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Indirect effects of agricultural pesticide use on parasite prevalence in wild pollinators

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ABSTRACT

Insect pollinators appear to be experiencing worldwide declines, a phenomenon that has been correlated both with exposure to chemical pesticides and disease prevalence. These factors have been found to have strong and often interacting negative effects on multiple pollinator species in laboratory based studies, however their interactions in the field are less clear. To try and understand the link between pesticide use on pollinator communities, and how this might impact on disease transmission, we took two complementary approaches. First, we undertook a series of pollinator surveys to assess the abundance and diversity of pollinator groups across British agricultural field sites subject to varying levels of pesticide use. We then screened the offspring of two taxa of tube nesting solitary bees (*Osmia bicornis* and *Megachile* spp.) for three parasite groups commonly associated with pollinators. We found lower pollinator abundance, group richness and diversity across agricultural sites associated with higher pesticide use. Specifically, there were fewer honey bees, hoverflies, solitary bees and wasps. Surprisingly, we found a lower prevalence of all three parasite groups in *O. bicornis* offspring reared in sites associated with higher pesticide use compared to lower pesticide use. We also found a lower prevalence of *Ascosphaera* but a higher prevalence of Microsporidia in *Megachile* offspring reared in sites associated with higher pesticide use compared to lower pesticide use. Together, our results suggest that agricultural sites associated with higher pesticide use may be affecting pollinators indirectly by disrupting community structure and influencing disease epidemiology and vectoring opportunities. This highlights the importance of understanding the interactions between pesticide use and disease in both managed and wild bee populations for the future mitigation of pollinator declines.

1. Introduction

Animal pollinators provide ecosystem services of environmental, agricultural and economic importance by pollinating an estimated 90% of all plant species, including essential agricultural crops (Kearns et al., 1998). European honey bees (*Apis mellifera*) are often cited as the most valuable agricultural pollinator. However, wild pollinators, such as wild bumblebees (*Bombus* spp), solitary bees, flies, wasps and Lepidoptera appear to pollinate certain (and prevalent) crops such as oilseed rape and orchard fruits more effectively (Velthuis, 2001; Breeze et al., 2011), by for example doubling fruit setting rates compared to the equivalent visitation rate by managed honey bees (Garibaldi et al., 2013). Indeed, wild bees contribute approximately the same value towards crop production as managed bees do (Kleijn et al., 2015). The increasingly evident role of wild insects in crop pollination has led to the suggestion that maintaining both the diversity and abundance of wild pollinators is crucial in meeting the mounting demands on the agricultural industry (Klein et al., 2003; Greenleaf and Kremen, 2006; Hoehn et al., 2008; Winfree et al., 2015). Unfortunately, multiple pollinator taxa are currently experiencing contracting ranges and reductions in species richness (Biesmeijer et al., 2006; Potts et al., 2010). This appears to be the result of a complex interaction between multiple stressors (Goulson et al., 2008; Bacandritsos et al., 2010; Ellis et al., 2010; vanEngelsdorp and Meixner, 2010). Understanding how stressors responsible for pollinator declines interact is therefore a key target both for improving their conservation in the wild and in supporting future global crop production.

A key driver of pollinator decline is believed by many to be the environmental stressors generated via agricultural intensification. For example, habitat fragmentation and landscape homogeneity in large-scale farm systems have been linked to reduced forage and nesting habitats required for wild bees as well as general biodiversity loss (Weibull and Östman, 2003). However, several studies suggest it is the combination of reduced quantity and diversity of flowering plants and

