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Research

Insect-Microbial Interaction

Landscape Composition and Fungicide Exposure Influence Host–Pathogen Dynamics in a Solitary Bee

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Abstract

Both ecosystem function and agricultural productivity depend on services provided by bees; these services are at risk from bee declines which have been linked to land use change, pesticide exposure, and pathogens. Although these stressors often co-occur in agroecosystems, a majority of pollinator health studies have focused on these factors in isolation, therefore limiting our ability to make informed policy and management decisions. Here, we investigate the combined impact of altered landscape composition and fungicide exposure on the prevalence of chalkbrood disease, caused by fungi in the genus *Ascosphaera* Olive and Spiltoir 1955 (Ascosphaeraceae: Onygenales), in the introduced solitary bee, *Osmia cornifrons* (Radoszkowski 1887) (Megachilidae: Hymenoptera). We used both field studies and laboratory assays to evaluate the potential for interactions between altered landscape composition, fungicide exposure, and *Ascosphaera* on *O. cornifrons* mortality. Chalkbrood incidence in larval *O. cornifrons* decreased with high open natural habitat cover, whereas *Ascosphaera* prevalence in adults decreased with high urban habitat cover. Conversely, high fungicide concentration and high forest cover increased chalkbrood incidence in larval *O. cornifrons* and decreased *Ascosphaera* incidence in adults. Our laboratory assay revealed an additive effect of fungicides and fungal pathogen exposure on the mortality of a common solitary bee. Additionally, we utilized phylogenetic methods and identified four species of *Ascosphaera* with *O. cornifrons*, both confirming previous reports and shedding light on new associates. Our findings highlight the impact of fungicides on bee health and underscore the importance of studying interactions among factors associated with bee decline.

Key words: Ascosphaera, Osmia, fungicides, pathogens, landscape

Bees are essential for the pollination of roughly 75% of global crops (Klein et al. 2007) and 87.5% of all angiosperm species (Ollerton et al. 2011). Consequently, these pollinators are critical for agricultural production and ecosystem function (Winfree et al. 2008, Garibaldi et al. 2013). Yet, the continued provision of these services is threatened, as mounting evidence suggests that numerous species are in decline (Biesmeijer et al. 2006, Potts et al. 2010, Cameron et al. 2011, Bommarco et al. 2012, Bartomeus et al. 2013). Many drivers of bee decline have been identified, including loss of floral resources due to land use change (Biesmeijer 2006, Scheper et al. 2014), pesticide exposure (Brittain et al. 2010, McArt et al. 2017b), and pathogen pressure (Colla et al. 2006, Meeus et al. 2011, Fürst et al. 2014, Ravoet et al. 2014). Though much of the existing literature has evaluated these drivers in isolation, increasing evidence suggests that the most lethal impacts to bees occur when these factors interact (Vanbergen 2013, Goulson et al. 2015, Potts et al. 2016, Meeus et al. 2018).

Land use change, specifically landscape simplification due to the expansion of urban and agricultural cover, can lead to a decrease in floral resource diversity and nesting resources (Scheper et al. 2015); this results in increased foraging distance (Lander et al. 2011, Redhead et al. 2016), and potential exposure to pesticides and pathogens. In fact, land use change has been found to increase pesticide exposure risk (Krupke et al. 2012, Pettis et al. 2013) and alter host–pathogen dynamics (Youngsteadt et al. 2015, Theodorou et al. 2016). Complex landscapes have been found to buffer some of the negative effects of pesticides (Park et al. 2015) and pathogens (Ricigliano et al. 2019) on bee species communities. The potential for pesticides and landscape simplification to interact and alter host–pathogen dynamics has yet to be explored in solitary bee populations, especially on the individual species level.

Bees come in contact with many different pesticides through both foraging and nesting. Many studies have focused on insecticides, though fungicides are often sprayed during crop bloom when bees are most active and have been increasingly implicated to impact bee health (Ladurner et al. 2005, Yoder et al. 2013, Bernauer et al. 2015). Fungicides are not only among the most widely applied pesticides both in terms of frequency and area (Garthwaite et al. 2013, Park et al. 2015, Atwood and Paisley-Jones 2017) but are also among the most commonly detected residues in bee pollen provisions (Pettis et al. 2012, McArt et al. 2017a). Fungicides were found to be the strongest predictor of range contractions and pathogen prevalence in declining bumble bee species across the United States (McArt et al. 2017b). Exposure to fungicides negatively impacts nutrition and virus loads in *Apis mellifera* Linnaeus 1758 (Apidae) (DeGrandi-Hoffman et al. 2017) and has a strong negative effect on the abundance and diversity of wild bees in agroecosystems (Park et al. 2015). In addition, fungicides have been found to negatively impact nest recognition in solitary bees (Artz and Pitts-Singer 2015). Fungicides have also been found to indirectly effect bee health mediated through their impacts on beneficial bee microbes that generally work to inhibit bee diseases (Yoder et al. 2011a,b, Mcfrederick et al. 2014).

Pathogens have also been implicated as significant drivers of bee species decline (Colla et al. 2006, Meeus et al. 2011). One pathogenic group of concern that remains understudied are fungi (Evison et al. 2012, Evison and Jensen 2018). Fungi have been identified as the most commonly detected pathogens in some important crop pollinating solitary bee genera such as *Osmia* and *Megachile* (Megachilidae) (Goerzen et al. 1992; Hedtke et al. 2015). *Ascosphaera* Olive and Spiltoir 1955 (Ascosphaeraceae) is of particular concern as it has been identified as an associate with bees in the following families: Apidae, Megachilidae, and Colletidae (Evison and Jensen 2018).

