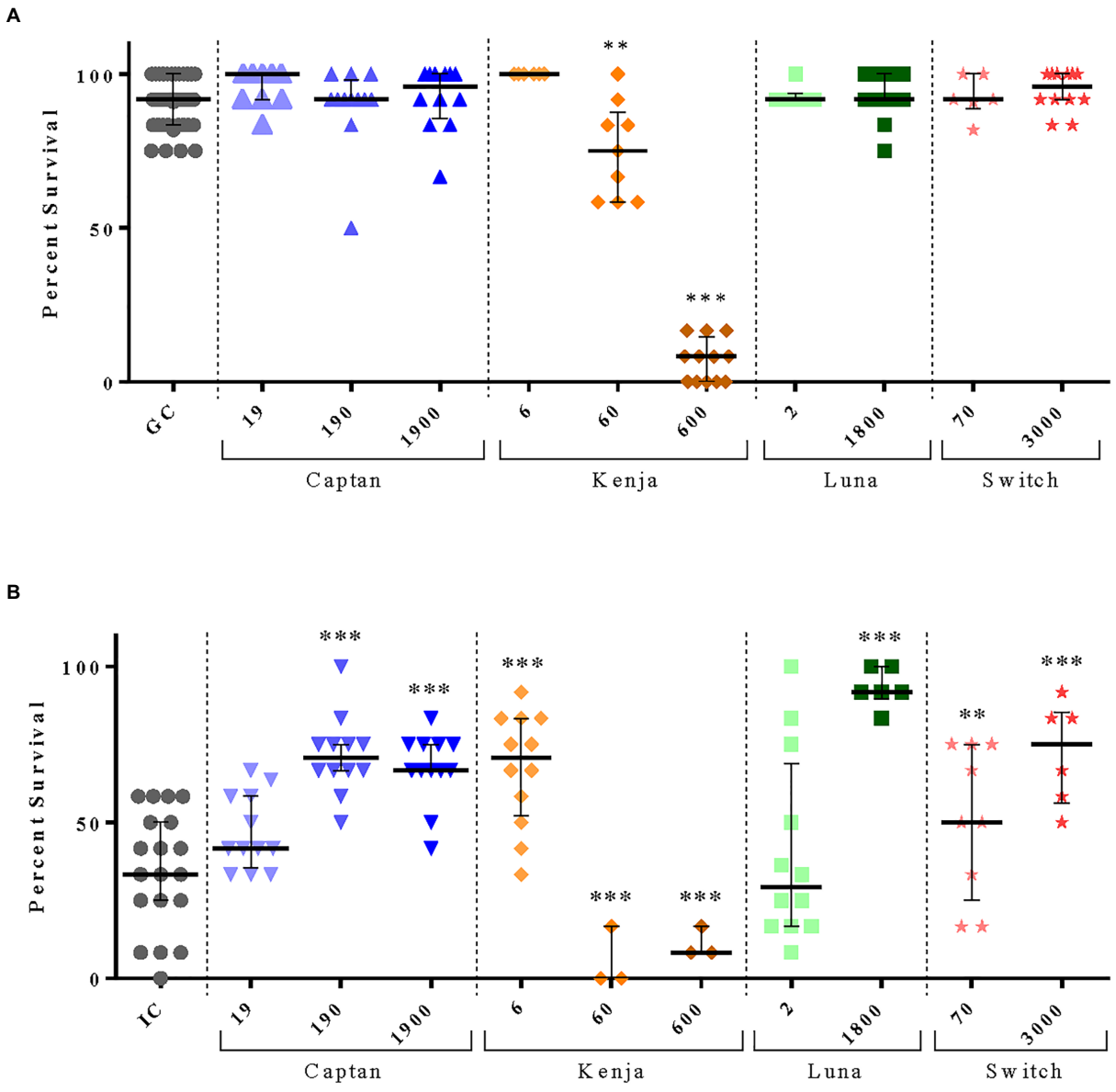

FIGURE 3
Effect of chronic fungicidal exposure on larval survival from European foulbrood disease *in vitro*. Honey bee larvae were reared *in vitro* for 6 days and chronically exposed to low, medium, and high concentrations of four different formulated fungicidal products. **(A)** Percent survival of 60–144 larvae chronically administered fungicidal product and compared to 501 grafting control (GC) larvae. **(B)** 36–144 larvae infected with 50CFU of *M. plutonius* bacteria, chronically exposed to fungicides, and compared to 228 infected control (IC) larvae. Each dot represents one replicate (n=12 larvae). Numbered categories on the x-axis represent the concentrations of the active fungicidal ingredient in ng/bee. Horizontal and vertical lines overlying the dots represent the median and interquartile range, respectively. **p<0.01, ***p<0.001, by a Pearson's chi-squared test.

from oral (or contact) exposure to field-relevant concentrations of the active fungicide ingredients in Captan, Luna, and Switch (Everich et al., 2009; Wood et al., 2020). While our highest tested concentrations of the active ingredients in Captan, Luna, and Switch reflected the highest reported residues of these active ingredients in pollen/bee bread and honey (Graham et al., 2021, 2022; Rondeau and Raine, 2022), the transfer rate of residues from pollen into royal jelly and worker jelly is believed to be low (Bohme et al., 2018, 2019; Milone et al., 2021), and thus, our concentrations are likely an over estimation of exposure. Although most fungicides detected in honey have systemic properties, fludioxonil is a non-systemic fungicide and its residues have never been reported in honey (Rondeau and Raine, 2022), therefore we used residue concentrations for pollen/bee bread in place of honey. As residue concentrations in pollen/bee bread are many folds higher than concentrations found in nectar/honey (Graham et al., 2021, 2022; Rondeau and Raine, 2022), this again illustrates our tested concentrations as a potential over exposure. On the other hand, water is also an important constituent in worker larval diet (McCune et al., 2021). As fungicides have been shown to accumulate in naturally occurring water sources (Zubrod et al., 2019), this may further contribute to increased fungicide exposure to honey bee larvae.

One limitation of this study is that we did not confirm fungicide exposure by measuring the concentration of active fungicide ingredients in the experimental diet. Accordingly, we cannot exclude possible fungicide degradation during diet preparation and freezing until time of use, or errors in manipulation that may have led to incorrect concentrations. Additionally, we only monitored larval survival until day 6 for treatment groups of larvae exposed to one, two, or three fungicidal

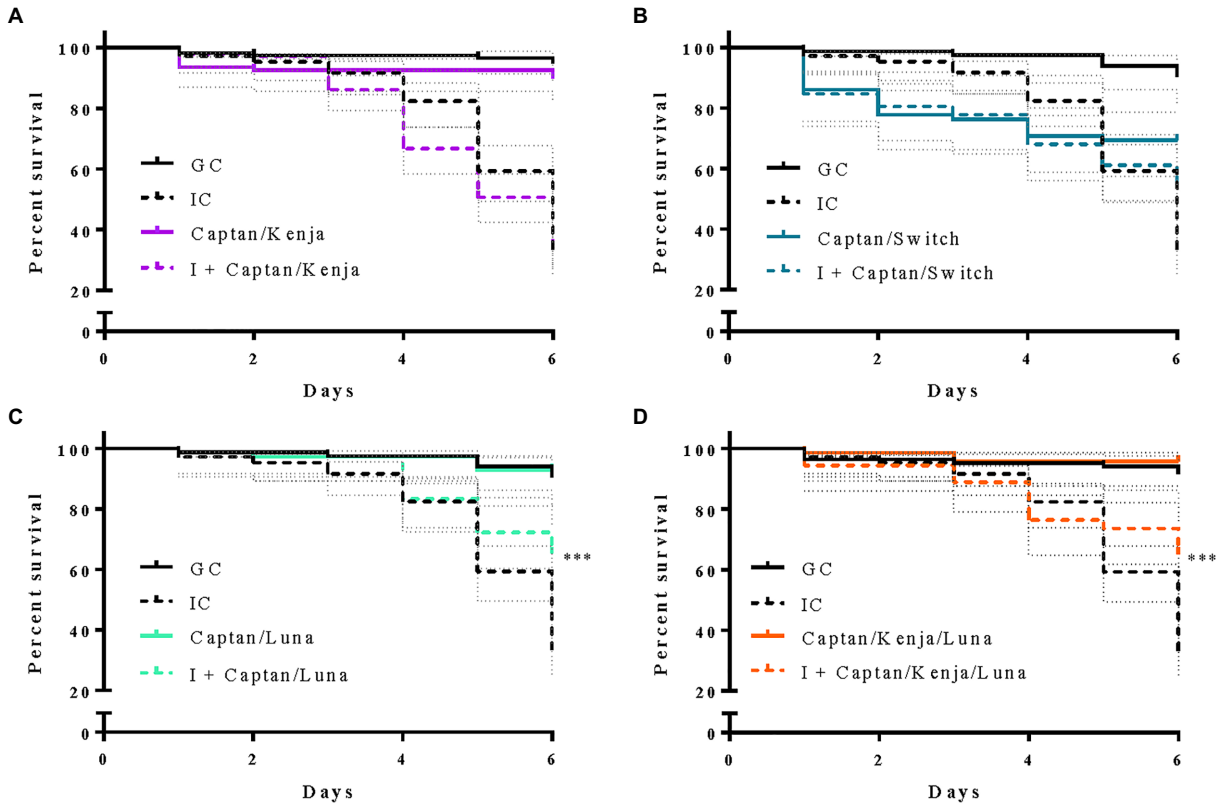

FIGURE 4
Effects of chronic exposure to combinations of fungicidal products on larval survival from European foulbrood disease *in vitro*. Solid and dashed lines represent percent survival±confidence interval (dotted grey lines) over 6days of *in vitro* rearing of 84–119 grafting control larvae (GC; solid black lines), 72–108 larvae chronically administered a combination of fungicide products (solid colored lines), 105 infected control larvae (IC; dashed black lines) inoculated with 50 colony forming units (CFU) of *M. plutonius* 2019 BC1, and 72–144 larvae that were infected (I) with 50CFU of *M. plutonius* 2019 BC1 and subsequently administered fungicide product combinations in their diet (dashed colored lines). Fungicide-exposed larvae received **(A)** high concentration Captan and low concentration Kenja, **(B)** high concentrations of Captan and Switch, **(C)** high concentrations of Captan and Luna, and **(D)** high concentration Captan, low concentration Kenja, and high concentration Luna. ***$p<0.001$, by a Mantel–Cox log rank test.

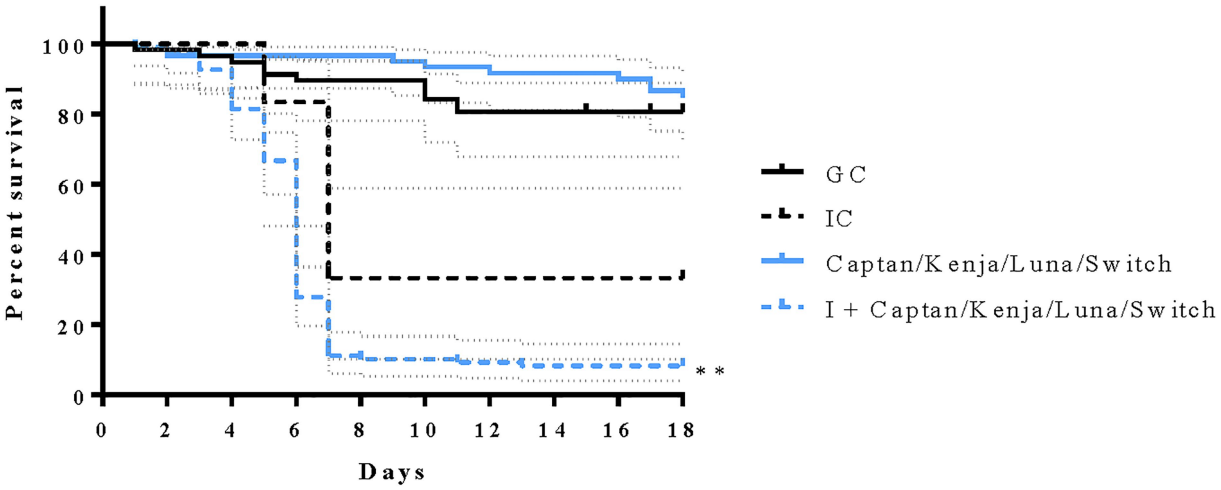

FIGURE 5
Effects of chronic exposure to four fungicidal products on larval survival from European foulbrood disease *in vitro*. Solid and dashed lines represent the percent survival±confidence interval (dotted grey lines) of honey bees until adult emergence for 58 grafting control larvae (GC; solid black line), 60 larvae chronically exposed to four fungicides simultaneously (solid blue line), 12 infected control larvae (IC; dashed black line) inoculated with 50 colony forming units (CFU) of *M. plutonius* 2019 BC1, and 108 larvae that were infected (I) with 50CFU of *M. plutonius* and subsequently administered fungicidal product in the diet (dashed blue line). Fungicide-exposed larvae received low concentrations of Captan, Kenja, Luna, and Switch. **$p<0.01$ by a Mantel–Cox log rank test.

products, thus limiting our ability to observe differences in pupal survival among treatment groups.

In the absence of *M. plutonius* infection, larval exposure to medium and high concentrations of Kenja™ 400SC (Kenja) were associated with a significant decline in larval survival compared to control larvae. Likewise, relative to infected controls (ICs), there was a significant decrease in survival when larvae were infected with *M. plutonius* and exposed to medium and high concentrations of Kenja. Isofetamid, the active ingredient in Kenja, is a newly developed fungicide and was only registered in Canada, United States, and Japan recently in 2014, 2015, and 2017, respectively (Umetsu and Shirai, 2020), and field-realistic residue concentrations of isofetamid in honey bee hive matrices have not been reported to date (Rondeau and Raine, 2022). Furthermore, Bellisai et al. (2021) found elevated residues of 3.65 mg/kg isofetamid in other fruit crops (whole raspberry plant) following foliar application of the product, emphasizing the need to further quantify field-realistic honey bee exposure to this fungicide during blueberry pollination.

Combined larval exposure to two or three fungicidal products did not significantly decrease larval survival from EFB compared to infected controls. Similar to our study, Prado et al. (2019) found that oral exposure to pyrimethanil had no negative effect on larval survival when combined with other fungicides. The exposure to low, non-toxic concentrations of fungicides (Lewis et al., 2016) that may not negatively impact immune function (O'Neal et al., 2018), are possible explanations for not observing negative effects on larval survival. Importantly, only selected combinations of fungicidal products commonly used during bloom in highbush blueberry production were tested in this study; we cannot exclude that other combinations of fungicidal products may impact larval survival from EFB.

However, when infected larvae were chronically exposed to a combination of four fungicide products with four different modes of action, there was a significant decrease in larval survival relative to infected controls. Given that multiple studies have reported the presence of ≥4 fungicidal residues in pollen samples (Rondeau and Raine, 2022), including those collected from blueberry-pollinating honey bee colonies (Graham et al., 2021; Guarna, 2021), these results provide a rationale for concern as we tested concentrations that were based on reported field-level concentrations or application rates. Other researchers have similarly found synergistic negative effects on larval survival after co-exposure to fungicides and insecticides (Wade et al., 2019; Wood et al., 2020), but to our knowledge, this is the first report of significant negative effects on larval survival following exposure to a combinations of fungicide products without insecticides. Decreased survival from pathogen infection in response to fungicide exposure may be explained by decreased immune function (O'Neal et al., 2018). Furthermore, proprietary ingredients present in these fungicide products may also contribute to the increased larval EFB mortality observed after exposure to multiple fungicides, as pesticide adjuvants have been previously implicated in enhancement of pesticide toxicity to honey bees (Mullin et al., 2015; Walker et al., 2022).

Surprisingly, larvae infected with *M. plutonius* and exposed the low concentration of Kenja or Switch, the medium concentration of Captan, and the high concentration of Captan, Luna, or Switch, had a significant increase in larval survival from EFB compared to infected control larvae. Likewise, an increase in survival was observed when larvae were infected with *M. plutonius* and exposed to combinations of high concentrations of Captan and Luna, and high concentrations of Captan, Luna, and the low concentration of Kenja. This observation may be explained by direct bacterial inhibition of the fungicide products on *M. plutonius*. While the antimicrobial properties of royal jelly have been previously reported to decrease *M. plutonius* viability in the diet (Takamatsu et al., 2017; Vezeteu et al., 2017; Floyd et al., 2020; Masood et al., 2022), the potential bactericidal effects of these fungicidal products on *M. plutonius* is unknown, and an area that warrants further investigation.

Our results demonstrate that chronic exposure of fungicide products used in highbush blueberry production only negatively impacts honey bee larval susceptibility to EFB *in vitro* when larvae are exposed to the four fungicidal products Captan, Kenja, Luna, and Switch combined, or when larvae are exposed to medium and high concentrations of Kenja. Accordingly, fungicidal exposure may be a driving force for the reported increase in incidence of EFB during blueberry pollination; however, comprehensive fungicide residue analysis is warranted, as well as the continued investigation of other host, pathogen, or environmental factors influencing the disease ecology of EFB.

5. Author's note

Honey bee pollination contributes significantly to blueberry production in Canada and the United States each year; however, outbreaks of European foulbrood disease is an evolving problem that threatens the supply of honey bee pollination services to the blueberry industry. Investigating the risk factors which contribute to EFB disease during blueberry pollination is an important step in safeguarding honey bee colony health and maintaining profitability of both beekeepers and blueberry growers. Our *in vitro* study suggests that fungicide products commonly used in highbush blueberry production may predispose honey bee larvae to disease, as exposure to medium and high concentrations of Kenja, and exposure to four fungicide products concurrently increased larval susceptibility to EFB. While further evaluation of field-relevant fungicide exposure for colonies pollinating blueberries is required, our study facilitates the understanding of pesticide risk to honey bees pollinating crops and contributes to the ongoing efforts to enhance sustainability of blueberry pollination in North America.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

SW, ES, JT, GW, MG, EG, and AR contributed to the conception and design of the study. JT, AC, and DL acquired the data. JT, AC, DL, FM, IK, CK, MZ, and SB contributed to animal care. IM assisted in bacterial protocols. LS assisted with fungicide preparation. MG contributed to sections of the manuscript. JT analyzed the data, drafted and revised the work. All authors contributed to the manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1073775/full#supplementary-material

SUPPLEMENTARY FIGURE 1
HSurvival of larval honey bees reared *in vitro* for until eclosion (6 days and fed diet containing the fungicidal products Captan or Kenja at concentrations 19,000 and 6000 ng/bee, respectively.) following fungicide exposure. Percent survival ± confidence interval of **(A)** 108 honey bee larvae exposed to 19,000 ng/bee of Captan and **(B)** 144 honey bee larvae exposed to 6000 ng/bee Kenja until eclosion compared to 48-72 grafting control larvae (GC). The tested concentrations of Captan and Kenja represent the larval exposure based on the application rate used during blueberry pollination to prevent fungal diseases such as anthracnose and botrytis fruit rot (Everich et al., 2009; Province of British Columbia, 2022; Mussen et al., 2004). ****$p < 0.0001$, by a Mantel-Cox log rank test.

SUPPLEMENTARY FIGURE 2
Effects of chronic exposure to fungicidal products on larval survival from European foulbrood disease (EFB) *in vitro* until eclosion or emergence. Percent survival ± confidence interval of honey bee larvae infected with *M. plutonius* (dashed lines) and exposed to low concentrations of Luna and Switch (colored lines). Infected (I), fungicide-exposed larvae were compared to infected control larvae (IC). Survival analysis of data from day 0-18 Larvae monitored until emergence **(A, C, E)** yielded had the same statistical relationship between infected experimental groups compared to survival analysis of data from day 0-as when the same data was truncated to 6 days **(B, D, F)**. *$p < 0.05$, ***$p < 0.001$, by a Mantel-Cox log rank test.

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Increased survival of honey bees consuming pollen and beebread is associated with elevated biomarkers of oxidative stress

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Introduction: Significant losses of honey bee colonies have been observed worldwide in recent decades. Inadequate nutrition is considered to be one of the factors that can reduce honey bee resistance to abiotic and biotic environmental stresses. Accordingly, we assessed the impact of food composition on worker bee survival.

Methods: Bees in cages were fed six different diets, and then their survival, levels of thiobarbituric acid reactive substances and protein carbonyl groups, catalase and lysozyme activities were evaluated.

Results and Discussion: After 17days of feeding, the lowest mortality was observed in the group of bees that received sucrose solution with the addition of willow pollen or artificial rapeseed beebread or artificial willow beebread (diets 4–6). The highest mortality was found in bees that consumed only sucrose solution (diet 1) or the sucrose solution supplemented with a mixture of amino acids (diet 2), which can be explained by the lack of vitamins and microelements in these diets. In the group of bees that received the sucrose solution with rapeseed pollen (diet 3), mortality was intermediate. To check whether the decrease in insect survival could be related to oxidative damage, we evaluated biomarkers of oxidative stress. Consumption of pollen (diets 3 and 5) and artificial beebread (diets 4 and 6) enhances protein carbonylation in worker bees. Feeding bees artificial beebread also resulted in increase in lipid peroxidation and catalase activity, which is probably due to the presence of hydrogen peroxide in the honey contained in beebread. Remarkably, the increase in biomarkers of oxidative stress was not accompanied by adverse but positive effects on insect survival. A lack of amino acids and proteins in the diet 1 did not cause oxidative stress, but led to an increase in lysozyme activity in hemolymph, a biomarker of immune system status. In conclusion, we believe that the increase in oxidative stress biomarkers we found do not indicate oxidative damage, but rather reflect the changes in redox balance due to consumption of certain dietary options.

KEYWORDS

catalase, honey bee, lipid peroxidation, lysozyme, nutrition, oxidative stress, protein carbonylation, biomarkers

Introduction

The western honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), is an important pollinator in natural and agricultural ecosystems (Potts et al., 2010; Garibaldi et al., 2017; Khalifa et al., 2021). Significant bee colony losses have been observed worldwide in recent decades (Gray et al., 2019, 2022). This issue has drawn particular attention to identifying factors that negatively affect honey bee health, and it is believed that poor nutrition may be one of them. A deficiency or a disturbed composition of dietary components can weaken the resistance of bees to stressors of abiotic and biotic nature and make them more sensitive to adverse weather conditions, infectious diseases, and the effects of environmental contaminants (Brodschneider and Crailsheim, 2010; Goulson et al., 2015; Tosi et al., 2017; Ptaszyńska et al., 2018; Wright et al., 2018; Branchiccela et al., 2019; Dolezal et al., 2019; Negri et al., 2019; Tong et al., 2019; Olate-Olave et al., 2021).

For normal activity of bee colony, all basic nutrients, i.e., proteins, fats, carbohydrates, vitamins, and minerals should be available (Paoli et al., 2014; de Groot et al., 2021). The nectar and honey derived from it are the main sources of carbohydrates, while pollen provides necessary proteins and lipids. Pollen also contains various carbohydrates, such as fructose, glucose, sucrose, polysaccharides, as well as vitamins, minerals, and polyphenolic compounds (Čeksterytė et al., 2008; Rzepecka-Stojko et al., 2015; Bogdanov, 2016; Arathi et al., 2018; Radev, 2018).

Honey bees, unlike other insects, are able to accumulate their own food reserves in the form of honey and beebread, a mixture of pollen and nectar or honey that also include some enzymes and micro-organisms. Beebread contains a large amount of proteins, lipids, free sugars, essential amino acids, mono- and polyunsaturated fatty acids, etc., and is the main food source for worker bees and larvae. The nutritional and biological qualities of beebread vary widely depending on the diversity of flora and the season of bee pollen collection (Urcan et al., 2017; Wright et al., 2018; Bakour et al., 2019).

The results of artificial feeding show that the nutrient composition of diets affects both the physiological state of individual bees and the health of the bee colony as a whole (Branchiccela et al., 2019; Castelli et al., 2020). Such parameters as the lifespan of worker bees (Di Pasquale et al., 2013; Arien et al., 2020), the size of the acini of the hypopharyngeal glands, the protein content in the hemolymph and intestines, the activity of glucooxidase (Alaux et al., 2010), the level of gene expression (De Grandi-Hoffman et al., 2021), etc. depend on the pollen composition. Nevertheless, the influence of pollen from different plant species on the lifespan of worker bees and the health of bee colonies is still insufficiently studied and requires further research.

In all aerobic organisms, even under optimal environmental conditions, reactive oxygen species (ROS), such as hydrogen peroxide, superoxide, and hydroxyl radicals, etc., are constantly generated in various compartments as an inevitable part of cell life. In animals, ROS are produced by respiratory chain in mitochondria and also as byproducts of different metabolic pathways (Barja, 1999; Drahota et al., 2002; Miwa and Brand, 2005; Murphy, 2009; Halliwell and Gutteridge, 2015). ROS levels are under the strict control of a complex system of antioxidant protection, which includes both enzymes and small-molecule non-enzymatic compounds, which contribute to ROS-splitting and reparation of oxidative damage (Corona and Robinson, 2006; Halliwell and Gutteridge, 2015; Jakubczyk et al., 2020; Irato and Santovito, 2021). Various external abiotic (extreme temperatures, dehydration, environmental pollutants, and radiation) and biotic (pathogens attack) stress factors induce excessive production of ROS causing an oxidative stress, i.e., imbalance between ROS formation and antioxidative defense mechanisms and leading to oxidative damage of proteins, lipids, and DNA, which in turn results in numerous violations of physiological functions and even cell death (Roelofs et al., 2008; Birben et al., 2012; Nikolenko et al., 2012; Halliwell and Gutteridge, 2015; Cervoni et al., 2017). Especially, a correlation between antioxidant system activity and longevity has been found in insects, including bees (Phillips et al., 1989; Arking et al., 2000; Corona et al., 2005).

In eukaryotes, ROS not only damage the cell, but also play numerous beneficial roles, serving as tools to combat infectious agents and as signaling molecules involved in the regulation of stress response, senescence, and immunity (Corona et al., 2005; Volkov et al., 2006; Aurori et al., 2013; Scialò et al., 2016; Cervoni et al., 2017; Foyer et al., 2017; Orčić et al., 2017).

Honey, pollen, and beebread contain both pro- and antioxidant compounds, the composition and concentration of which vary depending on the plant species that the honeybees pollinate (Čeksterytė et al., 2008; Rzepecka-Stojko et al., 2015; Bogdanov, 2016). Accordingly, it is to be expected that the level of ROS in bees can depend on the composition of the forage. However, very little is known about the relationship between the type of food, immunocompetence, the state of the antioxidant system, and oxidative stress in bees (Alaux et al., 2010; Li et al., 2012; Wheeler and Robinson, 2014). Therefore, the aim of our work is to evaluate the effect of the consumed feed (sucrose solution supplemented with amino acids, or pollen and beebread of rapeseed and willow) on survival, the state of the antioxidant system (lipid peroxidation, protein carbonylation, and catalase activity) and parameters of immunity (lysozyme activity) in honey bees.

Materials and methods

Experimental conditions and feeding of bees

The research was conducted in June 2018. Worker bees used for the experiment were obtained from three colonies of *Apis mellifera carnica* from the experimental apiary of the University of Life Sciences in Lublin (Poland). Combs with sealed brood in the late stages of pupal development were transferred from the hives to a thermostat (34°C and 60–70% relative humidity) and maintained until imago hatching. Bees hatched from the combs within 10 h were collected and reared in groups of 40 individuals in cages (12 cm × 12 cm × 3.5 cm) at 24°C and 60% relative humidity.

Firstly, all bees were fed 50% sucrose solution for 2 days for acclimatization. After that, the bees were fed *ad libitum* for 17 days on one of the six diets: diet 1–50% solution of sucrose (SS, control group); diet 2–10% solution of "Aminosteril N-HEPA 8%" (Fresenius Kabi Deutschland GmbH; 100 ml of "Aminosteril N-HEPA 8%" contain: L-Isoleucine 1.04 g, L-Leucine 1.309 g, L-Lysine monoacetate 0.971 g, L-Lysine 0.688 g, L-Methionine 0.11 g, N-Acetylcysteine 0.07 g, L-Cysteine 0.052 g, L-Phenylalanine 0.088 g, L-Threonine 0.44 g, L-Tryptophan 0.07 g, L-Valine—1.008 g, Arginine 1.072 g, L-Histidine 0.28 g, Aminoacetic acid 0.582 g, L-Alanine 0.464 g, L-Proline 0.573 g, L-Serine 0.224 g, and Glacial acetic acid 0.442 g) in SS; diet 3–10% rapeseed pollen in SS; diet 4–10% artificial rapeseed beebread (rapeseed pollen mixed with honey and fermented for 2 days) in SS; diet 5–10% willow pollen in SS; and diet 6–10% artificial willow beebread in SS. Rapeseed and willow pollen were obtained, respectively, from organic farming or nature conservation area. The amount of feed consumed and the number of dead bees were measured every 2 days.

Biochemical measurements

For biochemical measurements, the bees were taken on the 15th day of the feeding experiment, flash frozen in liquid nitrogen, and stored at −70°C. Biochemical parameters were assayed separately for head and abdomen. The frozen bees were dissected on ice, and then, for one sample, heads and abdomens from 12 individuals were pooled, ground in liquid nitrogen, and homogenized at 0–4° C using a Heildolph high-speed homogenizer (Germany) and different extraction buffers depending on the parameters examined.

The lipid peroxidation was assayed in head and abdomen by measuring the content of thiobarbituric acid reactive substances (TBARS; μmol/kg fresh weight; Placer et al., 1966) using RIPA-extraction buffer (150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 50 mM Tris HCl, and pH 7.4; Margotta et al., 2018). 100–120 mg of frozen tissues were homogenized at 0–4° C in 400 μl of RIPA-buffer, incubated on ice for 10 min, and centrifuged for 15 min at 12,000 *g*, 4°C. The supernatant was collected and the pellet was re-suspended in 400 μl of RIPA-buffer and centrifuged. Both supernatants were combined and mixed with 800 μl of 0.6% 2-thiobarbituric acid in 20% trichloroacetic acid. The control tube contained 800 μl of RIPA-buffer and 800 μl 0.6% TBA in 20% TCA. The sample and control tubes were incubated at 95°C for 60 min, cooled on ice, and centrifuged for 15 min at 12,000 *g*. The optical density of the obtained supernatants was determined at wavelengths of 532 and 600 nm and used to calculate the TBARS content.

The content of protein carbonyl groups (PCG; nmol/mg protein), a biomarker of protein carbonylation, was determined in abdomen using the DNPH (2,4-dinitrophenylhydrazine) method (Reznick and Packer, 1994). 100–120 mg of tissues ground in liquid nitrogen were mixed with 800 μl of extraction buffer (100 mM potassium phosphate, pH 7.4, 1 mM PMSF, and 1 mM EDTA), additionally homogenized on ice, and centrifuged for 20 min at 14,000 *g*, 4°C. 400 μl of to the obtained supernatant was added to (i) a sample tube containing 800 μl of 10 mM DNPH dissolved in 2 M HCl and (ii) a control tube containing 800 μl of 2 M HCl. After 60 min of incubation in the dark at 25°C, 800 μl of 40% TCA was added to both sample and control tubes. After 5 min of incubation on ice, the tubes were centrifuged for 15 min at 10,000 *g*. The pellets were washed three times with 1 ml of ethanol/ethyl acetate (1:1) mixture followed by centrifugation. Finally, the pellets were dissolved in 1 ml of 6 M guanidinium chloride for 30 min in the dark. The optical density of samples at 370 nm was determined.

Activity of catalase (CAT) was evaluated in head and abdomen by measuring the decrease in hydrogen peroxide content in the samples, applying the previously described method (Korolyuk et al., 1988; Buzduga et al., 2018). 100–120 mg of tissues ground in liquid nitrogen were mixed with six volumes of extraction buffer (100 mM potassium phosphate, pH 7.4, 1 mM PMSF, and 1 mM EDTA), additionally homogenized on ice, and centrifuged for 20 min at 14,000 *g*, 4°C. The supernatants were used for CAT activity estimation. Two samples, a blank and an experimental, were prepared. 100 μl of protein extract was added to 1.9 ml of reaction buffer containing 100 mM Tris–HCl (pH 6.8) and 70 mM H_2O_2. The mixture was stirred briefly, and 1 ml was immediately transferred to a tube (blank sample) containing 0.5 ml of a 4% solution of ammonium molybdate, which resulted in termination of reaction due to formation of colored complexes between ammonium molybdate and hydrogen peroxide. The remaining reaction mixture was incubated for 1 min at 25°C; thereafter the reaction was stopped by the addition of 0.5 ml of a 4% ammonium molybdate solution. The optical density of the samples at 410 nm was measured, and the content of hydrogen peroxide was calculated using a calibration curve. CAT activity was expressed in micromoles of hydrogen peroxide spited per minute per milligram of protein (μmol/min mg^{-1} protein).

The activity of lysozyme in hemolymph was quantified by the diffusion pit method (Snyder and Fritsch, 1984) with Lysozyme from chicken egg white (Sigma-Aldrich, United States) as the standard. Hemolymph was collected from bees as described previously (Borsuk et al., 2017). The samples were kept at −20°C until further analysis.

Protein concentration in the samples was determined according to the method of Bradford (1976).

Statistics

Each diet feeding experiment was performed three times for four pools of 40 bees per cage (i.e., for a total of 12 bee cages or 480 bees per diet). Biochemical measurements were performed in four analytical replicates for each feeding experiment. Differences in survival were determined using the log-rank test for the Kaplan-Meir survival curve. For amount of food consumed by bees and lysozyme activity, the significance of differences between control and each treatment was assessed using Duncan's one-way ANOVA test. For TBARS and PCG content and CAT activity, the Kruskal-Wallis test followed by the Mann–Whitney test was applied. *p* values less than 0.05 were considered statistically significant.

Relationships between survival of bees and biochemical parameters were evaluated using no parametric Spearman correlation analysis (Zar, 1996). All tests were performed with the statistical software Statistica 12.5.

Results

Consumption of different diets and survival rate of worker bees

In our experiment, the consumption of six dietary options by worker bees was compared. It was found that the bees consumed the least amount of sucrose solution supplemented with the amino acid-containing drug "Aminosteril," while solutions with rapeseed pollen or beebread as well as the control sucrose solution were most accepted (Figure 1).

The survival rate of the worker bees was evaluated depending on the composition of the different diets. The obtained data show that in the first 6–8 days after the start of the experiment, the survival rate of the bees in the control group, which only consumed sucrose solution (diet 1), was the highest (Figure 2). At the same time, the highest mortality was observed in individuals that consumed sucrose solution with the addition of rapeseed or willow pollen. However, with increasing duration of the experiment, a gradual increase in the mortality of bees receiving only sucrose solution or sucrose solution with the addition of amino acids mixture was observed. At the end, on day 18 from the beginning of the experiment, these last two feeding groups had the highest mortality, 88 and 91%, while the bees consuming the sugar solution supplemented with willow beebread, willow pollen, or rapeseed pollen showed mortality of 66, 70, and 72%. The experimental group that received rapeseed pollen had an intermediate mortality rate of 81%.

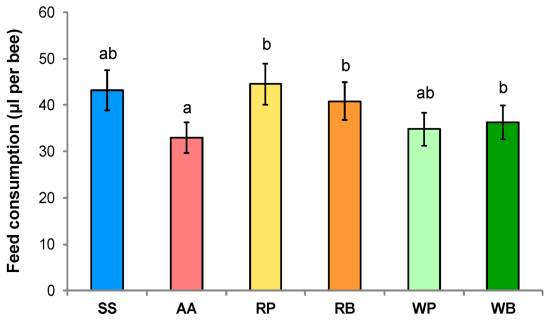

FIGURE 1
Consumption of different diets (µl per one bee). SS, sucrose solution (control group); AA, sucrose solution+amino acids; RP, sucrose solution+rapeseed pollen; RB, sucrose solution+rapeseed beebread; WP, sucrose solution+willow pollen; and WB, sucrose solution+willow beebread. Different letters indicate significant differences between the values (p<0.05).

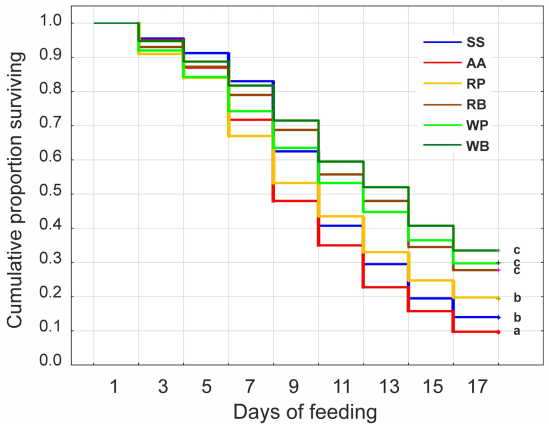

FIGURE 2
Kaplan–Meier survival curves for worker bees fed different diets for 17days. The administration of the diets was started 2days after the hatching of the bees. SS, sucrose solution (control group); AA, sucrose solution+amino acids; RP, sucrose solution+rapeseed pollen; RB, sucrose solution+rapeseed beebread; WP, sucrose solution+willow pollen; and WB, sucrose solution+willow beebread. Different letters indicate significant differences between the values (p<0.05).

Evaluation of stress biomarkers and activity of protective enzymes

Determination of TBARS content in the tissues of the head and abdomen revealed that this biomarker varies depending on the diet used. In particular, in the tissues of the head of bees whose diet included beebread, the TBARS content was the highest compared to other types of diet (Figure 3). At the same time, the content of TBARS when consuming rapeseed beebread was higher than that of willow beebread. In bees that were fed a sucrose solution with the addition of pollen or an amino acid mixture, the values of the biomarker studied were at the control level. A similar pattern was found in insect abdominal tissues. In general, the content of TBARS in the tissues of the head was higher than in the abdomen.

The content of PCG was determined only in the abdominal tissues of bees (Figure 4). It was shown that in insects that were fed a sucrose solution supplemented with pollen or beebread, this biomarker was higher than in the control group. At the same time, the level of protein carbonylation was higher in bees consuming willow beebread than rapeseed beebread. The lowest level of protein carbonylation was found in the insects that received a mixture of amino acids.

Measurement of CAT activity showed its increase in the tissues of the head of bees receiving a sucrose solution with the addition of an amino acid mixture, while the activity of the enzyme remained at the control level when pollen or beebread was consumed. However, in bees whose diet included beebread, the activity of CAT was about 14% higher than in bees that fed on pollen (Figure 5). In the tissues of abdomen, the activity of CAT was 3–4 times higher, depending on the applied diet, than in the tissues of head. In the abdomen, the highest activity of the enzyme was found in bees of the control group, whereas the lowest in those that consumed pollen. In the insects that received a diet containing a mixture of amino acids or beebread, the activity of CAT was higher than in bees consuming pollen, but lower (mixture of amino acids or rapeseed beebread) or equal (willow beebread) as in bees fed sucrose solution. Hence, similar to head tissues, consumption of beebread led to an increase in of CAT activity in abdomen.

The activity of lysozyme in the hemolymph of bees of the control group that consumed sucrose solution was found to be 4–9 times higher than in bees that received other diet variants. The lowest lysozyme activity was found in bees receiving willow pollen or beebread, but the difference in this parameter for different diets (except for the control group) was not statistically significant (Figure 6).

Correlation analysis

In order to reveal a possible relationship between survival of bees and biomarkers of oxidative stress, we applied correlation analysis (Table 1). A strong positive statistically significant relationship was found only in two cases: (i) the survival of bees when consuming different diets was correlated with the level of protein carbonylation and (ii) the level of lipid peroxidation in tissues of head correlated with that in abdomen.