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exposure to high levels agrochemicals that is driving pollinator declines (Nazzi and Pennacchio, 2014; Schmehl et al., 2014; Baude et al., 2016). While significant lethal and sub-lethal effects of certain agrochemicals, such as neonicotinoid insecticides, have been found in laboratory experiments (e.g. Cresswell, 2011; Lundin et al., 2015), there has been less evidence of such detrimental effects on pollinators by field-realistic exposure levels (Rundlöf et al., 2015). Some studies indicate no negative effects (Blacquiere et al., 2012; Nicholls et al., 2017), others indicate inconsistent sub-lethal effects (Woodcock et al., 2017), supporting the idea that prevailing environmental conditions are a key factor determining the lethality of agrochemicals in the field. As of the 1st December 2013, the European Commission initiated a restriction on the application of three major neonicotinoids (imidacloprid, clothianidin and thiamethoxam) on animal-pollinated crops throughout the European Union until there is more conclusive evidence as to whether these pesticides are causing unacceptable pollinator losses (European Commission, 2013). The effect of the memorandum on neonicotinoids is currently under review, but the general consensus remains that farming practises that involve high levels of their use pose a considerable threat to all wild pollinators (Wood and Goulson, 2017). Despite this consensus, the majority of studies on the effects of pesticides on pollinators have focused on honey bees and bumblebees, leaving a gap in knowledge on the effects of agrochemicals on wild pollinators (Blacquiere et al., 2012; Thompson, 2010; FERA, 2013; Lundin et al., 2015; Wood and Goulson, 2017).

Several studies have also correlated pollinator declines with the spread of pathogens and parasites (Goka et al., 2001; Otterstatter and Thomson, 2008; Meeus et al., 2011; Arbetman et al., 2012; Szabo et al., 2012). Again, the focus of research has largely centred on honey bees, and to a lesser extent bumblebee species. However, honey bees are generalist pollinators, which share their foraging sites with wild pollinators (Hudewenz and Klein, 2015). They are host to more than 70 different parasites (Morse and Flottum, 1997), and provide a significant reservoir of disease and potential for inter-species transmission, for example though shared flower patches (Graystock et al., 2015a). Indeed, several non-*Apis* UK pollinator species have been associated with a multitude of 'traditional' honey bee parasites (Evison et al., 2012; Fürst et al., 2014; Tehel et al., 2016; Villalobos 2016). Disease associations between honey bees and bumblebees (Fürst et al., 2014), and parasite spillover between commercially reared and wild pollinators (Graystock et al., 2013; Tehel et al., 2016) together suggest that inter-species transmission and/or novel vectoring routes are exacerbating the effects of disease driven pollinator decline. For example, co-infection in bumblebees by their neogregarine parasite *Apicystis bombi* and deformed wing virus (DWV), which is usually associated with honey bees, were shown to severely increase mortality (Graystock et al., 2015b). Damaging epidemics resulting from parasites switching between honey bee species, such as *Varroa destructor* (Mondet et al., 2014; Wilfert et al., 2016) and *Nosema ceranae* (Natsopoulou et al., 2015), are well documented and have taught us a great deal about emerging infectious diseases (EIDs) of honey bees, but their interactions with non-*Apis* species requires much more investigation.

The way in which parasites and pesticides interact may be a key reason for the contrasting results of studies investigating the effect of pesticides on pollinator health (e.g. Woodcock et al., 2017). Laboratory studies consistently suggest that exposure to pesticides increases the susceptibility of honey bees to disease, increasing mortality (e.g. Vidau et al., 2011; Wood and Goulson 2017), as well as causing harmful sub-lethal effects such as a reduced ability to sterilize colony and brood food (e.g. Alaux et al., 2010). There have also been reports of some insecticides, such as the carbamate Carbofuran, and the organophosphate Dimethoate, reducing the peak larval weights of honey bee larvae (Davis et al., 1988), which may have knock on effects in terms of immunocompromisation of adult honey bees (Yearsley et al., 2004). When adult workers of social species of bee are immunocompromised through exposure to pesticides, an increased susceptibility to disease, particularly to those that are commonly spread through shared foraging patches (Pettis et al., 2012, 2013; Wu et al., 2012), is likely to exacerbate its spread. For example, long range generalist foraging habits of honey bees, and high levels of intra-colony transmission predispose social species like these as superspreaders of disease, particularly if those hosts are already infected with other parasites (Vidau et al., 2011). Consequently, synergistic interactions between emerging infectious diseases (Natsopoulou et al., 2015) and pesticide exposure (e.g. Doublet et al., 2015) are likely to have serious consequences for wild pollinators such as solitary bees, but there is a dearth of information on how these factors might interact in wild populations.