Simultaneous pathogen and pesticide exposure has been found to have negative impacts on bee health in both laboratory and field studies (Pettis et al. 2012, Yoder et al. 2013, DeGrandi-Hoffman et al. 2015). Exposure to fungicide residues in pollen provisions may benefit bees by suppressing fungal pathogens. Recently, Evans et al. (2018) reported lower prevalence of *Ascosphaera* in *O. bicornis.* nesting on farms with high pesticide exposure. However, the incidence of chalkbrood infections has also been associated with fungicide exposure in commercial *A. mellifera* colonies (Yoder et al. 2013). Fungicide exposure may increase the incidence of fungal diseases through both direct and indirect mechanisms. Fungicides could directly compromise the host immune system (DeGrandi-Hoffman et al. 2015) leading to higher mortality of bees already exposed to pathogens. Effects of fungicides on pathogen prevalence may also be mediated indirectly through the microbial community. Many microbes in pollen provisions are known to play a beneficial role by altering the nutritional profile of provisions (Steffan et al. 2019) and by suppressing the growth of pathogens, including *Ascosphaera* (Gilliam et al. 1988). Beneficial fungi are thought to play an important role in inhibiting *Ascosphaera* infections (Gilliam et al. 1988, Mcfrederick et al. 2014). However, fungicides can reduce beneficial fungi in *A. mellifera* colonies (Yoder et al. 2011) and *Bombus impatiens* Cresson 1863 (Apidae) colonies (Steffan et al. 2017). Furthermore, beneficial microbes in pollen provisions are more susceptible to fungicides, including those like pyraclostrobin, which are commonly applied during crop bloom (Yoder et al. 2011a). By altering the community of beneficial microbes, fungicides may indirectly induce pathogen infection by both reducing the nutritional profile of the pollen provision and reducing the prevalence of microbes that potentially suppress pathogens. Additionally, it is important to mention that fungicides also have the potential to benefit the bees, as they could reduce their pathogen prevalence.

In this study, we examine the single and combined effects of landscape composition and fungicide exposure on the host–pathogen dynamics of the fungal genus *Ascosphaera,* the causative agent of chalkbrood disease, in the solitary bee *Osmia cornifrons* (Radoszkowski, 1887) (Megachilidae). The community of *Ascosphaera* species associated with *O. cornifrons* has not been well characterized, although prior work has detected up to five *Ascosphaera* species in adult and larval *O. cornifrons* (Hedtke et al. 2015). Here, we use phylogenetic methods to classify the community of *Ascosphaera* species present in *O. cornifrons* nests in apple orchards of New York. We then relate the prevalence of *Ascosphaera* and the incidence of chalkbrood disease to fungicide exposure and landscape composition.

Study System

We used *O. cornifrons* for our study because they inhabit artificial trap-nests, are polylectic, mass provisioning, and univoltine (Bosch et al. 2008). Individual female *O. cornifrons* establish nests in hollow cavities, including reeds, holes bored into wood, and artificial paper tubes, in which they sequentially provision an average of 10 cells divided by mud walls, with female offspring at the rear of the nest and male offspring at the front (Batra 1997, Bosch and Kemp 2002).

The genus *Ascosphaera*, the causal agent of chalkbrood disease, is commonly associated with bees. There are 28 known species of *Ascosphaera* and they are documented to have associations with 3 bee families (Klinger et al. 2013, Klinger et al. 2015, Evison and Jensen 2018). Not all *Ascosphaera* species cause chalkbrood infection in the nests of their hosts, as the genus has both saprophytic and pathogenic species (Bissett 1988), although it is unclear which species are harmful to solitary bees. Saprophytic species feed on pollen, mud, cocoons, frass, and other materials within the bee nests and it is not known whether these species are harmful to bee development. As a pathogen, *Ascosphaera* ascospores are ingested by larval bees through pollen provisions. Following ingestion, the ascospores germinate and hyphae penetrate through the hemocoel where they inhibit larval growth creating a chalk-like cadaver from which the common name of the disease, chalkbrood, is derived (Spiltoir 1955). *Ascosphaera* bee associations have been studied most in managed bees, such as *A. mellifera* and *Megachile rotundata* (Fabricius 1787) (Megachilidae) (Gilliam et al. 1988, Goerzen et al. 1992), whereas the impact of these fungi on other bee species is largely unexplored (Evison et al. 2012, Hedtke et al. 2015, Maxfield-Taylor et al. 2015).

Materials and Methods

Bee Collection

We placed ~100 overwintered adult bees, drawn from six source populations in Ithaca, New York, in nests at each of the 17 apple orchards in the Finger Lakes region (Fig. 1). Apple orchards varied in surrounding landscape composition and fungicide use. Nesting material consisted of new cardboard tubes with paper inserts (15.24-cm long × 7.5-mm diameter [crownbees.com]) housed in wooden nest blocks placed in a 250 l × 150 W × 200 D-mm wooden shelter and mounted to a post on the orchard edge at ~1.2 m from the ground. The nests were provisioned by adult female *O. cornifrons* between May and June of 2015 and completed nests were collected from the field in August 2015 and stored in ambient temperatures in a protected shed until November. From November