Discussion

Effect of diet on the lifespan of worker bees

In nature, foraging honey bees collect nectar and pollen from various flowers to provide the necessary nutrients for the colony. The main source of energy for bees are carbohydrates (fructose, glucose, sucrose, etc.), which are part of flower nectar and honey obtained from it. Also, a mandatory components of the diet must be pollen and beebread obtained from it, which provide bees with amino acids (especially with essential amino acids—EAAs), lipids, sterols, vitamins, and minerals (De Groot, 1953; Day et al., 1990; Herbert, 1992; Cook et al., 2003; Paoli et al., 2014; Bogdanov, 2016). Behavioral castes of adult worker honeybees differ in dietary requirements for EAAs/proteins and carbohydrates. In particular, young nurse bees need relatively more EAAs, while older forager bees, which have increased energy expenditure due to flight, consume relatively more carbohydrates (Crailsheim, 1990; Crailsheim et al., 1992; Paoli et al., 2014).

In our feeding studies, we determined the effect of different diets on lifespan, an important integral indicator reflecting the physiological state

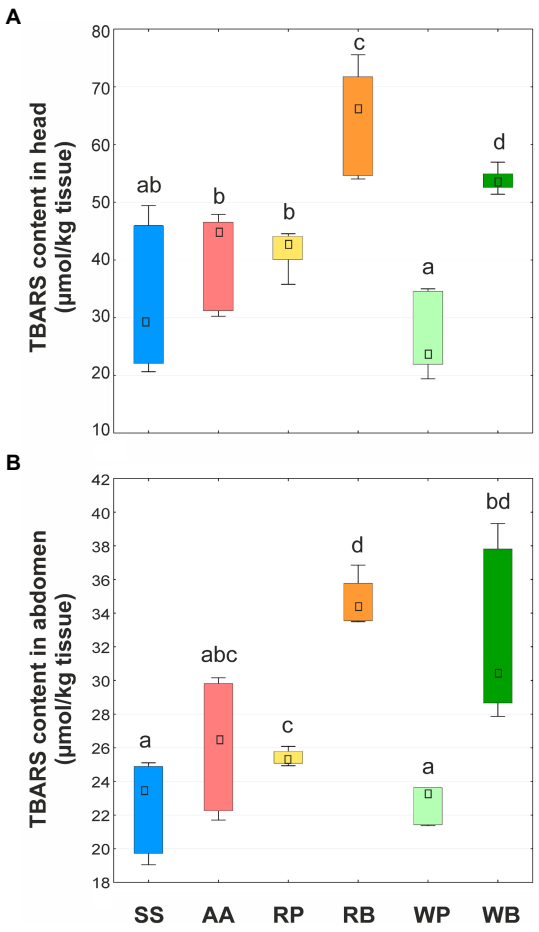

FIGURE 3
Content of thiobarbituric acid reactive substances (TBARS, µmol/kg tissue) in heads **(A)** and abdomens **(B)** of worker bees consuming different diets. SS, sucrose solution (control group); AA, sucrose solution+amino acids; RP, sucrose solution+rapeseed pollen; RB, sucrose solution+rapeseed beebread; WP, sucrose solution+willow pollen; and WB, sucrose solution+willow beebread. Boxes show first and third interquartile ranges with the dot denoting median. Different letters indicate significant differences between the values (p<0.05).

of bees. It was found that using a 50% aqueous sucrose solution (diet 1) to feed young worker bees ensured the highest survival rate within 6–8 days from the start of the experiment. Thereafter, however, the survival rate of the insects consuming this diet decreased and was the lowest on the 16th day after the start of the experiment. This effect can be explained by the gradual depletion of the pool of amino acids and other nutrients (vitamins and mineral elements) when using for feeding a sucrose solution without additives. It should be noted that adding a mixture of amino acids (diet 2) to the sucrose solution did not increase the survival rate of the bees. Moreover, when using this diet, the highest mortality compared to other feeding variants was observed starting from the 10th day (Figure 2). These results are consistent with the data that an optimal ratio between carbohydrate and protein content in the feed is necessary for high survival of worker bees, while consuming diets high in EAAs (Paoli et al., 2014) or proteins (Bouchebti et al., 2022) leads to a shortened life span of worker honey bees. Accordingly, it can be assumed that the concentration of amino acids we used in the diet 2 was too high and thus did not lead to an increase in the bee survival rate compared to the control group. Our results also show that the consumption rate of diet 2 was lower than that of the other diet variants (Figure 1), indicating that bees were trying to avoid this diet.

The highest survival rate of worker bees in our experiments was observed when consuming sucrose syrup with added pollen or beebread of rapeseed (*Brassica napus*) or willow (*Salix* spp.). These data further support the results of Di Pasquale et al. (2013, 2016) who found that adding pollen to sucrose syrup increased bee survival. However, Li et al. (2019) found that pollen supplementation did not significantly affect the lifespan of bees as long as their gut microbiome was not disrupted.

In our study, we chose for comparison pollen of rapeseed and willow since these plant species are ubiquitous in the Northern Hemisphere and represent an important source of nectar and pollen for honey bees and other pollinators (Čeksterytė et al., 2008; Ostaff et al., 2015). In particular, many species of willow begin flowering very early in spring, as the first wild pollinators become active following winter, and stop flowering by mid-May. A later-flowering willow species normally begins flowering in mid-May and stops flowering by mid-June (Ostaff et al., 2015). If willow pollen is particularly important for bees in spring, in the absence of other sources of amino acids and protein, the value of rapeseed pollen increases in summer, especially when bees are used to pollinate this crop. For instance, in Lithuania, beebread, collected in spring, contained almost equal parts of rapeseed and willow pollen, 45.1 and 41.8%, respectively. The content of rapeseed pollen in summer beebread was significantly higher, from 61.7 to 78.7%, while the content of willow pollen decreased, from 17.9 to 5.6% (Čeksterytė et al., 2008).

The nutritional value of pollen from different plant species depends on the protein and amino acid content, with the EAA content being particularly important (Day et al., 1990; Cook et al., 2003). In choice-test experiments, bees preferred pollen of rapeseed over that of field bean (*Vicia faba*) because rapeseed pollen contained a greater proportion of the most EAA, suggesting that rapeseed pollen is of greater nutritional quality (Cook et al., 2003). Our current results show that willow pollen has a nutritional value similar to that of rapeseed pollen, which agree well with the finding that positive influence of pollen consumption on honey bee health and survival was mostly independent of the pollen type (with the exception of *Cistus* spp. and *Zea mais* pollen; Di Pasquale et al., 2013, 2016).

It should also be noted that in our experiments, using rapeseed or willow beebread instead of pollen for feeding resulted in an increase in bee survival, although this difference was insignificant in the case of willow. This result can be explained by the fact that the artificial beebread contained honey in addition to pollen. Also, the beebread was fermented for 2 days (see the section Materials and methods), which could additionally affect its assimilation by insects. Hence, the exact mechanism of the beneficial effect of artificial beebread on bee survival compared to pollen is still unclear and needs further clarification.

Oxidative stress biomarkers in bees consuming of different diets

Previous studies have shown that extended longevity in insects correlates with enhanced expression of antioxidative defense system genes and increased activities of antioxidant enzymes, while defects of antioxidant system results in lifespan reduction (Phillips et al., 1989; Arking et al., 2000; Corona et al., 2005). Accordingly, it is believed that further investigation of ROS metabolism and antioxidative defense mechanisms can contribute to our understanding of bee survival and conservation of this species (Orčić et al., 2017). However, the effect of

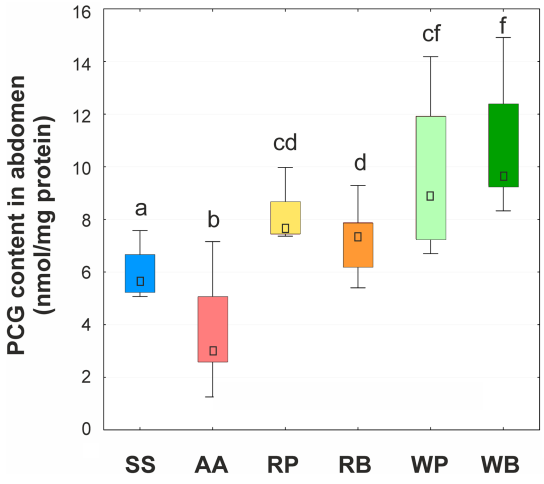

FIGURE 4
Content of protein carbonylic groups (PCG, nmol/mg protein) in abdomens of worker bees consuming different diets. SS, sucrose solution (control group); AA, sucrose solution+amino acids; RP, sucrose solution+rapeseed pollen; RB, sucrose solution+rapeseed beebread; WP, sucrose solution+willow pollen; WB, sucrose solution+willow beebread. Boxes show first and third interquartile ranges with the dot denoting median. Different letters indicate significant differences between the values (p<0.05).

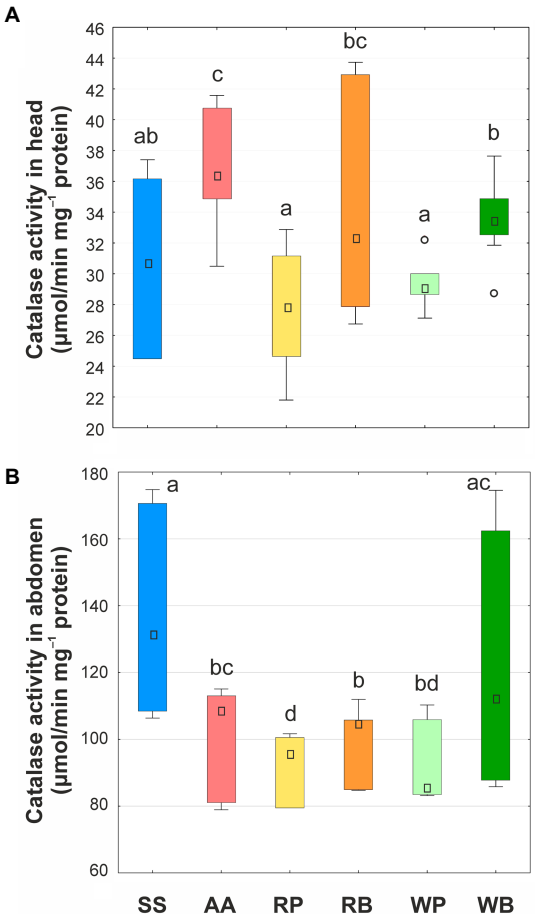

FIGURE 5
Catalase activity (µmol/minmg^{-1} protein) in heads **(A)** and abdomens **(B)** of worker bees consuming different diets. SS, sucrose solution (control group); AA, sucrose solution+amino acids; RP, sucrose solution+rapeseed pollen; RB, sucrose solution+rapeseed beebread; WP, sucrose solution+willow pollen; WB, sucrose solution+willow beebread. Boxes show first and third interquartile ranges with the dot denoting median. Whiskers encompass 90% of individuals, beyond which outliers are represented by circles. Different letters indicate significant differences between the values (p<0.05).

diet on the antioxidative status of worker bees is still poorly understood (Li et al., 2012; Wheeler and Robinson, 2014). Respectively, to assess the cell damage caused by production of ROS, we have measured two oxidative stress-related parameters, TBARS and PCG levels, which reflect membrane lipid peroxidation and the oxidation of cellular proteins, respectively (Levine et al., 1994; Reznick and Packer, 1994; Grotto et al., 2009; Cervoni et al., 2017).

Our results revealed that diet composition can significantly affect the intensity of lipid peroxidation in different body parts of worker bees. In particular, the consumption of sucrose solution with the addition of artificial willow and rapeseed beebread for 15 days led to an increase in the TBARS content, respectively, by 1.2–2.8 times in the head and 1.2–1.5 times in the abdomen compared to the bees receiving all other dietary variants, including pollen (Figure 3). Using the six experimental diets, a strong positive correlation was observed between TBARS levels in the head and abdomen (Table 1), indicating a systemic character of lipid peroxidation changes in different body parts of worker bees.

As mentioned above, the artificial beebread we used represents pollen mixed with honey and fermented for 2 days. Thus, the increase in lipid peroxidation in bees that consumed the artificial beebread must be caused by a compound absent from pollen but present in honey or produced in the fermentation process. Honey contains several substances that have pro-oxidant properties. The main components of honey are fructose (30–45%), glucose (24–40%), and sucrose (0.1–4.8%) those concentrations vary depending on the nectar source (White, 1957). According to our recent data, feeding worker bees a solution of fructose or a mixture of fructose and glucose resulted in an increase in lipid peroxidation compared to bees consuming glucose or sucrose solutions (Karavan et al., 2021). Besides, honey also contains 5 mM hydrogen peroxide, which has antimicrobial properties (White et al., 1963; Kwakman et al., 2010). This compound can activate oxidation of membrane phospholipids (Birben et al., 2012; Halliwell and Gutteridge, 2015). Thus, the presence of fructose and hydrogen peroxide seems to be a possible reason for the increase in lipid peroxidation in bees consuming artificial beebread.

Consumption of rapeseed beebread resulted in a slightly higher increase in lipid peroxidation than that of willow beebread. This can be explained by the different composition of the pollen of different plant species, in particular, the presence of different compounds that have pro- or antioxidant properties, such as carotenoids, flavonoids, anthocyanins, etc. (Rzepecka-Stojko et al., 2015; Bogdanov, 2016; Arathi et al., 2018; Radev, 2018). Especially, willow and rapeseed pollen differ in the content of polyunsaturated ω-3 and ω-6 fatty acids (Čeksterytė et al., 2008), tocopherols, ascorbate, polyphenols, etc. (Bogdanov, 2016).

Ingestion of rapeseed and willow pollen or beebread also resulted in an increase in another biomarker of oxidative stress and a PCG level in abdominal tissues (Figure 4). This result agrees with the data from Korayem et al. (2012), who showed an increase in hydrogen peroxide levels in worker bees in Egypt during the active season and suggested that this could be due to the intensive consumption of pollen and nectar, which contain phenolic compounds whose oxidation leads to increased ROS production (Thiboldeaux et al., 1998; Barbehenn et al., 2001; Mittapalli et al., 2007).

TABLE 1 Spearman rank order correlations among survival of bees and biomarkers of oxidative stress.

Variable	Survival, %	TBARS-Head	TBARS-Abdomen	PCG-Abdomen	CAT-Head	CAT-Abdomen
Survival, %	1					
TBARS-Head	0.257	1				
TBARS-Abdomen	0.314	**0.886**	1			
CO-Abdomen	**0.943**	0.143	0.143	1		
CAT-Head	−0.257	0.371	0.6	−0.486	1	
CAT-Abdomen	−0.257	0.143	−0.029	−0.314	0.429	1

TBARS-Head and TBARS-Abdomen, content of thiobarbituric acid reactive substances in head and abdomen; PCG-Abdomen, content of protein carbonylic groups in abdomens; and CAT-Head and CAT-Abdomen, catalase activity in heads and abdomens. Significant ($p < 0.05$) values are printed in bold.

In contrast to the increase in the TBARS content, the PCG level in the group of bees consuming pollen of both plant species studied did not differ from that in bees obtaining beebread. That is, the pro-oxidant compounds present in beebread enhance lipid peroxidation, but do not affect the carbonylation of proteins. Measurement of PCG levels also showed that consumption of rapeseed pollen caused a greater increase in this biomarker than willow pollen. This difference can be explained by the different composition of the pollen of the two species—see above.

In our feeding experiments, PCG levels were strongly correlated with survival (Table 1), suggesting that increasing the oxidative stress biomarker does not necessarily mean deleterious consequences for the bees. We believe that in our case, increased ROS levels when consuming a particular diet may reflect an overall activation of metabolism and/or ROS-dependent cellular signaling, resulting in increased bee survival. Such an interpretation agrees with the data of Scialò et al. (2016) that increasing mitochondrial ROS improves health and extends lifespan in *Drosophila*.

It should also be noted that the consumption of a mixture of amino acids (diet 2) led to a decrease in protein carbonylation. To explain this phenomenon, several mechanisms can be proposed: (i) Dietary amino acids protect cellular proteins from oxidative damage by interacting with ROS; (ii) Saturation of the cellular pool of amino acids promotes the synthesis of new protein molecules instead of ROS-damaged ones that undergo accelerated proteolysis; and (iii) Some amino acids have antioxidant properties and can affect the redox balance in the cell.

Activity of catalase

Cellular levels of ROS are under the control of the antioxidant system. One of the most important antioxidant enzymes is CAT, which splits hydrogen peroxide directly and is considered an biomarker of the general condition of the antioxidant system in insects (Corona and Robinson, 2006; Badiou-Bénéteau et al., 2012). Accordingly, the activity of this enzyme was determined in the next step of our research.

Ingestion of artificial beebread was revealed to cause a moderate (14%) increase in CAT activity in the head of worker bees (Figure 5), which can be considered a protective response to the consumption of diets containing hydrogen peroxide. Interestingly, an increase in the activity of CAT was also observed when consuming a mixture of amino acids, although no signs of oxidative stress (e.g., an increase in lipid peroxidation) were observed. Hence, this last effect requires further explanation.

Our measurements showed that for all diets used, CAT activity in the abdomen was significantly higher than in the head. It can be assumed that the high activity of CAT in the abdomen is necessary for the splitting of hydrogen peroxide, the formation of which is associated with the digestion of food or some other process characteristic of this part of the body. When the bees were fed different diets, the highest activity of CAT in the abdomen was observed in the control group of bees consuming the sucrose solution. The increase in CAT activity is thought to represent a component of the stress response activated by poor nutrition, namely depletion of the amino acid pool. Accordingly, when the "Aminosteril" or another source of proteins/amino acids was added to the diet, the activity of CAT became lower. Moreover, the lowest values of CAT activity were observed when pollen was consumed, i.e., with a more balanced diet. At the same time, when feeding with beebread, the activity of CAT in the abdomen increased, similar to that in the head.

It was shown recently that during wintering activity of CAT in worker bees was increased while activity of other antioxidative enzymes, superoxide dismutase (SOD), and glutathione transferase (GST) as well as lipid peroxidation were reduced indicating a decrease in production of ROS (Orčić et al., 2017). Considering our new results, it is tempting to speculate that the increase in CAT activity is necessary for the splitting of hydrogen peroxide contained in honey, consumption of which increases in winter compared to summer.

In winter, worker bees are relatively inactive and have a longer lifespan compared to summer. Accordingly, it was hypothesized that this lengthening of lifespan is associated with a decreased ROS production observed in winter, while the high activity of bees in summer is accompanied by an increase in ROS levels, which causes oxidative stress and leads to a shortening of lifespan (Münch et al., 2008; Orčić et al., 2017). Conversely, in our experiments, the lowest values of oxidative stress biomarkers were observed in bees that consumed only sucrose solution or sucrose solution with the addition of a mixture of amino acids and had the shortest lifespan. At the same time, bees that received pollen or beebread had the longest lifespan. Consumption of these dietary variants enhances lipid peroxidation and protein carbonylation in head and/or abdominal tissues of worker bees, which, however, was not detrimental but beneficial to insect survival.

We believe that the observed increase in biomarkers of oxidative damage does not reflect intracellular redox imbalance and oxidative stress, but is a direct consequence of dietary pro-oxidant intake, which is effectively compensated by antioxidant defenses. This example shows that the biomarkers of oxidative stress should be considered with caution: certain dietary options can induce a moderate oxidative damage, but do not have a negative impact on life expectancy.

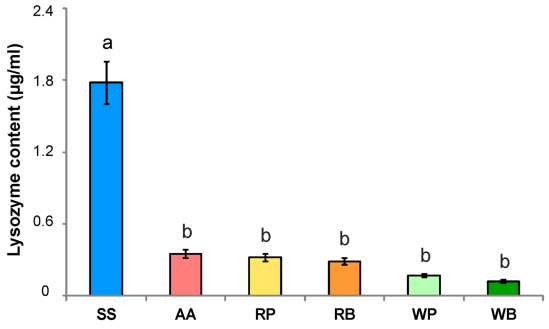

FIGURE 6
Lysozyme content (µg/ml) in the hemolymph of worker bees consuming different diets. SS, sucrose solution (control group); AA, sucrose solution+amino acids; RP, sucrose solution+rapeseed pollen; RB, sucrose solution+rapeseed beebread; WP, sucrose solution+willow pollen; WB, sucrose solution+willow beebread. Different letters indicate significant differences between the values (p<0.05).

Activity of lysozyme

Reactive oxygen species (ROS) are not only damaging compounds, but are also used by insects as a weapon to fight pathogens (Nikolenko et al., 2012; Orčić et al., 2017). Accordingly, it could be expected that changes in the redox state caused by different diets would affect the immunity of bees. Therefore, at the next stage of our research, we determined the activity of lysozyme, an enzyme possessing antibacterial and antifungal activity that is synthesized in fat body (Gillespie et al., 1997; Imler and Bulet, 2005). We found that the activity of lysozyme in the hemolymph of worker bees fed sucrose solution was significantly higher than after consumption of all other diets (Figure 6). Thus, the lack of amino acids/proteins is the main factor causing the increase in lysozyme activity, while other nutrients present in pollen and beebread have less influence on this biomarker.

Previously, the influence of different diets on the immune competence of honey bees was examined. Protein deficiency caused a significant increase in the percentage of granular hemocytes in hemolymph, a significant decrease in other types and lower metabolic activity of hemocytes, indicating a reduced ability to phagocytosis. Therefore, it was supposed that haemocyte concentration might increase in bees fed without protein to compensate for their decreased activity (Szymaś and Jędruszuk, 2003; Alaux et al., 2010). Also, a relative mass of fat body, the main site of antimicrobial peptide synthesis (Imler and Bulet, 2005; Alaux et al., 2010), and activity of glucose oxidase, which generates hydrogen peroxide and contributes to colony-food sterilization (White et al., 1963), were decreased in bees consuming a non-proteinaceous diet (Alaux et al., 2010). It was also shown that p-coumaric acid present in pollen specifically up-regulates select antimicrobial peptide genes, those expressions were compromised in bees consuming only sucrose (Mao et al., 2013). Taken together, these results show that the consumption of non-protein diets leads to a decrease in the immunocompetence of worker bees. Our new data suggest that the increase in lysozyme activity can be viewed as an alternative protective mechanism induced in the absence of adequate nutrition, namely amino acid and protein deficiencies.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

GB and RV conceived the ideas for this study. GB, RV, and LY designed the research program. GB, IP, LY, and MD conceived and designed the experiments. LY, VK, GB, and MD performed the experiments. LY, GB, IP, and VK analyzed the data. RV, LY, and IP wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Decision-making criteria for pesticide spraying considering the bees' presence on crops to reduce their exposure risk

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The risk of poisoning bees by sprayed pesticides depends on the attractiveness of plants and environmental and climatic factors. Thus, to protect bees from pesticide intoxication, an usual exemption to pesticide regulations allows for spraying on blooming flowers with insecticides or acaricides when no bees are foraging on crops. Nevertheless, decision-making criteria for farmers to assess the absence of bees on their crops remain under debate. To fill this gap, we present here a review of the literature and an analysis of weather conditions and environmental factors that affect the presence of bees on flowering crops that may be treated with pesticides, with the objective of proposing to farmers a series of decision-making criteria on how and when to treat. We conclude that the criteria commonly considered, such as ambient temperature, crop attractiveness, or distance from field edges, cannot guarantee the absence of forager exposure during pesticide sprays. Nocturnal sprays of pesticides on crops would be the most effective action to help farmers avoid unintentional acute poisoning of bees.

KEYWORDS

bees, pesticides, pollination, regulation, beekeeping, pollinators

1. Introduction

Before agricultural use, pesticides that protect cultivated plants against harmful organisms (e.g., insects and fungus) or undesired weeds, must be evaluated and approved by regulatory authorities. In the current regulatory framework of unintentional exposure to non-target organisms, such as bee pollinators, the effects of pesticides are assessed by standard tests (EPPO, 2010; EFSA, 2012). However, pesticide threats to bees persist despite regulatory efforts (Decourtye et al., 2019; Mancini et al., 2019; Sgolastra et al., 2020). Thus, additional direct measures applied to bees were initiated to protect these non-target species from poisoning when farmers use pesticides. For instance, French regulations stipulate that insecticides or acaricides bearing the « Bee label » have an exemption that allows for their application on flowering plants, provided that foraging bees are absent from the treated crops (JORF n°0271).[1] In Europe, the regulations specify that pesticide sprays not be applied during crop flowering or during foraging periods (Directive 2009/128/EC on Sustainable Use of Pesticides and

1 https://www.legifrance.gouv.fr/jorf/jo/2021/11/21/0271

related National Action Plans from 28 member states of the EU). When regulations prohibit the spraying of pesticides during the period of foraging activity of bees, the decision-making criteria to help farmers avoid unintentional poisoning of bees on crops remain unknown. This article aims to present an overview of the current knowledge on bee-crop interactions in order to clarify the decisions needed before the spraying of pesticides on flowering plants. In the first part, we recalled that regulations must protect a wide diversity of bee species that have different sensitivities to pesticides. In the second part, we assessed the limits of the current regulations to protect bees from pesticides by reviewing the exposure of bees to residues under real conditions. In the third part, based on the field data collected by the consortium of French apidologists, we analyzed the environmental and climatic conditions that would guarantee the absence of bees on agricultural crops. Finally in the fourth part, we investigated whether considering attractiveness of crops the exposure of bees to pesticides can be mitigated.

The two first parts set the scene and explain how we identified the two research questions which we then proceed to answer in the two following parts: (i) which factors related to the environment, the season and the climate best determine the foraging activity of bees and (ii) can we consider the attractiveness of cultivated plants as a criterion to help farmers in their decision? To address these questions, we have created a consortium of French apidologists who have developed their answers based on (i) previously published works and (ii) the analysis of their own datasets for which the methods have already been published. The answers will contribute to decision-making criteria for the use of pesticides on flowering plants in crops.

2. Regulatory obligation to protect all bees

While flies, beetles and butterflies take part in the pollination of cultivated flowering plants (Rader et al., 2016), bees represent the most important group of insect pollinators (Kevan and Baker, 1983). In collecting the pollen and nectar of flowers to feed themselves, and to feed their progeny, they passively transfer pollen between flowers, a phenomenon to which their pollen-harvesting morphology (pollen baskets and brushes) and feather-shaped hairs contribute (Michener, 2007). The biological and ecological traits of about 2000 bee species known in Europe (20.000 in the world) are very diverse in terms of social organization (even though most are solitary), feeding specializations (certain harvest their pollen from a single plant species, others are more generalist), mobility (from a few hundred meters to more than 10 km) and reproductive habitat type (even if the majority are ground nesting). While this wide diversity of bee species may be affected by pesticide use, the Western honey bee *Apis mellifera* L. is the only species to date considered in the evaluation of toxicity before marketing pesticides (SANCO, 2002). Beyond approval tests for marketing, information about the median lethal dose (LD_{50}) of pesticide active ingredients are mainly available for honey bees, sometimes for bumble bees (*Bombus* spp.), and much less frequently for other wild pollinators (Lewis et al., 2016; Lewis and Tzilivakis, 2019; Yasrebi-de-Kom et al., 2019). Moreover, wild solitary bees may be more sensitive than honey bees or other large bees (e.g., bumble bee species) to the effects of pesticides (Rundlöf et al., 2015; Azpiazu et al., 2021). For a given active ingredient, toxicity level can strongly vary between species. For instance, the median LD_{50} by contact for thiamethoxam is 0.28 µg.bee^{-1} for bumble bees and 0.024 µg.bee^{-1} for honey bees, while it's only 0.004 µg. bee^{-1} for solitary bees (Lewis et al., 2016; Lewis and Tzilivakis, 2019). Consequently, the application conditions of pesticides should consider the diversity of bees.

3. Bees' exposure to pesticides

3.1. Exposure to pesticides despite restrictive usage limits

In addition to work carried out in the United States (Mullin et al., 2010) and in the rest of Europe (Ghini et al., 2004; Bernal et al., 2010), the research shows that honey bees, honey, pollen and wax are regularly contaminated by pesticide residues (Chauzat et al., 2006, 2009; Lambert et al., 2013; Calatayud-Vernich et al., 2018; Tosi et al., 2018; Friedle et al., 2021). These works describe, in particular, considerable contamination by fungicides, insecticides and acaricides in the bees, as well as in the pollen and honey that they consume. The latter may be used by growers to protect their crops and by beekeepers to fight against the ectoparasitic mite *Varroa destructor*, a major thread for the honey bee colonies. The potential presence of synthetic anti-*Varroa* acaricide residues in the beeswax of the hive has driven the development of alternative management methods to fight against *Varroa* (organics acids, essential oils, genetic selection). The work of Lambert et al. (2013) showed that 72% of honey bees sampled ($n = 141$), 58% of pollen ($n = 128$) and 95% of honey ($n = 14$) collected contained at least one pesticide residue in western France. More precisely, five different fungicides were detected in the bees and nine in the pollen and honey. For the insecticides, 11 different residues were detected in the bees, whereas 10 or 15 were found in the pollen and honey, respectively. This interesting study showed that honey bees are regularly exposed to insecticides and the high concentrations measured for certain substances (for example, permethrin: 48 µg/kg per bee) suggest direct exposure to the bees during their foraging activity.

3.2. Co-exposure of bees to pesticides

The recent improvement in the sensitivity of chemical analysis methods allows for the detection of multiple substances. These advances allowed for the demonstration that the bees are simultaneously exposed to multiple pesticide residues. For example, Lambert et al. (2013) describe that, on average, honey bees are contaminated by 1.4 pesticides and the most contaminated bees contain up to 6 different pesticide residues. In the United States, extensive contamination was observed, on average, as 2.5 pesticide residues were found per honey bee sample and 25 pesticide residues in the most contaminated sample (Mullin et al., 2010). In France, the food supply of honey bees is also contaminated by multiple residues, as evidenced by studies conducted by regional beekeeping development organizations: 25% of the pollen collected by foragers is contaminated by at least 5 pesticide residues (Vidau, 2015; Table 1).

The frequent co-exposure of bees to pesticides, but also to other stress factors (for example, pathogens/pathogenic agents, nutritional deficiencies; Goulson et al., 2015) led the European Authority of Food Safety (EFSA) to recommend that the evaluation procedure for the toxicity of a phytosanitary product, before being marketed, should integrate tests that measure the effect of chemical co-exposure with another compound (ANSES, 2015; EFSA, 2019).

TABLE 1 Results of chemical analysis of 165 pollen samples collected in five French regions (Vidau, 2015).

Number of analyzed samples	165
Number of contaminated samples	72%
Number of samples contaminated by more than 5 residues	25%
Maximum number of residues by sample	11
Total number of residues detected	66
Number of insecticides detected	23
Number of fungicides detected	32
Number of herbicides detected	8
Number of growth regulators detected	3

This section illustrates, with examples, that current regulations are not sufficient to protect bees from pesticide exposure. In field conditions, bees are often exposed to mixtures of pesticides, which when applied individually have no toxicity, but might induce negative effects when tested as a cocktail (Prado et al., 2019). We propose that clarifying the criteria to ensure the absence of foragers on flowering crops would reduce this risk.

4. Criteria to ensure no foragers in flowering crops

Bees are commonly exposed to multiple pesticide residues everyday, in particular when they forage on treated crops. One question remains in order to prevent such direct exposures: what factors related to the environment, the season, and the climate, determine bee foraging on crops? Various factors could affect bee foraging activity. Ambient temperature is the meteorological factor most frequently cited in the literature on bee foraging activity. Relative atmospheric humidity shows less of an effect on foraging activity than temperature on honey bee workers' foraging activity and survival (Joshi and Joshi, 2010; Abou-Shaara et al., 2012, 2017). Overall, honey bees need to maintain their thoracic temperature within a certain range: 31–32°C during foraging (Heinrich, 1979). Beyond the honey bee, each bee species has specific ambient temperature thresholds (min. and max.) to maintain the body temperature and withstand the necessary energy costs (Stone, 1994). To do so, the body mass is a determining factor in insect thermoregulation (Heinrich, 1979; Stone et al., 1988; Herrera, 1990). The body temperature is greatly influenced by wind speeds and even a small increase in wind speed can result in a lowering of this parameter (Digby, 1955; Church, 1960). Hennessy et al. (2020) found an increase in wind speed of just 2.75 m/s resulted in a 37% decrease in floral visits. It is commonly accepted that more foragers are observed at wind speeds below 4 m/s (Rollin et al., 2013). In this article, we do not address in further depth the interest of wind speed as a criterion to reduce the risk of spraying pesticides, because these sprays are forbidden in the case of strong winds.

4.1. What is the relationship between foraging activity and ambient temperature?

Foraging activity of honey bees (number of flights per time unit) takes place within a wide range of temperatures, from 10 to 40°C (Abou-Shaara, 2014). Clarke and Robert (2018) found that 78% of the observed variations in honey bee activity was explained by variations in temperature and solar radiation related to cloud cover. The temperature threshold below which the honey bee can no longer forage varies according to the source: 6°C according to Tan et al. (2012), 7°C from Heinrich (1979), 9°C from Burrill and Dietz (1981), 12°C from Danka et al. (2006), and 16°C in tropical areas according to Joshi and Joshi (2010). Tan et al. (2012) observed an optimal foraging activity at around 20°C. For Burrill and Dietz (1981), the 9°C threshold, below which the honey bee does not fly, was independent of luminosity.

Corbet et al. (1993) studied the effect of climate on the foraging activity of different species of social bees: *Apis mellifera* and 5 species of bumble bees (*Bombus lapidarius, B. terrestris, B. lucorum, B. pascuorum* et *B. hortorum*). Corbet et al. (1993) and more recently Sanderson et al. (2015) and Clarke and Robert (2018) showed that the ambient temperature and the level of radiation were positively correlated with the foraging activity of these social bee species. These results were confirmed in Portugal and in the United Kingdom on phacelia and on flowering shrubs. Workers of *A. mellifera* and *B. lapidarius* start foraging at lower temperatures than workers of *B. terrestris*, *B. lucorum*, *B. pascuorun* and *B. horotorum*. Compared to the Western honey bee, most species of bumble bee are indeed known for having flying activity at lower ambient temperatures (Lundberg, 1980; Stone and Willmer, 1989; Corbet et al., 1993).

Some studies investigated the effects of multiple environmental variables on the foraging activity of honey bees. For instance, Burrill and Dietz (1981) showed correlations between foraging activity and temperature and solar radiation, two parameters that change inversely with relative humidity and atmospheric pressure. Overall, the optimal range of temperature for the foraging activity of the honey bee species (*Apis cerana*, *A. dorsata* and *A. mellifera*) ranges from 21.0 to 33.5°C (Usha and Devi, 2020). While foraging activity increases in a linear manner with temperature between 13 and 23°C, independently of the luminosity, other authors showed a quadratic effect of temperature on the foraging activity in the same *Apis* species (Danka et al., 2006; Abou-Shaara et al., 2012). In particular, the foraging activity increases with temperature up to 24°C and then decreases up to 30°C. A study on oilseed rape (*Brassica napus*) showed that between 27 and 45°C, the density of honey bees decreased as the temperature increased (Blažytė-Čereškienė et al., 2010). This inhibitory effect of rising temperatures would be particularly observed in pollen foragers (Cooper and Schaffer, 1985). It was also observed that the foraging activity of non-*Apis* pollinators (i.e., non-*Apis* bees and Syrphid flies) decreases with temperature, especially above 33°C (Usha and Devi, 2020).

New statistical analyses of the dataset of Rollin et al. (2013, 2019) show a significant nonlinear influence of temperature on the presence and abundance of bees in the sampled sites (red curves: Figure 1). In addition, the intensity of this effect varies as a function of the bee group considered (i.e., honey bees, bumble bees, and solitary bees) and the sampling period (April, May–June, July, September). In this study, field inventories of bee species (honey bees, bumble bees and solitary bees) were undertaken on different flowering plant covers and crops at the Plaine and Val de Sèvre (ZAPVS) research site, in the Poitou-Charentes region of France. Collections were conducted at an ambient temperature of ≥16°C and wind speed of ≤15 km/h (Rollin et al., 2013, 2019). The abundance of honey bee foragers (and of all bees) measured on flowers decreased at the highest temperatures. This effect seems less obvious for the bumble bees and solitary bees. The results from a study on the solitary bee species *Anthophora pauperata* follow the same trend.

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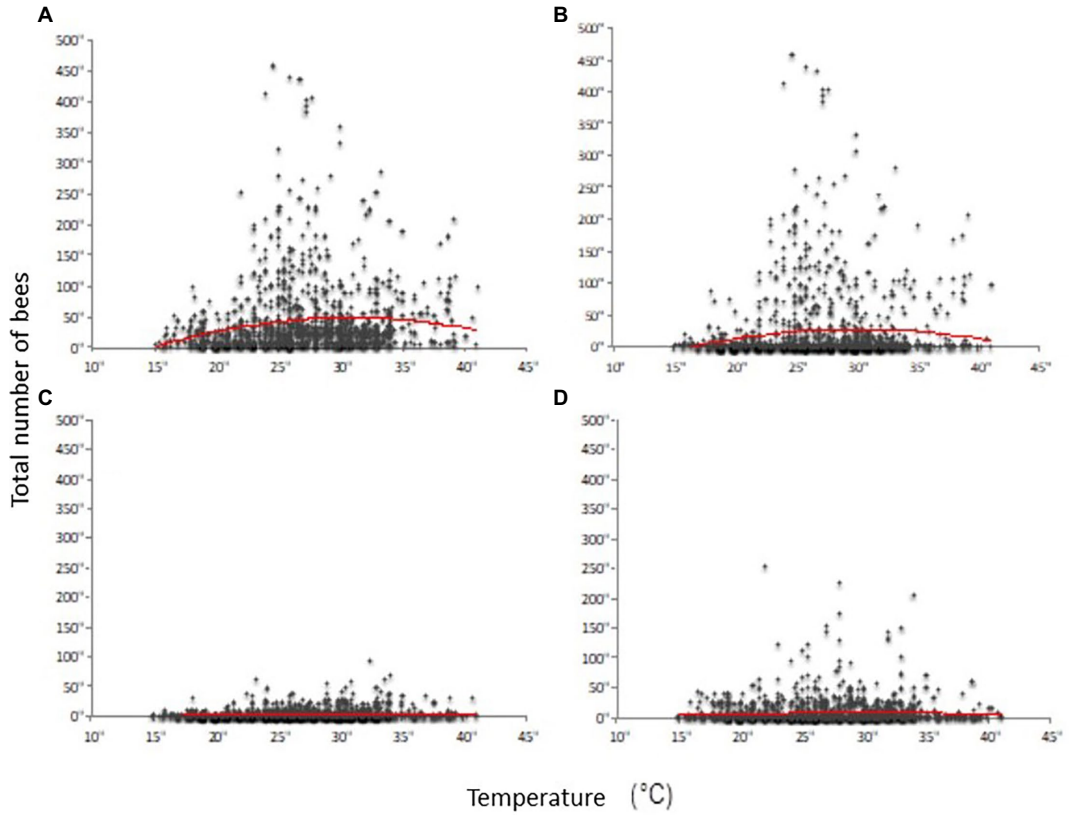

FIGURE 1
Abundance of bees on blooming plants as a function of ambient temperature. The *x*-axis is expressed in °C. The graphs show the abundance of all bees **(A)**, managed bees **(B)**, bumble bees **(C)** and wild bees other than bumblebees **(D)**. The points illustrate abundance data and the red curve illustrates the result of the mathematical model linking abundance data to temperature (polynomial function; Rollin et al., 2013).