Based on this information, here we aimed to start to disentangle the mechanisms underlying the documented pollinator declines by assessing, first, how differing levels of agricultural pesticide use impacts on the abundance, diversity and reproductive success of populations of British pollinators, and second, how this might influence the prevalence of parasites across wild bees in the same populations. We assessed the effect of level of pesticide use on wild pollinators using field surveys to measure general pollinator abundance, group richness and diversity. As an additional measure to the flying pollinator activity, we also measured the reproductive success of tube-nesting pollinator species, and the larval weight of their offspring (as an indicator of stable development and the production of healthy adults; Bosch and Vicens, 2002). Collecting tube-nesting pollinators as a method of assessing pollinator biodiversity is useful because they provide a small, interacting and reproducing community within the wider pollinator community (Tscharntke et al., 1998), and provide a more robust assessment of the local pollinator community than flying insect surveys alone can. We then measured the prevalence of three parasites previously associated with pollinators (Evison et al., 2012) across the same landscape, using tube-nesting solitary bees of the genus *Megachile* as a consistent way to sample the environment. These bees share a similar ecological niche to honey bees, as generalist pollinators (Hudewenz and Klein, 2015), so are a useful tool for detecting inter-species disease transmission across pollinator communities. Considering the potential impact of parasites on pollinator health, a deeper understanding of how pesticide use influences their prevalence in wild pollinators is invaluable.

2. Materials and methods

2.1. Field site selection and method overview

Twenty-three agricultural sites across Cambridgeshire and East Anglia were used in the study (Fig. 1), which was performed during 2012. This set of sites were selected from a larger database of field sites (Fig. S1) originally identified by the IPI AgriLand project (Linking agriculture and land use change to pollinator populations, BB/I000364/1; Supplementary material Section S4; Gillespie et al., 2017). The farms in this database are a randomised selection of farms that were chosen to encompass variation in four specific variables thought to be important in driving pollinator declines, yet were otherwise comparable (Gillespie et al., 2017). These variables were pesticide use, habitat diversity, floral resource availability, and managed honey bee colony density (see Gillespie et al., 2017 and Supplementary materials, Section S3 for specific details on how these were calculated). From the farms in the Cambridgeshire and East Anglia regions of this database, we selected the 23 sites used in this study from conventional farms only, based on their pesticide use figure. Pesticide use was estimated based on information from the UK Pesticide Survey, and was calculated by multiplying areas of different crop cover by recommended insecticide application, weighted by toxicity to honey bees (Supplementary materials, Section S4.1). We chose sites that differed in extremes of their pesticide use, and categorised 13 sites as high and 10 as low pesticide use, based on whether their estimated pesticide application levels fell above or below the mean pesticide use estimation figure (detailed in Tables S1.1.1 and S1.2). We used a series of survey protocols to assess