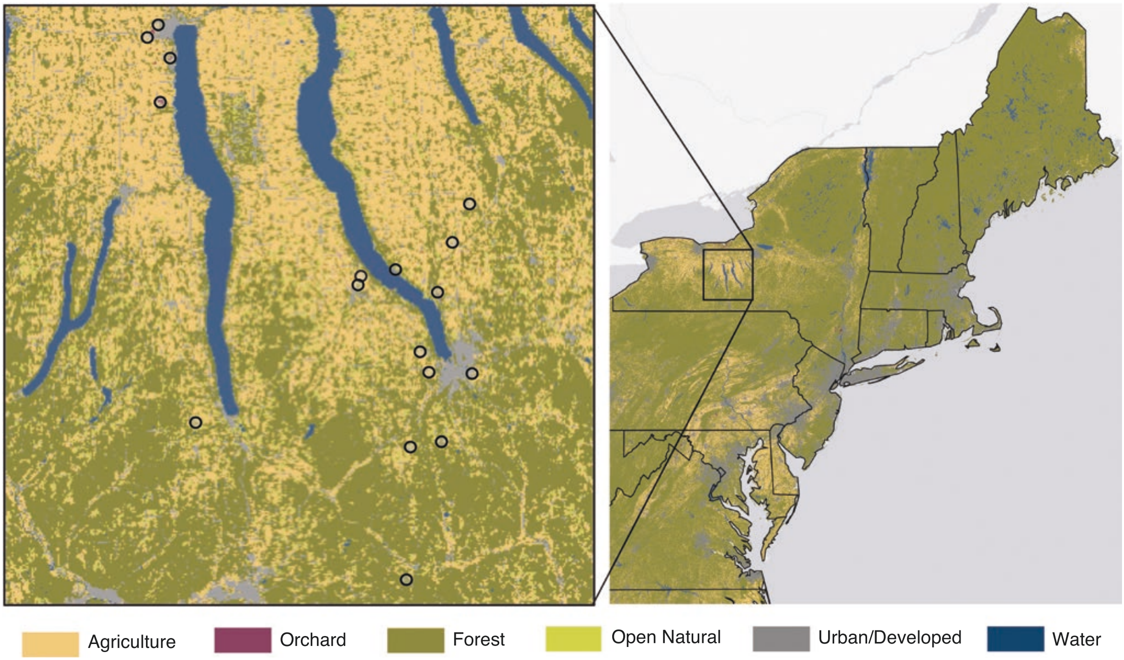

Fig. 1. A map of the Finger Lakes Region of New York State showing the 17 orchards in the study. Map colors indicate the cover of different land use types based on the 2015 Cropland Data Layer (USDA 2015)

2015 until this study was conducted in I-16, the nests were maintained in a refrigerator at 4°C.

Dissection of Nest Contents

An average of five randomly selected nests were dissected per site to estimate the prevalence of *Ascosphaera* in adult bees (via polymerase chain reaction [PCR]) as well as the incidence of chalkbrood infections that resulted in larval and pupal mortality. Sterile techniques were used to extract adult bees from their cocoons and place them in 1.5-ml Eppendorf tubes, which were stored at −80°C. Additional nest contents, such as cocoons, unconsumed pollen provisions, nest parasites, and *Ascosphaera*-infected larval cadavers, were also collected and stored the same way as adult *O. cornifrons*. We then quantified mortality due to chalkbrood based on visual confirmation of symptoms in cadavers (Supp Fig. 1 [online only]). The presence of *Ascosphaera* was assessed in overwintering adults and larval cadavers using molecular techniques, which are described below.

DNA Extraction

DNA was extracted from the whole metasoma (exterior and interior) of all adult bees or half of the remains of *Ascosphaera* infected larval cadavers. Extractions were carried out using a phenol:chloroform protocol (Danforth et al. 2011). PCR amplification was then conducted using primers AscoAll-F and AscoAll-R (James and Skinner 2005), which span ITS gene regions 1 and 2 and part of the 5.8S ribosomal RNA gene. We purified all positive PCR samples using ExoSAP (Exonuclease + Shrimp Alkaline Phosphatase; Affymetrix) and sequenced in one direction on an ABI 3730x1 capillary DNA Analyzer using Big Dye Terminator chemistry. Poor-quality sequences were excluded from further analysis as well as several relatively 'clean' sequences which had multiple ambiguous trace peaks potentially indicating mixed species infections. Mixed species occurrence in a host is known in *Ascosphaera* (Klinger et al. 2015); however, we were not able to isolate individual sequences in this study. Sequences used in further analyses are deposited in GenBank under accession numbers MT372910–MT372973.

Phylogenetic Analysis

We combined our sequences for the ITS+5.8S gene region with a reference database obtained from GenBank. Our reference database included all authoritatively identified *Ascosphaera* species currently available in GenBank plus 27 sequences obtained in previous studies of Osmia cornifrons in the Ithaca area (Hedtke et al. 2015) and three sequences from a study in Belgium (Ravoet et al. 2014). Sequences were aligned using the E-INS-i algorithm implemented in MAAFT v7 (Katoh and Standley 2013). The outgroup was assigned as *Eremascus fertilis,* Eidam 1883 (Eremascaceae) a sister taxon to Ascosphaeraceae (Klinger et al. 2013). The optimal model of sequence evolution (TN + F + R4) was determined in ModelFinder (Kalyaanamoorthy et al. 2017) and the final nucleotide alignment was then analyzed using maximum likelihood methods with ultrafast bootstrap (Minh et al. 2013) implemented in IQ- TREE (Nguyen et al. 2015) and accessed through the W-IQ-TREE portal (Trifinopoulos et al. 2016).

Fungicide Exposure

Fungicide data were collected for each of the 17 sites by analyzing the residue in pollen provisions of *O. cornifrons* (Centrella et al. 2020). Individual pollen provisions from a minimum of three nests per site were collected immediately following nest completion and aggregated into a composite sample containing equal amounts of pollen by weight per nest to better represent average pesticide exposure at the site and to ensure sufficient quantities of pollen for analysis. For each of the 17 sites, ~1 g of the composite pollen sample was extracted using a modified QuEChERS protocol (Eitzer et al. 2014) and concentrated to 1 ml for analysis by liquid chromatography/

mass spectrometry/mass spectrometry (LC/MS/MS). Full details of the pesticide analysis can be obtained from Centrella et al. (2020).