Increasing ambient temperature up to 25–30°C at midday did not lead to a reduction in foraging activity (Stone et al., 1999). On the other hand, a study on foraging by the bumble bee *Bombus terrestris* conducted in the greenhouse at high temperatures showed that the intensity of colony exit rates and the foraging activity of workers peaked at 25.7°C (during the morning). However, at an average temperature of 32.3°C, foraging activity decreased significantly by 70% and colony entrance traffic by 40% (Kwon and Saeed, 2003).

4.2. The effect of interactions between temperature/season/time of day

Devillers et al. (2004) used co-inertia analyses to look for statistical links between data on hive exit activity, recorded using an electronic counter, and temperature, overall sunlight, humidity, wind, and rain in the Rhone-Alpes region (France) between July and September. The existence of a co-structure between hive exit activity over 24 h and the temperature or overall sunlight was clearly established.

In the ZAPVS research site, the flying activity of thousands of honey bee workers was recorded using Radio Frequency Identification (RFID) from April to August. This life-long monitoring of bees records two bee life history traits, namely the number of exits per day and the duration of these trips (Requier et al., 2020). Similar to the previous study, flight activity (reflected by these two life traits) was significantly affected by the temperature and time of year (month). Exit activity is positively correlated with daily average temperature (Figure 2). But surprisingly, the exit activity recorded in September was not affected by temperature, i.e., the number of exits did not changed whether it was 12°C or 22°C. This month also showed a lesser effect of temperature on the duration of worker trips. One explanation of this activity pattern could be that this time of year (i.e., fall in September) was particularly dedicated to food reserve storage by the colonies in preparation for over-wintering. Interestingly, the daily flight activity of honey bees varied across the season (Figure 3). June is the period with longer daily activity, likely due to the fact that this month has a longer duration of daily sunlight. Another explanation relates to the foraging effort. Indeed, June is the period of food shortage for bees in such an intensive farming system (Requier et al., 2015, 2017; Timberlake et al., 2019), potentially affecting the need to increase the daily activity of foraging.

If ambient temperature is the most frequently cited criterion, the literature shows that it is difficult to determine a threshold to protect all bee species from pesticide spraying.

5. Crop attractiveness

Another question concerns the relationship between bee species and crops. Wildlife inventories carried out from April to August over 3 years (2010–2012) in the ZAPVS research site consisted of capturing and identifying to species around 30,000 bees foraging on flowers and on more than 800 plant covers (Rollin et al., 2013). The results revealed

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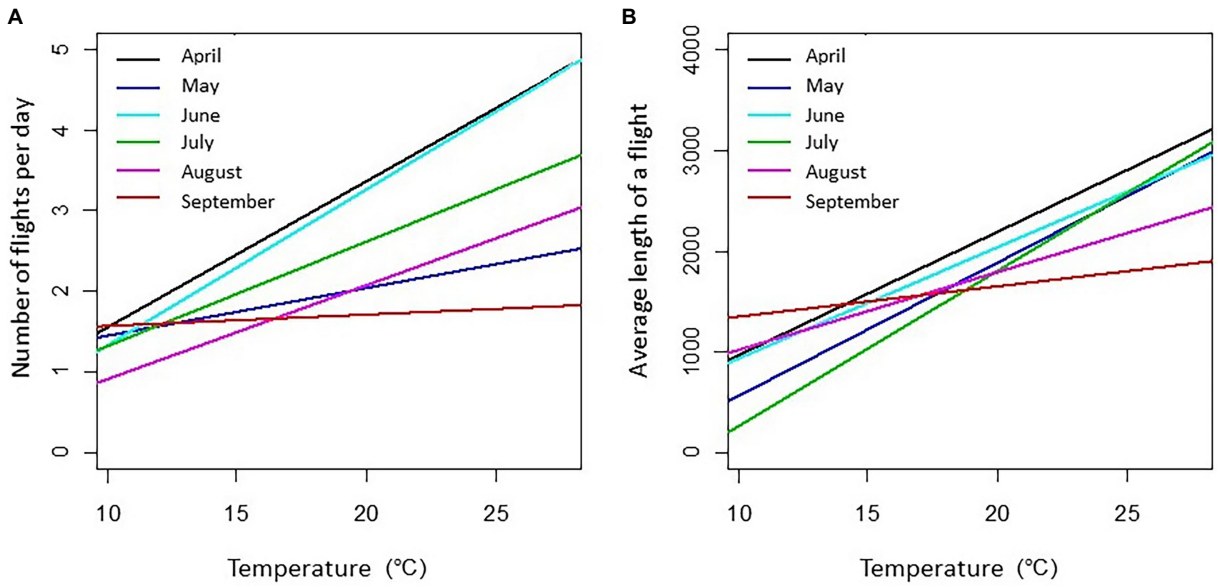

FIGURE 2
Mathematical model that predicts the number of honey bee exits from the hive according to ambient temperature. The curves depict the results of linear models, all significant, established based on recordings of RFID transponder-labelled workers (*n*=1,330 bees in total; Requier, 2013). **(A)** Number of exits by bee and by day. **(B)** Average duration of trips by bee and by day in second. The amplitude of daily temperatures recorded in April, May, June, July, August, and September were, respectively, 10.2–20.7, 12.9–27.6, 13.7–27.6, 15.1–26.1, 13.3–26.1, and 12.0–22.3°C. The number of recorded bees per month ranged from 90 to 308.

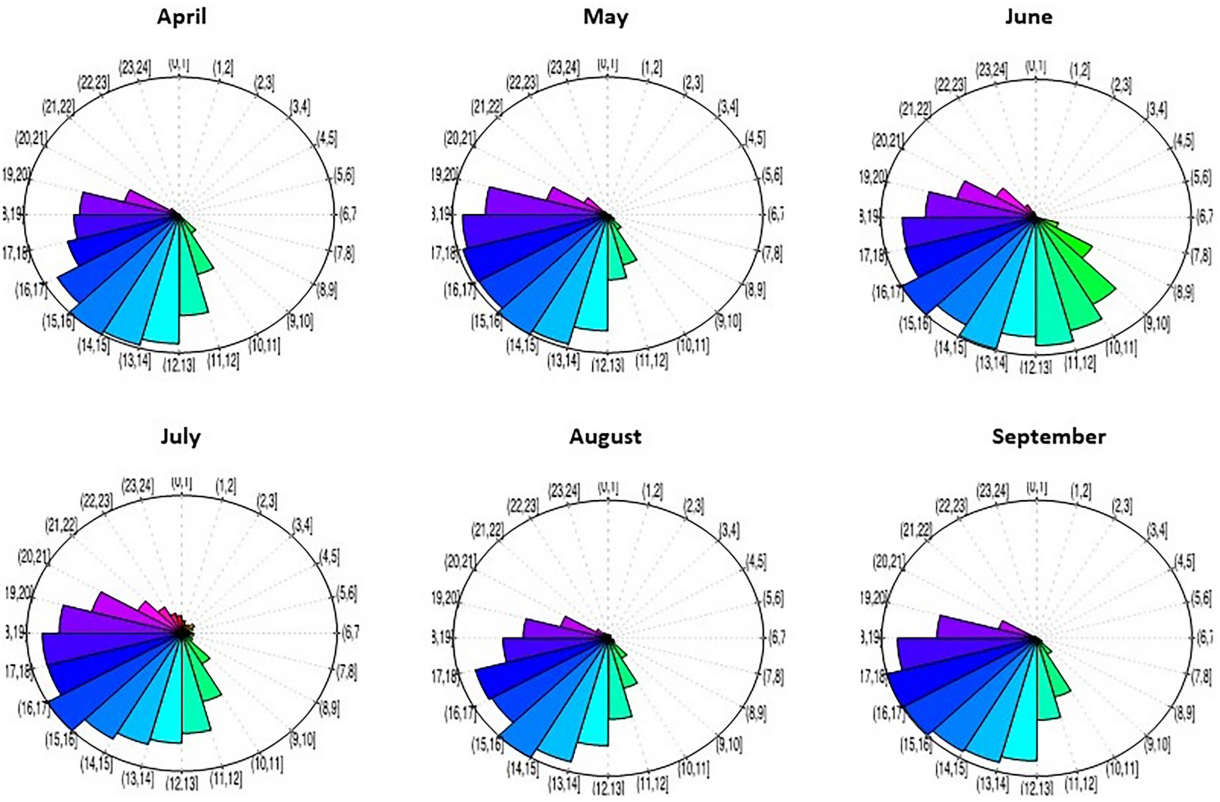

FIGURE 3
Honey bee exit activity from the hive by time of day. The circles depict the 24h of a day during which we recorded trips of RFID transponder-labeled workers (*n*=1,330 bees in total introduced at the beginning of every month between April and September by cohort of 90–308 individuals). The circle shows the maximum exit activity recorded per hour in the month (baseline from which the exit rate at other hours was calculated). The hours are those recorded by the RFID device- embedded computer (Requier, 2013).

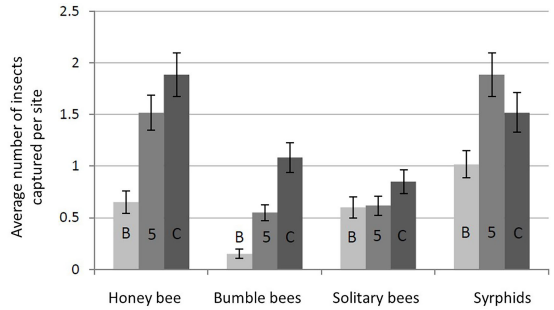

FIGURE 4
Average number of foraging bees according to position in the rapeseed plot. The bars represent the standard error. B: border, 5: at 5m from the border, C: center of the field plot (method described in Le Féon, 2010).

clear differences in the use of floral resources between groups of bees during the flowering of rapeseed and sunflower. Honey bees, and to a lesser extent, bumble bees, prefer to forage the flowers of rapeseed and sunflower over the wildflowers in the meadows and roadsides, whereas the opposite is true for solitary bees (Figure 4). Solitary bees were observed in greater numbers on wildflowers in meadows and edges, than in the rapeseed and sunflower fields. The diversity of bees on rapeseed measured 4 times less than that on the natural, herbaceous flora (Rollin et al., 2015). In contrast, bumble bees were more present on the flowers of oilseed crops than the other floral covers, but less markedly than the honey bees.

The wild bees use the crops as a food resource, like honey bees, but also as a nesting resource. In fact, certain ground-nesting bee species may nest in the soil within the crops (Shuler et al., 2005; Esther and Roulston, 2009). Nesting in the crop fields should be considered, because around 80% of solitary bees are ground-nesting species (Antoine and Forrest, 2021).

Nutritional resources, such as nectar, pollen, guttation drops, honeydew, and water, are found in cultivated fields and can be harvested by bees. To our knowledge, the use of the latter three resources by the bee has never been sufficiently studied to assess their use compared to the variability of factors such as the type of plant cultivated, the weather, the time of day, or the season.

5.1. How to classify cultivated plants according to their visits by bees?

For instance, in France, the ITSAP institute database[2], based on specialized expertise, yields a honey bee attractivity index for almost 50 crops, cover crops or mixture for honey-fallows. This database attributes three scores: weakly or not attractive (1), somewhat attractive (2), and very attractive (3). But this approach veers far from the reality since crop visits by bees show a variability that these qualitative values miss. Incidentally the quantitative data available on the nectar-producing potential of a plant species (i.e., the expected value of honey production for a given plant species in kilograms per unit of surface area) sufficiently convey this variability (Ion et al., 2018). For the cultivated species, the available databases show wide variability in their estimations:

- for oilseed rape (*Brassica napus* L.), the minimum noted is 67.5 kg/ha and the maximum is 325 kg/ha, a factor of approximately 5 between the two estimated values (Pierre et al., 1999; Ion et al., 2012). Baude et al. (2016) indicated an average of nectar sugar content of 394 µg/flower/day, with a standard deviation of 301;
- for pear (*Pyrus communis* L.), a species grown in different regions (flowering in March–April), bibliographic sources show nectar-producing potential ranging by a factor of 9 depending on the author – from 6 to 50 kg/ha, from Koltowski (2006) and Janssens et al. (2006), respectively;
- concerning cornflower (*Cyanus segetum* L.), a volunteer, crop-associated Asteraceae that flowers in June, its honey-producing potential is estimated between 60 kg/ha and 350 kg/ha (Koltowski, 2006), a difference of a factor of 6.

This variability can be explained by the method used, but also by the health and developmental state of the bees and environmental, agronomic (agricultural practices, crop variety), and pedoclimatic conditions (Ion et al., 2018). Another major limit of the indicator is that it does not integrate visits to plants by the bees for pollen. Additionally, the visit of crops by bees depends on other flowering plants available nearby (Henry et al., 2012; Rollin et al., 2013). A plant with an attractiveness labeled as medium will be better visited in the absence of alternative flowers.

5.2. Foraging of weeds in the crops

We previously saw that honey bees visit corn plots for the pollen of the farmed crops, but also use the weeds found in the crop. The ranking of cultivated plants, by their probable visitation by honey bees, has its limits, given that a farmed field, that produces no nectar nor pollen, can host attractive wild plant species. For example, the pollen of annual mercury (*Mercurialis annua* L.), a weed often observed in corn (*Zea mays* L.) crops, is consistently found in forager pollen pellets in the summer (up to 15% of the supply). But bees gather pollen from spontaneous plants in a variety of crops, not just corn (Bretagnolle and Gaba, 2015). This use is surprising in terms of the diversity of plants used, in frequency, and in the amount of pollen brought back to the hive. As such, 96 different pollens were catalogued in the samples from the ECOBEE long-term bee colony monitoring (Odoux et al., 2014) representing more than 30% of pollen yields during the corn flowering season (Requier et al., 2015).

A crop of cereal straw, having little interest in and of itself, will likely be visited by bee foragers if it contains weeds such as cornflower (*Centaurea cyanus* L.) or poppy (*Papaver rhoeas* L.). Poppy pollen can represent, in intensive cereal farming systems, 10% of the biomass of pollen harvested by honey bee colonies from April to September, making it the second-most harvested pollen after maize pollen (Requier, 2013; Requier et al., 2015). But the importance of the resources offered by weeds in crop fields is not reserved to intensive cereal farming systems. In July 2013, multiple pollen samplings were undertaken in an observation study during the lavender nectar flow using pollen traps ($n = 5$) and following eight apiaries in the Provence Drôme region (France). Palynological analysis of each sample revealed that the majority of pollen does not belong to the cultivated plants but to semi-natural (or volunteer) flora found in the inter-row

2 https://interapi.itsap.asso.fr/

areas. More precisely, the species of the genus Asteraceae (40%) and the herb plantain (*Plantago* spp.; 15%) are the most used floral species by the bees in a landscape that is primarily composed of vineyards (*Vitis vinifera* L.), cereal and lavender (*Lavendula spp.*). This pollen supply proved to be contaminated by 40 pesticide residues. Thus, taking into account the nature of the recovered pesticide residues and the application treatment calendars used during the month of July in this region, these observations suggest that the bees are overwhelmingly exposed to pesticides applied on the grapevines that contaminate the wildflowers traditionally found in the inter-row areas and on the field edges.

5.3. How are foragers spatially arranged within the fields?

The spatial distribution of foragers within the farmed plots is a frequently asked question by farmers. Measuring the foraging of honey bees along a distance gradient from the border of a field to its center, Sáez et al. (2012) found a reduction of 25% in the number of visits on capitulum inflorescences between 1 and 100 m. The density of the foragers is up to three times lower in the central rows of corn crop field than in the border rows (Thibord et al., 2015). But an analysis of these data calculating the number of foragers present on the surfaces shows that the center (surface subtracted from those corresponding to 8 m from border rows) of 10, 50, and 100 ha corn crop fields could host 75, 88, and 91% of observed, respectively. This result is also found in oilseed rape where the total abundance of pollinators, including the Western honey bee, syrphids and bumble bees (but not solitary bees), is greater in the interior of the fields than at the edges (Figure 4). The spatial distribution of bumble bee foragers favoring the center of the oilseed rape fields observed in France (research site: Pleine-Fougères, Bretagne) was confirmed by results obtained in Denmark (Calabuig, 2000); in this latter study, on the other hand, solitary bees were more abundant at the edges of the field. This result was confirmed in a study in France in which the distance to the field edge had a negative effect on the abundance of solitary bees such as the andrenids and *Nomada* species in oilseed rape fields (Bailey et al., 2014). These authors highlight a significant effect of the intertegular distance (the distance separating the base of the two wings of the bee) on the spatial distribution of different species of bees within the oilseed rape fields. This morphological trait measurement reflects the size of the individual and corresponds to an estimation of their flight capacity (Greenleaf et al., 2007). Larger bees (such as bumble bees) have a tendency to fly further into the middle of the rapeseed fields than smaller bees (such as *Andrena* and *Nomada*).

6. Conclusion

Whereas in many countries the spraying of insecticides and acaricides on flowering plants or during the foraging activity of bees is regulated, it is necessary to identify the decision-making criteria that could be used by farmers to protect bees. One option would be to choose a criterion depending on the crop type. For this, data are available for attributing a degree of visitation by the honey bee to cultivated plants (attractivity score, melliferous, or polliferous, potential). Offering crop-specific recommendations for pesticide use under all conditions has limits, for the following reasons:

- the degree of crop visitation has not been documented for wild bees as a whole;
- multiple factors (other than those related to the bee itself) will modulate bee visitation (crop variety, pedoclimate, cultivation practices, health status of the bees, presence of other flowers, pollinator diversity and bioagressors);
- the natural flora present in or near the farmed plots also represent a mode of pesticide exposure for the bees, managed or wild;
- concerning wild bees, they must have access to perennial nesting sites in agricultural landscapes, both for ground-nesting species (with appropriate undisturbed soil) and above ground-nesting species (e.g., with ligneous structures for stem-nesting species or leaf-cutter bees).
- As of today no criterion has been established that is true for all crops and bee species regarding the spatial distribution of foragers within plots. Such a criterion would null and void any decision-making treatment criterion that is based on the area to treat (for example, « do not treat *x* rows of crop *y* when flowering).

A second option that has been advanced is to employ meteorological criteria (for example, "treat if the temperature is lower than X°C"). While the temperature remains the meteorological parameter that explains a large part of the variability of foraging activity in bees, the notion of temperature threshold, beyond which this activity would be absent, is jeopardized by the significant effect of other factors related to the bees themselves (species, health, and developmental cycle) or to luminosity, wind, environment (quality, quantity, and location of resources), season (sunlight duration) and time of day (e.g., Burrill and Dietz, 1981; Woyke et al., 2003; Danka et al., 2006; Clarke and Robert, 2018). Our current knowledge is not sufficient at this time to define decision-making criteria that would combine these parameters together. For this reason, decisions based on meteorological criteria seem inappropriate to us.

French regulations stipulate that insecticides or acaricides bearing the « Bee label » have an exemption that allows for their application on flowering plants, provided that foragers are absent from the treated plots. For that, spraying only once the sun has set would significantly reduce the risk exposure of foragers to phytosanitary products. The goal of these regulations is to protect all bee species, namely managed honey bees raised by beekeepers, but also wild bees. Around 80% of bees are ground-nesting species, some of which may be affected by pesticide spraying on crops (Kim et al., 2006; Esther and Roulston, 2009), even if carried out at night. Moreover, nocturnal pollinators, which contribute to pollen transport of plants in agrosystems (Walton et al., 2020), would remain exposed to pesticides. The night spraying of pesticides will also not prevent the contamination of nectar and pollen that induces oral exposure of bees (Rortais et al., 2017) and the degradation of the quality of hive products (Mukherjee, 2009).

For some crops, the constraints on farmers related to the nocturnal spraying of pesticides on flowering plants are high: problems of safety at work, strain at work, and nuisance for the neighboring areas. Scientists need to prove the feasibility and sustainability of new practices with nocturnal application of pesticides. For that, they must test new work organizations with a research-intervention approach based on the cooperation of farmers.

Safeguarding bee biodiversity should be a priority, and thus, in the absence of less-dangerous alternatives to pesticides, the spraying of

pesticides should be carried out at night, even if the modalities of acceptable application by farmers often remain to be defined.

Author contributions

AD: coordination of the article. OR, FR, and CR: data acquisition and development of statistics and figures. FA, CV, OR, FR, CR, and MH: proofreading and expertise. FA, CV, OR, FR, CR, and MH: list of bibliographic references. AD, FA, and MH: coordination of projects producing the data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Planted pollinator habitat in agroecosystems: How does the pollinator community respond?

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Pollinators are important both ecologically and economically. Nonetheless, documented pollinator population decline threatens ecosystem functioning and human well-being. In response, conservation methods such as augmented pollinator habitat are becoming popular tools to combat pollinator losses. While previous research has shown added habitat can benefit bee communities, there are still aspects of the habitat implementation that require further research, particularly how this will impact bee communities in real-world settings beyond researcher-led efforts. In our study, we use a 2016 initiative mandating the planting of pollinator habitat on research stations across North Carolina, United States to act as an outdoor laboratory to investigate this exact question. From 2016 to 2018, we found significant increases in bee abundance and diversity. However, these increases depended on the quality of habitat, with areas of higher flower cover and diversity supporting larger, more diverse bee communities. Although the habitats positively supported bee communities, we found that resources within the habitats were lower later in the sampling season, highlighting the need of developing seed mixes that include late season resources. Weedy plants were documented to establish within the habitats, demonstrating the need for regular upkeep and maintenance of pollinator habitat in order to appropriately support bee communities. It is likely that planting pollinator habitat will not be a one-size-fits-all conservation solution, as bee species can respond differently to some habitat characteristics. Future long-term studies on pollinators will be important as natural fluctuations in bee populations may limit findings and many knowledge gaps on native bees still persist.

KEYWORDS

wild bees, pollinators, pollinator habitat, flower strips, agroecosystems, pollinator conservation

1. Introduction

Extreme losses of global biodiversity and animal biomass are receiving increasing attention (Dirzo et al., 2014; Ceballos et al., 2015). Insects have been the subject of several studies on this topic (e.g., Wagner, 2020) with staggering losses documented across the globe (Fox et al., 2014; Hallmann et al., 2017; Lister and Garcia, 2018). These losses will result in serious consequences to human well-being, as insects are integral parts of food-webs and provide critical ecosystem services such as decomposition, nutrient cycling, pest control, and pollination services (Losey and Vaughan, 2006; Dirzo et al., 2014; Noriega et al., 2018; Dainese et al., 2019; Goulson, 2019). The ecosystem service that has arguably received the most attention—in research, the public sphere, and politically (Wilson et al., 2017)—is pollination, and thus pollinators have gained prominence in empirical research and conservation biology [reviewed by Vasiliev and Greenwood (2020) and Silva et al.,

(2020)]. As pollinators are critical in both natural areas (Ollerton et al., 2011) and agricultural systems, there have been growing efforts to support pollinator populations to limit further population and diversity losses.

A large focus for pollinator conservation efforts has been to protect and augment foraging habitat. Previous research from across the globe has shown that increased habitat—whether in natural areas, hedgerows, or planted flower plots—can support pollinator populations (Morandin and Kremen, 2013; Blaauw and Isaacs, 2014; Williams et al., 2015; Widhiono and Sudiana, 2017; Buhk et al., 2018). However, much remains to be understood about implementing such habitat. It has been shown that the benefits to pollinators from habitat implementation can change depending on the specific context (Kremen et al., 2018), scale (Scheper et al., 2015; Wood et al., 2015), and insect group of interest (Antonio Sanchez et al., 2019). Moreover, different plants can affect results (Warzecha et al., 2018; Mallinger et al., 2019) and even pollinator health (Giacomini et al., 2018), leading to efforts to develop seed-mix recommendations through empirical research. Of further curiosity is how researcher-led habitat implementation may differ when compared to real-world scenarios. As has been noted in citizen science projects, participant effort can differ from person-to-person, over time, and in response to external factors (Lewandowski and Oberhauser, 2017; Lynch-O'brien et al., 2021). While some areas of the world, particularly Europe, have government organized and endorsed programs (Rotchés-Ribalta et al., 2021) that may result in more uniform and sustained efforts of habitat implementation, the United States does not have such programs on a large-scale. It is critical that we investigate all aspects surrounding this conservation method further to ensure its effectiveness, limit any unintended consequences, and prevent wasted time and resources during implementation.

In 2016, the North Carolina Department of Agriculture & Consumer Services (NCDA&CS) mandated that all NCDA&CS and NC State University Experimental Agricultural Research Stations plant pollinator habitat as part of a program titled "Protecting NC Pollinators." To our knowledge, this is the first state-wide government-led program of its kind in the United States. As part of the initiative, each research station allocated one or more areas for pollinator habitat and have continuously maintained it since. We used this 'outdoor laboratory' to investigate how adding pollinator habitat into the agroecosystem affects the pollinator community in a real-world setting beyond researcher-led efforts. Previously at the same habitats, we documented the effect of added habitat on soybean yield (Levenson et al., 2022) and investigated interspecific pathogen occurrence among various bee species within the habitat (Levenson and Tarpy, 2022a). Here we explore how different aspects of the habitat affected pollinator community composition through two main research questions: *1. How does the pollinator community as a whole respond to newly established habitat?* and *2. How do environmental factors affect different aspects of the pollinator community?*

2. Materials and methods

2.1. Site description

All research stations (hereafter referred to as "stations") are at least 4.02 kilometers apart, with an average distance of 57.8 km (SD ± 36.2 km) (Table 1). These stations are distributed across three geographic regions of the state: coastal, piedmont, and mountains (Supplementary Table S1). While all stations planted the pollinator habitats (hereafter referred to as "habitat") in fall 2015 or early spring 2016, the size and within-station location of the habitat varied across sites as each station was independently responsible for habitat establishment and maintenance. The habitat was seeded using commercially available seed mixes from American Meadows[1]—including the southeast seed mixes, zinnias, cosmos, sunflowers, and buckwheat—and was reseeded every 1–2 years (Supplementary Tables S2, S3). Although the habitat was actively maintained by the stations, many weed species appeared in the habitat throughout the duration of the study (Supplementary Table S3). While many stations planted multiple habitat plots throughout the property, we only sampled at one per station. The sampling location at each station remained the same throughout the duration of the study with the exception of one station that moved the sampling location in 2017 due to a major rainstorm event (new sampling plot was 225 meters away). The sampled habitats were between 0.1 and 1 acres (Supplementary Table S1).

TABLE 1 Reports station name, NCDA number assignment, coordinates of the sampling habitat location, and whether hand net sampling occurred at a given station.

Station	Latitude	Longitude	Hand Net Sampling
1. Border Belt	34.41299	−78.7925	Yes
3. Castle Hayne	34.32306	−77.9171	No
16. Caswell	35.28005	−77.6124	Yes
2. Central Crops	35.66792	−78.5105	Yes
4. Clinton	35.02558	−78.2787	Yes
5. Cunningham	35.30539	−77.5797	No
19. Lake Wheeler	35.72580	−78.6751	Yes
6. Mountain	35.48539	−82.9685	Yes
7. Mountain Horticulture	35.41842	−82.5584	Yes
8. Oxford	36.30438	−78.6155	Yes
9. Peanut Belt	36.13140	−77.1733	Yes
10. Piedmont	35.69722	−80.6220	Yes
11. Sandhills	35.19546	−79.6840	Yes
18. Umstead	36.15644	−78.7688	No
14. Upper Mountain	36.39970	−81.3096	Yes
15. Upper Piedmont	36.37823	−79.6905	No

All stations participated in bee bowl sampling. More information about the research stations can be found at https://www.ncagr.gov/research/locations.htm.

2.2. Sampling methods

Sampling occurred at 16 stations across 3 years (2016–2018) utilizing two sampling methods: bee bowls and hand netting. At all 16 stations, three bee bowls per side (one painted blue, one yellow, and one

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white) were placed 5-meters apart along the perimeter of the habitat; bee bowl color order within each side was randomized. Following the protocol from Droege (2008), these samplings occurred once a month for 4 months [hereafter referred to as 'season' and categorized into spring, early summer, late summer, and fall] from 9 am to 3 pm during peak bloom at the habitat (roughly May – September) on days when temperatures were above 15°C. At the end of the sampling day, the contents of all bowls were combined into one falcon tube filled with 75% isopropyl alcohol and stored at 4° C until further processing.

At 12 stations, additional samples were collected using hand netting as close as possible to the same day as each bee bowl sample (Table 1). During these netting samplings, 2–3 people collected along haphazard transects (Hayes et al., 2019) throughout the habitat for 1 h. The time of day the sampling occurred shifted for each event in order to avoid any temporal bias. Each specimen collected was placed into an individual 1.7 mL centrifuge tube, transported back to the lab on ice, and then stored at −20°C until further processing. At each netting sampling event, a measure of flower cover and flower diversity was taken. As described in Levenson and Tarpy (2022a), 'cover' measured the percentage of the habitat in bloom at the time of sampling and was scored as low (0–30%), medium (31–50%), or high (50% or higher); 'diversity' measured the number of different plant species in bloom at the time of sampling and was scored as low (100–80% of the habitat in bloom with one flower species), medium (79–60%), or high (59% or less) (Figure 1 and Supplementary Table S1).

2.3. Identification of samples

Samples were identified using Discover Life (Ascher and Pickering, 2016) and Levenson and Youngsteadt (2019), keeping samples on ice throughout the identification process to allow for subsequent analysis in associated research projects. We identified samples to the lowest level of identification possible, with most specimens identified to species except for specimens in the genera *Andrena* and *Lasioglossum*. All samples are stored in the Tarpy Laboratory at North Carolina State University.

To answer research question two – *How do environmental factors affect different aspects of the pollinator community?* – we categorized each genus by size (small, medium, large), nesting material (ground, cavity, wood/stem), and pollinator type (generalist, specialist, parasitic) (Supplementary Table S4). Information to make these categorizations was drawn from Mitchell (1960), Michener (2007), Gibbs (2011), Ascher and Pickering (2016), Levenson and Youngsteadt (2019), and Fowler and Droege (2020). While these categorizations could differ between some species within each genus, categories were selected to represent most–if not all–species within a given genus.

2.4. Statistical analysis

As bee bowl sampling yielded much smaller and less diverse sample sizes of the bee communities, we did not use these data during analysis and only report our findings from this sampling method below descriptively. Instead, we used our hand netting dataset only for analysis. All analyses were conducted in RStudio (version 4.1.3, R Core Team 2022).

2.4.1. How does the pollinator community as a whole respond to newly established habitat?

We ran models using the *lme4* package with the glmer function (Bates et al., 2015) followed by a post-hoc Tukey's analysis using the *multcomp* package (Hothorn et al., 2008). We conducted χ^2 contingency analyses in base R. To evaluate bee abundance response to the planted habitat, we used a generalized linear mixed model with a negative binomial distribution

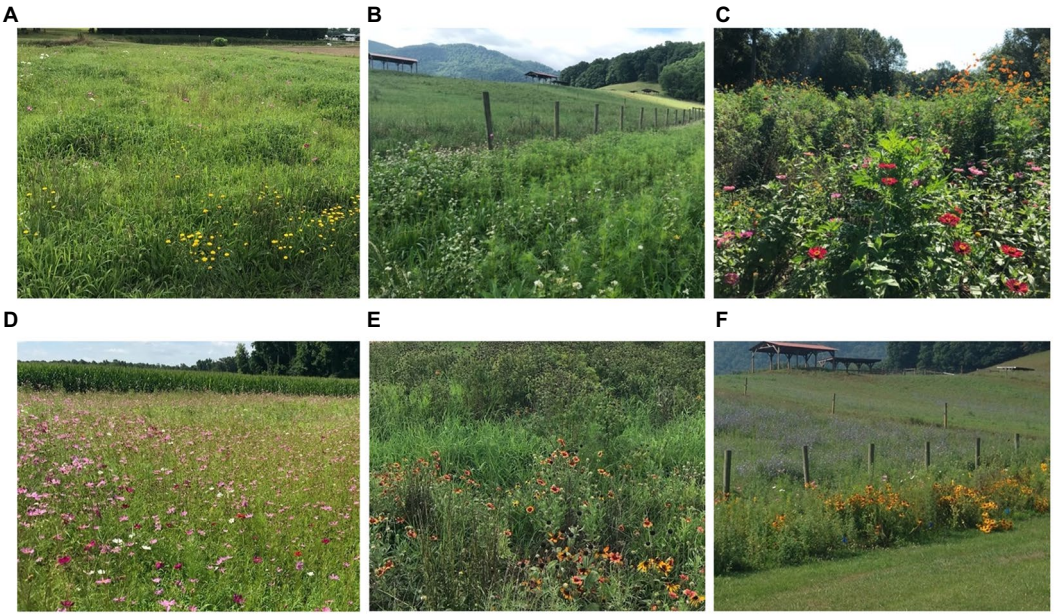

FIGURE 1
Examples of various levels of plot cover **(A–C)** and plot diversity **(D–F)**. Picture **A** (Central Crops 2018) represents a low flower cover, Picture **B** (Mountain 2018) represents medium flower cover, and Picture **C** (Peanut Belt 2018) represents high flower cover. Picture **D** (Border Belt 2018) represents low flower diversity, Picture **E** (Caswell 2018) represents medium flower diversity, and Picture F (Mountain 2016) represents high flower diversity.

and included season, year, flower cover, flower diversity, the acreage of the sampled habitat, the number of habitat plots planted per station, and the number of managed honey bee hives present as predictor variables (Supplementary Table S1); as well as region as a random effect. To evaluate genus richness response to the planted habitat, we used the same model as described above but with a Poisson distribution. These models were selected using AIC criterion. During the model selection process, we explored using genus richness versus species richness as a response variable. Outputs and conclusions did not change between the two variables; thus, we conducted all analyses using genus richness to preserve replication and sample size. To test for changes in the habitat itself over the course of the study, we conducted χ^2 contingency analyses comparing flower cover and diversity across the sampling season and sampling years.

2.4.2. How do environmental factors affect different aspects of the pollinator community?

We used fourth corner analysis (Dray and Legendre, 2008; Grab, 2018) to compare specific bee traits, habitat characteristics, and environmental factors of the study using the *mvabund* (Wang et al., 2012) and *lattice* (Sarkar, 2008) packages. To best fit the data, we used the negative binomial family, where appropriate, and ran 1,000 bootstrap replications. Information used during this analysis is reported in Table 1 and Supplementary Tables S1, S4, S5. Again, data was analyzed at the genus level. Variables that were not found to significantly impact the pollinator community under 2.4.1 were removed for this analysis.

3. Results

In total, we collected 16,038 bees: 11,896 from hand netting and 4,142 from bee bowl sampling (Supplementary Table S5). We found 38 different genera across the entire state of North Carolina (with at least one new genus found each year of sampling) and 128 different species (Figure 2). However, the actual number of species is likely higher as not all specimens were identified down to species. Three genera–*Halictus* (30%), *Lasioglossum* (22%), and *Bombus* (17%)–together accounted for 69% of all collected samples; *Apis mellifera* only accounted for about 4% of the collected samples. A few species were rare within our study, only being documented at one location or even one sampling event (e.g., *Nomia nortoni* was only found at Border Belt, *Melitoma taurea* at one time point at Mountain, one individual of *Augochloropsis metallica* at one time point at Mountain Horticulture, and one individual of *Perdita bradleyi* at one time point at Sandhills; Figure 2).

3.1. How does the pollinator community as a whole respond to newly established habitat?

We found that bee abundance increased over time (Table 2), with the most bees collected in 2018 (Z (127) = 4.58, $p < 0.001$; Tukey = b) as compared to the reference year of 2016. Most bees were collected in the early summer (Z (127) = 2.10, $p < 0.05$; Tukey = a) compared to all other sampling time points with spring as the reference season. Flower cover of the habitat significantly influenced bee abundance, with the fewest bees found at low flower cover (Z (127) = −5.71, $p < 0.001$; Tukey = a) with high flower cover as the reference level. Flower diversity showed a similar trend but was not significant. The sampling habitat acreage, number of habitat plots planted per station, and the number of honey bee colonies stocked per station had no influence on bee abundance (all $p > 0.08$).

Bee genus richness also increased over time (Table 3); however, it was highest in 2017 (Z (128) = 3.00, $p < 0.005$; Tukey = b), then 2018 (Z (128) = 2.75, $p < 0.05$; Tukey = b), and lowest in 2016 (Tukey = a). Genus richness decreased across the sampling season with the lowest richness found in the fall (Z (128) = −2.00, $p < 0.05$; Tukey = a) as compared to the reference season of spring. Flower cover and flower diversity both significantly influenced genus richness, with the lowest richness found at low flower cover (Z (128) = −2.89, $p < 0.005$; Tukey = a) and low flower diversity (Z (128) = −2.08, $p < 0.05$; Tukey = a); high flower cover was the reference level for both variables. Habitat acreage, number of habitat plots planted per station, and the number of honey bee colonies stocked per station had no influence on bee genus richness (all $p > 0.22$).

Both flower cover and flower diversity within the habitat was found to significantly change across the sampling season [χ^2 (6) = 15.2, $p < 0.05$] and [χ^2 (6) = 13.3, $p < 0.05$], respectively) with both measures decreasing across the sampling season. Neither flower cover nor diversity significantly changed across years of the study [χ^2 (4) = 1.02, $p = 0.91$ and χ^2 (4) = 2.39, $p = 0.67$, respectively].

3.2. How do environmental factors affect different aspects of the pollinator community?

Much of the variation in bee community functional traits was influenced by variables beyond the planted habitat. Most of the variation in the bee community and its trait categories changed across the state when comparing stations (Figure 2) and regions (Figure 3), respectively. Bee community functional traits also changed across the sampling season with more ground nesting and parasitic bees, but less small bees, detected in early summer. While there were some changes in traits across years of the study, these effects were minimal.

Characteristics of the planted habitat effected a small number of bee community functional traits. Fewer ground nesting bees were detected when flower cover was high. At low flower diversity, more cavity nesters and specialist pollinators were detected.

4. Discussion

Overall, the planting of pollinator habitats on these agricultural research stations positively supported the bee communities over time; we saw a significant increase in abundance and genus richness within the habitat over the course of our study. While the small acreage of the habitats may limit our ability to detect signals that could be extrapolated to global impacts of pollinator habitat, our study confirms the more localized effects of small habitat plots within finite landscapes and that pollinator communities are positively affected by their establishment. While the habitat supported bee communities over time, we found the quality of the habitat was critical to their success. Habitat with higher flower cover supported higher bee abundance and richness, as has also been documented in previous research (summarized in Kowalska et al., 2022). Similarly, higher flower diversity supported a higher genus richness. It is encouraging that even small acreages of habitat (1 acre or less) can positively support local bee communities, suggesting that any amount of habitat restoration is better than none and thus efforts should be made to support bee communities wherever and however possible, especially in agroecosystems.