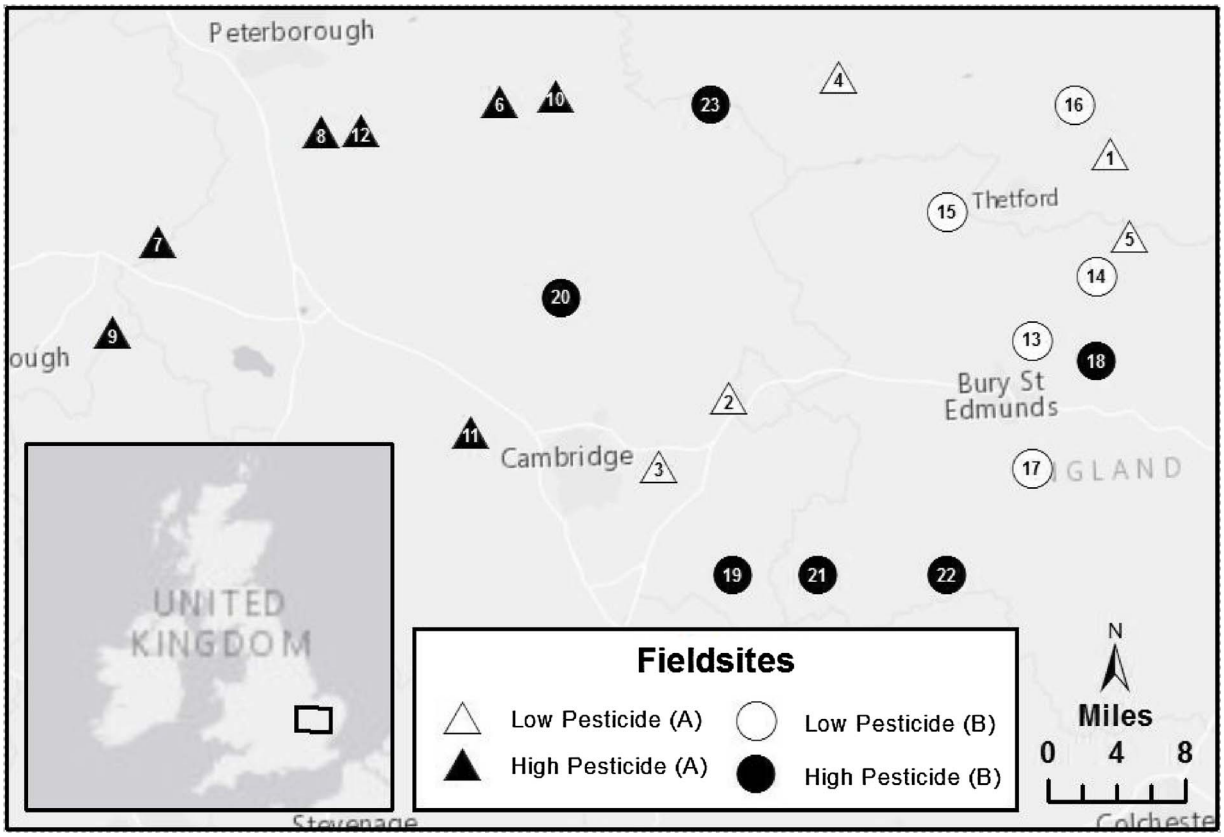

Fig. 1. The location of the 23 field sites across Cambridgeshire and East Anglia (inset map shows the location within UK). Group A sites (detailed in Table S1.1) are represented by triangular markers, and Group B sites (detailed in Table S1.2) are represented by circular markers. Sites associated with low pesticide use are represented by open markers, and high pesticide use by filled markers.

abundance, richness and diversity of pollinators (Section 2.2) at 12 of the sites (which we refer to as Group A sites; Fig. 1, Table S1.1.1). At these sites, land owners were surveyed to qualitatively assess the types of pesticide they were using (Table S1.1.2), and abiotic conditions were recorded during flying pollinator surveys, and local flowering plant surveys were taken in the immediate area surrounding survey sites, both of which were included as co-variates in analyses on the effect of the level of pesticide use (high or low) on local pollinator abundance, richness and diversity. We used a separate sampling protocol to assess the prevalence of parasites amongst two species of tube-nesting bees (Section 2.3) at the remaining 11 sites (which we refer to as Group B sites; Table S1.2). No local information was recorded at these sampling sites, but the remaining three landscape scale variables provided by the AgrilLand data set (Gillespie et al., 2017) that were associated with each site (habitat diversity values derived from land cover maps [Section S4.2], floral resource availability calculated from published values of nectar production [Section S4.3], and honey bee colony density estimated from UK Governmental 'BeeBase' records [Section S4.4]), were instead used as co-variates in analyses on the effect of pesticide use (high or low) on parasite prevalence.

2.2. Pollinator and flowering plant surveys (Group a sites)

We used a series of surveys to assess how the abundance, group richness and diversity of pollinators across the Cambridgeshire and East Anglia area differed across sites associated with high and low pesticide use. Flying insect surveys allowed us to assess local pollinator presence, and placement of tube-nests (fig. S2) around the sites allowed us to assess the reproductive success of a variety of species of solitary tube-nesting species across these sites by providing nesting cavities to collect their brood. The tube-nest arrays consisted of 33 cardboard tubes of five different aperture sizes (4, 5, 6, 8 and 10 mm diameter) which accommodate multiple nesting species. During May, three tube-nests were placed at each of the 12 Group A sites and were collected in July. This time period allowed for an adequate assessment of species with variable breeding season lengths to be collected. Between placement and retrieval, tube-nests were left undisturbed, apart from two monitoring visits, during which flying pollinator surveys were conducted. The monitoring visits were approximately 18 days apart, but were adjusted to correspond with the most suitable weather to observe pollinator foraging activity, including low wind speeds and minimum mean daily temperatures of 13 °C (Pollard and Yates, 1994). Flying pollinator surveys were conducted by taking counts of all bumblebees, honey bees, hoverflies, lepidopterans, solitary bees and wasps that were observed foraging within a 1 × 5 m area surrounding the tube-nest during a 20-min period (Brittain et al., 2010a). Temperature, wind speed and a 'weather' variable (weather conditions were classed as either raining, overcast or sunny) were also recorded. Counts were taken while the surveyor stood in a location that allowed the area surrounding the tube-nest to be observed in all directions. Recorded pollinators were categorised into the six groups using Field Identification Guides (O'Toole and Shields, 2007), and those that could not be identified on site were captured, photographed and stored in ethanol for categorisation later. The species richness of animal-pollinated flowering plants within the same 1 × 5 m area was also surveyed (Ebeling et al., 2008). After 72 days in the field, all these Group A tube-nests (36) were removed from the field sites and returned to the lab to assess the reproductive success of the species using the tube-nests, by counting the number of developing brood items and calculating their peak larval weight (calculations described in Section 2.4).