Landscape Composition

Landscape composition surrounding each of the orchard study sites (Fig. 1) was characterized at 500-, 750-, 1,000-, and 1,250-m radii from the nest location using the 2015 National Agricultural Statistics Service New York State Cropland Data Layer (USDA-NASS 2016) in ArcGIS 10.1 (ESRI 2011). These radii were chosen because they correspond to the range of expected foraging distances predicted by Greenleaf et al. (2007). We estimated the cover of four surrounding landscape types: 1) urban, which includes any developed areas, 2) agricultural, which includes all crop categories as well as forage and pasture, 3) open natural habitat, which includes shrub-land and wetlands, and 4) forest.

Bioassay

To explore the interaction between *Ascosphaera* and fungicide exposure in a controlled laboratory setting, we conducted a laboratory assay on *O. cornifrons* eggs and larvae collected in May 2016 from three suburban sites in the Ithaca, New York, United States. In total, 30 nests were collected within 24–48 h of completion and brought to the laboratory where they were carefully opened. Eggs and developing larvae were separated from their pollen provisions and maintained as sibling groups in separate 90 × 15-mm Petri dishes for up to 2 h. During this time, pollen provisions from all nests were homogenized using a mortar and pestle. The average pollen provision weight across all nests was 0.1895 g, thus this same quantity of pollen was allocated to individual wells of 96-well microtiter plates, leaving alternate wells within a row and alternate columns empty (Supp Fig. 2 [online only]). Eggs and larvae were exposed to fungicides and pathogens in a crossed factorial design with four treatments: 1) fungicide, 2) *Ascosphaera*, 3) *Ascosphaera* + fungicide, and 4) control. There were 44 replicates per treatment. The fungicide treatment group contained pollen provisions that were dosed with the fungicides pyraclostrobin at 363 ppb and boscalid at 513 ppb dissolved in acetone to replicate the average ppb of these compounds recovered from pollen collected in apple orchards from our study (Centrella et al. 2020). We used pyraclostrobin and boscalid because they were frequently detected at high levels across our samples and both compounds are present in the commonly used commercial product Pristine (BASF), which is recommended for control of powdery mildew and apple scab during the orchard flowering period. Additionally, this combination of fungicides has been found to alter pathogen loads in honey bees (DeGrandi-Hoffman et al. 2015) and behavior of *Osmia* (Artz and Pitts-Singer 2015) in previous studies. The *Ascosphaera* treatment group contained pollen provisions that were inoculated with 100,000 *Ascosphaera* ascospores in 1.6-µl double-deionized water (ddH$_2$O). Spore solutions were derived by sonicating five *Ascosphaera* laden larval cadavers from the field study described above in ddH$_2$O at 15 Hz for 10 min. The fungicide + *Ascosphaera* treatment group contained pollen provisions with both *Ascosphaera* ascospores and fungicides at the same doses as described in above treatments. The control treatment group contained pollen provisions that were not inoculated with pathogens or fungicides. Water and acetone were added at equal levels across all four treatments. After 1 h, acetone and excess water had evaporated, and individual eggs and larvae were added to each well. Microtiter plates with treatments were maintained in darkness at 24°C and 45% humidity. Developmental progress was recorded daily until they spun cocoons or died. Sources of mortality were noted including presence of white mycelium, fungal hyphae, ascospores, and spore cysts, indicative of *Ascosphaera* infection. The bioassay was terminated in June after 2 mo, at which point nearly all individuals had either perished or developed to adulthood.

Statistical Analysis

All analyses were conducted in R version 3.3.3 (R Core Team 2017). We used generalized linear mixed effects models (Bates et al. 2014) with a binomial error distribution and log link function to assess the effects of landscape composition, fungicide exposure, and their interaction on the incidence of chalkbrood in larval *O. cornifrons*. In each model, the random effects' structure included nests within the site to account for nonindependence of samples taken from the same nests and for multiple nests screened per site. We built candidate models by first evaluating Variance Inflation Factors and concluded that only one land cover type per scale could be included in each model. We then evaluated each single cover candidate model with a fungicide main and interaction terms via the dredge function in R and reported top models based on Δ2 Akaike Information Criterion corrected for small sample size. Additional candidate models are reported in Supp Table 1 (online only). The same procedure and model structures were used to evaluate the effects of landscape composition, fungicide exposure, and their interactions on the prevalence of *Ascosphaera* in adult *O. cornifrons* based on PCR screening (Supp Table 2 [online only]). Data from the factorial bioassay were analyzed with a binomial mixed effects model including both the main effects of fungicide addition and *Ascosphaera* addition, as well as their interaction. Random effects were nested to include source population and stage (egg or larva) at the start of the bioassay.