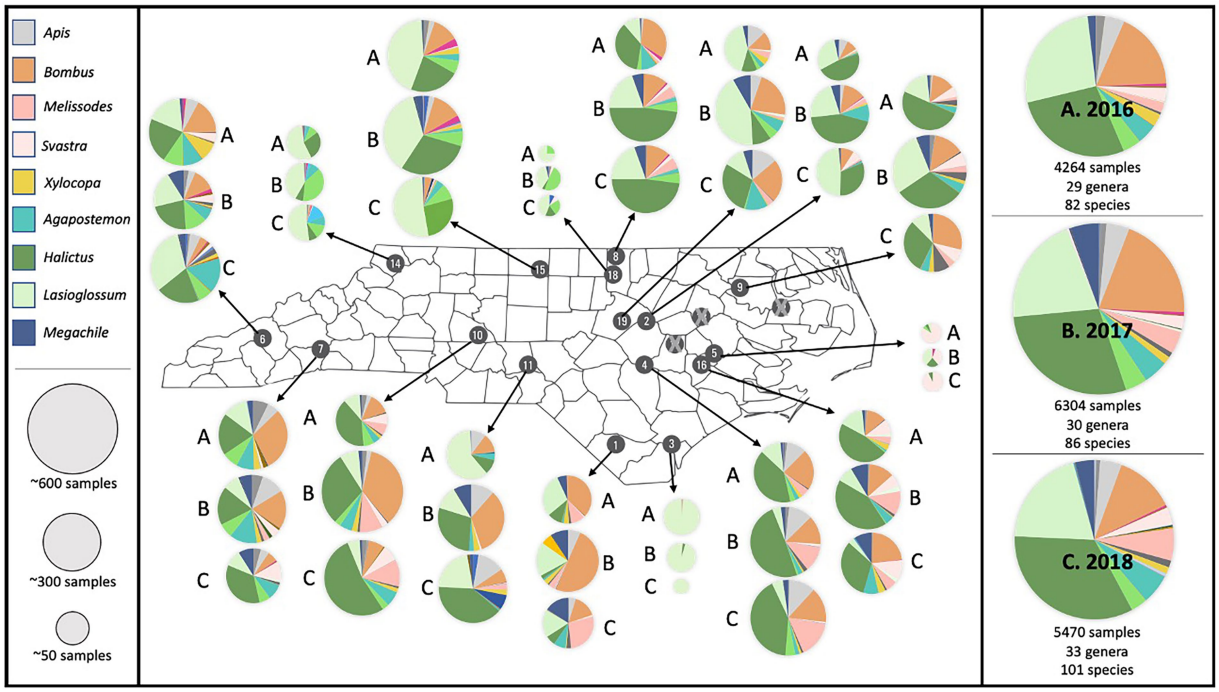

FIGURE 2
Map of the bee abundance and genus richness results for each station across all 3 years. Pie charts represent relative abundance collected at each station and display the most commonly occurring genera across each year. The entire dataset for each year is shown along the right-hand side. The top three genera–*Halictus* (30%), *Lasioglossum* (22%), and *Bombus* (17%)–accounted for 69% of all collected samples.

TABLE 2 Output from generalized linear mixed model and Tukey's post-hoc analysis with abundance as the response with a negative binomial distribution.

Variable		Mean	Std. dev.	Estimate	*p*-value	Tukey
Year	2016	60.28	45.01			a
	2017	97.23	44.29	0.54	<0.001	b
	2018	97.60	55.15	0.55	<0.001	b
Season	Spring	90.06	43.61			ab
	Early summer	103.71	48.87	0.29	0.036	a
	Late summer	75.94	53.26	−0.07	0.652	b
	Fall	70.49	53.49	0.06	0.667	ab
Flower cover	Low	57.05	46.05	−0.69	<0.001	a
	Medium	105.07	54.96	−0.13	0.315	b
	High	105.07	39.23			b
Flower diversity	Low	68.14	47.53	−0.22	0.076	a
	Medium	84.08	46.72	−0.14	0.339	a
	High	105.70	50.43			a
Other	Habitat acreage			0.03	0.203	
	Number of habitat plots			0.44	0.266	
	Number of managed honey bee hives			−0.00	0.320	

The mean habitat acreage was 0.34 acre (std. dev. = 0.17), mean number of plots was 3.13 (std. dev. = 2.31), and the mean number of managed honey bee colonies was 9.23 (std. dev. = 20.60). The reference level for each variable is shown in grey.

We found that flower cover and flower diversity at the habitat significantly changed across the sampling season, with both of these measures generally decreasing over time, findings which are mirrored in Morandin and Kremen (2013). Since late-season resources are extremely important for bee populations (Couvillon et al., 2014; Park and Nieh, 2017), adding more plant species that flower later in the

TABLE 3 Output from generalized linear mixed model and Tukey's post-hoc analysis with genus richness as the response with a Poisson distribution.

Variable		Mean	Std. Dev.	Estimate	*p*-value	Tukey
Year	2016	6.51	2.84			a
	2017	8.40	2.78	0.23	0.003	b
	2018	8.17	3.00	0.21	0.006	b
Season	Spring	8.54	2.80			ab
	Early summer	8.86	3.05	0.08	0.338	b
	Late summer	7.17	2.89	−0.11	0.223	ab
	Fall	6.20	2.42	−0.19	0.045	a
Flower cover	Low	6.25	2.96	−0.22	0.004	a
	Medium	8.86	2.47	−0.01	0.867	b
	High	8.65	2.59			b
Flower diversity	Low	6.41	2.87	−0.16	0.038	a
	Medium	7.75	3.07	−0.09	0.330	a
	High	9.20	2.31			a
Other	Habitat acreage			0.17	0.495	
	Number of habitat plots			0.01	0.390	
	Number of managed honey bee hives			−0.00	0.358	

The means and standard deviations for the 'other' variables are as reported under Table 2. The reference level for each variable is shown in grey.

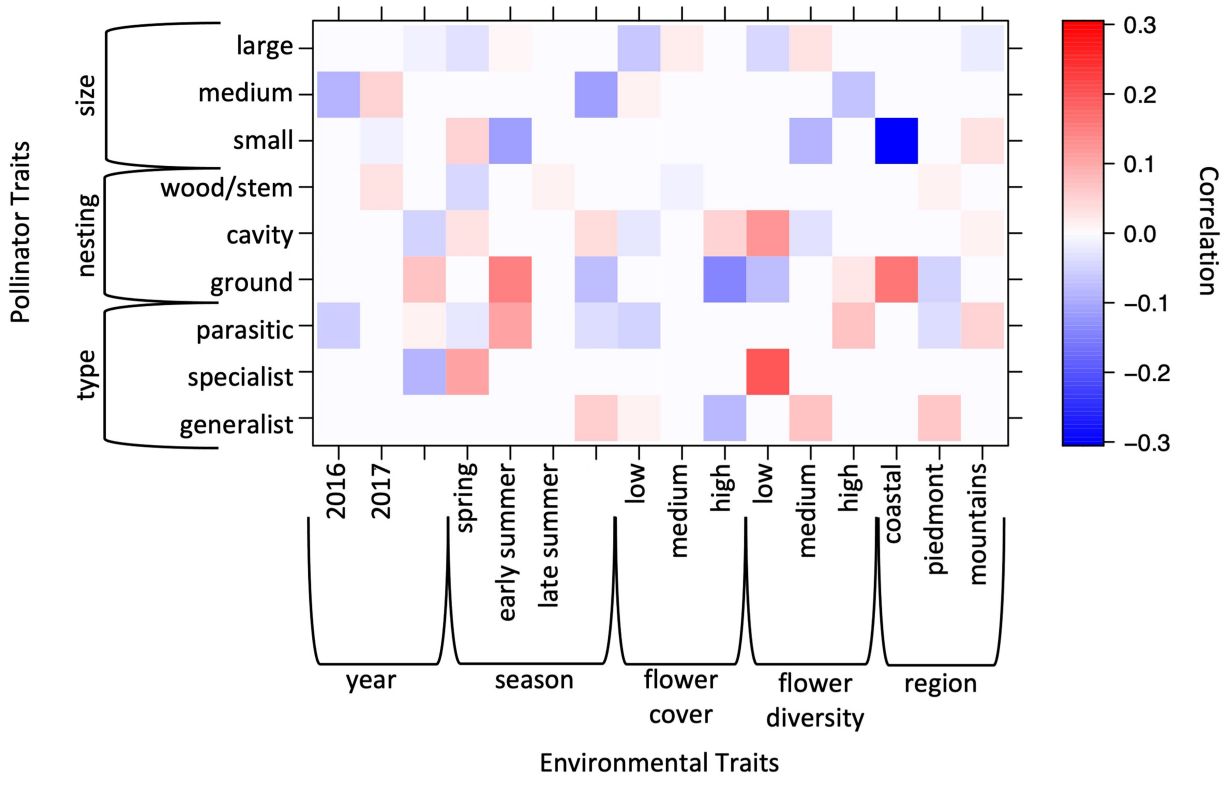

FIGURE 3
Output from fourth corner analysis showing correlations between habitat characteristics and bee functional traits. Positive correlations are shown in red negative correlations are shown in blue. Larger numbers show stronger correlations.

year to seed mixes used in pollinator habitat establishment would be particularly beneficial; especially considering late-season is precisely the time of year when we documented the fewest resources. While we did not detect a significant change in these habitat measurements over the years of our study, we documented many plant species that likely naturally established within the habitat and

were not a part of the seed mixes used by the NCDA&CS (Supplementary Table S3). While some of these flowering plants may also play a key role in supporting bee communities, many are considered weedy or invasive. It is known that fast-growing, high-propagating plant species establish during times of disturbance (González-Rivero et al., 2016), such as establishing pollinator habitat (Piqueray et al., 2019; Kowalska et al., 2022). This is of concern when establishing pollinator habitat, as these plants may outcompete other flowering species that are desired to have establish within planted pollinator habitat. In our study, several research station managers had difficulties with grasses and other weeds from soil seed banks overtaking the habitat. If left unmanaged, this could aid in the spread of unwanted plants or eventually no longer support bee communities, depending on how the plant community within the habitat shifts overtime. This underscores the need to continuously maintain planted pollinator habitats to ensure bee communities are sufficiently supported with quality resources (see also Kennedy et al., 2013) and that unwanted plant species are not spread.

We found that much of the variation in bee communities, as measured by functional traits, was from environmental factors beyond the habitat (e.g., location and time of year). However, we did document that flower cover and flower diversity had an impact on some community measures. This suggests that planting pollinator habitat will not be a 'one-size-fits-all' solution for all bee species, even though bee communities as a whole were supported in our study. Since analyses in this study were conducted using specimen identifications at the genus level, it will be important for future work to look at specimen identifications at the species level. As reported in Levenson (2021), there is evidence that different species will respond differently to habitat characteristics. Specifically, it was noted that managed *Apis mellifera* responded oppositely to flower diversity compared to the overall wild bee community, potentially due to differences in their foraging behavior. Thus, if a specific bee species is the focus of a particular conservation effort, we suggest that planted habitat should be tailored to better support the functional traits of said species.

Future research would benefit from continued long-term studies on bee communities, particularly studies conducted over a longer period of time than our 3-year study. While we were not testing for global increased abundance over time, there is some evidence of fluctuations across years, something that is expected to naturally occur in communities (Franzen and Nilsson, 2013). As such, longer-term monitoring in the future will reveal the true trajectory of these communities despite natural fluctuations. Continuous monitoring of bee populations should be a priority, as changes in the landscapes can have immediate impacts on bees (as shown here). Additionally, there were several species within this dataset that were only documented at one station or during one sampling event. Without wide-spread, long-term monitoring we will not be aware of which bees are present in an area, let alone which populations are being threatened. As our agricultural system becomes more reliant on pollinators (Aizen et al., 2009) but pressures on their populations intensify (Goulson et al., 2015; Koh et al., 2016), resources toward conserving bee populations will only become more important. Although, North Carolina was the first state in the US to implement a government-led program such as this, our findings should serve as encouragement for future government initiatives across the country to restore habitat in agricultural systems and to support monitoring programs of such habitat. Findings from this study should be used to better design and implement habitat in future plantings so as to best support pollinator populations.

Data availability statement

The datasets presented in this study can be found in the article, Supplementary materials, and in online repositories via the Dryad Digital Repository https://doi.org/10.5061/dryad.w6m905qsq (Levenson and Tarpy, 2023).

Author contributions

HL and DT equally contributed to experimental design. HL was responsible for sample collection, data processing, analysis, the initial write up, and figure creation, with DT supporting these efforts. DT was responsible for acquiring funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1060834/full#supplementary-material

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Seasonal differences in *Varroa destructor* population growth in western honey bee (*Apis mellifera*) colonies

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Varroa destructor is a major threat for apiculture worldwide. A successful approach to control this parasite must include the application of effective treatments at the correct time. To understand the effect that treatment timing has on *Varroa* populations at different seasons, we conducted an experiment using a dataset comprising two separate field trials over multiple years, both trials containing four apiary sites composed of 20 honey bee colonies across an area representative of north central Florida environments. Before the start of the season, colonies were treated with two acaricides simultaneously to bring the *Varroa* populations to ~0.25 mites/100 bees. Following treatment, we monitored the mite populations monthly via alcohol washes. Our results show that the temporal efficacy of *Varroa* treatments varies across seasons. We observed that it takes about 4–5 months after treatment in winter and spring for mite populations to return to the standard economical threshold (3 mites/100 bees). Nevertheless, there is a steeper increase in mite populations (<3 months to exceed the economic threshold) after treating colonies in summer and fall. The level of infestation that leads to colony collapse and the rate of colony decline also varied by season. To our knowledge, this is the first study evaluating seasonal effects on *Varroa* population growth and the first model of *Varroa* population growth in Florida, USA. Our results serve as a foundation for *Varroa* treatment models, aiding beekeepers in the future as a part of a holistic approach to control this devastating honey bee parasite.

KEYWORDS

***Apis mellifera*, *Varroa*, population, honey bee, infestation, season, survival**

1. Introduction

For decades, beekeepers around the world have struggled to control *Varroa destructor*, an ectoparasitic mite, in their honey bee (*Apis mellifera* L.) colonies (Rosenkranz et al., 2010). The pest now has a worldwide distribution and can be found nearly everywhere honey bee colonies are managed (Boncristiani et al., 2021). *Varroa*'s impact is primarily linked to its ability to vector and transmit a large number of viruses (Genersch and Aubert, 2010;

Traynor et al., 2020), severely weakening honey bee colony strength (Budge et al., 2015), and resulting in widespread colony losses (Brutscher et al., 2016; Gray et al., 2020).

Different non-chemical and chemical treatments are used by beekeepers to reduce *V. destructor* populations below the economic threshold, which is typically considered as 3 mites/100 adult bees (Jack and Ellis, 2021). The more time a colony spends above the economic threshold, the more likely it is to experience both increased viral infection and colony mortality (Kulhanek et al., 2021). Each year, beekeepers must decide which *V. destructor* treatment regimens are best to use in their management setting, this being based on several factors (Thoms et al., 2019; Underwood et al., 2019; Steinhauer et al., 2020). The most obvious factor is the efficacy of the treatment, which can vary under specific circumstances (Jack and Ellis, 2021). Weather conditions, particularly temperature and precipitation, have been observed to play a significant role in the efficacy of several *V. destructor* treatments (Beyer et al., 2018; Steube et al., 2021). Natural chemical treatments are particularly responsive to ambient temperature conditions, as volatile chemicals release gases based on the temperature where they are placed (Imdorf et al., 1995; Gracia et al., 2017). Even honey bee behaviors, such as grooming behaviors to remove *V. destructor*, can be affected by weather (Currie and Tahmasbi, 2008).

Beekeepers have historically relied heavily on synthetic chemical treatments to control *V. destructor* (Roth et al., 2020), but the efficacy of these treatments has become limited due to resistance issues (Haber et al., 2019). Some have observed treatment efficacy to vary according to location and season in which it is applied (Currie and Gatien, 2006; Gracia et al., 2017). As *V. destructor* can only reproduce within the capped brood cells containing honey bee pupae, the mites spend a significant amount of their lives inside these cells (Rosenkranz et al., 2010). Thus, the timing of chemical treatments is also essential for sustaining *V. destructor* control (Delaplane and Hood, 1997; Gatien and Currie, 2003), as the effectiveness of some treatments is considerably reduced if mites are hidden within the capped brood cells at time of application (Kraus and Berg, 1994; Rosenkranz et al., 2010; Al Toufailia and Ratnieks, 2018).

Another factor beekeepers must consider when attempting to control *V. destructor* is the amount of time required to apply the treatment. Some non-chemical treatments can be effective (Ellis et al., 2001; Wantuch and Tarpy, 2009; Kablau et al., 2020), but often require too much time. This makes such treatments unpopular among commercial beekeepers (Underwood et al., 2019). The length of time the treatment must remain in the hive is also an important factor to consider, as most chemical treatments are not labeled for use while honey supers are present on the hive (Honey Bee Health Coalition, 2018). To be successful with sustainable *V. destructor* control, beekeepers must consider all these variables in relation to beekeeping activities such as honey production, queen rearing, package bee production, and commercial pollination. It would be extremely valuable to beekeepers to have a decision tool to aid them in their selection of *V. destructor* treatments according to their own location and beekeeping situation.

Before a *V. destructor* control decision tool could be created, researchers need to understand the relationship between *V. destructor* population growth and the individual mite treatments. Many researchers have explored the complex dynamics of *V. destructor* population growth (reviewed by Fries et al., 1994; DeGrandi-Hoffman and Curry, 2004; Coffey et al., 2010; Ratti et al., 2012; DeGrandi-Hoffman et al., 2016). Some have created elaborate growth models which include several factors such as honey bee brood rearing, acaricidal efficacy, mite reproductive rates, and the total number of foragers with mites to name a few (reviewed by Fries et al., 1994; Wilkinson and Smith, 2002; DeGrandi-Hoffman and Curry, 2004; DeGrandi-Hoffman et al., 2016). Nevertheless, after thorough research, an extensive study evaluating the effect of season has not been conducted to our knowledge. By knowing the seasonal growth rate of *V. destructor* populations, one may be able to predict how long after treatment it takes mite populations to return to pretreatment levels.

It is necessary to create a model of natural *V. destructor* population growth by season as a first step toward the creation of a decision tool. Herein, we observed natural *V. destructor* population growth rates in north central Florida throughout multiple years and compared the growth rates by season. We hypothesized that *V. destructor* population growth would vary by season, with the fastest rate of growth happening in the summer and fall seasons, as that is when the greatest amount of capped brood is present in colonies. We made this hypothesis based on the knowledge that the population dynamics of *V. destructor* and honey bees are interwoven, as the mites can only reproduce within capped brood cells (Rosenkranz et al., 2010).

2. Materials and methods

2.1. Experimental design

Two separate trials were conducted over multiple years. In both trials, groups of honey bee colonies were maintained in four different apiaries in north central Florida, all within 32 km of the University of Florida's Honey Bee Research and Extension Laboratory (HBREL), Gainesville, FL (29°37′38″ N 82°21′23″ W). During each trial, a specific apiary was assigned to a designated calendar season. The apiary sites were as follows: (1) North Gainesville, FL (29°44′01″ N 82°16′31″ W), (2) Citra, FL (29°24′36″ N 82°08′48″ W), (3) Hawthorne, FL (29°35′24″ N 82°08′36″ W), and (4) HBREL (29°37′38″ N 82°21′23″ W). Trial 1 was initiated in January 2018 and trial 2 was initiated in April 2020. A map of the apiary locations assigned to each season for both trials is shown in **Figure 1**.

2.2. Hive configuration

At the start of each trial, apiaries contained 20 healthy honey bee colonies of European-derived honey bee stock. The genetic lineage of honey bees used in this study likely derived primarily from *Apis mellifera ligustica* stock, though we made no effort to use a specific stock. Honey bee stocks used in the U.S. are usually mixed-race (Schiff and Sheppard, 1995; Delaney et al., 2009). The bees used in this study were of the same genetic origins for both trial 1 and trial 2. Colonies were maintained in 10-frame Langstroth hives consisting of a single deep hive body and a solid bottom board. Brood combs were all a standard

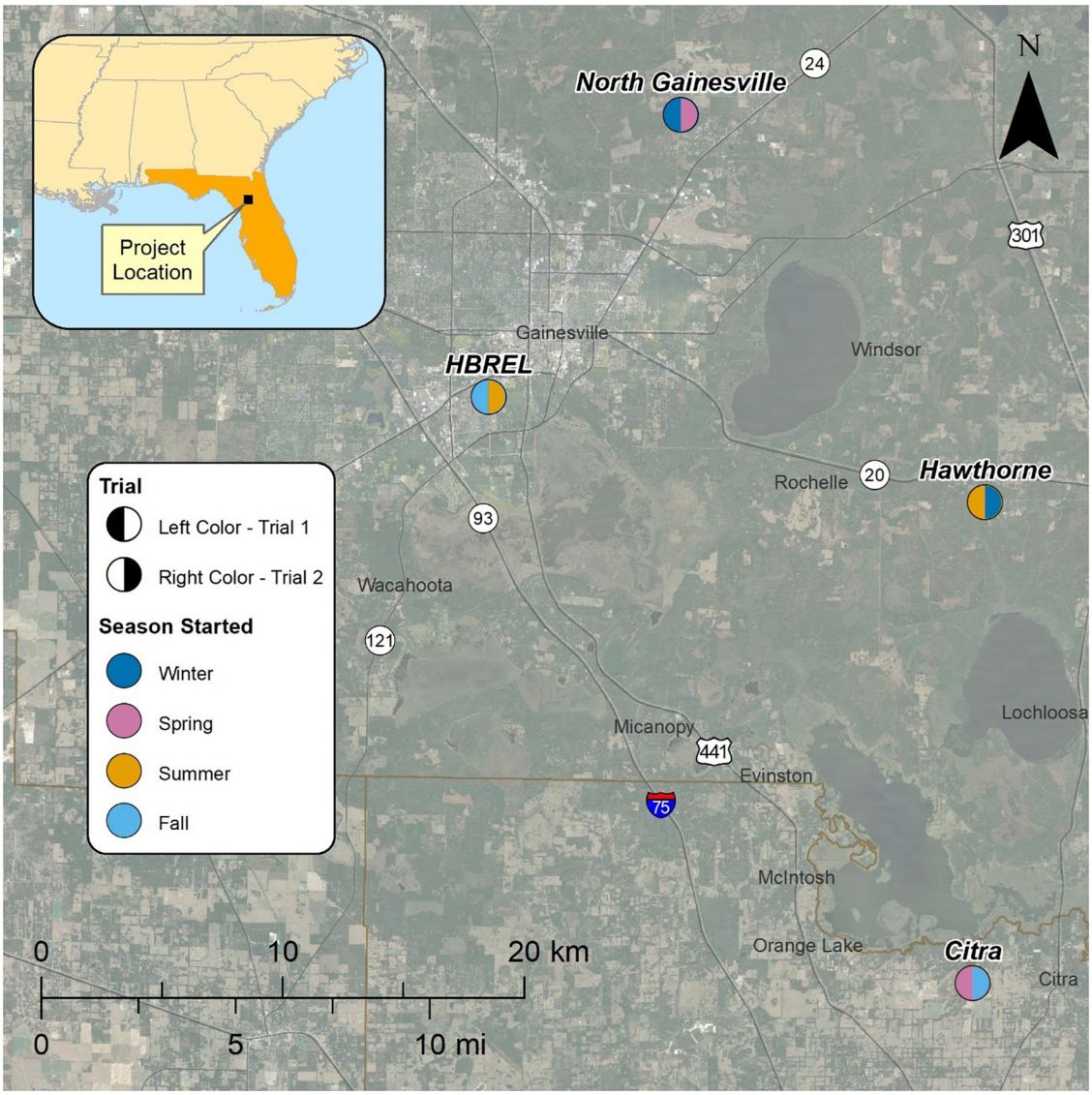

FIGURE 1
Satellite image of apiary locations used in both trials (image by Google Earth). Figure legend indicates the assigned season and trial used in each location.

size and contained Plasticell foundation (Dadant & Sons, Inc., Hamilton, IL, USA). Colonies were equalized prior to the start of the experiment to ensure that each colony was of similar size and strength (approximately nine frames of bees and six frames of brood).

2.3. Honey bee colony management

Prior to the start of each season, colonies within the assigned apiary were treated with acaricides to bring the *V. destructor* populations to an average of 0.25 mites/100 bees (high of 0.6 mites/100 bees). All colonies were treated with amitraz via Apivar® strips (Véto-pharma, New York, NY, USA) for 3 weeks instead of the recommended 6-week period to maintain appropriate timing of the seasonal groups. However, we do not believe that this reduced treatment period negatively affected the reduction of mites, as colonies were also simultaneously treated with 4 g of oxalic acid (OA) dihydrate (Sigma Aldrich, St. Louis, MO, USA) via vaporization. We used the commercially available ProVap 110® vaporizer (OxaVap LLC, Manning, SC, USA) to vaporize the OA, sealing the hive entrance and all cracks around the nest to limit the escape of the vapor as per Jack et al. (2021). Colonies received OA treatment once per week, for up to 3 consecutive weeks. This process was repeated for the colonies in each apiary prior to the beginning of their respective seasons. Once the experimental colonies' *V. destructor* populations were ~0.25 mites/100 bees, no further treatments were administered. All experimental colonies were managed according to best management practices that are common for this region (feeding bees when necessary, swarm control, etc.), with the exception of applications of additional miticides to control growing *V. destructor* populations. To maintain the integrity of the study, no brood combs were shared between colonies, even within the same seasonal

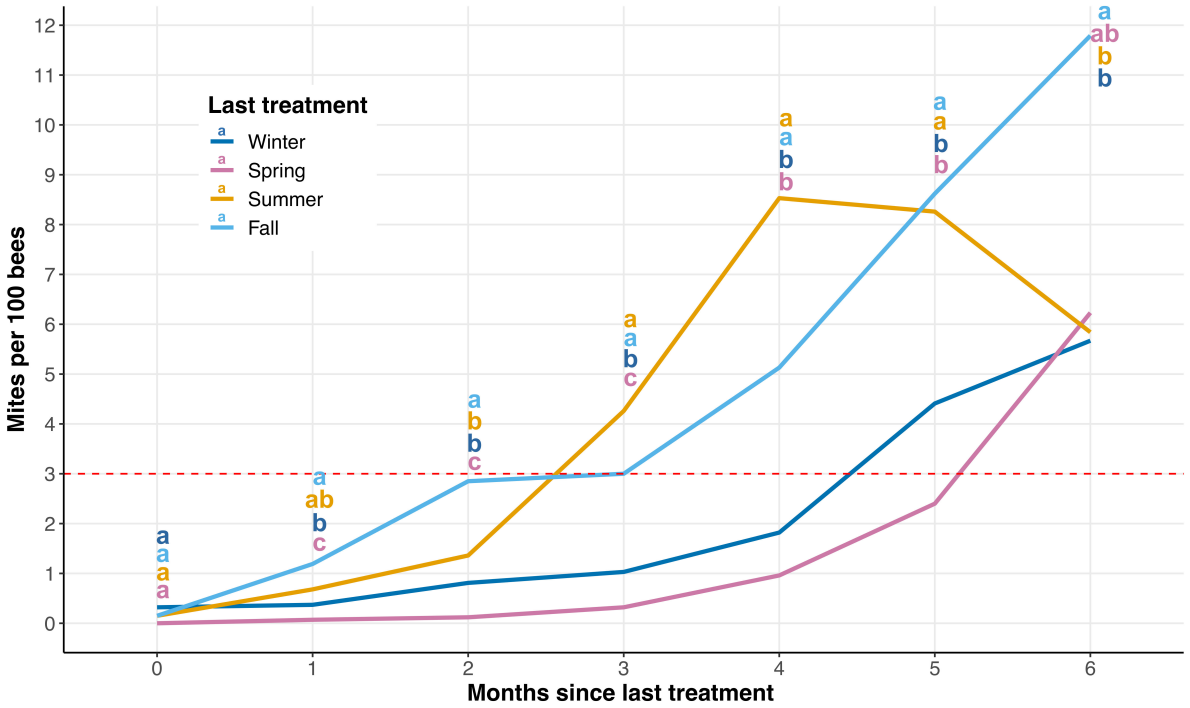

FIGURE 2
Average *Varroa destructor* population growth expressed as number of mites/100 bees after 6 months for each season. These results combine data from both trials 1 and 2. The dashed red line represents the standard economical threshold of 3 mites/100 bees. There was a significant impact of season treatment was administered on the mites/100 bees ($P \leq 0.05$). Significant differences between means are indicated with different letters ($\alpha = 0.05$; a > b > c).

cohort. Furthermore, small hive beetle (*Aethina tumida*) traps were added to all colonies to reduce the effects of beetle damage. All experimental colonies were treated with oxytetracycline (Tetra-B Mix 2X®, Dadant & Sons, Inc., Hamilton, IL, USA) to minimize the potential of foulbrood outbreaks. The number of surviving colonies was recorded each month, given there was some colony mortality. Colonies were considered dead once there were no more adult bees to sample or if the health of the colony would have been significantly impacted by the sampling. As colony populations began to decline, they were fed sugar syrup when needed and entrance reducers were placed on hive entrances to limit robbing. We believe that the colony mortality observed in this study was a result of the high *V. destructor* populations and associated virus loads rather than from the treatment regimen, as few colonies in this study died within 2–3 months after treatment.

2.4. *Varroa destructor* population monitoring

Varroa destructor population growth was monitored for every colony monthly using alcohol washes according to the technique described in Dietemann et al. (2013). Each month, 200–300 bees were collected from the brood area and a ratio of # mites/100 bees was calculated for each sample. Monitoring of *V. destructor* continued for each group until the mite population peaked and then declined for 2 consecutive months, or until all of the colonies within a cohort died. A decline in *V. destructor* populations indicates that the colonies with severe infestations are collapsing and the remaining colonies are in similar danger. Ending the study after 2 months of consecutive mite population decline allowed us to rescue the remaining colonies before their collapse.

2.5. Statistical analyses

All analyses were performed using the *glmer* function from the package lme4 (Bates et al., 2015) implemented in the R platform (R Core Team, 2022). Models for each analysis are described below. Graphical visualizations were obtained using ggplot2 (Wickham, 2016).

2.5.1. *Varroa destructor* population growth model

The main interest with our present research was to observe the effect of season on *V. destructor* infestation. For that, the following generalized linear mixed model was used (i.e., model 1):

$$\bar{y} = \mu + X_1 t + X_2 se + X_3 t * se + Z_1 h + e$$

where, $\bar{y}$ is the responsible variable (i.e., # mites/100 bees), μ is overall mean, t is the effect of time since last treatment, se is the effect of last season treated, $t \times se$ *is* the interaction effect between time since last treatment and the last season treated, h is the effect of each hive evaluated (hiveID), and e the vector for the residual error. X_1, X_2, X_3, and Z_1 are the incidence matrices for time since last treatment, last season treated, the interaction between time since last treatment and last season treated, and hiveID, respectively.

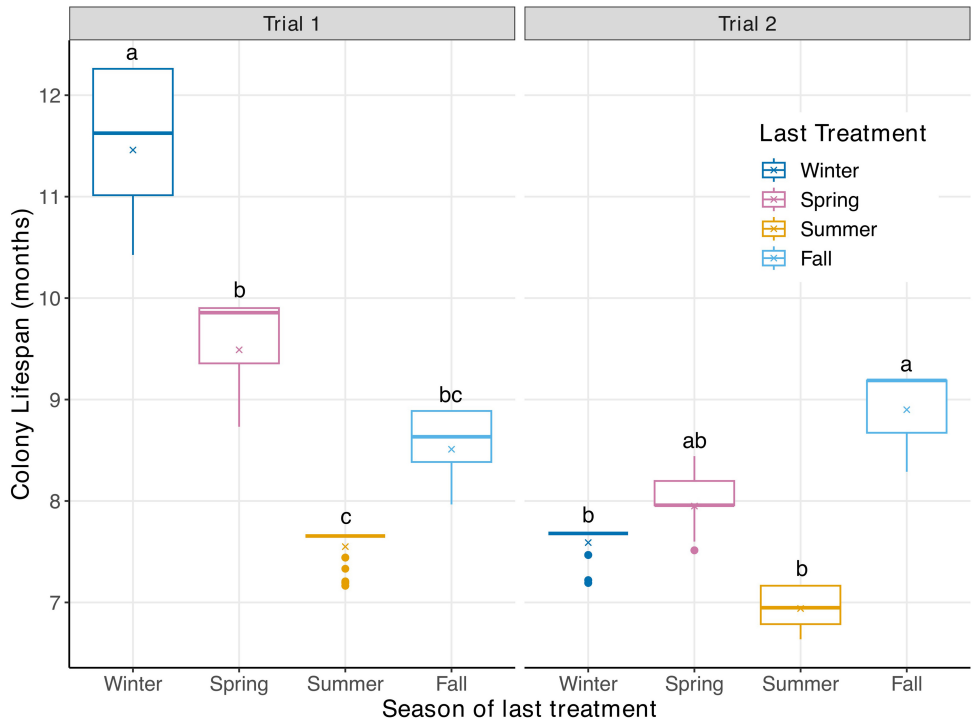

FIGURE 3
Box plots illustrating the assumed lifespan distribution for colonies (in months) in the season in which treatment were applied for both trials. The × indicates the mean survival for colonies within that seasonal cohort. There was a significant impact of season the mite treatment was administered on colony lifespan within trial ($P \leq 0.05$). *Post-hoc* tests were performed within trial and significant differences between means are indicated with different letters ($\alpha = 0.05$; a > b > c).

Only hiveID was not considered as a fixed effect, to account for the independent variances of each hive evaluated. The analysis was implemented using the package lme4 (Bates et al., 2015). Autocorrelation regarding the repeated measures was accounted in the model and a Poisson distribution was assumed, given the nature of the data and its skewed distribution. As colony mortality began to be significantly impacted after 6 months, creating significantly unbalanced data, seasonal *V. destructor* population growth was only analyzed considering the first 7 months of data. Preliminary analysis was performed to select the best model to be used in this analysis (i.e., model 1). For this, nested generalized linear mixed models were tested and comparisons were made considering model fit parameters (AIC, number of parameters, significance in the ANOVA model comparison; data not shown). From these results, we identified that *V. destructor* population growth per season was not dependent upon trial ($P = 0.998$) and that the addition of trial effect turned the model singular, which could be indicative of overfitting. Thus, trial effect was not considered in model 1.

2.5.2. Colony lifespan model

The following generalized linear mixed model was used to infer the effect of last season treated on hive lifespan.

$$\bar{y} = \mu + X_1 T + X_2 se + X_3 T * se + Z_1 T : h + e$$

In this model, $\bar{y}$ is the responsible variable (i.e., hive's lifespan), μ is overall mean, T is the effect of trial, se is the effect of last season treated, $T \times se$ *is* the interaction effect between trial and the last season treated, T:*h* is the effect of each hiveID nested on trial, and e the vector for the residual error. X_1, X_2, X_3, and Z_1 are the incidence matrices for trial, last season treated, the interaction between trial and last season treated, and hiveID, respectively. As in the previous model, only hiveID was considered as a random effect, accounting for the independent variances of each hive evaluated. Given the nature of the responsible variable (i.e., counting data), a Poisson distribution was assumed for the analysis.

2.5.3. *Post hoc* analyses

In order to verify significance between the factors tested in each analysis, post hoc tests assuming Sidak correction for multiple comparisons were performed ($\sigma = 0.05$), using functions implemented in the package emmeans v. 1.7.5 (Lenth, 2022).

3. Results

3.1. *Varroa destructor* population growth by season

Varroa destructor population growth per season was not dependent upon the trial ($P = 0.998$), so data from both trials were combined for subsequent analyses. *V. destructor* population growth data became unbalanced 6 months post-treatment for all seasons due to colony mortality (**Figure 2** and **Supplementary Figure 1**). Thus, we only included the first 6 months post-treatment in our analyses. There were no significant differences

in starting *V. destructor* levels each season after treatments were administered ($P > 0.05$). Yet, there was strong evidence to support the observation that *V. destructor* population growth varies depending on which season the treatment was applied (**Figure 2** and **Supplementary Table 1**).

There are some notable observations when examining *V. destructor* population growth by season. First, when mite populations are reduced in the winter and spring seasons, *V. destructor* population growth rates remain below the economic threshold (3 mites/100 bees) for 4–5 months, respectively (**Figure 2**). Second, *V. destructor* populations rapidly increased after the summer and fall seasons' treatments, extending beyond the economic threshold less than 3 months after treatment (**Figure 2**). Third, mite populations peak only 4 months after a summer treatment before significant colony mortality is observed, causing a decline in average *V. destructor* populations. Meanwhile *V. destructor* populations may be sustained at higher levels 5–6 months after a fall treatment, likely due to a buildup of bees during the spring (**Figure 2**).

3.2. Colony lifespan by season

There was a significant interaction between colony lifespan per season and trial effect ($P < 0.05$, **Supplementary Table 2**); therefore, data from each trial were analyzed separately, and interpretations were made separately within each trial. The colony lifespan during trial 2 was generally shorter than that during trial 1 (**Figure 3** and **Supplementary Table 2**). In trial 1, colonies treated during summer had the shortest of all colony lifespans, averaging 7.5 months of survival post-treatment. Regardless, there is no evidence that survival after fall treatment was different than that after summer at 8.5 months survival post-treatment. Colonies treated in the winter season had significantly longer survival post-treatment during trial 1 than did any other group, with an average colony surviving 11.5 months. In trial 2, treatment in the summer and winter seasons ultimately led to the quickest mortality, with colonies averaging only 6.9 and 7.6 months of survival post treatment, respectively. The colonies that survived the longest during trial 2 were treated in the fall and spring seasons, surviving 8.9- and 8-months post-treatment, respectively.

4. Discussion

We evaluated an extensive and robust dataset composed of 160 honey bee colonies to understand *V. destructor* population growth seasonally. Our main goal was to generate information that can guide beekeepers as a part of a holistic approach to control this devastating honey bee parasite. Our results not only confirm the importance of seasonal effects on the efficacy of treatments for the mite, but they can also be used as a natural model of *V. destructor* population growth for north central Florida, and possibly colonies kept in similar climates. Additionally, we believe that the results of this study demonstrate the importance of regular *V. destructor* monitoring and treatment by beekeepers, as colony survival is often less than 1 year for untreated colonies in Florida. We anticipate that the benefits and information described in our study can be applied to optimize treatment and control for *V. destructor* in Florida and can help guide studies and research for the control of this parasite worldwide.

Although much has been written related to *V. destructor* population growth, to our knowledge, this is the first attempt to determine *V. destructor* population growth seasonally after treatment. As *V. destructor* population growth is closely tied to honey bee brood rearing (Wilkinson and Smith, 2002), it is not surprising that mite levels were able to rebound rapidly after treatment in the summer and fall seasons when honey bee brood is plentiful in Florida (**Figure 2**). However, somewhat surprising is the significantly slower *V. destructor* population growth after treatment in the spring when compared to that in winter (**Figure 2**). One might predict that in colonies where mites were reduced to near-zero in the winter, *V. destructor* populations would be delayed in growth due to the lack of honey bee brood required for reproduction. Interestingly, mite levels were still significantly lower in the spring than in all other seasons 3 months after treatment, making this the longest period during which mite populations were maintained below the economic threshold (**Figure 2**). Dolezal et al. (2016) demonstrated how the environment in which colonies are placed can influence the nutritional physiology of the colony, thus directly affecting *V. destructor* presence in a given environment. As honey bee colonies in the spring season have access to more floral resources, this nutritional advantage may have allowed the bees to defend or guard against *V. destructor* reproduction or reinfestation better than presumably nutrition-deficient colonies treated in winter.

Information regarding seasonal *V. destructor* population growth is applicable to beekeepers who struggle to maintain mite populations below economic thresholds (Jack and Ellis, 2021; Brodschneider et al., 2022). It appears that reducing *V. destructor* populations in the spring season is important for long lasting mite control. An effective reduction of mite populations in the spring could provide beekeepers sufficient coverage through the spring and summer months, effectively reducing the likelihood of necessary treatments during the major nectar flows for most temperate regions. Winter is also an effective season to treat for *V. destructor*, providing beekeepers with coverage through the spring season. However, even after an effective winter treatment, mite populations could still return to economic thresholds by the spring season. In this case, the beekeeper has a difficult decision; either they interrupt their colonies' honey production during a major nectar flow or they delay treatment until after honey supers have been removed. If beekeepers are not able to reduce the *V. destructor* population below the economic threshold of 3 mites/100 bees, their colonies are likely to succumb to viral infection (Kulhanek et al., 2021).