The tube-nests were dismantled in the lab. The inner cardboard tubes were removed from the outer structure and any occupied

cardboard tubes were dissected to reveal the brood cells. Developing brood were removed from the brood cells using soft forceps and placed individually in Petri dishes, along with any remaining food provisions and a sample of the partitioning material constructed by the insect. Weight measurements were taken following a similar protocol to Bosch and Vicens (2002): first, an empty 1.5 ml Eppendorf tube was weighed using a high-precision Mettler Toledo AX26 DeltaRange microbalance and the egg, larva, pupa or cocoon was then added to the Eppendorf tube. If present, the remaining food provisions from the brood cell were added. From these measurements, it was possible to calculate individual weights for the brood and remaining food. Once weighed, the Eppendorf tubes containing the brood and remaining food were pierced to provide an air hole and stored in a temperature-controlled room at 24 °C (Abbott et al., 2008) to continue development into adulthood in case of further need of identification.

2.3. Molecular screening for parasites (Group B sites only)

Alongside our surveys of the Group A sites, a separate sampling protocol was used to assess how different levels of pesticide use might affect disease transmission amongst the same populations of pollinators. To do this, a separate set of tube-nest arrays were placed at the 11 Group B sites. These arrays consisted of a single cardboard tube size (8 mm) and each were seeded with 10 pupae of the Megachilid solitary bee species *Osmia bicornis*. Megachilid bees show natal nest preference (e.g. Pitts-Singer, 2007), so this technique allowed us to use the bees to sample the environment for any parasites that they might acquire via their natural foraging for nectar and pollen, which they collect to mass provision their offspring. This way we could assess the prevalence of parasites picked up during foraging (i.e. via a horizontal transmission route) and spread amongst their offspring (i.e. via a vertical parasite transmission route). Being generalist pollinators (Hudewenz and Klein, 2015), this meant we were effectively sampling their entire foraging range (~2 km diameter around each tube-nest (Gathmann and Tscharntke, 2002)). During April, four tube-nests were placed at each of the 11 Group B sites and collected in September. This time period maximised our sampling over the breeding season of Megachilid bees. These 44 tube-nests were left undisturbed the entire time they were in the field, and upon retrieval were stored at 4 °C for subsequent parasite screening (detailed below). Despite being seeded with *Osmia bicornis,* some tube-nests attracted other solitary tube-nesting species. The two solitary bee taxa that were collected most frequently and consistently from the surveyed Group A sites were *O. bicornis* and a *Megachile* leaf-cutting bee spp. (see Results), the parasite screen was therefore performed only on these two groups. This also removed bias in low numbers of hosts per species, which may have skewed our assessment of parasite prevalence (Jovani and Tella, 2006). All the developing *O. bicornis* and *Megachile* individuals extracted from the Group B tube-nests were first weighed to assess if level of pesticide use in the area they were reared may have influenced larval development. The *O. bicornis* offspring (which overwinter as pupae) had their entire abdomen removed. The abdomen only was used to extract DNA for screening as the parasites being assessed in this study were most likely to be found in the gut (Evison et al., 2012). The *Megachile* offspring overwinter as larvae, so the entire body was used to extract DNA for screening.