Results

Ascosphaera Species

Using phylogenetic analysis, we assigned our *Ascosphaera* sequences from adult and larval *O. cornifrons* to four known species, including *Ascosphaera subglobosa, Ascosphaera naganensis, Ascosphaera callicarpa*, and *Ascosphaera proliperda* (Fig. 2). We detected *A. subglobosa* in 14 samples from live adults; *A. naganensis* was found in 6 adults and 5 cadavers; *A callicarpa* was found in 10 adults and 4 cadavers, whereas *A. proliperda*, which was the most frequently detected, was found in 5 adults and 19 cadavers. Fungal sequences from four adult individuals did not fall into a specific clade. Two were closely related to *A. naganensis* and BLAST with ~85% percent identity to the *A. naganensis* type sequence. The third strain in this clade was identified from *O. bicornis* in Belgium (Ravoet et al. 2014) and had 96% identity to the *A. naganensis* type sequence. The other two sequences were in a sister clade to *A. callicarpa* and *A. xerophila* but both BLAST with greater percent cover (~98%) and identity (~86%) with *A. callicarpa.*

Effects of Fungicides and Surrounding Landscape Composition on *Ascosphaera*

The cover of the two dominant habitats, open natural habitat at the 500-m scale and forests at the 1,000- m scale, both impacted the proportion of chalkbrood mortality in the larvae across sites. Neither cover was correlated with fungicide residues detected in the pollen provisions ($r = -0.41$, $t = -1.74$, $P = 0.10$; $r = 0.16$, $t = -0.65$, $P = 0.51$). The proportion of chalkbrood larval cadavers decreased with increasing open natural habitat cover ($F_{(1,16)} = 6.94$,

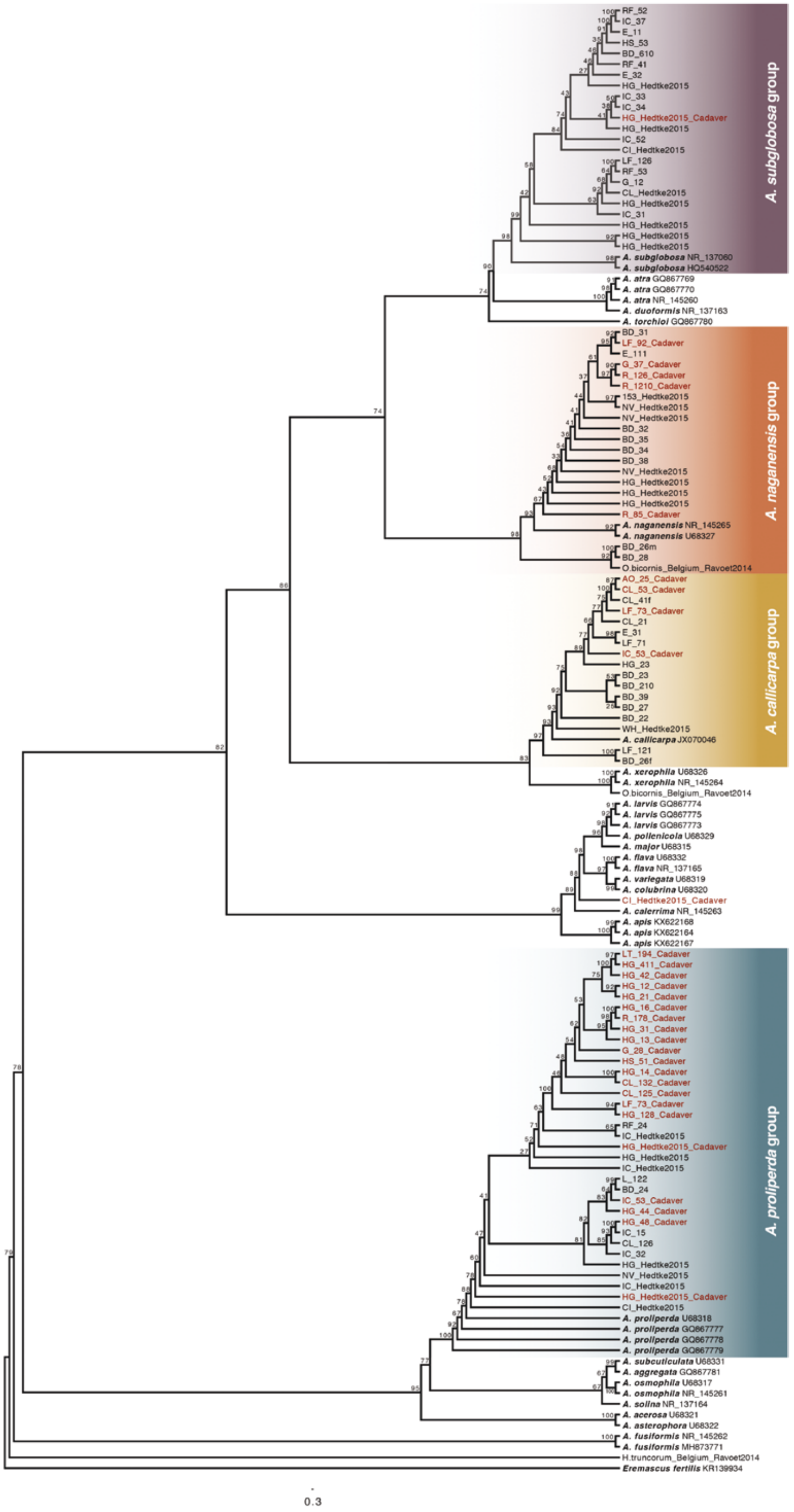

Fig. 2. Maximum-likelihood estimate of *Ascosphaera* species based on ITS sequences. Numbers at nodes represent bootstrap proportions. Sequences generated from this study are represented by a two-letter code for their site of origin and a sample number. Additional sequences from Ravoet *et al.* (2014) and Hedtke *et al.* (2015) are included. Cadaver sequences are shown in red. Colors indicate species assignment.

P = 0.009; Fig. 3a). Additionally, the total cover of forest at the 1,000-m scale interacted with fungicides to alter chalkbrood incidence ($F_{(1,16)}$ = 12.02, P = 0.003). At sites with low forest cover, increasing fungicide exposure was associated with an 85% increase in chalkbrood incidence, whereas sites with average to high forest cover showed no increase in chalkbrood (Fig. 3b).