While reducing *V. destructor* populations in summer and fall seasons may be important, the benefits of doing so could be very short-lived. A reduction of mite populations in summer only resulted in about 2 months of coverage for the beekeeper, meaning that another treatment in fall would be necessary. Unfortunately, reducing mite levels in fall again only provides 2 months of coverage, requiring another treatment in winter. Thus, it appears that multiple treatments are likely necessary if mite populations reach economic thresholds in the summer and fall seasons. Frequent treatments such as these can, increase the likelihood that the mites develop resistance to chemical treatments and the cost

TABLE 1 This table provides a proposed treatment (trt) schedule based on the month (January–December) that beekeepers apply their first treatment (light green).

	January	February	March	April	May	June	July	August	September	October	November	December	January	February	March	April	May	June	July	August	September	October	November	December	January	February	March	April	May	June	July	August	September	October	November	Total
January trt	X					X			X			X					X			X			X													7
February trt		X						X			X					X					X			X												6
March trt			X						X			X					X			X			X													6
April trt				X						X			X					X			X			X												6
May trt					X			X			X					X						X			X												6
June trt						X			X			X					X			X			X					X									7
July trt							X			X			X					X			X			X					X								7
August trt								X			X					X					X			X					X								6
September trt									X			X					X			X			X					X									6
October trt										X			X					X			X			X					X			X					7
November trt											X					X						X			X					X			X				6
December trt												X					X			X			X					X					X				6

The intervals indicated in this table are derived from the data illustrated in Figure 2. The "Total" column shows the number of treatments made in a 2-year cycle following this schedule. The bold line is to differentiate between year 1 and year 2.

X - Initial treatment.

X - Subsequent treatment based upon previous treatment.

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of controlling *V. destructor* to beekeepers. However, the need to reduce *V. destructor* populations in the summer and fall months is crucial, as viral titers tend to be at their highest and colonies are most severely impacted during these seasons (Highfield et al., 2009; Dainat et al., 2012; Traynor et al., 2016).

Varroa destructor treatments can vary widely in cost, with some treatments, for instance oxalic acid, costing less than others, such as the synthetic compound Apivar™. Thus, optimizing treatment efficacy and reducing the frequency of treatments could provide the beekeeper with considerable savings. Based on the *V. destructor* population growth data presented in **Figure 2**, we created **Table 1** to provide a better understanding of when a beekeeper could expect to apply miticides for the control of the pest. Depending upon when the treatment regime is started, a beekeeper may need to apply one additional treatment over the period of 2 years. Perhaps for a hobbyist beekeeper, that would not equate to significant savings, but it might for a large commercial operation. However, we created **Table 1** to be used as a reference for when colonies may need treatment, as treatment efficacy can be region-specific and not all treatments can be applied at all times of the year (Jack and Ellis, 2021). Thus, beekeepers should not stay on a strict treatment regimen but should closely monitor *V. destructor* populations to determine treatment timing.

The difference in colony lifespan post-treatment between the two trials was stark (**Figure 3**). For instance, survival was greatest in trial one after treatment in the winter. In trial 2, the winter treatment survival was similar to that of the summer group, with both being low. It is possible that these two winter treatments were affected differently by their location, as colonies receiving the winter treatment in trial one were located in the northern Gainesville apiary and while in trial 2, they were located at the Hawthorne apiary. Both apiary sites are ∼24 km apart, yet the floral resources available at the Hawthorne apiary are more plentiful than those at the northern Gainesville apiary during the late spring and summer months. Nutritionally, colonies in the Hawthorne apiary may have been better able to handle *V. destructor* after a winter treatment than were those at the Gainesville apiary. It is also possible that viral titers differed between colonies at the two sites, leading to the different responses of mite populations to winter treatments at both apiaries. While we believe that the bees used in this study were of the same genetic origins for both trial 1 and trial 2, slight genetic differences may have existed between the two populations. It is possible that these genetic differences of the bees used in both trials could have led to varying rates of death after exposure to elevated *V. destructor* levels. Weather differences, or other environmental parameters could have played a role. Unfortunately, we cannot determine the impact of location, genetics, virus load or weather on colony survival as we were only able to conduct two trials for this experiment and did not collect all the data necessary to make these determinations.

There are other variables that would likely impact *V. destructor* populations and warrant additional exploration. These variables could include temperature, frost-free days, rainy/dry seasons, nectar flows, and growing seasons. As mite population growth is closely tied to honey bee brood rearing (Wilkinson and Smith, 2002), the same climatic conditions that increase brood rearing likely also increase *V. destructor* populations. Ultimately, we can only use our results to predict the *V. destructor* population outcomes in north central Florida. However, beekeepers managing hives in areas with similar rates of brood rearing could use our work to assist with predictions of their own colonies' *V. destructor* population growth. Therefore, regional or countrywide honey bee brood surveillance would be helpful for predicting mite population growth with greater resolution. Such a surveillance program for the southeastern USA is currently underway (G. Williams, personal communication, University of Auburn).

Beekeepers are in desperate need of effective controls to use against *V. destructor*. As the development of new controls can take many years, it is essential that beekeepers utilize existing treatments more efficiently. We believe that the research presented herein helps us better understand the seasonal efficacy of *V. destructor* treatments and could potentially aid in the development of a mite control decision tool for beekeepers. More effective timing of treatments could reduce the frequency of treatments, thereby reducing the likelihood of *V. destructor* development of resistance to a given miticide. Additional research related to *V. destructor* population predictions and modeling is essential for long-term, sustainable management of this devastating honey bee parasite.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

CJ and JE: study conception and design, and interpretation of results. CJ: data collection. CK and IB: data analysis and interpretation of results. CJ, CK, IB, and JE: draft manuscript preparation. All authors reviewed the results, contributed to the article, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1102457/full#supplementary-material

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Effects of geographic origin and temperature on survival, development, and emergence of the managed pollinator *Osmia lignaria*

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Introduction: The blue orchard bee, *Osmia lignaria*, is a widespread North American native pollinator that can be employed for commercial fruit and nut crop production. The largest supplies of these bees are collected in the wildlands ssssof Utah and Washington, United States. How *O. lignaria* from different geographic regions respond to current recommended management practices or translocation to novel environments is not fully understood.

Methods: Utah- and Washington-originated *O. lignaria* were reared in laboratory incubators under two thermal regimens: (1) constant temperatures used to manage bees through immature development, adult winter dormancy, and for spring emergence, and (2) hourly fluctuating temperatures programmed to mimic the natural daily (24 h) thermal cycles of the nearest cherry orchard growing region through their life cycles.

Results: In comparison to rearing bees at orchard temperatures, we found that rearing bees at a constant temperature increased survival and shortened egg–adult development periods. Washington bees were more adversely affected by the consistent warm rearing temperatures than were Utah bees, possibly due to their adaptations to Washington's relatively moderate climate. At orchard temperatures, Utah bees suffered high prepupal and pupal mortality, while Washington bees suffered high pupal and adult mortality. These late life stages coincided with the hottest maximum temperatures in their respective thermal regimens. Adult females from both states naturally emerged in synchrony with local bloom time, but their emergence period overall was prolonged compared to bees in the constant thermal regimen that were induced to emerge at orchard bloom times.

Discussion: Our data support that bees originating from cool montane habitats of different U.S. states suffer from the warmer climatic conditions at lower altitudes of their respective crop-growing regions. A better understanding of optimal management temperatures for *O. lignaria* from different geographic regions and the effect of bee origin and temperature on survival and development timing is needed for best managing these pollinators when they are translocated or when climate change results in increased temperatures during bee development periods.

KEYWORDS

blue orchard bee, solitary, Megachilidae, phenology, environmental stressors, genes × environment, ecophysiology

1. Introduction

Bees serve a critical ecosystem function as pollinators and are vital for the production of pollinator-dependent crops (Klein et al., 2007; Ollerton et al., 2011; Khalifa et al., 2021). Numerous studies have noted declines in health and numbers of both managed and wild bees (e.g., LeCroy et al., 2020; Osterman et al., 2021; Zattara and Aizen, 2021) due to stressors, including loss of habitat, pests and pathogens, pesticides, and climate change (Brown and Paxton, 2009). Climate change is predicted to alter the environment in both managed and natural ecosystems in ways that pose a substantial threat to solitary bees. Also, management practices used to rear, supply, and employ commercially available bees in agricultural lands can have negative consequences for managed bees (Pettis and Delaplane, 2010). Environmental and management stressors often occur in tandem or interact so that effects are additive, synergistic, or antagonistic (Meeus et al., 2018).

Bee management and commercialization vary by species. Honey bees, *Apis mellifera* L., and bumble bees, *Bombus* spp., (Hymenoptera: Apidae) are the most well-known commercially available bees for crop pollination. Commercial honey bee colonies are propagated in apiaries, and colonies are transported sometimes very long distances for migratory pollination services, honey production, and winter dormancy. Colonies of several *Bombus* species are reared in commercial facilities and shipped, or otherwise transported, for pollination services in field or enclosed crops, such as tomatoes, various berries, and other fruits (Velthuis and van Doorn, 2006). Typically, commercial *Bombus* spp. colonies are not managed for self-propagation, and, therefore, new colonies must be purchased each year. Solitary, cavity-nesting bees are also available as commercial pollinators and require unique management practices. The native blue orchard bee, *Osmia lignaria* Say, and the introduced alfalfa leafcutting bee, *Megachile rotundata* F., (Hymenoptera: Megachilidae) are pollinators for tree fruit, nut, and berry crops and for alfalfa seed and hybrid canola seed production, respectively, in North America (Torchio, 1985, 2003; Bosch et al., 2006; Pitts-Singer and Cane, 2011; Artz et al., 2013; Andrikopoulos and Cane, 2018; Horth and Campbell, 2018; Pitts-Singer et al., 2018). *Osmia bicornis* and *O. cornuta* are used as managed orchard pollinators in Europe (Sedivy and Dorn, 2014; Krunić and Stanisavljević, 2006). *Osmia cornifrons* is propagated in Japan (Maeta et al., 1990; Matsumoto and Maejima, 2010) and was introduced into the eastern U.S. for pollination and has been spreading across North America since the 1980s (Cane, 2003; LeCroy et al., 2020; MacIvor et al., 2022).

Ideally, managed solitary bees are propagated in provided nesting materials on-farm or in open landscapes where preferred forage is naturally available. Bee progeny contained in nesting materials can be moved to other locations for storage (under controlled temperatures as needed), processing, and preparation for future use or sale. However, solitary bee reproduction results in population increase only in certain regions or crops and is highly variable from year to year. To fulfill the demand for large numbers of pollinators over a short blooming period, cocooned bees are shipped to novel environments outside of their natal regions.

Osmia lignaria naturally occupy montane riparian forests in western North America where nesting materials (e.g., exit holes of boring beetles in trees and moist soil from winter snow melt) and preferred foraging resources (such as willow, maple, and rosaceous trees) are found (Levin, 1957; Rust, 1974; Bosch and Kemp, 2001; Tepedino et al., 2022). In the United States, much of the commercial supply of *O. lignaria* is trapped by placing nesting materials in wildland sites with native populations (primarily in the Rocky Mountain and Pacific Northwest regions). Sometimes, bee ranchers reintroduce a portion of their harvested bees and enhance bee forage at the native sites to prevent overharvesting and increase propagation. Harvested bees usually are removed from nests as cocooned adults. Surplus bees are sold and transported in large numbers to homeowners and orchardists.

The market for *O. lignaria* as pollinators has substantially increased since commercial management was initiated in the 1970s (Torchio, 2003). When *O. lignaria* are used to pollinate orchard crops, they are typically transported to regions of lower altitudes that have favorable growing conditions (weather, soils, accessibility, etc.) for the crops. Thus, translocated *O. lignaria* often experience warmer, possibly drier climatic conditions than those of their natal locations, even when used in the U.S. state of origination. Studies of the movement of solitary bee populations outside of ranges from which they are propagated are sparse and, thus, the impacts of moving bees to new climates and landscapes are unknown or poorly understood.

For our study, we were interested in the separate and interactive effects of geographic origin of *O. lignaria* (trapped from wildlands) and the temperatures they experience during a life cycle in local growing regions when they are used as managed pollinators. Unlike for social bees, solitary bee development, reproduction, and overwinter survival are directly influenced by ambient temperature because there is no ability to regulate temperatures via the protection of a colony. For example, populations of *O. lignaria* from different geographic origins have been shown to exhibit regional differences in survival and developmental biology when exposed to common, but non-natal temperatures (Sgolastra et al., 2012; Pitts-Singer et al., 2014). Phenological differences, such as emergence timing, can be assumed to have a genetic basis if responding to climate in a different manner under the same climactic conditions. These populations can undergo local selection when responding to changes in natal climatic conditions (Bosch et al., 2008). When bees are translocated to a warmer climate or used for orchard pollination earlier than their natural phenology, high mortality occurs if bees are not managed under artificial conditions (i.e., removed from the field and stored in a climate-controlled shed or refrigeration unit). If left at ambient temperatures, bees become adults so early in the summer that they deplete their fat reserves before winter (Sgolastra et al., 2011; Pitts-Singer et al., 2014). Understanding *O. lignaria* ecophysiology throughout its range, especially considering climate change predictions, will improve best management practices for using bees from various geographic regions according to the location and timing of crop bloom.

We chose to explore the interaction of bee origin and temperature on *O. lignaria* survival, development, and timing of emergence from Utah and Washington because these are the main locations from which bees are currently collected from wildlands to sell in North America. We reared bees from Utah and Washington in laboratory incubators under two thermal regimens: (1) "constant" – one temperature through immature development, one for winter dormancy, and one for adult emergence, and (2) "natural" – hourly fluctuating temperatures programmed to mimic the natural daily (24 h) thermal cycles of the nearest cherry orchard growing region for

the entire bee life cycle (Supplementary Figure S1). The former treatment was a shared "common garden" in which bees from both populations experienced the same, managed temperature conditions during their spring–summer immature development, during their time as adults in cocoons in the fall and winter, and during induced adult emergence (for two temporally separate pollination events) in spring. This treatment allowed for a direct comparison of observed variables by origin only. The latter treatment was specific to bee origin and served to compare observed variables at fluctuating (natural orchard) temperatures to the same outcomes at constant temperatures for each bee population. Understanding at what life stage(s) and at what temperatures *O. lignaria* from geographically distinct locations differ in their survival, development, and emergence will aid in the development of best management practices for relocating and sustaining bees in novel, orchard environments.

2. Materials and methods

2.1. Bee collection

In spring 2019, suitable nesting material for *O. lignaria* was deployed in open landscapes to collect immature bees from two climatically distinct locations: near Logan in northern Utah (41.798, −111.650; 1,675 m) and near Leavenworth in central Washington (47.482, −120.656; 500 m) (Supplementary Figure S2; PRISM Climate Group, 2022). Bundled cardboard tubes lined with paper straws (diameter = 7.5–8 mm, length = 16.2 cm, end of tube closed with plastic plug) were sheltered in a corrugated plastic box (length by width by height = 22 × 17 × 26 cm); boxes were hung from tree branches facing south to southeast at least 1.5 m from the ground. Wild bees could construct nests in straws, and individual nests could be collected and kept intact for transport and experimentation. To increase nesting in the provided materials, a formulation (decanoic acid dissolved in ethyl acetate) of a patented chemical attractant was prepared and applied to the open end of the nesting tubes (Pitts-Singer et al., 2016).

Nests were checked periodically for bee activity and completion. Recently provisioned (≤2-week old) *O. lignaria* nests were collected and shipped (WA bees) or transported (UT bees) immediately to the USDA ARS Pollinating Insects Research Unit in Logan, UT. The paper straw nests were sliced longitudinally and visually inspected to select only cells with an egg, a first stadium larva inside the egg chorion, or a recently hatched second stadium larva (Figure 1 and Table 1). Bees older than the second stadium were left in their cells but not further observed or used in the study. All cells were kept within the paper straw nests and held on corrugated cardboard trays (to prevent nest movement) (Supplementary Figure S3) by treatment throughout immature development. Leaving cells in the paper straws was intended to reduce artificially-induced mortality due to moving the provision masses with eggs into rearing chambers, such as well plates, that may cause provisions to dry out or mold or may not provide suitable space for cocoon-spinning (Kopit et al., 2022).

2.2. Bee sample size and treatments

Nests contained 1–11 cells. Nests from each location were alternately assigned to one of two treatments, constant or natural thermal regimen, until at least 300 cells were available for each bee origin and temperature treatment. Therefore, all treatments contained cells that held female and male bees. In total, 1,432 *O. lignaria* nest cells were used in the experiment, split between four treatment groups: (1) UT bees exposed to a constant thermal regimen (UT-constant, $n = 373$), (2) WA bees exposed to the same constant thermal regimen as UT bees (WA-constant, $n = 369$), (3) UT bees exposed to a natural thermal regimen that mimics a cherry production zone near their collection site (UT-natural, $n = 325$), and (4) WA bees exposed to a natural thermal regimen that mimics a cherry production zone near their collection site (WA-natural, $n = 365$) (Supplementary Figures S1, S4). Each treatment consisted of approximately 60 nests (UT-constant = 63, WA-constant = 60, UT-natural = 62, WA-natural = 59), and nests each contained a mean of six cells (mean ± SE: UT-constant = 5.9 ± 2.5, WA-constant = 6.1 ± 1.8, UT-natural = 6.2 ± 2.2, WA-natural = 6.2 ± 2.4). Additionally, cells from all positions within nests were used (nests had up to 11 cells) with an equal number of cell positions being represented within each treatment and population.

The nests in the constant thermal regimen were held in a common environmental test chamber (Forma Scientific Dual Temperature Cabinet, Marietta, OH) set to 26°C (Bosch and Kemp, 2000, 2001;

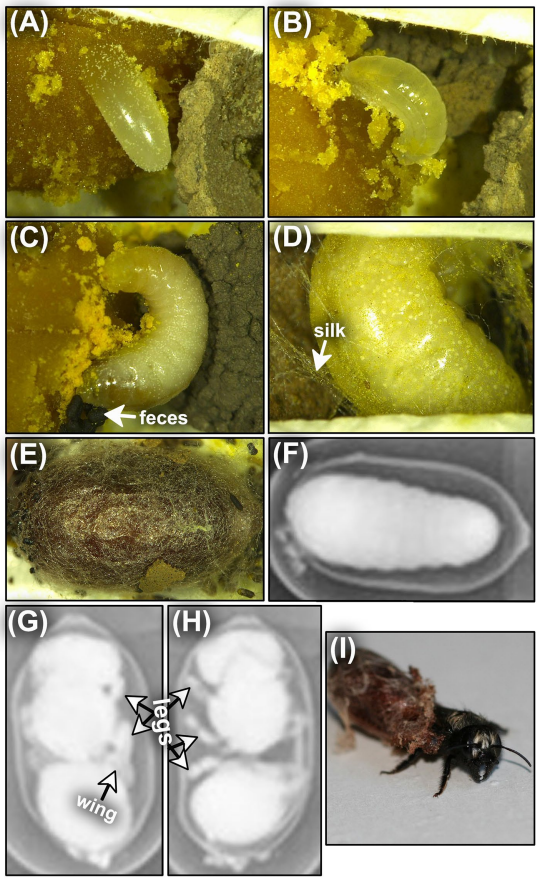

FIGURE 1
Optical and radiographic images of *Osmia lignaria* egg **(A)**, second stadium larva **(B)**, fifth stadium larva **(C,D)**, cocooned prepupa **(E,F)**, pupa **(G)**, cocooned adult **(H)**, and emerging adult **(I)**. See Table 1 for details.

TABLE 1 Life stages used to track *Osmia lignaria* phenology, with diagnostic characteristics used for delineation.

Life stage – Experimental details	Diagnostics	Figure panel (s)
Egg – Cells with only eggs or recently (<48 h) hatched larvae were selected.	The developing embryo and subsequent first larval stadium are contained within the egg chorion (Torchio, 1989).	Figure 1A
Second stadium larva – Hatched larvae with only little or no pollen in the gut were selected.	Once eclosed, second stadia are nearly translucent, having little to no pollen in the gut. Larvae begin to feed within 24 h of hatching (Torchio, 1989).	Figure 1B
Fifth stadium larva – We recorded the first day that fecal pellets and silk strands were observed.	The first fecal pellets are deposited within 24 h of molting to the fifth stadium (Torchio, 1989). Cocoon building is initiated <24 h after the pollen provision is consumed (Helm et al., 2017). Larvae make circular motions to weave fine, white silk strands, produced from salivary glands near the mouth, around its body. Cocoons darken in color and harden with each layer.	Figures 1C,D
Prepupa – Completed cocoons marked the start of the prepupal stage.	Cocoon building is complete when exterior is dark and rigid. Prepupae (cocooned fifth stadium larvae) undergo a month-long dormancy (Bosch and Kemp, 2001). By radiographic image, prepupae first appears C-shaped, then straightens prior to metamorphosis.	Figure 1E
Pupa – Radiographic images revealed metamorphosis.	Three-segmented pupae are easily distinguished from the grub-like prepupal form after metamorphosis. The legs of pupae are held close to the body and developing wings appear opaque laterally and posteriorly from the thorax.	Figure 1F
Cocooned adult – Radiographic images were used to determine day of adult eclosion.	After adult eclosion, fully sclerotized legs may splay laterally, and developed wings are translucent. Thorax and head capsule are well defined.	Figures 1G,H
Emerged adult – When bees were fully egressed from the cocoon.	Bees initiate emergence by chewing a hole in the cocoon, using their mandibles, to crawl out.	Figure 1I

Bees originating from Utah and Washington were reared within intact nests during immature development, and then transferred to gel capsules once cocoons were completed to continue tracking development *via* radiographic imaging.

Orchard Bee Association, 2021) throughout their immature development period (Supplementary Figures S1, S4 and Supplementary Table S1). Thirty days after adult eclosion (mean date by bee origin), bees were cooled over a 2-week period by subjecting them to 19°C for 1 week and then 11°C for the next week, requiring a second incubator to accommodate differences in developmental timing (similar to Orchard Bee Association, 2021). At the end of 2 weeks, bees were placed at a winter storage temperature of 4–5°C where they remained until the following spring (Bosch and Kemp, 2000, 2001). These management steps were made to avoid an excessively long (>45 days) pre-wintering period, which can cause high winter and pre-emergence mortality (Bosch and Kemp, 2004; Bosch et al., 2008). As possible, UT and WA bees for the constant treatment remained in the same incubator.

The natural thermal regimen reflected the use of "local" bees as pollinators in their regional orchard environments. Nest cells collected from a UT mountain site were placed in an incubator programmed for temperatures in a UT cherry orchard as the UT-natural regimen. Another incubator held nest cells from a WA mountain site and was programmed for temperatures in a WA cherry orchard as the WA-natural treatment (Percival Intellus Control System, Percival Scientific, Inc., Perry, IA, United States) (Supplementary Figure S4). For each bee origin, the natural thermal regimen mimicked daily (24 h) temperature cycles of the nearest orchard growing region for which data were available, starting at the time of cherry bloom. The programmed diel temperature cycles were updated weekly to match average hourly temperatures from a previous 6-year period (2012–2017) from weather stations in Provo, Utah (40.216, −111.716; 1,370 m; approx. 170 km from the bee collection location) and Wenatchee, Washington (47.397, −120.201; 378 m; approx. 35 km from the bee collection location) (Supplementary Figure S1; MesoWest, 2021). Bees remained at these natural temperature cycles throughout immature development, winter dormancy, and adult emergence the following spring (or until death) (Supplementary Figure S1).

The timing of tart cherry bloom in central Utah and sweet cherry bloom in the Columbia Basin of Washington is mid-April (United States Department of Agriculture National Agricultural Statistics Service, 2006). For the UT-natural and WA-natural treatments, the programmed diel temperature cycle for Week 1 of our experiment corresponded to mean hourly temperatures during 16 April (Day 1) to 22 April (Day 7) in each location (Supplementary Table S1). Week 2 of the experiment corresponded to the following seven dates, and so on. Since *O. lignaria* were collected from higher altitude wildlands than orchard landscapes and are naturally active in collection locations after cherry bloom, nests used in this study were made and collected after 16 April. UT bees (eggs or second stadium larvae) were obtained and assigned to experimental treatments between actual calendar dates of 14–21 May, and WA bees between actual dates of 07–14 May. For ease and clarity, we have reported dates in terms of weeks and days since the start of the experiment or dates which correspond to the timing of cherry bloom (i.e., the artificial timeline).

2.3. Mortality and development

Each bee cell was visually inspected (with the aid of a compound microscope, when necessary) three times per week (on Monday, Wednesday, and Friday) to document mortality and timing of immature life stages until larvae completed cocoon spinning (similar

to Pitts-Singer et al., 2014). Because the date of oviposition was unknown, the first life stage date recorded for all study specimens was the second stadium larva, after eclosion from the egg chorion; this served as a reliable starting point for comparing treatment effects on development periods for each immature life stage (Figure 1 and Table 1). Once cocooned, bees were considered prepupae and transferred to gelatin capsules. Digital radiographic images (12 s exposure at 24 kVp; computed radiography high-resolution system by Faxitron X-Ray LLC, Linconshire, IL) were taken three times per week (Monday, Wednesday, Friday) to determine the development periods for the prepupal, pupal, and adult stages and to record mortality at any cocooned life stage. Mortality was indicated by the failure to develop to the subsequent life stage (e.g., a bee died in the egg stage if a larva did not eclose from the egg chorion, or died in the prepupal stage if metamorphosis was incomplete).

2.4. Spring emergence

2.4.1. Incubation for adult emergence for constant regimen treatment

The UT- and WA-constant treatments were each further subdivided for two post-winter incubation events (Supplementary Figure S4). One subset was for incubating cocooned UT and WA bees in mid-March to simulate management for pollinating California cherry orchards; this treatment reflects a common real-world scenario in which bees are translocated because pollination demand in California is high and the natural abundance of *O. lignaria* is low or absent. The other subset was for incubating bees on two dates in mid-April to simulate pollinating cherry orchards in their natal regions so that UT bees were timed for cherry bloom in UT, and WA bees were timed for cherry bloom in WA.

2.4.2. Adult emergence for natural regimen treatment

Prior to incubation, the test chamber containing UT-constant and WA-constant bees was raised from 4 to 7°C for 5 days to prime bees for subsequent rapid emergence, which is a practice used by some bee managers. However, because some males emerged at 7°C (see below), the temperature was cooled back down to 5°C; males that emerged prematurely were excluded from further statistical analyses. Cocooned bees were moved to a 24°C incubator to induce emergence as if used for pollination in cherry orchards at the assigned times (Supplementary Figures S1, S4 and Supplementary Table S1), and checked daily for emergence, i.e., when the adult bee had chewed out of its cocoon but remained in the gelatin capsule.

Bees in the natural thermal regimens remained at mean daily temperature cycles of their region of origin to reveal emergence timing in the absence of temperature management. Bees were checked daily once natural temperatures reached 10°C to document the timing of adult emergence.

2.5. Statistical analysis

Prior to formal analysis, all Generalized Linear Mixed Models (GLMM) underwent an Akaike Information Criterion (AIC) model selection process, where treatment, bee origin, nest number, cell position (categorical) and sex (when appropriate) were analyzed. Nest number was always treated as a random effect, while all other factors were treated as fixed effects. The AIC model selection showed best fit for models using bee origin and treatment type as predictor variables and with nest as a random factor. All analyses were performed using R.3.1.2 (R core team) and R packages lme4 (Bates et al., 2015) and arm (Gelman and Su, 2022). *Post hoc* tests were performed using the lsmeans – Tukey analysis (Lenth, 2016).

2.5.1. For mortality and development

To compare mortality during immature development with respect to bee origin as a binary output (i.e., dead or alive), we performed a generalized linear mixed effects model (GLMM). We examined mortality as a response variable and bee origin and treatment as our predictor variables; we treated nest as a random effect. We performed this model with a binomial distribution.

To investigate immature (larva, prepupa, pupa) and mature (cocooned adult) mortality with respect to bee origin, we performed two GLMMs using a Poisson distribution, one for each treatment type (managed and unmanaged). Prior to analyses, data was checked for over-dispersal using a Goodness-of-fit model. In these models, the sum of dead individuals at each life stage per nest were used as our response variable. Life stage and bee origin was the predictor variables, and nest was a random effect.

To look for effects of thermal regimen and bee origin on duration of immature development, we performed two GLMMs separated by sex (i.e., a male model and a female model) comparing each life stage development. Female and male bees were analyzed separately because life stage duration has previously been shown to vary by sex (Bosch et al., 2000; Sgolastra et al., 2012; Pitts-Singer et al., 2014). To determine sex, we visually inspected bees after emergence. Cocoons containing dead pupae and un-emerged adults were also dissected to determine the sex, when possible. Sex could not be determined for prepupae and some pale (unsclerotized) pupae, nor for bees that died in the egg or larval stages.

2.5.2. For spring emergence

To reveal any effects of bee origin on (temperature-induced) timing of spring emergence, we performed two GLMMs (one for each treatment). For the managed treatment, we examined the number of days it took *O. lignaria* adults to emerge in spring of year 2 as a response variable and bee origin as our predictor variable; we treated nest number as a random effect. For the unmanaged treatment, we analyzed the number of days between adult eclosion and adult emergence in spring of year 2 as a response variable and bee origin as our predictor variable, with nest as a random effect.

3. Results

3.1. Mortality and development

Mortality was higher in the unmanaged treatments than in the managed treatments ($Z = 6.33$, $df = 3$, $p < 0.001$). When reared at constant 26°C, significantly fewer UT bees died (16%) prior to adult eclosion than did WA bees (25%; $Z = 2.91$, $df = 3$, $p = 0.022$). Mortality was statistically similar between UT and WA bees at the egg ($Z = -1.141$, $p = 1.000$), prepupal ($Z = 2.064$, $p = 0.467$), and pupal

($Z = 1.147$, $p = 1.0000$) life stages, but was significantly higher for WA bees at the larval stage ($Z = -3.746$, $p = 0.002$) (Figure 2).

The percent mortality during immature development was significantly higher for UT (86%) and WA (43%) bees reared at the natural thermal regimen compared to the constant 26°C treatment (UT bees: $Z = 12.24$, $df = 1$, $p < 0.001$; WA bees: $Z = 10.23$, $df = 1$, $p < 0.001$), and tended to increase from early to late immature life stages (Figures 2, 3). Compared to the constant treatment, mortality was eight times higher for UT-natural prepupae and pupae and was five times higher for WA-natural pupae. Due to high mortality of UT-natural bees prior to reaching the adult stage, their sex was unknown for later comparisons of life stage duration (Supplementary Figure S5).

Mortality during the cocooned adult life stage was similar for both populations ($Z = 0.247$, $df = 1$, $p = 0.618$; Figures 2, 3) in the constant thermal regimen (i.e., when bees were managed during the pre-wintering, wintering, and emergence periods). For UT bees, adult mortality was significantly higher in the managed constant thermal regimen than the natural thermal regimen ($Z = 19.81$, $df = 1$, $p < 0.001$); however, as noted previously, immature mortality for UT-natural bees was high, and few bees survived to the cocooned adult life stage for statistical comparisons. For WA bees, adult mortality was significantly lower in the constant compared to the natural thermal regimen ($Z = 54.91$, $df = 1$, $p < 0.001$).When bees from both regions were reared at constant 26°C, immature development (from the second stadium to adult eclosion) was shorter for WA bees than for UT bees; this difference was significant for females ($p < 0.001$, $\Delta = 4.42$ days) but not for males ($p = 0.09$, $\Delta = 1.82$ days) (Figures 3, 4 and Supplementary Tables S2, S3). For both bee populations and sexes, immature development was completed in 68–74 days. The duration of larval and prepupal stages

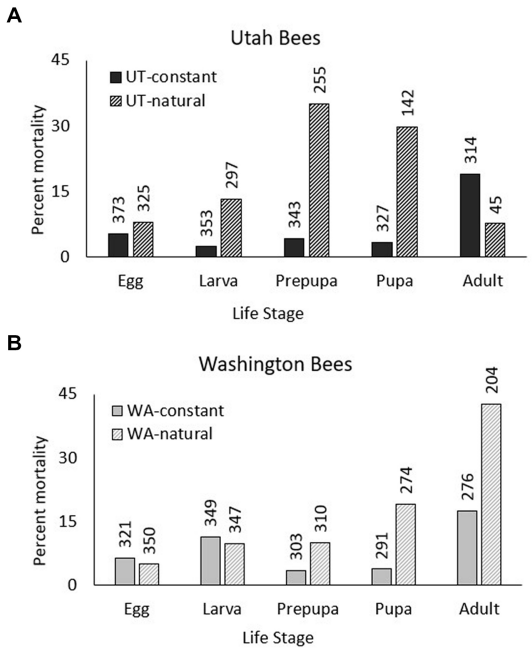

FIGURE 2
For Utah- **(A)** and Washington-sourced **(B)** *Osmia lignaria* by thermal regimen, the percent of reared bees that died at each immature (egg, larval, prepupa, pupa) and mature (cocooned adult) life stage. The number of bees in each sample is noted at the top of each bar.

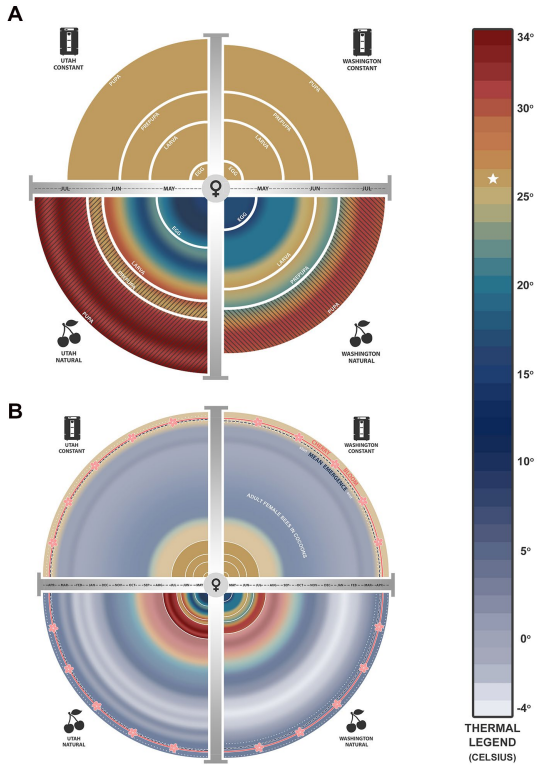

FIGURE 3
For a study of development and adult emergence of *Osmia lignaria*, Utah (left) and Washington (right) bees were reared using: (1) a constant thermal regimen in incubators (Top), and (2) a regimen of mean annual temperatures from a cherry orchard near their natal origins (UT-natural and WA-natural) (Bottom). **(A)** Mean life stage durations (width of band) at constant and maximum natural temperatures (color-coded) across months since the timing of Utah and Washington orchard bloom (mid-April) for female bees from egg (date of collection) through adult eclosion. Hatched lines indicate life stages where mortality was significantly high. **(B)** Thermal conditions for adult female bees in cocoons after eclosion (inner disc) until females chewed out of cocoons (emergence=dashed lines; duration of emergence=between dotted lines). Mean emergence day is weighted for when most bees emerged. For the constant regimen (Top), the temperature was lowered 30days after adult eclosion to provide a moderate pre-wintering period and lowered further for a long overwintering period. Constant temperature was increased to induce adult emergence in time for mid-April orchard bloom. No modifications were made to natural orchard temperatures. Average bloom date is indicated by the outer "blossom" circle. (Graphic created by Erica J. Brus).

were not statistically different by bee origin; however, the pupal stage was significantly shorter for WA bees compared to UT bees.

With respect to thermal regimen, development was shortened for both UT and WA bees when reared at the warmer constant temperature compared to their respective natural thermal regimens (Figures 3, 4 and Supplementary Tables S2, S3). This difference was significant for WA females ($\Delta = 7.56$ days) and males ($\Delta = 4.91$ days) but not for UT females ($\Delta = 2.52$ days) nor males ($\Delta = 1.54$ days). For both bee populations and sexes in the natural thermal regimens, immature development was completed in 72–78 days. The larval period prior to defecation at the fifth stadium was significantly reduced in bees reared at constant 26°C by about 13 days for UT females and 11 days for WA females; similar results were found for males. By contrast, we found that the prepupal stage was

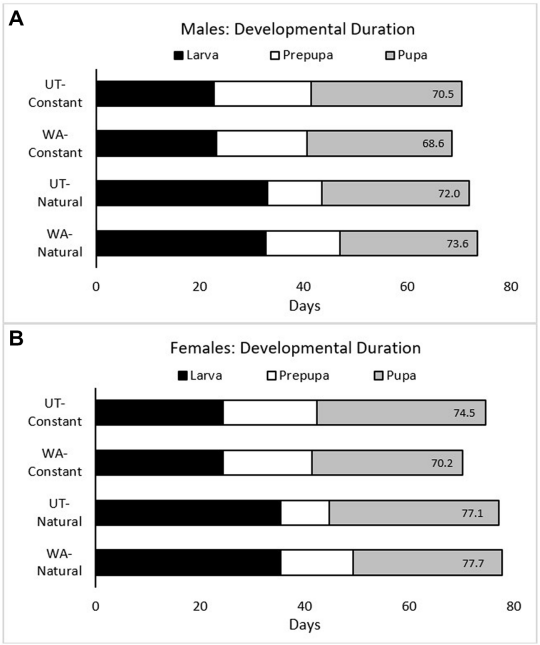

FIGURE 4
For male **(A)** and female **(B)** *Osmia lignaria* by treatment (bee origin–thermal regimen), the mean number of days spent in each immature life stage (larva, prepupa, and pupa). The mean number of days from the larval second stadium to adult eclosion is noted at the inner end of each bar.

significantly prolonged for bees reared at the constant compared to the natural thermal regimens, for both UT females ($\Delta = 7.71$ days) and WA females ($\Delta = 2.70$ days); similar results were found for males. Overall, thermal regimen had little effect on the duration of the pupal stage for both bee origins. Similar trends in the durations of immature stages were observed from analyses of all data including individuals whose sex was undetermined (Supplementary Figure S3).

Mean rearing temperatures during immature development were higher in the constant thermal regimen (26°C) compared to the natural thermal regimen for both bee sources (Figures 3, 5). Since temperatures in the natural thermal regimens slowly increased through spring and summer, the effect of temperature was greater during the larval period than for the prepupal and prepupal period. For the UT-natural regimen, mean temperatures were 12–22°C during larval development, 19–22°C during prepupal development, and 19–26°C during pupal development. For WA-natural bees, mean rearing temperatures were cooler than UT-natural temperatures during larval development (12–18°C) and similar during prepupal and pupal development. However, maximum temperatures exceeded 26°C by the end of the larval period in the UT-natural treatment and during the prepupal period for the WA-natural treatment.