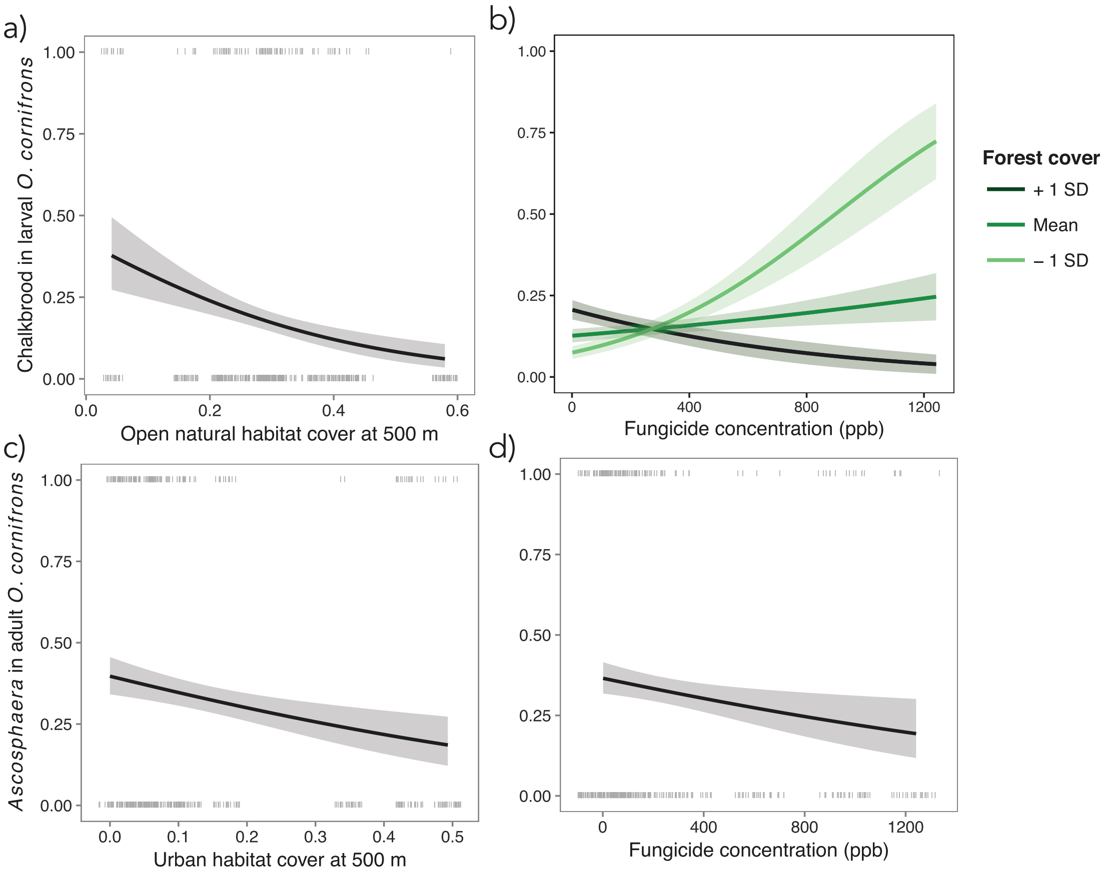

Fig. 3. Probability of *Ascosphaera* in *Osmia cornifrons* by life stage (larvae vs adult) in relation to landscape composition and the concentration of fungicides detected in pollen provisions. a) the incidence of chalkbrood infection in larvae was negatively associated with cover of open natural habitats at the 500-m scale, whereas b) forest cover at the 750-m scale moderated the impact of fungicides. In adults, c) *Ascosphaera* prevalence was lowest at sites with high urban cover and d) high fungicide exposure.

In adults, the cover of urban habitat at the 500-m scale was associated with a decrease in proportion of *O. cornifrons* that screened positive for *Ascosphaera* via PCR ($F_{(1,16)}$ = 6.16, P = 0.024; Fig. 3c). Compared with the increase in chalkbrood observed in larvae, fungicide exposure was associated with an opposite effect on the proportion of adult *O. cornifrons* that screened positive for *Ascosphaera*, which declined at sites with greater fungicide levels ($F_{(1,16)}$ = 4.93, P = 0.041; Fig. 3d).

Bioassay

We found no significant interaction between the addition of *Ascosphaera* ascospores and the fungicides pyraclostrobin and boscalid ($F_{(1,16)}$ = 1.16, P = 0.28). However, there was a significant main effect where *Ascosphaera* inoculation was associated with increased *O. cornifrons* mortality ($F_{(1,16)}$ = 7.03, P = 0.007) and the application of fungicides also increased mortality of *O. cornifrons* ($F_{(1,16)}$ = 3.89, P = 0.048), suggesting an additive effect of fungicides and fungal pathogen exposure on larval mortality (Fig. 4).