3.2. Spring emergence

3.2.1. Incubation for adult emergence for constant regimen treatment

For both bee populations and sexes in the constant thermal regimen, the mean day of adult eclosion since the time nests were placed in incubators was during Week 12 (Days 79–84); the number of development days observed here was longer than the number of days in Figure 4 because it includes the time needed for eggs (of unknown age when collected) to hatch to the second stadium. Development from egg to second stadium was greatly affected by temperature (Figures 3, 5). For the UT-natural and WA-natural treatments, the mean day of adult eclosion was during Week 16 (Day 106) and Week 14 (Day 93), respectively. Unlike the constant thermal regimen in which bees were gradually cooled to wintering temperatures (4°C) 30 days after adult eclosion (Week 16), bees in the natural thermal regimen experienced warm pre-wintering and wintering temperatures for a longer period. Mean temperatures reached 4°C during Week 33 (Day 225) for the UT-natural treatment and during Week 31 (Day 211) for the WA-natural treatment.

For the cocooned bees in the constant thermal regimen placed at 7°C (from 4°C) prior to incubation, 3 female and 90 male UT bees and 1 female and 31 male WA bees emerged prematurely and were excluded from data analyses on adult emergence timing (Supplementary Table S1). When one subsample of bees was managed (incubated at 24°C) for March orchard bloom (UT bees on Day 339 and WA bees on Day 333), over 90% of UT and WA bees to emerge did so within the first and second day after incubation (Figure 6 and Table 2). Therefore, the duration of the cocooned adult stage was 257 days for UT males and 255 days for UT females; for WA bees, males were cocooned adults for 252 days and females for 253 days.

When the other subsample of bees was incubated in April (UT bees on Day 365 and WA bees on Day 360) to mimic the same timing of orchard bloom in their respective regions, 88 and 98% of UT and WA bees, respectively, emerged within 1–2 days. Therefore, the duration of the cocooned adult life stage was 284 days for UT males and 281 days for UT females; for WA bees, males were cocooned adults for 272 days and females for 271 days.

3.2.2. Adult emergence for natural regimen treatment

When UT and WA bees were reared at natural orchard temperatures of their respective regions, they emerged over a much longer period than bees in the constant regimen treatment (Figures 3, 5B and Table 2). Bees in the UT-natural group emerged over a 32-day period, 85% of which emerged between Day 350 (peak male emergence) and Day 366 (peak female emergence). Bees in the WA-natural group emerged over a 26-day period, 95% of them emerged between Day 344 (peak male emergence) and Day 360 (peak female emergence).

4. Discussion

Our study revealed the effects of *O. lignaria* origin and rearing temperature on development and survival. Use of the common, constant temperature regimen for UT- and WA-sourced bees allowed for isolating the differences between the populations in the absence of temperature variation. Use of the natural orchard temperatures allowed a comparison of development and survival for each population at both the constant and natural temperatures to reveal impacts of realistic uses of bees as pollinators in their respective localities. Insect development, activity, reproduction, and survival are strongly influenced by ambient temperature. Increased temperatures have been shown to shorten developmental periods, increase activity, and result

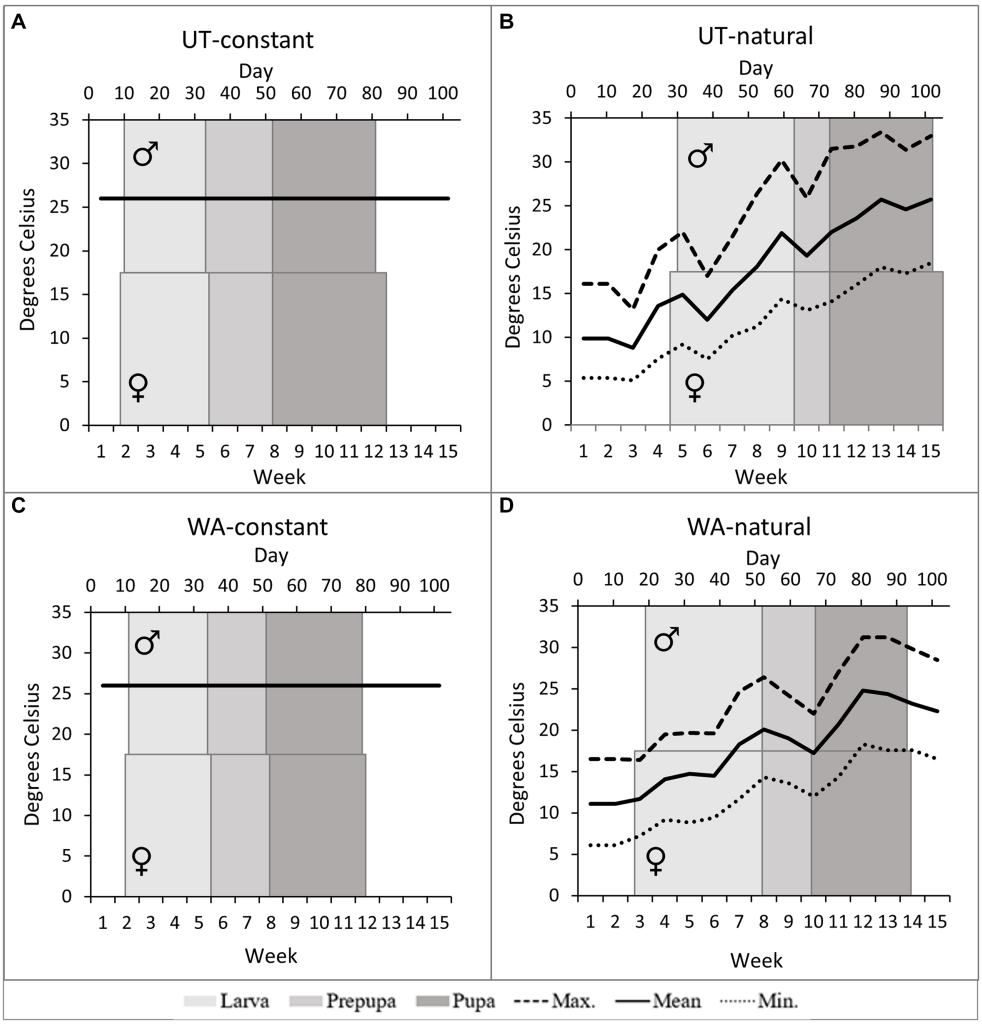

FIGURE 5
The programmed temperatures and mean duration of male and female Osmia lignaria immature developmental stages by treatment **(A)** UT-constant, **(B)** UT-natural **(C)** WA-constant, & **(D)** WA-natural.

in high survival and reproduction in *Osmia* (Bosch and Kemp, 2003, 2004), but of course extreme temperatures, or those outside a species' thermal limits, are harmful (Martin and Huey, 2008; McCabe et al., 2019). However, responses to ambient temperature can vary by species and by populations within species (Colinet et al., 2015; Jarimi et al., 2020; Orr et al., 2021; Porter, 1988; Rebaudo and Rabhi, 2018; Wilson, 2019). Our work corroborates these two former statements. Understanding how temperature affects solitary bees managed to provide pollination services can help to not only assure population survival, but also inform how to use temperature management for timing adult bee emergence with crop bloom, especially when the crop is located where climate differs from that of the bees' origin (Bosch et al., 2000; Sgolastra et al., 2011; Pitts-Singer et al., 2014).

At the constant warm temperature, young UT and WA larvae fed and grew very quickly. The orchard temperatures of both states during larval development times fluctuated but were much lower than 26°C, meaning that larval growth was slowed under cool conditions. Conversely, the prepupal period was prolonged at constant 26°C compared to those at orchard temperatures. During the prepupal and pupal stages, the mean orchard temperature never reached 26°C, but maximum orchard temperatures exceeded 30°C and most likely shortened these life stage durations. Warm temperatures have been known to speed up development and/or increase mortality; both are consistent with our results (Bosch and Kemp, 2000).

Overall bee mortality from egg to adult emergence under the constant thermal regimen was low. The level of larval mortality in this study (<20% total) was similar to a previous laboratory study with UT bees held at 22 and 26°C (Bosch and Kemp, 2000). Interestingly, we found mortality was 11% for larval WA bees, but less than 3% for larval UT bees. We suggest that WA bees may be more adversely affected by warmer than natural spring and early summer conditions than are UT bees. Pitts-Singer et al. (2014) exposed offspring of UT and WA populations to temperatures simulating a California almond orchard that included hotter maximum temperatures than either of the natural temperature regimens used in this study. That study also found a difference in larval mortality according to parental bee origin with mortality for WA male larvae being lower than for UT male larvae, but mortality for WA female larvae being higher than for UT female larvae.

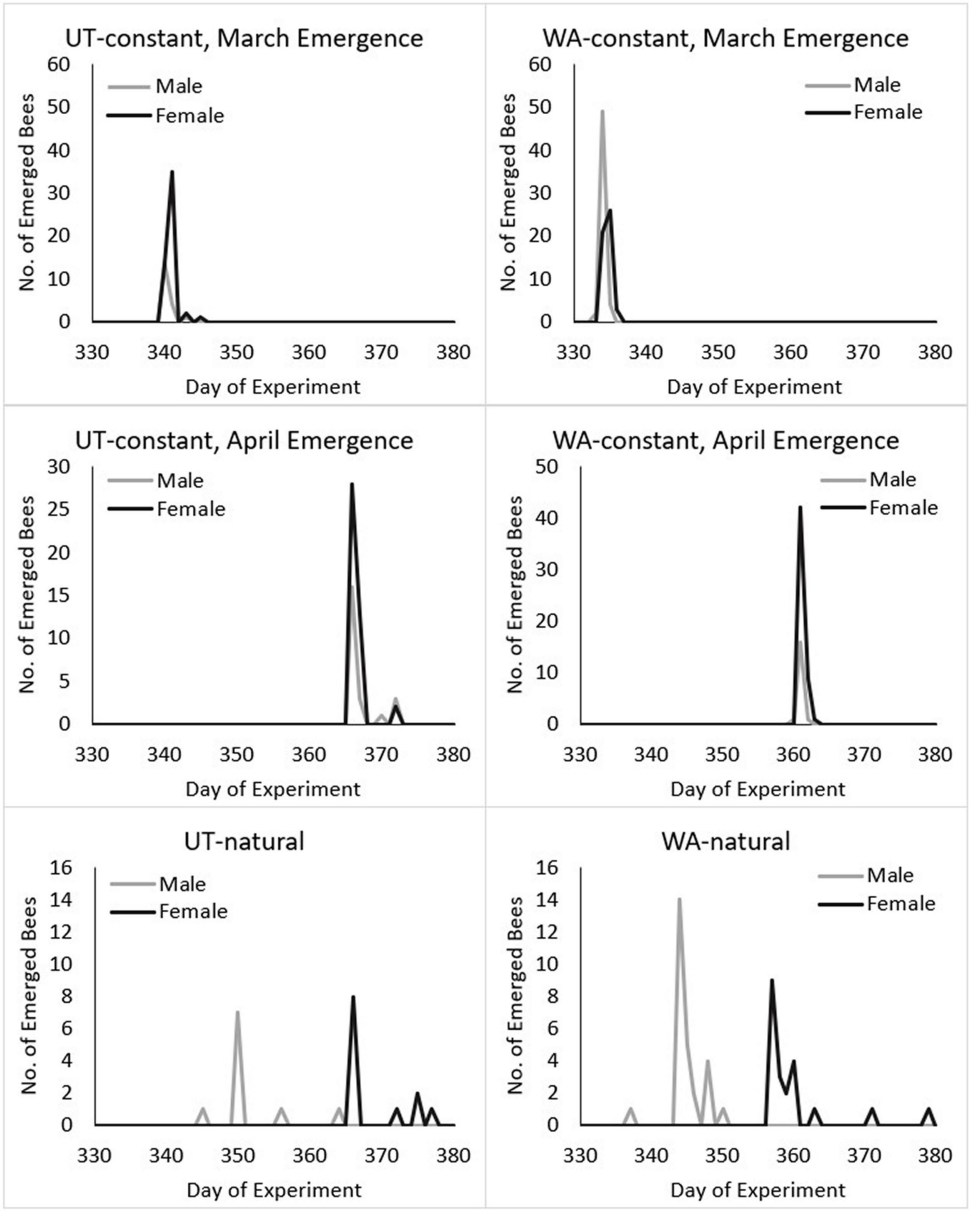

FIGURE 6
By treatment, emergence of *Osmia lignaria* females and males in spring of year 2. For the constant thermal regimen, bees were incubated (at 24°C) to induce emergence in mid-March (UT bees on Day 339 and WA bees on Day 333) approximately 1 month earlier than the pollination season in which they were born the previous year, and in mid-April (UT bees on Day 365 and WA bees on Day 360) on the same pollination timeline of their natal orchard. Bees in the natural thermal regimens were not induced to emerge via artificial warm temperatures but remained at simulated outdoor orchard temperatures; if kept for pollination in their natal orchard locations, target emergence period would be around Day 365 given similar timing of crop bloom. Prior to incubation, 3 female and 90 male UT bees emerged prematurely (at 7°C), and 1 female and 31 male WA bees emerged prematurely and are not included here.

At natural orchard temperatures, mortality was higher overall than when bees were raised at a constant temperature. The greatest mortality was in the life stages that experienced the highest maximum temperatures, which were the prepupal and pupal stages for UT bees and the pupal and adult stages for the WA bees. Bosch and Kemp (2000) found that some bees from northern UT failed to complete prepupal dormancy only at the lowest temperatures tested (constant 18°C). However, our UT-natural bees experienced a range of temperatures that were well above and just below 18°C. Although there was a decline in UT temperatures during the prepupal stage, it was only about as cool as the hottest temperature during the WA prepupal stage. McCabe et al. (2022) also found that maximum temperatures were likely to predict prepupal and pupal mortality in megachilids in natural montane environments.

Differences by bee parental origin in timing of development under fluctuating temperatures also resemble the results found by Pitts-Singer et al. (2014). In this study and the previous one, WA bees took slightly longer to reach adulthood than UT bees, and the duration of

TABLE 2 Summary data and statistics for ANOVA comparisons of the number of days for *Osmia lignaria* to emerge from the cocooned adult life stage.

Thermal regimen	Bee source (No. of bees)	Days from adult[1]	Days from incubation[2] or Bloom time[3]	Duration (days)	Statistics (*df*=1)
Male bees					
Constant – March Bloom	UT (19)	258.4	1.4	4	$z = 1.009$
	WA (55)	253.0	1.0	3	$p = 0.915$
Constant – April Bloom	UT (24)	284.0	2.1	7	$z = 3.36$
	WA (18)	283.9	1.0	3	$p = 0.011$
Natural	UT (9)	249.0	−13.3	20	$z = 1.037$
	WA (27)	251.1	−15.1	14	$p = 0.316$
Female bees					
Constant – March Bloom	UT (51)	255.9	1.9	6	$z = 0.988$
	WA (50)	254.6	1.6	3	$p = 0.825$
Constant – April Bloom	UT (44)	280.6	1.6	7	$z = 2.787$
	WA (52)	280.2	1.2	3	$p < 0.001$
Natural	UT (12)	263.4	3.9	12	$z = 0$
	WA (21)	263.3	−0.1	23	$p = 0.989$

[1]Mean days from the start of the cocooned adult stage. [2]For the constant thermal regimen, mean days from incubation. [3]For the natural thermal regimen, mean days from the start of cherry bloom, Day 365; a negative number indicates emergence prior to bloom.

the UT prepupal period was much shorter and pupal period longer than the durations of those life stages for WA bees. Potentially, this quicker development to adulthood for WA bees could lead to lethal or sublethal effects if care is not taken to properly manage bees from different origins. Bosch et al. (2010) found that when bees remained at warm temperatures in their adult stage for greater than 30 days, their fat bodies were depleted, and adults were less likely to emerge. There has also been evidence that bees with smaller body sizes (perhaps due to quick development) are poor pollinators (Jauker et al., 2016). Therefore, by not understanding the development timing of these bees, farmers and bee managers could be releasing suboptimal pollinators in their orchards.

Managing bees via artificial conditions to time their release for commercial pollination is thought to be more effective than allowing for natural emergence (Bosch and Blas, 1994; Bosch et al., 2000, 2008; Bosch and Kemp, 2000). In our study, the use of a constant thermal regimen for both an early and late cherry bloom event resulted in synchronous and quick bee emergence from cocoons. Management recommendations for commercial populations are to allow for at least 180 days of winter temperatures to obtain narrow peaks of bee emergence (Bosch and Kemp, 2001); the cocooned adults in this study received >240 days of winter temperatures (4°C). The natural emergence of bees from both UT and WA occurred very close to 1 year since they were collected as eggs, which was around the time of natural orchard bloom. These bees did not experience temperatures at or below 4°C of wintering until approximately 150 days before they emerged in spring. This short wintering period may be the reason for the window of natural adult emergence being wider than for the managed bees. Males in the natural thermal regimen emerged slightly ahead of cherry bloom, and females emerged more or less in synchrony with the timing of cherry bloom. Naturally emerging bees would benefit from supplemental, early blooming floral resources to sustain early emerging male bees prior to female emergence. After crop bloom has ceased, females (some just recently out of cocoons) could continue nesting if floral resources are available (Boyle et al., 2020).

Overall, the effect of the various timings of higher-than-average temperatures implies that using bees in climates with temperatures warmer than, or at the extreme ends of, their native temperature ranges can be detrimental for developing bees. Scenarios where temperature extremes are experienced by *O. lignaria* are realistic, as in California for almond pollination when bees are left to develop there. If bees from Utah and Washington produce offspring in California during February almond bloom, then many of those progenies are unlikely to survive if not managed for much of the year under controlled temperatures. Proper bee management strategies will also be needed if climate change predictions of a 3–8°C increase in Earth's temperature over the next 80 years are realized (Allen et al., 2018). Therefore, this current baseline understanding of temperature effects on *O. lignaria* and other pollinators and how different populations/species respond to new and warming conditions is needed for future management and decision making.

These results provide baseline expectations for the performance of bees as pollinators in areas where they presumably are regionally adapted to climatic conditions. Practical management implications are that bees used for orchard (or other crop) pollination should be moved during larval development periods (>7–9 weeks after nesting, before larvae make cocoons and enter the prepupal stage) to a location where they can avoid high temperatures such as the maximum 30°C that bees experienced in this study. Maintaining bees at a constant temperature, such as 26°C, prior to reaching adulthood might also better synchronize adult eclosion so that the timing for winter storage is easy to predict and accommodate, especially when the target, pollinator-dependent crop blooms in early spring.

The differences in development between the UT and WA populations at constant and natural thermal temperatures may indicate a genes × environment (G × E) interaction. Our work indicates that each population genotype is responding to environmental conditions (in this case, temperature) in a different way. This G × E interaction is important to understand especially when translocating managed bees to novel climates. Honey bee populations have been shown to have different behavioral responses when moved to a common environment (Costa et al., 2012). As the climate continues to warm, different *O. lignaria* population genotypes may be better adapted to tolerating increased temperatures, making some populations more optimal than others to serve as pollinators. Further research is needed to understand whether geographically distinct bee populations present different physiological thermal tolerances, differences in developmental timing of specific life stages, or differences in ability to adapt to novel environments over generations, when extreme climatic conditions are experienced, such as prolonged or shortened summer quiescence as prepupae and earlier or later spring adult emergence.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MS, TP-S, and DA: conceptualization. MS: data curation and investigation. MS and LM: formal analysis. TP-S and DA: funding acquisition and supervision. MS and TP-S: methodology and project administration. TP-S: resources. MS, TP-S, and LM: visualization and writing—original draft. MS, TP-S, LM, and DA: writing—reviewing and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Impact of landscape configuration and composition on pollinator communities across different European biogeographic regions

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Introduction: Heterogeneity in composition and spatial configuration of landscape elements support diversity and abundance of flower-visiting insects, but this is likely dependent on taxonomic group, spatial scale, weather and climatic conditions, and is particularly impacted by agricultural intensification. Here, we analyzed the impacts of both aspects of landscape heterogeneity and the role of climatic and weather conditions on pollinating insect communities in two economically important mass-flowering crops across Europe.

Methods: Using a standardized approach, we collected data on the abundance of five insect groups (honey bees, bumble bees, other bees, hover flies and butterflies) in eight oilseed rape and eight apple orchard sites (in crops and adjacent crop margins), across eight European countries (128 sites in total) encompassing four biogeographic regions, and quantified habitat heterogeneity by calculating relevant landscape metrics for composition (proportion and diversity of land-use types) and configuration (the aggregation and isolation of land-use patches).

Results: We found that flower-visiting insects responded to landscape and climate parameters in taxon- and crop-specific ways. For example, landscape diversity was positively correlated with honey bee and solitary bee abundance in oilseed rape fields, and hover fly abundance in apple orchards. In apple sites, the total abundance of all pollinators, and particularly bumble bees and solitary bees, decreased with an increasing proportion of orchards in the surrounding landscape. In oilseed rape sites, less-intensively managed habitats (i.e., woodland, grassland, meadows, and hedgerows) positively influenced all pollinators, particularly bumble bees and butterflies. Additionally, our data showed that daily and annual temperature, as well as annual precipitation and precipitation seasonality, affects the abundance of flower-visiting insects, although, again, these impacts appeared to be taxon- or crop-specific.

Discussion: Thus, in the context of global change, our findings emphasize the importance of understanding the role of taxon-specific responses to both changes in land use and climate, to ensure continued delivery of pollination services to pollinator-dependent crops.

KEYWORDS

habitat heterogeneity, intensity gradient of land-use, pollinators, standardized approach, European biogeographic regions

Introduction

Flower-visiting pollinating insects provide a vital ecological service, contributing to the pollination of both wild and cultivated plants (Ollerton et al., 2011). However, in recent decades, a decrease in the abundance and diversity of insect pollinator taxa has been recorded across different regions of the world (IPBES, 2016). Across Europe, between 37 and 65% of wild bee species are considered of conservation concern (Patiny et al., 2009; Nieto et al., 2014; Bretagnolle and Gaba, 2015), and a decline in bumble bee species richness has been documented for the last 60 years (Goulson et al., 2008). According to the European Red List of Butterflies, up to 10% of the butterfly species are considered threatened or nearly threatened (van Swaay et al., 2010, 2011; Warren et al., 2021), and over one-third of the European hover fly species are threatened as well (IUCN–International Union for Conservation of Nature, 2022). Scenarios of global change project further loss of butterflies (Settele et al., 2008), hover flies (Miličić et al., 2018), and bumble bees (Rasmont et al., 2015), supported by observed responses to historic climate change (Kerr et al., 2015).

These declines of pollinators are caused by multiple stressors (e.g., pesticides, climate change related factors, pathogens, invasive and alien species), with anthropogenic land use often considered as the main threat to flower-visiting insects (Goulson et al., 2008; Winfree et al., 2009; Potts et al., 2010; Scheper et al., 2013; Proesmans et al., 2021). Two main aspects of land use have been shown to affect biodiversity: compositional and configurational heterogeneity (Fahrig et al., 2011). The composition of the landscape describes the amount and the diversity of habitats or land use types that constitute the landscape; whereas the configuration of the landscape defines its spatial arrangement, e.g., the number and distribution of patches and their shapes, or their connectivity (Seppelt et al., 2016).

Landscapes characterized by high compositional heterogeneity are more diverse, and might offer additional foraging and breeding resources, and thus may support higher numbers of species (Dunning et al., 1992; Flick et al., 2012). In addition, high configurational heterogeneity enhances landscape connectivity, providing crucial structural elements for the movement of species and their orientation within the landscape, with positive consequences for population dynamics (population genetic structure and demography) and community interactions (Steffan-Dewenter and Tscharntke, 1999; Becher et al., 2016; Dominik et al., 2018; Hass et al., 2018). Thus, landscapes that are heterogeneous in both their composition and configuration are expected to support higher biodiversity, e.g., flower-visiting insects communities, by facilitating their dispersal providing extra nesting sites, and positively affecting the temporal and spatial distribution of floral resources (Steffan-Dewenter et al., 2002; Kremen et al., 2007; Fahrig et al., 2011; Cole et al., 2017; Senapathi et al., 2017; Hass et al., 2018). Conversely, habitats with low composition and configuration heterogeneity are usually associated with phenomena like fragmentation, habitat loss and degradation, which can result in the decrease of resource availability (Senapathi et al., 2017) and have negative impacts on flower-visiting insect movement, diet, reproduction, survival, and interaction with plants (Day, 1991; O'Toole, 1994; Steffan-Dewenter and Tscharntke, 1999; Gathmann and Tscharntke, 2002; Hadley and Betts, 2012).

Although the conversion of semi-natural land to intensive agriculture leads to habitat loss with adverse consequences for flower-visiting insects (Senapathi et al., 2017), the landscape surrounding cultivated crops may still support insect communities, depending on its composition and configuration (Steffan-Dewenter et al., 2002; Rundlöf et al., 2008a; Cranmer et al., 2012; Kennedy et al., 2013; Bourke et al., 2014). Semi-natural landscape features, hedgerows and field margins in particular, can promote insect diversity by providing additional food or nesting resources, and facilitating the movement of individuals between patches (Marshall and Moonen, 2002; Fahrig, 2003; Bengtsson et al., 2005; Hole et al., 2005; Jonason et al., 2011). Mass-flowering crops can also be attractive to flower-visiting insects, by offering food resources with short-term beneficial effects for pollinators (Westphal et al., 2003; Jauker et al., 2012; Holzschuh et al., 2016), while floral strips, hedges, bushes and field margins can fill

nutritional gaps outside the blooming periods of these crops (Timberlake et al., 2019; Bottero et al., 2021).

Despite overall negative impacts of agricultural intensification on insect communities, different taxa may respond differently to landscape heterogeneity and land-use intensity due to the disparities in their diet, behavior, floral resource preferences and, nesting, and overwintering sites (Gathmann and Tscharntke, 2002; Fenster et al., 2004; Cane et al., 2006; Klein et al., 2012). A recent meta-analysis analyzing the combined effects of edge density length and percentage of semi-natural habitat on the abundance of different arthropod taxa across Europe, showed that the responses were highly context dependent (Martin et al., 2019). In large-scale studies covering multiple biogeographic regions, contrasting responses of landscape heterogeneity on insect communities may also be a result of varying weather conditions (daily temperature, wind, rain) and climate (annual temperature and precipitation, precipitation seasonality). Weather and climatic conditions can either have direct effects, by affecting the survival and fitness of individuals, or indirect effects, by impacting the availability of foraging resources and the phenology of both insects and plants (Vicens and Bosch, 2000; Brittain et al., 2013; Lawson and Rands, 2019; Martinet et al., 2021), with consequent cascading impacts on plant-pollinator interactions (Hegland et al., 2009; Vasiliev and Greenwood, 2021). While the responses of insects to these effects are mostly taxon-specific, they also differ according to the temporal (daily, seasonal, between years) and spatial scale, as landscape structure can buffer climate impacts (Papanikolaou et al., 2017; Herrera, 2019; Zoller et al., 2020; Ganuza et al., 2022). However, studies investigating the complex suite of landscape and environmental factors that influence flower-visiting insect communities over a larger (e.g., European) biogeographic scale are still scarce.

In this study, we investigated the impact of both the composition and configuration of the landscape on the abundance of several broad taxonomic groups of flower-visiting insects, in 128 crop dominated sites across Europe. At the landscape scale, we hypothesized that more heterogeneous landscapes, with a larger proportion of less-intensively managed habitat (i.e., non-crop and non-urban), and less isolated habitats sustained a greater abundance of flower-visiting insects. At the field scale, we hypothesized that mass-flowering crops and orchards adjacent to the sites could supplement flower-visiting insect abundance, by providing them with additional resources at the beginning of the spring season. In the face of climate change, we also investigated the effects of weather and climate on the abundance of different pollinator insect groups, distributed across multiple biogeographic regions.

Methods

Experimental design

Eight countries were selected within the PoshBee site network (https://poshbee.eu/; Figure 1), representing four of the main European biogeographic regions – Switzerland (CHE) and Germany (GER) for the Continental zone; Italy (ITA) and Spain (ESP) for the Mediterranean zone; Britain (GBR) and Ireland (IRE) for the Atlantic zone; and Estonia (EST) and Sweden (SWE) for the Boreal zone. In each country, we selected 16 sites to encompass a gradient of land use intensity: eight sites containing annual crops – winter-sown oilseed rape (OSR; *Brassica napus*) – and eight sites with perennial crops – apple orchards (APP), for a total of 128 sites (Figure 1; Hodge et al., 2022). The land use intensity gradient was defined by the proportion of cropland and orchards within a 1 km radius of the center of the sites (Hodge et al., 2022). We ensured a minimum distance of 3 km between the sites to avoid overlapping landscape buffers and violation of spatial independence for subsequent analyses (Hodge et al., 2022). Because of the large geographic range, and differences in cultivation patterns across this range, field sizes varied considerably: apple orchards varied between 0.32 and 45 hectares, while oilseed rape crops varied between 0.5 and 135 hectares (Hodge et al., 2022). A significant confounding factor in these studies was the presence of three honey bee (*Apis mellifera*) colonies and three bumble bee (*B. terrestris*) colonies in each site, and three *Osmia bicornis* trap nests in some of the sites, at the time of sampling – as sentinel colonies for other studies conducted at the same time in the PoshBee site network (Hodge et al., 2022). These sentinel colonies were introduced to the landscape immediately prior to blooming and removed immediately afterwards (Hodge et al., 2022), and thus will not have had long-term effects in the local pollinator community composition. However, they might have contribute to the insect survey data for these three pollinator groups. The statistical section describes how these biases were addressed during the analyses.

Insect surveys

We recorded the presence of five groups of obligate flower-visiting insects – honey bees (*Apis mellifera*), bumble bees (*Bombus* spp.), bees other than honey bees and bumble bees – here called solitary bees (despite the fact that some of them might be primitively eusocial, communal or kleptoparasitic species; Hymenoptera, Apoidea, Anthophila), hover flies (Diptera, Syrphidae), and day-flying butterflies (Lepidoptera).

Insects belonging to the five groups were recorded along four transects per site. Two transect were placed in the center of the focal crop field or orchard and two along the respective margins. The two transects in the center of the crops were at least 30 m apart, at least 30 m from the edges of the field and as close as possible to the center of the field. The two transects on the margins were performed on the actual field borders (e.g., strips along the side of the crop, hedgerows, ditches, stonewalls, etc.). We surveyed the field borders rather than the edges of the crop itself, because our aim was to focus on landscape-level features, rather than to analyze variation within the crop field. Each transect was 50 m long and 2 m wide and walked for 5 min on three occasions during the main crop flowering period – at the beginning, peak and towards the end of flowering, resulting in a maximum of 12 transect walks per site. Transect walks were conducted from the 1st of April 2019 (oilseed rape in Ireland) to the 7th of June 2019 (oilseed rape in Germany; see Supplementary Table S1). Insect surveys were only performed during suitable weather conditions, and between 10.00 am and 4.00 pm (see Mahon and Hodge, 2022). Due to unfavorable weather conditions or the difficulties accessing the center of the crops at specific growing stages, 1,295 transect walks (84%) were completed (out of a possible total of 1,536). Transect walks were performed in a non-destructive manner (Hodge et al., 2022), which prevented a species-level identification, but allowed for the assessment of taxon-specific abundances.

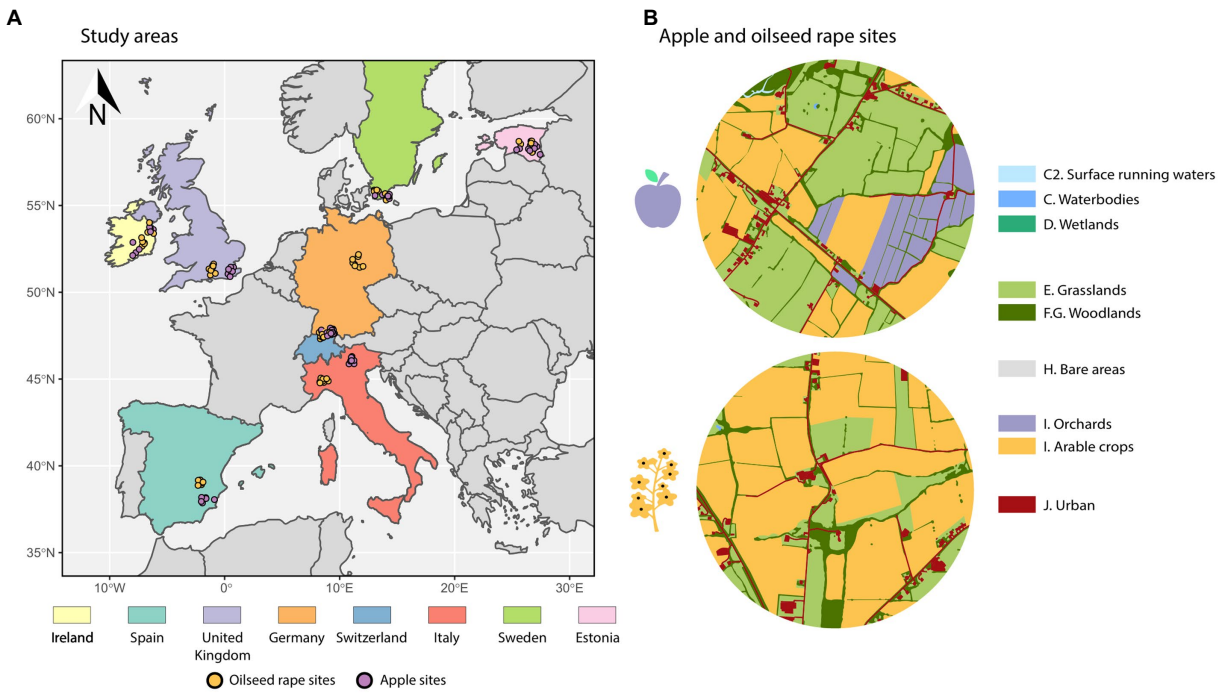

FIGURE 1
(A) Location of the 128 sites – oilseed rape sites (orange dots) and apple sites (purple dots) across the eight countries. **(B)** Examples of mapping land cover features within 1-km radius buffers around apple and oilseed rape sites.

Landscape heterogeneity

At the field scale, we identified the habitat type surrounding the focal sites, based on categories defined by the EUNIS habitat classification system (Davies et al., 2004), and recorded the number of the site borders with adjacent mass-flowering crops and orchards (such as apple orchards, oilseed rape crops, horticulture other than apples, and other types of crops, e.g., peas) *in situ*.

At the landscape scale, we quantified different aspects of landscape heterogeneity by calculating multiple metrics that best describe habitat composition and configuration. First, all landscape features were manually digitalized at a 1:2500 scale within a 1 km radius around the sampling sites (Figure 1), using a combination of head-up digitizing remote sensing data provided by World Imagery (ESRI) and GIS tools (ArcGIS Pro 2.4.1, ESRI). Following the EUNIS habitat classification reference, we classified all land cover categories into nine final categories: Surface Running Waters, Waterbodies, Wetlands, Grasslands (including both managed grassland for livestock, and semi-natural grassland), Woodlands (including also hedgerows, shrub plantations, lines of trees and gardens), Bare Areas, Orchards, Cropland, and Urban Areas (including different types of sealed areas such as roads and cities), see below and Figure 1. Although, the EUNIS reference offers a detailed classification of each land-cover that best defines ecological habitats, we harmonized and reclassified the land cover categories in accordance with the habitat requirements of flower-visiting insects. Therefore, woodlands and hedgerows were combined into the same land-use cover class, under the assumption that they both positively benefit flower-visiting insects, by providing potential additional nectar, pollen or nesting resources (Marshall and Moonen, 2002; Marini et al., 2012a; Alison et al., 2022). In contrast, sealed areas (urban areas of different intensity gradient) were grouped within the same land use type, as they may be an impediment to the survival of flower-visiting insects.

As a measure of compositional landscape heterogeneity, we measured the proportion of cropland, orchards, urban areas and less-intensively managed habitats (aggregation of wetland, woodlands and grasslands habitat types; Supplementary Table S2). Given the resolution of the habitat classification in our study, it was not possible to distinguish between highly managed grasslands (including pastures and silage fields) and semi-natural meadows, and between commercial forestry and woodlands, thus these land-uses were aggregated into less-intensively managed habitats. In addition of the proportion of cropland, orchards, urban areas and less-intensively managed habitats, we calculated a measure of landscape diversity (Shannon diversity index, SHDI) using all nine final land-cover categories (see Supplementary Table S2). Landscape diversity is generally perceived as a critical aspect of landscape heterogeneity, as many arthropods may be associated with a single land use category (e.g., pollinators respond positively to semi-natural habitats).

As a measure of configurational landscape heterogeneity, we used the number of patches (NP) for orchards and cropland, as a proxy for the fragmentation of those habitats (Supplementary Table S2). In addition, we calculated the Interspersion and Juxtaposition Index (IJI), which describes how the different land use types are mixed together in the landscape; and habitat isolation (using the coefficient of variation of Euclidean Nearest-Neighbor distance – ENN), which calculates the distance between near patches belonging to the same land use type – calculated separately for cropland, orchard and less-intensively managed patches. We did not include edge density in our analyses, despite the established use of this measure for the assessment

of configuration heterogeneity of the landscape, as it was strongly correlated with the proportion of less-intensively managed habitat. Configurational and compositional landscape metrics were calculated with the R package "landscapemetrics" (Hesselbarth et al., 2019).

Weather and climate parameters

Temperature was measured in the field during each sampling, at 1 m above the ground level in the shade, using a thermometer (Supplementary Table S2). For each site, long-term climate parameters (30 years averages from 1970 to 2000, spatial resolution approximately 1 km^2), related to multi-annual temperature and precipitation variables (such as the annual mean temperature and precipitation, or the precipitation seasonality which expresses the variation in monthly precipitation over the year), were extracted from the WorldClim database (v2.1; Fick and Hijmans, 2017; Supplementary Table S2).

Statistical analyses

The impacts of landscape structure, weather and climatic conditions on the abundance of the different insect groups were assessed using generalized linear mixed effects models (GLMM) with a Poisson distribution and a logit link, using country as a random factor (Supplementary Table S3). Because of the disparity in the insect surveys performed between countries (Supplementary Table S1), we pooled insect count data per site and used the number of visits to each site as an offset in the GLMMs.

Due to the co-occurrence of other experiments at the moment of sampling, notably the presence in each site of three honey bee hives, three *Bombus terrestris* colonies and three *Osmia bicornis* trap nests (Hodge et al., 2022), we did not directly compare the different groups of insects in an overall model. Instead, we used separate models for each of our response variables: total number of insects (excluding *A. mellifera*), *A. mellifera*, *Bombus* spp., solitary bees, hover flies, butterflies, and for the two types of crops (oilseed rape and apple). We removed *A. mellifera* counts from the "total Insects" group because of their high abundance in the samples (72%; Figure 2), boosted by three sentinel honey bee colonies nearby and the pervasive presence of beekeeping in many of the landscapes. The remaining 28% of the Total Insects record was split more-or-less evenly between hover flies, butterflies, bumble bees and solitary bees, including possible contributions by the two other experimentally placed sentinel bee species (*B. terrestris* and *O. bicornis*). We tested for multicollinearity between our initial set of explanatory variables, by using the variance inflation factor (VIF). A total of 18 variables were included as explanatory variables in the initial models (Supplementary Table S3). Because of the potentially strong impact of climate on the phenology of crop plants (Hegland et al., 2009), we considered potential interactions between annual temperature and the number of mass-flowering crops and orchards in the area adjacent to the sites, and landscape diversity, as well as an interaction between annual precipitation and mass-flowering crops and orchards (Supplementary Table S2).

In case of overdispersion, we added an observer term to the random structure (Harrison, 2014). To avoid overfitting, we limited the maximum number of terms to 6 (*ca.* 10% of data points). If the model failed to converge and the variance of "country" as random factor was negligible, we removed" country" as a random factor from the model. For each analysis, the final selection of the best model was conducted following a multimodel inference approach (Burnham and Anderson, 2002); dredge function of the MuMIn R package (Multimodel inference approach). Finally, we ran model diagnostics to test if all statistical requirements were met for each model and checked for spatial autocorrelation ("check_model" and "testSpatialAutocorrelation" functions in "performance" and "DHARMa" packages (Lüdecke et al., 2021).

To test for differences in total insect abundances between the two crop types (oilseed rape and apple), we used generalized linear mixed effects models (GLMM) with a Poisson distribution and a logit link, for each insect taxon, where crop was the independent variable and the country as a random intercept.

All analyses were performed using R software Version 1.3.1093 (R Core Team, 2020). We used the libraries "ggplot2" and "effects" for the construction of the graphs (Wickham, 2016; Fox and Weisberg, 2019), and the libraries "vegan" and "RcolorBrewer" for building the PCA plot (Oksanen et al., 2022). The library "hclust" was used to check for collinearity among variables. The models were built under the library "lme4" (Bates et al., 2015).

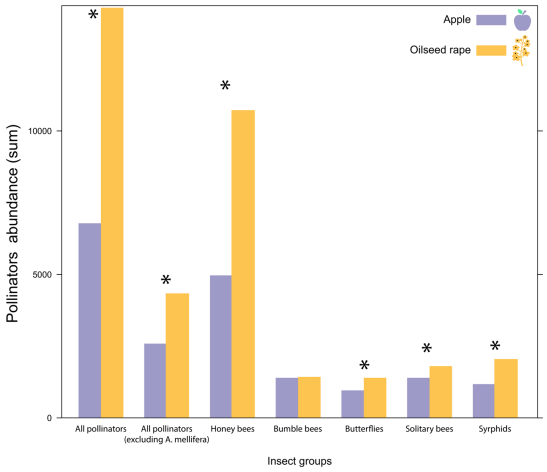

FIGURE 2
The total number of flower-visiting insects recorded across all sites (All pollinators), excluding honey bees (All pollinators (excluding *A. mellifera*)) and each insect group (*Apis mellifera*, Bumble bees, Butterflies, Solitary bees and Hover flies), according to crop type (purple=apple orchard sites, orange=oilseed rape sites). Asterisks show significant (p <0.05) differences between crop types.

Results

A total of 19,632 insects were recorded in our study across the two crops (6,122 in apple sites and 13,510 in oilseed rape sites; Figure 2). Honey bees (*A. mellifera*) were by far most abundant, accounting for the 72.44% of all individuals recorded (4,270 in the apple sites and 9,951 in the oilseed rape sites; Figure 2). Hover flies were the second most abundant group in our record, accounting for 8.68% of the individuals (428 in apple sites and 1,276 in oilseed rape sites; Figure 2), solitary bees contributed to 8.30% of the individuals (612 in apple sites

and 1,017 in oilseed rape sites), while bumble bees and butterflies, respectively, comprised 6.57 and 4.02% of the samples (631 bumble bees in apple sites and 658 in oilseed rape sites; 181 butterflies in apple sites and 608 in oilseed rape sites; Figure 2). Total insect abundances and abundances of all taxonomic groups were significantly higher in oilseed rape sites than in the apple sites (all $p < 0.001$; Figure 2), except for bumble bees ($p = 0.6$; Figure 2). The extent to which the survey records were augmented by the sentinel honey bee colonies and *O. bicornis* trap nests at the center of the sites is not known. Wild and feral colonies of the bee species are also very common throughout the biogeographic range covered by the sites (hence their choice as sentinel species) and indistinguishable from sentinel-derived bees. However, since the number of bees in these sentinel colonies was rigorously standardized across sites prior to placement (Hodge et al., 2022), the numerical basis for their presence in the survey records is essentially identical for the sites. The actual presence of sentinel bees in the survey records is therefore primarily subjected to the same landscape and climate factors as their wild conspecifics, for the purpose of statistical analysis. Of course, any beekeeping around each of the 128 sites may have biased the number of non-sentinel honey bees at the sites, as function of the number of colonies within range, augmented by social recruitment to the site's focal crop in competition with other floral resources: factors that are neither known nor can be modelled reliably. For this reason honey bees are analyzed separately throughout the study.

Landscape composition

At the field scale, the responses of insects to the number of mass-flowering crops adjacent to the site were taxon-specific (Figure 3). Bumble bee abundance was positively correlated with the extent of mass-flowering crops and orchards surrounding the apple sites ($p < 0.001$; Tables 1, 2; Figure 3). However, both honey bees and hover flies abundance was negatively correlated with mass-flowering crops and orchards surrounding the oilseed rape sites (respectively $p = 0.01$ and $p < 0.001$; Tables 1, 2; Figure 3). Butterfly abundance in the apple sites showed a moderate decline with the increase of mass-flowering crops and orchards in the surrounding landscape ($p = 0.07$; Tables 1, 2).

A high proportion of orchards in the landscape surrounding the apple sites was negatively correlated with total insect abundance (excluding honey bees; $p < 0.001$; Tables 1, 2; Figure 3), bumble bees ($p < 0.001$; Tables 1, 2) and solitary bees ($p < 0.001$; Tables 1, 2). Honey bees were slightly more abundant in sites surrounded by a higher proportion of orchards, although this result was not statistically significant ($p = 0.08$; Tables 1, 2). Beekeeping is popular near fruit orchards, both for hobby and for pollination services, and could well have generated this marginal effect. The proportion of urban area negatively influenced hover flies in the apple sites ($p = 0.02$; Tables 1, 2). As expected, a positive relationship between the proportion of less-intensively managed areas and number of flower-visiting insects was found, though the effect was only observed in oilseed rape sites. The total number of insects (excluding honey bees) increased with the increasing proportion of less-intensively managed areas ($p = 0.002$; Tables 1, 2; Figure 3). Similar patterns were observed between the proportion of less-intensively managed areas and the abundance of bumble bees and butterflies ($p = 0.004$ and $p < 0.001$ respectively; Tables 1, 2).

High landscape diversity in the surrounding landscape increased the abundance of hover flies ($p = 0.02$; Tables 1, 2). Solitary bees were also positively influenced by landscape diversity, although this was only found in oilseed rape sites ($p = 0.09$; Tables 1, 2).

Landscape configuration

None of the selected explanatory variables describing landscape configuration explained insect abundance, except for habitat isolation (ENN). Contrary to our hypothesis, isolation of less-intensively managed habitat patches was positively correlated with abundance of bumble bees in apple sites ($p < 0.001$; Tables 1, 2). On the other hand, isolation of orchard patches was negatively correlated with the abundance of honey bees in apple sites ($p = 0.01$ Tables 1, 2). Honey bees in oilseed rape sites showed a positive correlation with the isolation of cropland patches ($p < 0.001$; Tables 1, 2). A positive relationship between the isolation of cropland and butterflies was also found in apple sites ($p < 0.001$; Tables 1, 2; Figure 3). However, the opposite was found for butterflies in oilseed rape sites, where abundance declined with increasing isolation of cropland patches ($p = 0.04$; Tables 1, 2; Figure 3).

Weather and climate parameters

Daily temperature, annual temperature, annual precipitation, and precipitation seasonality played a role in shaping insect abundance. Although the positive effect of daily temperature only emerged in oilseed rape sites, annual temperature, annual precipitation and the precipitation seasonality affected insect abundance in both crop types, albeit the effect was positive or negative depending on the insect group and crop type (Tables 1, 2; Figure 3).

The total number of insects in oilseed rape sites was positively correlated with both daily and annual temperatures (both $p < 0.001$; Tables 1, 2), but was negatively correlated with annual precipitation ($p = 0.01$; Tables 1, 2; Figure 3). In contrast, annual precipitation had a positive relationship with insect abundance in apple sites, albeit non-significantly ($p = 0.08$; Tables 1, 2; Figure 3).

By analyzing the responses of different insect groups to weather and climatic conditions, we found that daily temperature was positively correlated with the number of honey bees, solitary bees and butterflies (respectively $p = 0.003$, $p = 0.004$, $p = 0.02$; Tables 1, 2), while the responses of insects to annual temperature were mostly taxon-specific. The abundance of solitary bees in apple and oilseed rape sites, and hover flies and butterflies in oilseed rape sites were positively correlated with annual temperature ($p < 0.001$, $p < 0.001$, $p < 0.001$ and $p = 0.02$; Tables 1, 2), whereas the abundance of bumble bees was negatively correlated with annual temperature in both crop types ($p < 0.001$; Tables 1, 2; Figure 3). Moreover, a positive interaction of annual temperature and landscape diversity on the abundance of solitary bees in oilseed rape sites was found ($p = 0.002$; Table 1), i.e., that positive effects of landscape diversity were even stronger under warmer climates and vice versa.

The effect of annual precipitation on insect abundance varied across crop type and insect group (Tables 1, 2). The abundance of honey bees and solitary bees in apple sites were positively correlated with increasing annual precipitation ($p < 0.001$; Tables 1, 2). On the

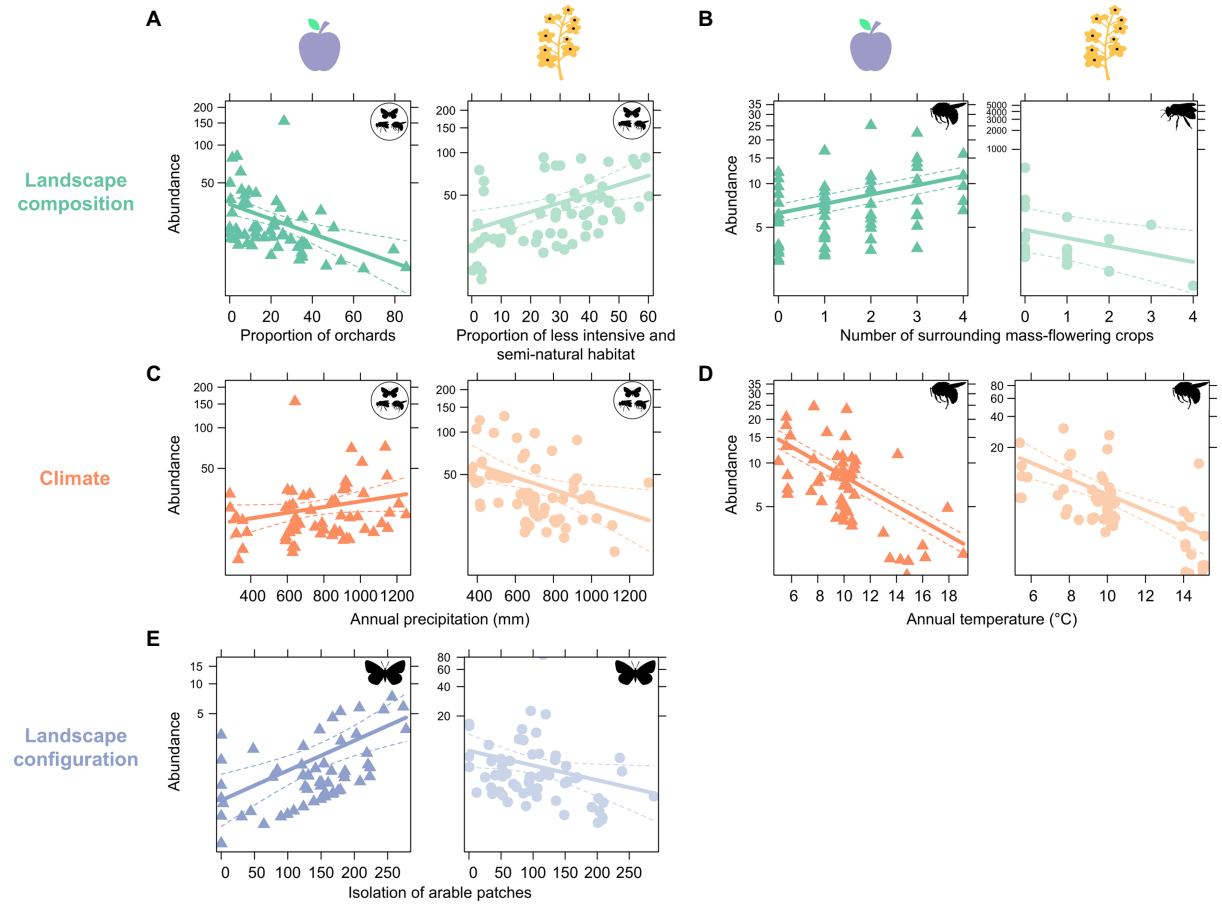

FIGURE 3
GLMM representing relationships found in both apple and oilseed rape sites between **(A)** the proportion of orchards or less intensive and semi-natural habitat, and abundance of all pollinators, **(B)** number of surrounding mass-flowering crops and abundance of bumble bees and honey bees, **(C)** annual precipitation and abundance of all pollinators, **(D)** annual temperature and abundance of bumble bees, and **(E)** isolation of arable patches and abundance of butterflies.

other hand, solitary bee and butterfly abundances in oilseed rape sites responded negatively to annual precipitation (respectively $p = 0.002$ and $p < 0.001$; Tables 1, 2). Finally, precipitation seasonality was negatively correlated with the abundance of bumble bees in both apple and oilseed rape sites ($p < 0.001$ and $p = 0.02$; Tables 1, 2), although it was positively associated with the abundance of honey bees and butterflies in apple sites ($p < 0.001$; Tables 1, 2).

Discussion

Impact of landscape composition and configuration on insect abundance

Our results demonstrate that both the composition and configuration of the landscape, such as landscape diversity, the presence of less-intensively managed land, but also the complexity and connectivity of the landscape, were major drivers of flower-visiting insect abundances in agriculturally dominated landscapes. As expected, a higher proportion of less-intensively managed habitats was found to support higher numbers of flower-visiting insects. However, we found these effects to be highly context dependent, in regard to both taxon and crop-type. Because we used a non-destructive sampling method (Hodge et al., 2022), our analyses only considered insect abundance in very broad taxonomic categories. While previous studies found hover flies responses to surrounding habitat structure can vary within these broad groups, probably due to scale-dependent ecological requirements of species, particularly within the species rich groups of hover flies and solitary bees (Stanley et al., 2013), we were not able to investigate species-level patterns, except for honey bees.

In addition, differences related to crop-type emerged in relation to insect abundance, with more individuals recorded in oilseed rape crops, compared to apple orchards – with the exception of bumble bees. Oilseed rape crops are known to be highly attractive to bees, and the pollen diet of some species (e.g., honey bees and red mason bees) consists predominantly of mass flowering crops when available (Holzschuh et al., 2013; Stanley and Stout, 2013). However, bumble bees also include other plant species in their diet, even when mass-flowering crops are abundant (Kovács-Hostyánszki et al., 2013). Similarly, apples are pollinator-dependent, attracting a wide diversity of insects (Russo et al., 2015; Burns and Staney, 2022; Gamonal Gomez et al., 2023). However, their nectar production is lower compared to oilseed rape flowers, and some groups of insects (i.e., honey bees) have been shown to be attracted away from apple orchards, when oilseed

TABLE 1 The results from the mixed effect models (GLMM models testing the relationship between landscape and climatic variables and insect abundance in each crop – apple (APP) and oilseed rape (OSR).

Variable	Crop	Insect group	Estimate	Standard error	*Z*-Value	*p*-value |>Z|
Orchard area (%)	APP	All groups	−0.259	0.071	−3.653	<0.001***
		A. mellifera	0.191	0.109	1.748	0.08
		Bumble bees	−0.244	0.003	−81.14	<0.001***
		Solitary bees	−0.379	0.002	−177.9	<0.001***
Urban areas (%)	APP	Hover flies	−0.257	0.112	−2.288	0.02*
Less-intensively managed and semi-natural habitats (%)	OSR	All groups	0.2643	0.0873	3.029	0.002**
		Bumble bees	0.3050	0.1078	2.828	0.005**
		Butterflies	0.5863	0.1449	4.048	<0.001***
Landscape diversity (SHDI)	APP	Hover flies	0.2646	0.1139	2.323	0.02*
	OSR	Solitary bees	0.2531	0.1504	1.683	0.09
Isolation of orchard patches	APP	*A. mellifera*	−0.2430	0.0966	−2.516	0.01*
Isolation of cropland patches	APP	Butterflies	0.5331	0.1412	3.776	<0.001***
	OSR	*A. mellifera*	0.3284	0.0909	3.614	<0.001***
		Butterflies	−0.2305	0.1126	−2.047	0.04*
Isolation of less-intensively managed and semi-natural patches	APP	Bumble bees	0.2756	0.003	91.75	<0.001***
Adjacent mass-flowering crops and orchards	APP	Bumble bees	0.1933	0.003	64.35	<0.001***
		Butterflies	−0.2545	0.1410	−1.804	0.07
	OSR	*A. mellifera*	−0.2341	0.094	−2.491	0.01*
		Hover flies	−0.2505	0.0026	−96.536	<0.001***
Daily temperature (°C)	OSR	All groups	0.2197	0.0784	2.802	0.005**
		A. mellifera	0.3516	0.1185	2.966	0.003 **
		Solitary bees	0.4054	0.1417	2.861	0.004**
		Butterflies	0.2678	0.1164	2.300	0.02*
Annual temperature (°C)	APP	Bumble bees	−0.3429	0.003	−114.10	<0.001***
		Solitary bees	0.3331	0.0021	156.6	<0.001***
	OSR	All groups	0.2734	0.0808	3.384	<0.001***
		Bumble bees	−0.5060	0.1109	−4.562	<0.001***
		Solitary bees	0.6169	0.1480	4.168	<0.001***
		Hover flies	0.7222	0.0026	278.230	<0.001***
		Butterflies	0.2507	0.1082	2.316	0.02*

(Continued)

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TABLE 1 (Continued)

Variable	Crop	Insect group	Estimate	Standard error	Z-Value	*p*-value |>Z|
Annual precipitation	APP	All groups	0.12057	0.06986	1.726	0.08
		A. mellifera	0.8155	0.1207	6.754	<0.001***
		Solitary bees	0.3548	0.0021	166.7	<0.001***
	OSR	All groups	−0.2046	0.0833	−2.456	0.01*
		Solitary bees	−0.4871	0.1563	−3.116	0.002**
		Butterflies	−0.5528	0.1257	−4.396	<0.001 ***
Precipitation seasonality	APP	*A. mellifera*	0.5088	0.124	4.104	<0.001***
		Bumble bees	−0.2362	0.003	−78.64	<0.001***
		Butterflies	0.8481	0.1454	5.832	<0.001***
	OSR	Bumble bees	−0.2387	0.1047	−2.279	0.02*
Annual temperature (°C) * landscape diversity (SHDI)	OSR	Solitary bees	0.4247	0.1345	3.157	0.002**

The significant codes are: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1.

rape crops were co-occurring in the vicinity (Quinet et al., 2016; Carruthers et al., 2017; Osterman et al., 2021). On the other hand, oilseed rape crops can attract some insects species from the surrounding landscape, given their abundant floral resources and high flower density (Hoyle et al., 2007; Rollin et al., 2013; Vrdoljak et al., 2016; Woodcock et al., 2016; Magrach et al., 2017; Van Reeth et al., 2018). Another explanation for the different number of insects recorded in the two crops could be related to management. Previous studies showed that apple orchards were associated with high level of pesticides (with fungicide contributing to over the 98% of the total pesticides residues in pollen collected by bees; Šlachta et al., 2020). Although generally not toxic to bees (Rondeau and Raine, 2022), some fungicides can negatively impact the behavior and fitness of honey bees [European Commission Implementing Regulation EU, 2018, (2018/1865 of 28 November 2018); Liao et al., 2019]. Moreover, some pollinator groups might have been attracted to the target crops by other species of plant growing within and along the margins of the fields, despite the pollen and nectar resources offered by the target crops during sampling. The composition and abundance of these non-crop plants may also differ between sites, as a result of fundamental differences in cultivation between our two focal crops; oilseed rape being an annual field crop and apples a woody orchard crop. In fact, previous studies show that some insect groups are more abundant along the margins of the cultivation rather than in the center of the crop itself (e.g., butterflies and hover flies; Bottero et al., 2021). Similarly, the larvae of butterflies and some hover fly species feed on plant tissues (particularly the larvae of *Pieris* butterfly species that favour Brassicacea), whilst other hover fly larvae are saprophagous or predatory (Speight et al., 2010). Thus butterfly and hover fly abundance in crops may be determined by factors other than the availability of floral resources.

Less-intensively managed habitats

Our results show that the abundance of different groups of pollinators increased with the proportion of less-intensively managed habitats and with habitat diversity, confirming that heterogeneous habitats can support beneficial insects in agricultural landscapes, likely by offering a greater diversity of food and resources (Rundlöf et al., 2008b; Marini et al., 2012b; Nayak et al., 2015; Raderschall et al., 2021; Martínez-Núñez et al., 2022). Different pollinator taxa have different ecological and physiological requirements, and even individuals of the same species might benefit from diets based on a diversity of plant species (Cane and Sipes, 2007; Eckhardt et al., 2014; Bertrand et al., 2019), and during different stages of their life cycle (Erhardt, 1985; Erhardt and Mevi-Schütz, 2009; Meyer et al., 2009). Therefore, less-intensively managed and semi-natural habitats might fill nutritional gaps at specific times of the year, such as at the end of the abundant, yet temporally constrained flowering period of mass-flowering crops (Timberlake et al., 2019; Bottero et al., 2021). The less-intensively managed habitats in the landscape surrounding the fields, may also play an important role in promoting pollinators. For instance, Maurer et al. (2022) reported that different types of semi-natural features (meadows, floral strips in the cultivated crops, hedgerows) have a different impact on the richness and the abundance of different flower-visiting insects, depending on the insects' needs and the time of year they are active. Similarly, the presence of floral strips in cultivated crops promoted bumble bees across seasons (Bommarco et al., 2021), while hover flies and butterflies were shown to favour the

TABLE 2 Summary of the positive ("+") and negative ("−") effects of landscape and climate variables on the abundance of the six groups of pollinators.

		Orchards (%)	Urban (%)	Less intensive & SNH (%)	SHDI	MFC	ENN Orchards	ENN Less intensive & SNH	ENN Arable	Daily T °C	Annual T°C	Annual precipitation	Precipitation seasonality
	All taxa	−										+	
	Honey bees	+					−					+	+
	Bumble bees	−				+		+			−		−
	Solitary bee	−									+	+	
	Syrphids		−		+								
	Butterflies					−			+				
	All taxa			+						+	+	−	
	Honey bees					−			+	+			
	Bumble bees			+							−		−
	Solitary bee				+					+	+	−	
	Syrphids					−					+		
	Butterflies			+					−	+	+	−	

The first column shows the different pollinators groups (All pollinators, excluding honey bees; Honey bees; Bumble bees; Solitary bees; Hover flies; and Butterflies). The upper part of the table shows the interactions found in the apple sites, while the bottom part the ones in the oilseed rape crops. Orchards (%) = proportion of orchards; Urban (%) = proportion of urban areas; Less intensive & SNH (%) = proportion of less-intensively managed and semi-natural areas; SHDI = landscape diversity; MFC = number of mass-flowering crops and orchards; ENN Orchards = isolation of orchard patches; ENN Less intensive & SNH = isolation of less-intensively managed and semi-natural; ENN Arable = isolation of cropland patches; Daily T°C = daily temperature (temperature recorded at the moment of the sampling); Annual T°C = annual temperature; Annual Precipitation; and Precipitation Seasonality. Only significant relationships (value of p <0.05; in black) and those representing a trend (0.05 < value of p <0.1; in grey) are shown in the table.

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crops' flowering margins (Bottero et al., 2021). Similarly, the diversity and growth stages of the plants present in the floral strips can support pollinator communities in cultivated crops, ultimately promoting pollination services in agricultural landscapes (Albrecht et al., 2020). In addition, Raderschall et al. (2021) showed that higher crop diversity (and semi-natural habitats) may support bumble bee density in agricultural landscape.

Highly managed crop and urban habitats

Urban areas and highly managed crops such as orchards decreased the abundance of different groups of flower-visiting insects. The negative impact of anthropogenic habitats on insect communities is likely to be related to habitat disturbance and/or management intensity (McKinney, 2008; Vanbergen and Initiative, 2013). An increase in the proportion of both cropland and orchards adds to the overall intensification burden throughout the landscape, through agrochemical inputs and reduced nesting opportunities, not only in the actual cropland fields and orchards, but also outside of these, due to crop rotation and the persistence and dispersal of agrochemicals through soil and groundwater to areas beyond their initial application.

Apple orchards are usually subjected to intensive application of plant protection products to maximize crop value (Damos et al., 2015). As a result, they may directly lead to declines in pollinator abundance, or precipitate their departure from target crop sites to the surrounding areas – the latter may be particularly true for honey bees and bumble bees, which are known for their long foraging distances (Beekman and Ratnieks, 2000; Knight et al., 2005; Carvell et al., 2012). Many of the adjacent patches in our apple sites were mass-flowering crops, including oilseed rape and other orchards, which may have caused a dilution of flower-visiting pollinators for high floral rewards in the vicinity, especially when these mass-flowering crops bloom at the same time (Stanley and Stout, 2013; Riedinger et al., 2015; Holzschuh et al., 2016; Grab et al., 2017; Bänsch et al., 2021; Osterman et al., 2021). However, in contrast to our results, Osterman et al. (2021) did not observe a shift in bumble bee abundance when apple sites were surrounded by oilseed rape, but found more solitary bees in apple sites. These differences could be explained by the different types of crops surrounding our sites, though we do not have the necessary information regarding the cultivar nor the intensity of inputs used in the surrounding crops.

Mass-flowering crops and orchards adjacent to the sites

Competition for better floral rewards between different crop types may also explain the negative relationships found between the presence of mass-flowering crops and orchards in the adjacent patches and the abundance of both honey bees and hover flies at the focal site. The proximity of competing mass-flowering crops and orchards in the vicinity of a site could lead to the dilution of pollinators (Robinson et al., 2022). Alternatively, low plant richness in mass-flowering monocultures may explain the low abundance of pollinators found in mass-flowering dominated landscapes. Indeed, butterflies, bees and hover flies require different plants to properly complete their life cycle (Erhardt, 1985; Erhardt and Mevi-Schütz, 2009), and thus can strongly benefit from the presence of semi-natural habitats that offer a greater diversity of floral and nesting resources (Steffan-Dewenter et al., 2002; Rundlöf et al., 2008a, 2008b; Nayak et al., 2015). On the other hand, bumble bees seemed to profit from the presence of mass-flowering crops in the vicinity, likely due to their longer foraging ranges combined with the highly attractive nature of these crops, which corroborates the findings of previous studies (Holzschuh et al., 2013; Stanley and Stout, 2013).

Habitat configuration

Although the spatial arrangement of crop fields and other habitats has been shown to promote insects in agroecosystems (Martin et al., 2019), habitat isolation was the only configurational landscape metric that influenced the abundance of flower-visiting insects. The effects of habitat isolation on pollinators in our study sites appeared to be highly context dependent. In oilseed rape fields, more honey bees were found when the nearest croplands were further away, as opposed to butterflies which were more abundant when croplands were close by. We found the opposite trend in apple sites, where more honey bees were found when the nearest orchard was close, and more butterflies when the nearest cropland was further away. Contrary to our expectations (Fahrig, 2013; Perović et al., 2015), we found more bumble bees in apple sites when the less-intensively managed habitat patches were further away.

The opposing trends observed for honey bees and butterflies may be due to the differences in their foraging behavior and ranges, the composition of the landscape surrounding the sites, and the crop's attractiveness in regards to flower rewards. Honey bees can forage over large distances, and are known to be central-place foragers that recruit individuals to more rewarding patches (Seeley, 1995; Dyer, 2002). The placement of honey bee hives is usually managed by beekeepers to optimize both access and proximity to a diversity of high-yielding floral resources, especially in highly managed crops such as apple orchards. Thus, the high number of honey bees found in our apple sites may be explained by the presence of numerous honey bee hives managed by beekeepers, especially since many other orchards were found in the vicinity, and may have influenced the abundance of other insect groups. Butterflies on the other hand are part of a much more diverse group that is influenced by a number of factors mostly related to foraging behavior, mating opportunities and oviposition resources at the patch and landscape level (Dover and Settele, 2009). Butterflies generally benefited from the isolation of the cropland in apple sites, suggesting that cropland offered poor rewards to butterflies, as opposed to less-intensively managed habitats. Similarly, we did not assess overall crop diversity and thus lack the information about specific crops in the vicinity of oilseed rape and apple sites. Although honey bees seem to favour oilseed rape sites that are further away from croplands, we can only presume that our oilseed rape sites were in landscape dominated by less attractive crops for honey bees, e.g., cereal fields. In contrast, oilseed rape fields may need to be less-isolated to attract butterflies, suggesting that the temporary boost of early floral resource pulse provided by mass-flowering crops are not sufficient to support butterflies in more intensive landscapes.

Impact of weather and climate variables on flower-visiting insects

In the context of general concern about the impact that heat waves, droughts, and changes in temporal dynamics (including precipitation seasonality) can have on flower-visiting insects, our study collected important information about the responses of different

groups of pollinators at a European level, albeit the relationships were highly context dependent.

As expected, the abundance of several taxa of flower-visiting insects decreased with increasing annual precipitation and precipitation seasonality. On the other hand, only bumble bees responded negatively to annual temperature.

Daily and annual temperature

Our results showed that both daily and annual temperatures positively influenced the abundance of most of the studied insect groups, as could be expected given that most insects are ectotherms and more active during warmer day periods. Fewer bumble bees were found when annual temperatures were higher though; as temperate species, they are generally more suited to northern latitudes in Europe and lower temperatures (Rasmont and Iserbyt, 2010-2014). Changes in temperature, especially when rising above specific levels and during the developmental stages of the species, can negatively affect flower-visiting insects by impacting foraging activities, fertility, morphology (wing and tongue length and body size), colony productivity and development time, and survival (Tepedino and Parker, 1986; Weidenmüller et al., 2002; Radmacher and Strohm, 2010; O'Neill et al., 2011; Holland and Bourke, 2015; Miller-Struttmann et al., 2015; Gerard et al., 2018a,b; Martinet et al., 2021). Moreover, higher temperatures are linked to earlier emergence of flower-visiting insects, which can have repercussions on plant-pollinators interactions (Hegland et al., 2009). Furthermore, higher temperatures are often related to drought, extreme weather phenomena, and to changes in seasonality with possible adverse consequences on plant communities and the resulting cascading effects on food resources (Lawson and Rands, 2019; Höfer et al., 2021). In the face of climate change, a better understanding of the relationships between pollinator abundance and temperature is crucial, given the risk that higher temperatures may result in more homogeneous pollinator communities, likely associated with higher dispersion rates, with a consequent decrease of the species pool (Ganuza et al., 2022).

Annual precipitation and precipitation seasonality

Precipitation can directly affect insects, e.g., their flight mechanism and sensory signals, but also indirectly affect their food resources (Lawson and Rands, 2019). It is also responsible for nectar dilution and pollen damage in some plant species, but the corolla shape and the position of nectaries, nectar spurs and anthers can facilitate the protection of pollen and nectar from rain or drought (Lawson and Rands, 2019). Although both apple and oilseed rape flowers are characterized by an open corolla, we found contrasting responses of insects to annual precipitation in both crop types, suggesting that the differences in landscape composition and configuration, rather than direct impacts of precipitation on food resources, are more important in shaping pollinator communities. Apple sites were surrounded by a higher proportion of both diverse and less-intensively managed habitats, and associated with a lower isolation between semi-natural patches. Such landscape structures might facilitate access to different flower resources, e.g., when pollen was damaged, or when the nectar of the mass-flowering crops was diluted. The contrasting effects of precipitation on different taxonomic groups might be explained by morphological differences in body size and wing structure of the different taxa (Lawson and Rands, 2019), or indirectly mediated through forage resources. Flowers pollinated by butterflies usually have more dilute nectar, while bee-pollinated ones show higher sugar concentrations (Pyke and Waser, 1981; Baker and Baker, 1983; Lawson and Rands, 2019), suggesting that the different responses to the precipitation seasonality on butterflies might be related to taxa preferences for nectar resources. However, changes in floral communities related to different climate event may also be responsible for a shift in flower-visiting insect community.

Conclusion

The adoption of a standardized insect sampling protocol across 128 structurally different sites characterized by different climatic and weather conditions, and the decision to focus on multiple groups of insects, allowed us to properly account for context dependency when disentangling the effects of landscape heterogeneity and climate on pollinator communities at a European level. Despite being constrained to a single flowering season, and due to logistical constraints in conducting the study at the European scale, our study offers important insights on the combined effects that climate and landscape structure have on flower-visiting insect communities. Overall, our results indicate that heterogeneous landscapes, characterized by diverse and less-intensively managed habitats, with low levels of patch isolation, can have a positive impact on the communities of flower-visiting insects, even when the landscape is dominated by intensive agricultural land use. Conversely, structurally simple landscapes will likely be associated with a loss of flower-visiting insects (Senapathi et al., 2017).

Moreover, our study offers new evidence about the importance of both weather and climate parameters on shaping flower-visiting insect communities across Europe. This is particularly relevant in the context of climate change, which will have direct or indirect repercussions on insects and plants communities in the next few decades. Furthermore, due to the strong impact of climate on pollinators shown in this study, we recommend including weather and climate parameters in studies investigating pollinator communities, notably in regard to different biogeographic ranges and fluctuating weather patterns. Additionally, future studies that aim to generate a better understanding of the impact of landscape configuration on insect population dynamics should also focus on the natural structural elements present in the landscape, which have previously been shown to play a major role in influencing insects, especially in an intensive agricultural context (Dover and Sparks, 2000; Marshall and Moonen, 2002; Cranmer et al., 2012).

Broadly, our take-home message is that despite some taxonomic variation, landscape simplification negatively affects some important pollinating insect taxa. In addition, our results show a negative impact of high temperature on bumble bee abundance. Taken with other studies, which have reported similar findings for other taxonomic groups, there may be widespread implications of landscape simplification and climate change on multifunctionality and the delivery of multiple ecosystem services (Mooney et al., 2009; Dainese et al., 2017; Martin et al., 2019; Le Provost et al., 2021). Together, these findings support the implementation of land-use plans and policies to preserve heterogeneity and semi-natural features at a field and landscape level in Europe, to sustain the communities of beneficial

insects in agricultural landscapes. For example, increasing the amount of less-intensively managed and semi-natural habitats in landscapes characterized by oilseed rape cultivation, could promote pollinator abundance in oilseed rape crops. On the other hand, in habitats dominated by apple orchards, decreasing the total orchard area, and/or increasing crop diversity and the number of types of mass-flowering crop, could have a positive impact on pollinating insect communities. As well as helping to reverse decline and restore pollinator populations, which are key global and European biodiversity targets, this could have knock-on benefits for other taxa and the restoration of biodiversity more broadly in agriculturally-dominated landscapes across Europe.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MA, MB, PR, CD, SH, KI, A-MK, MM, VM-L, SP, MR, OS, and JSt: study design. MB, PR, A-MK, MM, OS, CC, JM, MA, MR, SP, and JSt: funding and resources. MA, EA, IB, MB, CC, PR, JM, GP, SH, AK, VM-L, SP, PM, RR, MR, OS, and JSt: protocol development. MA, IB, EC, CC, PR, GP, CD, DDU, SH, A-MK, AK, VM-L, PM, HP-P, MR, JSc, OS, DS, GT, and ET: data collection. IB, CD, OS, and JSt: analysis and interpretation of results. IB, CD, and JSt: draft manuscript preparation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1128228/full#supplementary-material

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Deformed wing virus prevalence in solitary bees put to the test: an experimental transmission study

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Virus spillover from managed to unmanaged bees and vice versa may be one mechanism driving colony losses of the former and declines of the latter. There is clear evidence that the ubiquitous Deformed wing virus (DWV) is a major driver of honey bee (*Apis mellifera*) colony mortality. Although DWV has been detected in the solitary bee *Osmia bicornis*, data on DWV infectivity and virulence from solitary bees are scarce. Here, we used microinjection to investigate whether DWV genotype A (DWV-A) obtained from honey bees can replicate in *O. bicornis*. DWV-A titers and intermediate strand analyses suggest that DWV-A does not replicate in *O. bicornis* and thus is probably not infectious for this solitary bee species. Interestingly, the data demonstrate that DWV-A recovered from *O. bicornis* 16 days post-microinjection remains infectious for *A. mellifera*. Therefore, despite the lack of apparent virulence of DWV in this solitary bee species, *O. bicornis* has the potential to act as a virus spillover host and may contribute to increased colony losses of managed honey bees and declines in populations of other managed or unmanaged bee species.

KEYWORDS

***Apis mellifera*, host shift, *Osmia bicornis*, pathogen spillover, pollinators, wild bee**

1 Introduction

Emerging infectious diseases (EIDs) are of considerable concern to both human and animal health and may pose a risk to ecosystem services such as insect pollination, ultimately leading to potentially detrimental consequences for economic gains and food security (Potts et al., 2010; Vanbergen and Initiative, the I.P, 2013; Potts, et al. 2016a; Potts et al., 2016b). The managed Western honey bee (*Apis mellifera*), the most economically valuable managed pollinator species, is well described to suffer from a multitude of such emerging diseases, pests, and parasites (Ellis and Munn, 2005; Rosenkranz et al., 2010; Goulson et al., 2015; Neumann et al., 2016). There is clear evidence that these pathogens are

key players of unsustainably high losses of managed colonies over the last decades (Neumann and Carreck, 2010). However, other bee populations, managed or unmanaged, are also of dire concern (Potts, et al. 2016a; Tehel et al., 2016). Not only because pathogen spillover from managed honey bees may contribute to reported declines of other populations, but also because these may act as pathogen reservoirs and thus potential hosts for spillback to managed honey bees (Proesmans et al., 2021). This may thereby perpetuate a vicious cycle of colony losses and pollinator population declines (Rhyan and Spraker, 2010; Fürst et al., 2014; Graystock et al., 2016; Potts et al., 2016b).

Pathogen transmission among bee populations can occur through multiple routes, but recently attention has increased regarding spread through the shared use of flowers (Durrer and Schmid-Hempel, 1994; Chen et al., 2006a; Chen et al., 2006b; Burnham et al., 2021; Proesmans et al., 2021). Unsurprisingly, a wide range of pathogens reported from managed honey bees has been detected in other bees' populations, underlining the commonality of pathogens jumping from one host species to another (host shift) (Fürst et al., 2014; Tehel et al., 2016; McMahon et al., 2018; Alger et al., 2019; Figueroa et al., 2019). This process can be unidirectional or bidirectional: from an initial host spilling over to a novel host, and/or from a novel host spilling back to the initial host, i.e. reverse spillover, or "spill-back" (Woolhouse, 2001). Although the susceptibility of a novel host can be driven by pathogens previously infecting other closely related phylogenetic hosts, another avenue for the development of novel EIDs may be through environmental pressures, such as widespread pesticide use and habitat loss and degradation, that serve as concurrent stressors and contribute to increased susceptibility to pathogens (Longdon et al., 2014; Retschnig et al., 2015; McMahon et al., 2018; Straub et al., 2019; Straub et al., 2022). However, empirical data on the relative importance and interplay of different drivers remain scarce and direct evidence on the type of spillover scenario is lacking (Rigaud et al., 2010; Evison et al., 2012; Straub et al., 2022). In all cases, whether or not a host species resists the infection depends on multiple interacting factors involved in the triad of "host–pathogen–environment" (Rhyan and Spraker, 2010). The emergence of such infectious diseases within bee communities has been suggested as an important driver contributing to wild bee decline (Potts et al., 2010; Manley et al., 2015; Graystock et al., 2016; Potts et al., 2016a; Tehel et al., 2016; Grozinger and Flenniken, 2019).