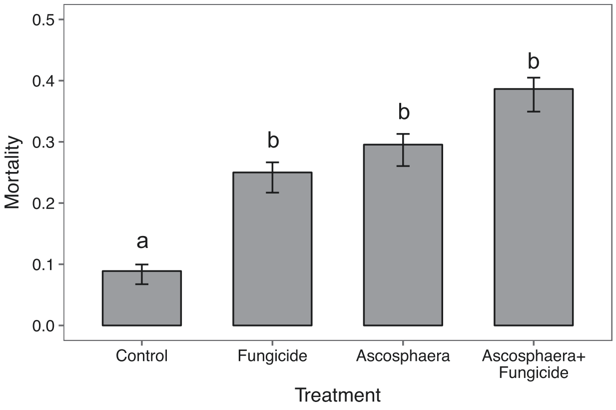

Fig. 4. Impact of *Ascosphaera* and fungicide exposure on larval mortality in laboratory-reared *Osmia cornifrons*. Mean (±SEM) probability of mortality for larva reared in 96-well microtiter plates and fed control pollen or pollen exposed to sublethal doses of Pyraclostrobin and Boscalid, *Ascosphaera* ascospores (100,000) or both.

Discussion

Our study aimed to better understand the *Ascosphaera* species community associated with *O. cornifrons* and how potential threats such as land use change and fungicide exposure impact host–pathogen dynamics. We found that landscape composition surrounding apple orchards had direct effects on *Ascosphaera* prevalence and interacted with fungicides to alter chalkbrood incidence. Furthermore, we found additive effects of fungicide exposure and *Ascosphaera* on larval *O. cornifrons* mortality. Our findings demonstrate that environmental factors have the potential to act independently and in combination to influence host–pathogen dynamics in solitary bee populations.

Ascosphaera Species

Our study found four species of *Ascosphaera* associated with *O. cornifrons* adults and cadavers. A previous study of *O. cornifrons* in New York apple orchards, which sampled five sites in common with the current study, found the same four species of *Ascosphaera*, although at different frequencies (Hedtke et al. 2015). Differences in species composition suggests a variable community of *Ascosphaera* over space and time within apple orchards of New York state. Similar to Hedtke et al. (2015), there were some strains with close matches to known species that did not fall unambiguously within a known-species group and bootstrap support across many of the nodes was low. As noted by Hedtke et al. (2015), the markers used in this study were designed for broad detection within the genus and not for phylogenetic inference. Additionally, our understanding of the relationships within *Ascosphaera* are recent (Klinger et al. 2013) and new species continue to be discovered (Wynns et al. 2012, 2013). Therefore, we encourage caution in interpreting species identities presented here as they may represent undescribed species or described species for which sequence data are unavailable.

Although our study was not intended to determine saprophytic or pathogenic lifestyles of the *Ascosphaera* species in *O. cornifrons*, the clustering of cadaver sequences in particular clades is suggestive of pathogenicity. Cadaver sequences were clustered in all clades but *A. subglobosa*, suggesting that it is saprophytic in *O. cornifrons* as hypothesized by Wynns et al. (2012) for another host, *M. rotundata*. While all three other species groups contained sequences from cadavers, only *A. proliperda* had a high proportion (19 of 25 samples), suggesting pathogenicity. The species *A. proliperda* is a known pathogen on *M. rotundata*, another introduced commercial bee in the family Megachilidae, whereas *A. naganensis* and *A. callicarpa* were previously hypothesized to be saprophytic (Klinger 2015). *Ascosphaera proliperda* was not as prominent in *O. cornifrons* in the Hedtke et al. (2015) study; our cadaver amount was larger by a factor of 10, suggesting that there could be cross infection of this pathogen from other commercial solitary bees. The lifestyles of *A. callicarpa* or *A. naganensis* are not clear since both had adults and cadavers present; however, our study is the first to report cadavers associated with *A. naganensis*. Skou (1988) first described *A. naganenis* as a saprophyte from *O. cornifrons* in Japan and it was likely introduced to the United States with the bee from its native habitat (Hedtke et al. 2015), yet formal studies have not been done to confirm this. Hedtke et al. (2015) previously reported a single sequence matching *A. callicarpa* from the same regions. This species was previously described as a saprophyte on the larval frass of the solitary bee *Chelostoma florisomne* L. and was only recently described on the island of Zeeland, Denmark (Wynns et al. 2013). The United States strains may therefore represent a sister taxa to *A. callicarpa*. The lifestyles of these *Ascosphaera* species should be studied as it relates to each bee they are associated with, as some fungal species reported as saprophytic have demonstrated pathogenesis in laboratory assays (Klinger et al. 2015).

Landscape Composition and *Ascosphaera*

Increasing landscape complexity is often associated with increased pollinator abundance, and diversity (Kennedy et al. 2013, Connelly et al. 2015). Our findings suggest that some of these effects may be mediated by a reduction in pathogen-associated mortality, as we found that increasing cover of open natural habitats and urban habitats around apple orchards led to a decrease in chalkbrood incidence in larval *O. cornifrons* and *Ascosphaera* prevalence in adult *O. cornifrons*. Open natural habitats, which include grassy meadows, wetlands, and old fields, have been found to provide diverse floral resources for bees (Williams and Kremen 2007), thus promoting better nutrition, and subsequently, improved immune function (Alaux et al. 2010, Di Pasquale et al. 2016). Polyfloral pollen diets have been found to increase longevity of *A. mellifera* exposed to parasites (Di Pasquale et al. 2013). Similarly, experimentally increasing the protein to sugar ratio in larval diets dramatically reduced the susceptibility of *M. rotundata* to chalkbrood infection (Vandenberg 1994). It is also possible that these habitats provide greater exposure to a diversity of beneficial microbes (Mcfrederick and Rehan 2019) leading to lower prevalence of pathogens, such as *Ascosphaera* (Gilliam et al. 1988).