The worldwide-distributed Deformed wing virus (DWV) is among the most harmful pathogens associated with managed honey bees (Martin et al., 2012; Neumann et al., 2012). DWV is a positive-sense, single-stranded RNA virus (family *Iflaviridae*, genus *Iflavirus*) that causes notable clinical symptoms (deformed wings), as well as deleterious effects on foraging and life span in honey bees (de Miranda and Genersch, 2010; Möckel et al., 2011; Dainat et al., 2012; Benaets et al., 2017). It is considered to be among the major drivers of honey bee colony collapses, especially because of its efficient vectoring by the ubiquitous ectoparasitic mite *Varroa destructor*, which can generate disease epidemics within individual colonies (de Miranda and Genersch, 2010; Neumann et al., 2012; Wilfert et al., 2016; Yañez et al., 2020). This efficient vector activity is based on the injection of DWV particles directly into bee pupae or adult bees by the feeding mite, which is the most efficient method of horizontal transmission known so far (Chen et al., 2006b; Möckel et al., 2011; Yañez et al., 2020). Furthermore, *V. destructor* has been demonstrated to serve as a biological vector for DWV genotype B (DWV-B) based on viral titers (Di Prisco et al., 2016) as well as DWV intermediate strand analyses, both used as tokens of positive strand RNA virus replication (Ongus et al., 2004; Gisder et al., 2009; Posada-Florez et al., 2019; Posada-Florez et al., 2020). The increased presence of DWV in honey bees due to efficient vectoring thus poses a threat of cross-species virus transmission (Martin and Brettell, 2019).

Deformed wing virus has been detected in a wide range of other species, including several species of bumble bees (*Bombus* spp.) and solitary mason bees (*Osmia cornuta* and *O. bicornis*) (Mazzei et al., 2014; Ravoet, 2014; Martin and Brettell, 2019; Yañez et al., 2020). However, the mere detection of a virus is nonsynonymous with actual replication within its host; it may rather reflect that an individual has ingested or carries viral particles that are not actively replicating (Evison et al., 2012; Tehel et al., 2016). In fact, the production of minus-strand intermediates in positive-strand RNA viruses has been regarded as a prerequisite of pathogenicity for overt DWV infections (Yue and Genersch, 2005; Gisder et al., 2009). Replication of DWV was detected via intermediate strand analyses in several bumble bee species (*Bombus* spp.), and several studies have demonstrated experimentally that DWV can exploit bumble bees as a host (Singh et al., 2010; Li et al., 2011; Levitt et al., 2013; Fürst et al., 2014; Radzevičiūtė et al., 2017). Thus, DWV is increasingly considered to be a multihost pathogen (Zhang et al., 2012; Fürst et al., 2014). In solitary bees, few studies have similarly demonstrated prevalence of DWV as well as its minus-strand intermediate (Ravoet, 2014; Radzevičiūtė et al., 2017). However, controlled experimental infection scenarios with unequivocal evidence that DWV can exploit solitary bees as a host is largely lacking. Furthermore, the role of a potential reverse shift scenario for viruses from other bee species back to managed honey bees remains largely unexplored. Such research is needed to expand on the potential role of virus spillover for the health of both managed and unmanaged bees.

Here, we investigated through a series of virus infectivity assays whether DWV that has been propagated in honey bee pupae can replicate in the solitary bee *Osmia bicornis* after abdominal microinjection directly into hemolymph. Presence of replication was assessed by viral titers measured by quantitative polymerase chain reaction (qPCR) and performance of intermediate strand assays. In addition, we tested whether inocula taken from previously microinjected *O. bicornis* remain infectious to honey bees by reintroduction of inocula into the hemolymph of honey bee pupae. Our results demonstrate that although DWV does not appear to overtly replicate in *O. bicornis*, inocula harvested 16 days post-microinjection from *O. bicornis* remained infectious once reintroduced into the hemolymph of honey bees. Our results demonstrate that *O. bicornis* has the potential to act as a virus spillover or spillback host for DWV despite the lack of obvious replication, and may contribute to increased colony losses of managed honey bees and declines in populations of other wild bee species.

2 Materials and methods

2.1 Treatment solutions

Sealed Western honey bee (*Apis mellifera*) worker brood combs were sampled from a local colony at the Institute of Bee Health in Bern-Liebefeld, Switzerland. Then, Deformed wing virus (DWV) treatment and control solutions were prepared via standard propagation in pink-eyed honey bee pupae (de Miranda et al., 2013). Five honey bee pupae per treatment were microinjected with 2 µl of a DWV (107 viral copies) or PBS buffer (Phosphate Buffered Saline; pH 7.4), respectively, and incubated at 34.5°C for five days. Afterwards, DWV treatment and control solutions were prepared by homogenization of pupae in 500 µl PBS buffer. 100 µl of chloroform was added and the solution was centrifuged at 13,000 rpm for 10 minutes. Supernatants were collected and stored at −20°C until use. To inactivate possible DWV particles in control PBS solution, it was incubated for 15 min at 65°C (Lelie et al., 1987). DWV titers in the solutions were quantified with standard quantitative polymerase chain reaction (qPCR) as detailed below (de Miranda et al., 2013; Evans et al., 2013). The bees used (N = 5) to create the DWV treatment solution for subsequent infections in *Osmia bicornis* were tested for variant identity using specific PCR assays (see Section 2.4) for two dominant European variants (DWV genotype A (DWV-A) and genotype B (DWV-B)) (Kevill et al., 2017).

2.2 *Osmia bicornis* infections

O. bicornis cocoons were purchased from WAB-Mauerbienenzucht, Konstanz, Germany, and stored at 4°C until experimental start. Adult bees emerged individually in 1.3 L round plastic cylinders (Ø = 110 mm, height = 160 mm) sealed with multifilament netting (Lanz-Anliker AG, Rohrbach, Switzerland) in a climate-controlled room at 25° C equipped with a sunlight simulation system at the research station of Agroscope, Zürich, Switzerland (Sandrock et al., 2014). Each cage was supplied with sugar water (50% (w/v)) in 0.2 ml Eppendorf® tubes and pollen (Sonnentracht Imkerei GmbH, Bremen, Germany, Petri dish, Ø = 30 mm) ad libitum. Pollen was gamma ray irradiated (Leoni Studer Hard AG, Däniken, Switzerland) prior to use to limit potential pathogen interference via contamination (Sandrock et al., 2014). Two days after emergence, 90 bees (34 males and 56 females) were randomly assigned to each of the two treatments ("*O. bicornis* DWV"; N = 46; 17 males and 29 females or "*O. bicornis* PBS Control"; N = 44; 17 males and 27 females). This time span from emergence and treatment enabled the bees to adapt to the cage, recover from eclosion, defecate and feed (Dmochowska-Ślęzak et al., 2015). For each treatment, bees were microinjected between the third and the fourth tergite with 2µl of DWV treatment (107 viral copies) or PBS control solutions that were the product of the procedure mentioned in Section 2.1 (de Miranda et al., 2013) using a Hamilton syringe with 26-gauge needle (0.45 mm) (Human et al., 2013). To facilitate the handling of bees, they were cooled for two hours at 4° C, then ice-chilled for 3 minutes prior to microinjection (Ebadi et al., 1980; Frost et al., 2011; Chen et al., 2014). After microinjection, individuals were returned to their cages in the climate chamber. Pollen and sugar water provisions were refilled every three days. Mortality was assessed daily. The experiment was terminated 16 days post-treatment, and all surviving individuals were freeze-killed and stored at −20° C until molecular analysis was conducted (Chen et al., 2007). Three individuals from each treatment were tested for DWV variant identity using specific PCR assays (see Section 2.4) for two dominant European variants (DWV-A and DWV-B) (Kevill et al., 2017). Twelve *O. bicornis* bees were frozen immediately after microinjection with DWV treatment solution to confirm the baseline concentration of the virus-inoculated bees ("DWV inoculum"; Supplementary Table 1).

2.3 *Apis mellifera* infections

Prior to experimental infection, a total of 19 untreated *A. mellifera* pupae were selected from two local colonies in Liebefeld, Switzerland and molecularly screened to determine naturally occurring baseline levels of DWV ("*A. mellifera* No Treatment," Supplementary Table 2).

To confirm the infectivity of the DWV particles in the DWV treatment solution administered to *O. bicornis*, pink-eyed worker pupae (*A. mellifera*), which were not infested by the parasitic mite *Varroa destructor*, were then collected and randomly assigned to treatment groups (*A. mellifera* DWV, N = 21); *A. mellifera* PBS control, N = 20). Pupae were microinjected intraabdominally with the solutions prepared as previously described (de Miranda et al., 2013). Six individuals from both treatment groups were freeze-killed immediately after microinjection at −20° C until molecular processing to assess initial virus levels following microinjection (see Supplementary Table 2).

To test whether DWV particles recovered from *O. bicornis* remain infectious for *A. mellifera*, a new treatment solution was prepared by extracting virus particles from previously DWV-treated *O. bicornis* bees (N = 3) 16 days post-injection as previously described. Inoculation by microinjection in additional pink-eyed worker pupae ("*A. mellifera* DWV from *Osmia*") was performed as detailed before using pupae (N = 21) from the same two colonies as previously mentioned (de Miranda et al., 2013). Six individuals were freeze-killed immediately after microinjection at −20° C until molecular processing to assess initial virus levels (see Supplementary Table 2).

All remaining *A. mellifera* pupae from the treatment groups "*A. mellifera* DWV" (N =15) and "*A. mellifera* DWV from *Osmia*" (N = 15) were incubated at 34.5°C and ≥ 50% relative humidity and darkness for five days, then stored at −20°C until molecular analysis was conducted. DWV quantification was performed by quantitative PCR as detailed below (de Miranda et al., 2013; Evans et al., 2013).

2.4 RNA extraction, reverse transcription and quantitative PCR

Individual bees were crushed in 2 ml Eppendorf® tubes with 5 mm metal beads in a TN buffer (100 mM Tris, 100 mM NaCl, pH 7.6) and homogenized for 1 min by 25 1/s frequency using a Retsch® MM 300 mixer mill (Evans et al., 2013). TN buffer volume added depended on the weight of the bee to achieve a concentration of 0.5 mg/µl (Supplementary Tables 1, 2). Fifty µl of homogenate were used for RNA extraction. RNA was extracted with the NucleoSpin® RNA II kit (Macherey–Nagel) following the manufacturer's recommendations. The extracted RNA was eluted in 60 µl of RNase-free water and stored at −80°C until further processing (Evans et al., 2013). An exogenous internal RNA reference, the Tobacco Mosaic Virus (TMV), was introduced into each sample during the RNA extraction to monitor the efficiency of RNA purification and cDNA synthesis steps (Tentcheva et al., 2006). Reverse transcription was performed by using M-MLV RT enzyme (Promega) with 2.4 µg of RNA template, 1 µg of random hexamer primers, 200 Units of reverse transcriptase, in 25 µl of final reaction volume (de Miranda et al., 2013). The cDNA synthesis was performed in a Thermocycler (Biometra) with a PCR cycling profile of 5 min incubation at 70°C and 60 min at 37°C. The quantitative PCR reactions were prepared with the KAPA SYBR® FAST Universal qPCR kit (KAPA Biosystems) with 3 µl of tenfold-diluted cDNA, 0.24 µl (0.2 µM) of forward and reverse specific primers, 6 µl of 2X KAPA SYBR® green reaction mix, in a total of 12 µl final reaction volume (de Miranda et al., 2013). Primers used for DWV and TMV are detailed in Table 1. Each sample was performed in duplicate. Each plate included no-template negative controls and four positive controls per primer pair obtained from ten-fold serial dilutions of purified PCR products that function as standard curves (Bustin et al., 2009). The reaction was processed in an ECO™ Real-Time PCR machine (Illumina) and the qPCR cycling profile consisted of 3 min incubation at 95°C and 40 cycles of 3 sec at 95°C for denaturation, 30 sec at 57°C for annealing and extension, and data collection. To verify the specificity of the qPCR products, the amplification was followed by a melting curve analysis by reading the fluorescence at 0.5°C increments from 55°C to 95°C.

Viral titers were calculated from qPCR output data and standard curves adjusted by the various experimental dilution factors to arrive at an estimated DWV genome copies per microgram of RNA (Yañez et al., 2012), which were then log-transformed (Supplementary Table 1). All DWV-A titers are reported as log-transformed means ± standard deviations (see Section 3). For DWV variant identity, the relative number of DWV-A or DWV-B genome copies per microgram of RNA are reported as a percentage of the sum of DWV-A and DWV-B genome copies per microgram of RNA (Table 2).

2.5 DWV-A intermediate strand analysis

The presence of the DWV-A intermediate strand RNA was assessed as a token of viral replication in *O. bicornis* and *A. mellifera* by strand-specific RT-PCR (Yue and Genersch, 2005). The analyses were conducted following standard procedures (de Miranda et al., 2013) for all three DWV treatment groups ("*A. mellifera* DWV", "*O. bicornis* DWV", "*A. mellifera* DWV from *Osmia*") in two separate reactions by first tagging the RNA intermediate strand during the cDNA synthesis using a "Tagged" primer, then by specifically amplifying it using a "Tag" primer (Table 3). Intermediate strand validation controls, labelled as "No Tag," were run in parallel for the detection of potential unspecific strand amplification (false positives). Those controls do not include "Tag" primers in the PCR reactions and ensure the effective removal of "Tagged" primer during the purification process. RNA was converted to cDNA using a Superscript® III reverse transcriptase (Invitrogen) following the manufacturer's recommendations with 1 µl of DWV 3F tagged primer (Table 3), 1 µl of 0.01M dNTP mix (Bioline), 4 µl of 5X first strand buffer, 1 µl of 0.1M DTT, 1 µl (200 Units) of reverse transcriptase, in 20 µl of final reaction volume (de Miranda et al., 2013). The reaction was processed in a thermocycler (Biometra) with the following PCR cycling profile: 5 min at 65°C; then 10 min at 25°C and 60 min at 50°C, followed by 15 min at 70°C. The high temperature used for the reverse transcription improves specific strand amplification by reducing secondary structures. The resulting cDNA was purified results using the NucleoSpin® Gel & PCR Clean-up kit (Macherey-Nagel) and eluted in 30 µl of elution buffer. Purified tenfold-diluted cDNA was amplified by using the same conditions as conventional PCR (see above) with MyTaq™ kit (Bioline). A Tag oligonucleotide was used as forward primer and DWV4-R1 was used as reverse primer (Table 3). In addition, other PCR reactions without the Tag primer ("No Tag") were run as a control for inactivation and efficient removal of the excess of DWV 3F tagged primer via purification after reverse transcription. The thermal cycling profile consisted in 2 min incubation at 95°C and 35 cycles of 20 sec at 95°C for denaturation, 20 sec at 42°C for annealing, and 30 sec at 72°C for extension. The PCR products were purified and analysed

TABLE 1 Primers used for the relative quantification of DWV.

Target	Primer	Sequence	Size (bp)	Reference
DWV-A	DWV-F8668	TTCATTAAAGCCACCTGGAACATC	136	Yañez et al., 2012
	DWV-B8757	TTTCCTCATTAACTGTGTCGTTGA		
TMV	TMVQ1-fwd	TGTAGCGCAATGGCGTACAC	55	Tentcheva et al., 2006
	TMVQ1-rev	CATGCGAACATCAGCCAATG		

TABLE 2 Viral variant identification by relative viral titers detected by quantitative PCR for DWV-A and DWV-B in inoculation solution, PBS-Control or DWV-treated *O. bicornis*.

Treatment group	DWV-A log-mean ± SD (Relative %)	DWV-B log-mean ± SD (Relative %)
Inoculation solution (*N* = 5)	9.13 ± 2.14 (99.99%)	4.18 ± 1.87 (0.01%)
O. bicornis PBS-Control (*N* = 3)	1.05 ± 0.49 (0.02%)	4.85 ± 0.42 (99.98%)
O. bicornis DWV (*N* = 3)	6.41 ± 0.03 (99.80%)	3.69 ± 0.14 (0.20%)

Titers are reported as means of log-transformed genome copies per bee with standard deviations. Relative percentages calculated from the number of DWV-A or DWV-B genome copies detected per microgram of RNA prior to log transformation. N refers to the number of individuals from each treatment group screened.

by electrophoresis on a 1.2% agarose gel, stained in 30% GelRed® Nucleic Acid Gel Stain bath for 30 min, and visualized under UV light.

2.6 Statistical analysis

All statistical analyses and figure preparation were performed using R Statistical Software (v4.2.1, R Core Team, 2022).

Survival analyses of *O. bicornis* bees were performed using the packages "survival" (Therneau and Grambsch, 2000) and "survminer" (Kosinski et al., 2020) to calculate and create Kaplan-Meier cumulative survival curves for each treatment group. Bees that survived until the experimental endpoint and were freeze-killed were censored from the survival analysis. Significant variation between treatment groups and sex were performed separately using the survdiff() function (rho = 0). Pairwise testing for any significant different variation was done using the pairwise_survdiff() function (rho = 0) with Bonferroni corrected p-values (Bonferroni, 1936; Figure 1).

To test whether viral titers could be explained by sex in *O. bicornis*, a simple linear regression model (lm) from the "stats" package (R Core Team, 2022) was conducted with log-transformed viral titers as a dependent variable and sex (male/female) as an independent variable for *O. bicornis* treatment groups (PBS control and DWV). Titer data for *O. bicornis* was pooled by treatment group regardless of sex. Then, a Shapiro-Wilk's test was performed on the log-transformed viral titers for all *O. bicornis* and *A. mellifera* treatment groups (Figure 2; "DWV inoculum," "*O. bicornis* PBS control," "*O. bicornis* DWV," "*A. mellifera* PBS Control," "*A. mellifera* DWV," and "*A. mellifera* DWV from *Osmia*") using the shapiro.test() function and indicated non-normality ($p < 0.05$). As such, a Kruskal-Wallis test was done using the kruskal.test() function. A post-hoc pairwise Wilcoxon test for significant differences between all possible pairwise combinations was done with the pairwise.wilcox.test() function, with Bonferroni adjusted p-values (Figure 2).

3 Results

3.1 DWV variant identity

The virus-strain specific PCR for two predominant European variants (DWV genotypes A (DWV-A) and B (DWV-B)) showed that DWV inoculation solution propagated in *Apis mellifera* and further used for infection assays in *Osmia bicornis* consisted integrally of DWV-A (99.99%; Table 2).

In *O. bicornis* PBS-injected controls, very low titers of DWV-A were detected (log mean 1.05 ± 0.49), whereas DWV-B comprised 99.98% of the variant composition (log mean 4.85 ± 0.42). Oppositely, DWV-treated *O. bicornis* had relatively high titers of DWV-A (log mean 6.41 ± 0.3, N = 3) compared to DWV-B (log mean 3.69 ± 0.14), thus representing 99.80% of the variant composition as DWV-A (Table 2), similar to the inoculation solution.

3.2 Mortality of *O. bicornis*

A total of 56 *O. bicornis* survived until experimental end, with 29 PBS-Control individuals (7 males, 22 females) and 27 DWV-treated individuals (6 male, 21 female). *O. bicornis* males treated with DWV showed significantly higher mortality compared to *O. bicornis* PBS Control treated females (Figures 1A, B; Kaplan-Meier log-rank test, $p = 0.0046$). However, PBS Control females and PBS

TABLE 3 Primers used for the detection of intermediate strand DWV-A in *O. bicornis* and *A. mellifera*.

Primer	Sequence	Reference
DWV 3F tagged	agcctgcgcaccgtggGGATGTTATCTCCTGCGTGGAA	Gauthier et al., 2007
Tag	agcctgcgcaccgtgg	Yue and Genersch, 2005
DWV4-R1	TGTCGAAACGGTATGGTAAACT	This study

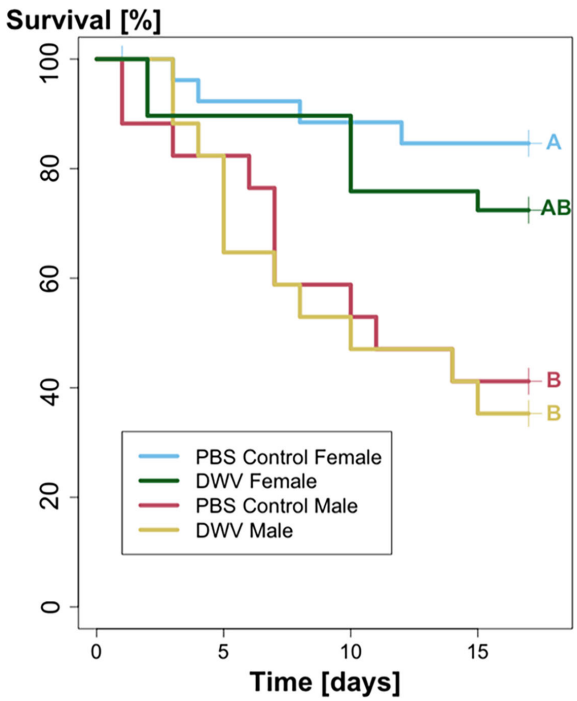

FIGURE 1
Kaplan-Meier survival analysis of *Osmia bicornis*. Both male PBS Control (N = 17) and DWV (N = 17) treatment groups showed significantly higher mortality than PBS control females (N = 27), whereas DWV treated females (N = 29) showed no significant differences from other treatment groups. Significant differences are marked by different letters **(A**, **B)**; Kaplan-Meier log-rank test, $p < 0.05$).

Control males demonstrated significantly different lifespan outcomes (Kaplan-Meier log-rank test, p = 0.0023), suggesting that sex (male vs. female), rather than treatment (PBS vs. DWV), contributed to differences in mortality.

3.3 DWV viral titers

The screening for background levels of DWV-A in the two local colonies selected to serve as the source of *A. mellifera* individuals for the present study demonstrated low titers in screened, untreated individuals expressed at the logarithmic scale of genome copies per μg of RNA with log means of 4.05 ± 0.36 (N = 19) (Supplementary Table 2; "*A. mellifera* No Treatment"). Individuals from both colonies were then used for subsequent creation of inoculation solution and for further experimental virus transmission assays.

For *O. bicornis*, viral titers between males and females were not significantly different (lm, t-value = −1.076, p = 0.287), indicating that sex did not influence viral titer outcomes. As such, males and female *O. bicornis* were pooled based on treatment group for further analyses. DWV-A was detected in DWV-treated *O. bicornis* bees ("DWV inoculum") that were frozen immediately after treatment at a log-mean of 6.48 ± 0.11 (N=12), demonstrating successful inoculation with DWV by the procedure of microinjection (Figure 2). Furthermore, microinjection with PBS control solution in *O. bicornis* ("*O. bicornis* PBS Control") and *A. mellifera* ("*A. mellifera* PBS Control") resulted in low levels of DWV-A with titer log means of 3.68 ± 0.42 (N = 44) and 3.55 ± 0.92 (N = 14), respectively, suggesting that the stress of microinjection did not contribute to the development of infection.

O. bicornis treated with DWV ("*O. bicornis* DWV") showed a significantly higher titer with a log mean of 5.55 ± 0.70 (N = 46) (Figures 2B, C) of DWV-A than those treated with PBS control solution ("*O. bicornis* PBS Control"). However, this value was significantly lower than the initial inoculum level (Figures 2A–C), suggesting a lack of overt infection with DWV-A. In comparison, *A. mellifera* treated with DWV showed significantly higher levels of DWV-A than both the initial inoculum ("DWV inoculum") and *A. mellifera* treated with PBS control solution ("*A. mellifera* PBS Control"), with a log mean of 8.89 ± 0.52 (N = 15), consistent with establishment of infection (Figures 2A–E). Furthermore, *A. mellifera* treated with DWV that had been recovered from *O. bicornis* ("*A. mellifera* DWV from *Osmia*") showed similar levels of DWV-A suggestive of infection with a log mean of 8.80 ± 0.54 (N = 15).

3.4 DWV intermediate strand analysis

Strong, clear bands representing DWV-A were visualized using gel electrophoresis following the intermediate strand-specific RT-PCR assay in *A. mellifera* pupae five days after microinjection with DWV treatment solution, indicating that the microinjected virus particles were infective (Figure 3). In contrast, *O. bicornis* bees 16 days after microinjection with DWV treatment solution showed only very faint bands. For those *A. mellifera* pupae microinjected with DWV particles recovered from previously DWV-

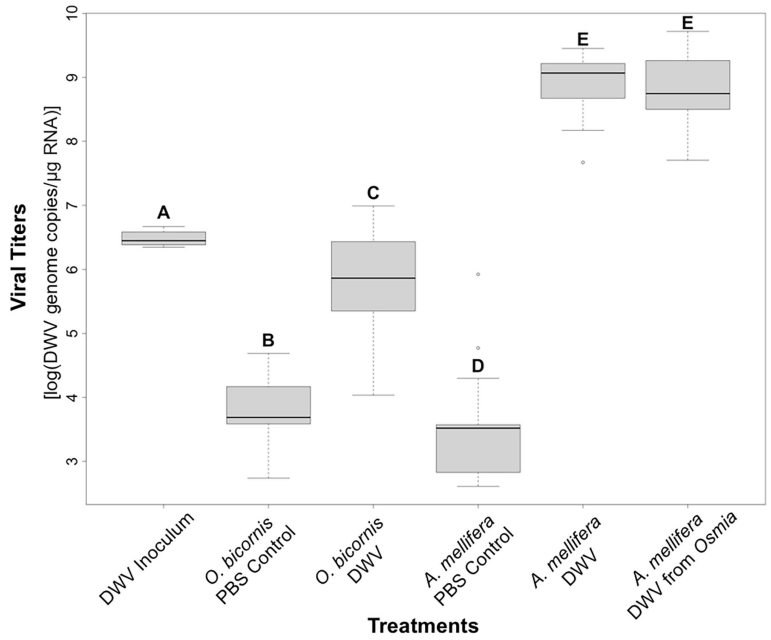

FIGURE 2
DWV-A titers expressed as log transformed DWV-A genome copies per microgram of RNA across the six treatment groups. Different letters **(A–E)** indicate significant differences between treatment levels (*Post-hoc* pairwise Wilcoxon test, $p < 0.05$; "DWV inoculum": the initial microinjected DWV-A titer in *O. bicornis*, N = 12; "*O. bicornis* PBS control": *O. bicornis* microinjected with DWV-free PBS solution, N = 44; "*O. bicornis* DWV": *Osmia bicornis* microinjected with DWV, N = 46; "*A. mellifera* PBS Control": *A. mellifera* microinjected with DWV-free PBS solution N = 14; "DWV *A. mellifera*": *Apis mellifera* microinjected with DWV, N = 15; "*A. mellifera* DWV *Osmia*": *Apis mellifera* microinjected with DWV originating from previously infected *O. bicornis*, N = 15). A five number summary is visually displayed in each box whisker plot: 1) minimum value, 2) first quartile, 3) median, 4) third quartile, and 5) maximum value.

microinjected *O. bicornis*, the intermediate strand DWV-A RNA was clearly detectable, indicating active replication. Intermediate strand validation controls ("No Tag," Figure 3), displayed no visible bands, indicating an efficient removal of the DWV 3F tagged primer, ruling out the possibility false-positive results.

4 Discussion

The viral infectivity assay employed in our study offers the stimulation of an extreme infection scenario for the studied solitary bee species, *Osmia bicornis*, through an artificial microinjection with a high number of Deformed wing virus genotype A (DWV-A) copies of genome (106 genome copies per microgram of RNA). This viral infectivity assay offers two important advantages to test for infectivity of a virus within a new host by (1) overcoming of the natural physical barriers and physiological antiviral defenses to viral infections, thereby enabling the virus to rapidly spread into the host's body and (2) negatively affecting the expression of immune response genes (Yang and Cox-Foster, 2005; Möckel et al., 2011; Yañez et al., 2012; Ryabov et al., 2014).

In support of apparent infection, as was demonstrated by DWV microinjected *A. mellifera* in our study ("*A. mellifera* DWV"), an increase in viral titers for DWV-A (Figure 2) and the presence of strong, clear bands by intermediate strand analysis representing replication of DWV-A (Figure 3) are expected. In contrast, DWV treated *O. bicornis* ("*O. bicornis* DWV") showed slightly yet significantly lower viral titers compared to starting levels ("DWV Inoculum"), and only faint bands were present by intermediate strand analysis. In the case of active viral replication, we would expect viral titers to increase after inoculation, which was not observed. Instead, the viral titers in microinjected *O. bicornis* were detected at a slightly yet significantly lower level to the original virus inoculum, suggesting that DWV-A particles visualized by the intermediate strand assay may be residual post-microinjection. However, a very low rate of DWV-A replication in *O. bicornis* cannot be excluded.

Although pupae of *A. mellifera* were used as positive controls to confirm the infectivity of the experimental DWV particles according to the standard method of virus propagation, newly emerged adults were used for experimental transmission of DWV to *O. bicornis* (de Miranda et al., 2013). Data from the literature in adult *A. mellifera* suggest an inoculation range of 104 to 108 DWV particles for covert, low-level infections, and 1010 to 1011 DWV particles for overt, high-level infections (Highfield et al., 2009; Zioni et al., 2011; de Miranda et al., 2013; McMahon et al., 2018). DWV-treated *A. mellifera* in the present study demonstrated titers within this range, which is consistent with previous findings (Ryabov et al., 2014). Interestingly, our results for *O. bicornis* demonstrate a DWV particle range that matches the range described for an asymptomatic honey bee with a covert infection (104 to 108 DWV copies of genome), which is likely due to experimental DWV injection. These findings are similarly consistent with reported DWV low-level infections in *Bombus* spp., with an

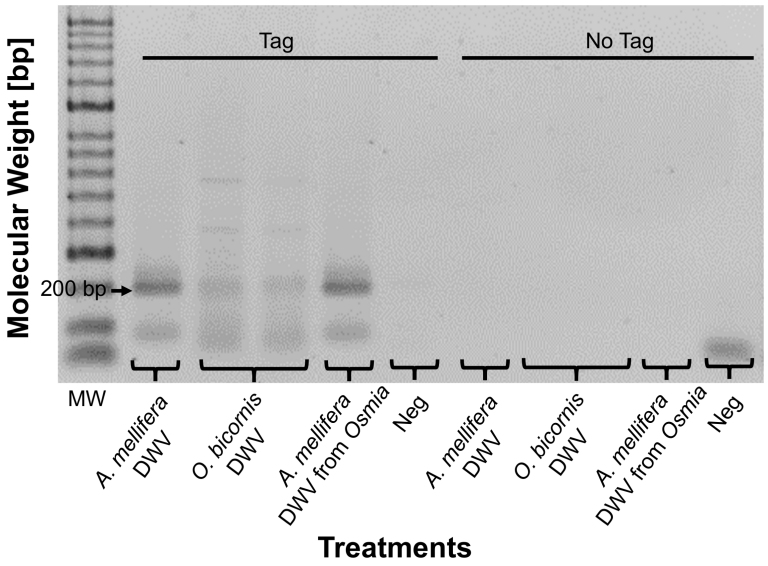

FIGURE 3
Intermediate strand assay for replication of DWV-A in three treatment groups "*A. mellifera* DWV," "*O bicornis* DWV" and "*A. mellifera* DWV from *Osmia*," as well as a no-template negative control "Neg." Positive results are shown with the presence of a 200 bp sized band representing DWV-A. Validation controls ("No Tag") for the detection of potential unspecific strand amplification (false positives) are displayed. (MW = molecular weight size marker; bp = base pairs).

estimated range between 104 to 106 DWV particles (McMahon et al., 2018).

Interestingly, *A. mellifera* treated with DWV sourced from previously microinjected *O. bicornis* ("*A. mellifera* DWV from *Osmia*") showed similarly high titers of DWV-A as *A. mellifera* injected with DWV that had been propagated in honey bee pupae ("*A. mellifera* DWV"). This suggests that although DWV did not cause an overt infection in *O. bicornis*, the virus remains viable and infectious to *A. mellifera*, even sixteen days post-microinjection. Although the virus particles do not appear to actively replicate in *O. bicornis*, residual particles from microinjection may remain in a latent state and become virulent upon microinjection into an optimized host, i.e., *A. mellifera*. Furthermore, the presence of only faint bands representing DWV-A for DWV-treated *O. bicornis* ("*O. bicornis* DWV") on the intermediate strand assay could be potentially explained by the presence of inoculum remnants, whose source was DWV-A propagated in honey bee pupae. The mechanism of this ability of DWV-A to not cause overt infection in *O. bicornis*, yet become infective upon inoculation into its optimized host, *A. mellifera*, up to sixteen days post-inoculation, remains to be understood. Should a natural scenario occur in which *O. bicornis* may serve as a source of latent DWV-A particles that have the potential to become infectious to an optimized host such as *A. mellifera*, there may be implications for repercussions on other managed or unmanaged bee communities in terms of pathogen transmission.

How can a solitary bee species such as *O. bicornis* become infected with DWV in nature? Although this remains unclear, it has been suggested that virus uptake occurs per os via a food-borne transmission, likely via shared flowers (Chen et al., 2006a; Chen et al., 2006b; Singh et al., 2010; Ravoet, 2014; Radzevičiūtė et al., 2017; Burnham et al., 2021; Keller et al., 2021). Though DWV is a key pathogen in managed honey bees and spillover to other species, e.g. bumble bees, has been reported repeatedly, more data are required before deriving general conclusions on the role of spillover of viruses contributing to solitary bee decline (Fürst et al., 2014; Alger et al., 2019; Gusachenko et al., 2019; Tehel et al., 2020; Burnham et al., 2021; Cilia et al., 2021). Our results are in line with data of field survey study, in which intermediate strand RNA of DWV was detected in only one solitary bee species (*Andrena haemorrhoa*) (Fabricius, 1778), but not in a range of other analyzed solitary bee species, including *O. bicornis* (Radzevičiūtė et al., 2017). In contrast, several *Bombus* spp. have displayed intermediate strand RNA of DWV (Radzevičiūtė et al., 2017; Alger et al., 2019). A lower infectivity and virulence of DWV in solitary bees compared to social bees could be explained by several factors.

For example, solitary bees cannot rely on social immunity and must therefore entirely rely on individual immune responses (Wilson-Rich et al., 2009; Meunier, 2015). These individual immune responses may be better developed compared to workers in social insects, which can be considered analogous to somatic cells. Therefore, losses of individual workers can be compensated for as long as the germ line remains intact (Evans, et al., 2006; Straub et al., 2015). Furthermore, differential gut microbiota enabling the host to fight against pathogens may also play a role (Engel et al., 2016; Keller et al., 2021). In any case, there appear to be significant differences between host species and their susceptibility to DWV infections (McMahon et al., 2018). Furthermore, variations arise both between and within RNA viruses due to the

high mutation rates and ample opportunity for local strains to adapt to novel hosts (Daszak et al., 2000; Parrish et al., 2008; Gisder et al., 2018; Paxton et al., 2022).

For example, intermediate strand RNA of Black queen cell virus (BQCV), another RNA virus, has been detected in *Anthophora plumipes*, several *Bombus* spp., *Xylocopa* spp., *Vespa velutina*, the stingless bee *Melipona colimana*, and in *O. bicornis* (Radzevičiūtė et al., 2017; Mazzei et al., 2019; Morfin et al., 2021). Since BQCV transmission is not attributed to an efficient biological vector, as is largely the case with DWV in honey bees and the ubiquitous ectoparasitic mite *Varroa destructor*, exploring transmission dynamics in BQCV and other RNA viruses that are not associated with an efficient vector may be a better predictor for their possible role in novel hosts (Neumann et al., 2012).

Nonetheless, these results indicate that DWV has the potential to cause overt infection in *A. mellifera* when sourced from *O. bicornis*, potentially posing an additional threat to other managed and unmanaged bees in terms of DWV transmission. This could cause a spillback scenario for DWV and possibly for the other honey bee viruses detected in *O. bicornis* (Radzevičiūtė et al., 2017). Interestingly, the variant identity of DWV that predominated in the present study was DWV genotype A (DWV-A). A recent study by Paxton et al. (2022) has highlighted the worldwide replacement of DWV-A by DWV genotype B (DWV-B). Thus, further research is needed to investigate to which extent results regarding infectivity of *O. bicornis* and its potential role with regard to spillover obtained in here for DWV-A may differ for the recently increasingly DWV-B. Given the abundance of RNA viruses identified in populations of both managed and unmanaged bees and the subsequent potential of virus spillbacks, it is prudent to take such a scenario also into account for managed honey bee health.

5 Conclusions

Because the mere detection of DWV in managed or unmanaged bee species is not a reliable sign of a host shift, survey data should ideally be accompanied by controlled infection scenarios. In our study, we demonstrate for the first time through experimental transmission that Deformed wing virus genotype A (DWV-A) does not obviously replicate in *Osmia bicornis* as a novel host. Nevertheless, DWV-A particles-maintained infectivity for *A. mellifera* within *O. bicornis* up to sixteen days post microinjection. Therefore, this solitary bee species has the potential to serve as a transient spillover host, which may ultimately contribute to colony losses and diminishing populations of wild bee species more detrimentally affected by DWV infections, as is observed for several bumblebee species. More survey and controlled infection data are required from a range of species and viruses to draw general conclusions on the role of virus spillover and spillback for the health of both managed and unmanaged bees.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

Ethical approval was not required for the study on animals in accordance with the local legislation and institutional requirements.

Author contributions

Conceptualization, PN and OY. Data curation, AS, NB and OY. Formal analysis, AS, NB, AB and OY. Funding acquisition, PN. Investigation, AS and NB. Methodology, NB and OY. Project administration, OY, MA and PN. Supervision, OY, MA and PN. Validation, AS and OY. Visualization, AS and AB. Writing – original draft, AS, NB, OY and PN. Writing – review and editing, AS, NB, OY, AB, MA and PN. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1122304/full#supplementary-material

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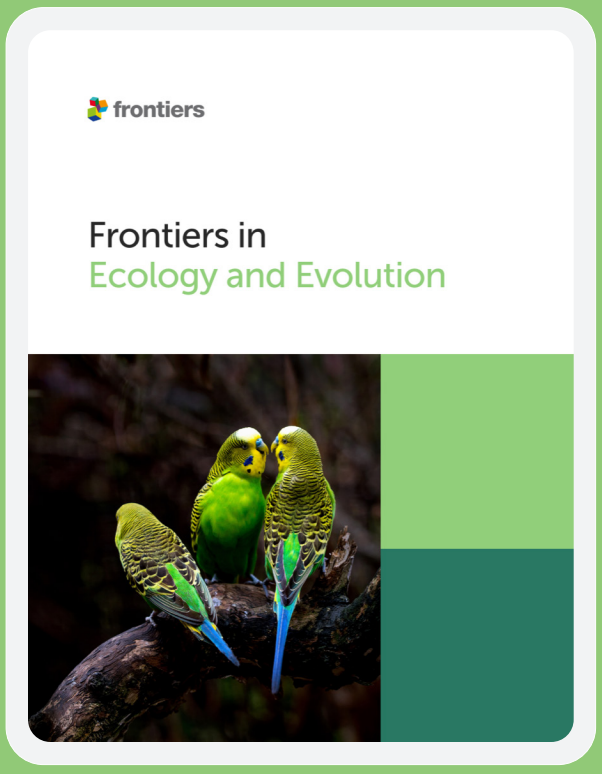

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