Fungicides

On a global scale, fungicides are among the most widely used pesticides to defend plants and agricultural crops against pathogens (Belsky and Joshi 2020). As mentioned above, fungicides often negatively impact nontarget organisms, particularly bees, in nearly all life stages. Compared with other fungicides pyraclostrobin and boscalid, the two in our study, are frequently investigated with relation to bee health; however, the total amount of those studies is still <30, with a strong bias towards social bees (Cullen et al. 2019). The lethality of fungicides has been found to increase when they are acting concurrently with other pesticides (Wade et al. 2019); sublethal effects are less studied but may have a greater impact due to the often indirect modes of action of fungicides on bees. Fungicides have five primary modes of action that depend on the class, such as inhibition of membrane components, protein synthesis, and respiration (Yang et al. 2011). Pyraclostrobin and boscalid are in different classes, strobilurin, and anilide, respectively, yet both act on transport chains associated to the mitochondria (Anonymous 2002, Bartlett et al. 2002, Stammler 2008), allowing increased susceptibility to other stressors, interference with gut microbiota and composition, change in immune function, and mortality (Cizelj et al. 2016, Batista et al. 2020, Carneiro et al. 2020). Boscalid has been found to decrease bee pollen consumption and digestion and increase virus titers (DeGrandi-Hoffman et al. 2015), appetite change may also explain death of larvae in our bioassay. Pyraclostrobin impacts on *A. mellifera* depend on the lifestage, which coincides with our competing findings on combined effects in *O. cornifrons* larvae and adults. In adults, pyraclostrobin decreases mitochondrial function, reduces longevity, and causes morphological impairments (Fisher et al. 2017, Zaluski 2017, da Costa Domingues et al. 2020, Nicodemo et al. 2020). Larvae exposed to pyraclostrobin have been found to survive yet have cytotoxic effects on the midgut as adults; this particular relationship intensifies with pathogen exposure (Tadei 2020). Ascospores proliferate in the bee gut; the finding of Tadei (2020) could explain the discovery of living adults with pathogenic *Ascosphaera* species; future studies isolating the gut from the cuticle prior to extraction would be important to test this mechanism.

Fungicides and *Ascosphaera*

Although prior studies have reported the presence of fungicides in bee pollen provisions (Pettis et al. 2013, McArt et al. 2017a), few have addressed the impact on fungal pathogen prevalence. Recently, Evans et al. (2018) found the prevalence of *Ascosphaera* in *Osmia bicornis* (Linnaeus,1758) pupae was lower in agricultural sites associated with higher levels of pesticide use; we found the same trend with respect to fungicide exposure on *Ascosphaera* prevalence in *O. cornifrons* adults. The pattern observed here and in Evans et al. (2018) would be expected if fungicides increased mortality of bees in the larval stage when already exposed to *Ascosphaera*. In this case, if larvae exposed to fungicides and *Ascosphaera* are removed from the population through increased chalkbrood mortality during this life stage, potentially fewer adults would develop with ascospores in their gut or cuticle at sites with high fungicide exposure. We note that *Ascosphaera* prevalence is likely to be conservative in our study, given that adults were removed individually from their cells and not allowed to emerge naturally (Vandenberg et al. 1980). Alternatively, the pattern of lower *Ascosphaera* prevalence among adult *O. cornifrons* may be a result of shifting *Ascosphaera* species composition, where sites with high fungicide toxicity have greater prevalence of pathogenic species. Nothing is known about interactions between pathogenic and saprophytic *Ascosphaera* species and their relative susceptibility to fungicides, which would be a promising avenue for future study.

Landscape Composition and Fungicide Interactions

The impact of fungicides on larval chalkbrood incidence were strongly dependent on surrounding forest habitat cover. Specifically, sites with greater fungicide exposure and low forested land cover had the highest prevalence of chalkbrood in *O. cornifrons* larvae. Similar to open natural habitats, forest habitat provides rich floral resources (Batra 1985), particularly in the early spring before canopy closure. The importance of forest habitat and woody plants in providing alternative pollen sources for bees has been increasingly recognized (Wood et al. 2018). A recent study of orchard pollinator communities in the same region found that natural habitats, including forest, can buffer the abundance and diversity of pollinator communities against pesticide exposure including fungicides (Park et al. 2015). Our findings suggest additional mechanisms (pathogen suppression) by which natural habitats might buffer pollinator communities from the negative effects of pesticides. Together, these findings highlight the importance of maintaining natural and seminatural habitats on farms for bee population health.

Bioassay

There is mounting evidence of the negative effects of fungicide on host–pathogen dynamics in bees (Pettis et al. 2013, Yoder et al. 2013, McArt et al. 2017b). Yet, it is often unclear whether pathogen and fungicide exposure act independently or in combination to influence bee health and ultimately mortality. Results from our bioassay experiment show that *Ascosphaera* and fungicide exposure impacted larval mortality additively. Although there have been studies on fungicides impacting *Osmia* sp. (Ladurner et al. 2005, Artz and Pitts-Singer 2015), this is the first study to detect additive effects of fungicides and pathogens on *O. cornifrons*. The additive nature of these effects suggests that the fungicide compounds we explored will not, on their own, either inhibit or amplify the impact of *Ascosphaera* on the rate of chalkbrood infection in *O. cornifrons* larvae; but rather, other factors such as those that vary with land use change will have a greater impact on host–pathogen dynamics.

Conclusion

As policies governing pesticides and land use continue to be implemented, it is important to understand how environmental stressors interact to impact host–pathogen dynamics of bees, whose pollination services are essential to maintaining floral diversity and supporting ecosystem function. It is especially necessary that we work to understand the factors impacting solitary bee species, because they have been understudied in comparison to social bee species. Our results provide further evidence for the emerging role of fungicides as a significant factor influencing solitary bee health. Our study also emphasizes the potential for forested landscape surrounding farms to buffer the negative effects of fungicides and pathogens on bee health.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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