We screened each individual for *Wolbachia*, *Ascosphaera* and Microsporidia. *Wolbachia* is a genus of intracellular bacteria that is thought to infect over half of all insect species (Hilgenboecker et al., 2008) and has the potential to disrupt the colony dynamics for social bees and population dynamics for solitary bees by manipulating the sex ratios of its hosts, or by negatively affecting host survival (Werren, 1997). *Ascosphaera* and Microsporidia are commonly associated with bees, particularly honey bees, and have been implicated in colony losses across the globe (e.g. Cox-Foster et al., 2007; Higes et al., 2009). *Ascosphaera apis* is an obligate fungal brood parasite of *Apis mellifera*, causing a common disease known as chalkbrood (Aronstein and Murray, 2010), but solitary bees are also associated with *Ascosphaera* infections (Anderson et al., 1998). The Microsporidia include the genus *Nosema,* which causes dysentery in the workers of several bee species (Paxton et al., 1997; Otti and Schmid-Hempel, 2007; Plischuk et al., 2009), and important EIDs such as *Nosema ceranae* (Fürst et al., 2014).

The sample was homogenized and total DNA and RNA was extracted in 300 µl 10% Chelex by heating to 95 °C for 20 min and centrifuged for 8 min at 4000 rpm. PCR amplification was carried out using ABI 3700 thermal cyclers in 10 µl volumes containing 1 µl Chelex supernatant, 0.2 µl of each forward and reverse primer, 2 µl PCR buffer and 0.05 µl of 5U/µl Taq (Promega). Reactions contained primer specific quantities of 25 mM $MgCl_2$ and 10 mM dNTPs and made up to 10 µl with ddH_20. To check the quality of the extraction, each sample was amplified at the CO1 gene using *LCO-Hym/HCOout* primers (Folmer et al., 1994; Prendini et al., 2005) with 1.5 µl $MgCl_2$ and 1 µl dNTPs, with an initial denaturation of 2 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 45 s at 50 °C and 2 min at 72 °C, and a final extension step of 72 °C for 7 min. All extractions that amplified successfully were then screened for the presence/absence of 1) *Ascosphaera* using the *AscoAll1/AscoAll2* primers (James and Skinner, 2005) with 1 µl $MgCl_2$ and 1.5 µl dNTPs, with an initial denaturation of 10 min at 94 °C followed by 30 cycles of 45 s at 94 °C, 45 s at 62 °C and 1 min at 72 °C, and a final extension step of 72 °C for 5 min. 2) Microsporidia using the *V1f/530r* primers (Terry et al., 2004) with 1.5 µl $MgCl_2$ and 0.5 µl dNTPs, with an initial denaturation of 1 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C, and a final extension step of 72 °C for 7 min. 3) *Wolbachia* using *CoxA f/r* primers (Baldo et al., 2006) with 1 µl $MgCl_2$ and 1 µl dNTPs, with an initial denaturation of 2 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 45 s at 55 °C and 2 min at 72 °C, and ending with a final extension step of 72 °C for 7 min. PCR products were visualised under UV using 1% agarose gels stained with ethidium bromide and compared to a 100 bp size ladder. Positive and negative controls were included in every PCR.

2.4. Statistical analyses

All statistical analyses were performed using R v3.1.3 (R Core Team, 2013) and all averages reported are mean ± standard error. We used mixed models that allowed us to account for sample size bias and complex structuring within the data set (Paterson and Lello, 2003). All the fixed effects within the models were assessed using stepwise model comparisons from the full model to assess their importance for the model fit, but the final significance effect of pesticide level reported is derived from the full model including all the fixed terms (no interactions). Supplementary material (Section S3) lists details and results of every test performed.

During surveys performed at the Group A sites, fewer pollinators were recorded on survey days where rainy conditions prevailed compared to survey days when overcast and sunny conditions prevailed ($\chi^2_2 = 22.9$; $P < 0.001$). As such, any data collected during rainy conditions were removed prior to performing statistical analyses. This left a total of 90 pollinator surveys (30 surveys at low pesticide sites and 60 surveys at high pesticide sites) across the three visits. The Simpson's index was used to calculate a pollinator diversity value for each site, Simpson's $[1\text{-}]D = 1\text{-}\Sigma(n/N)^2$, where n is the abundance of a specific pollinator group, and N is the abundance of all pollinators per site. Simpson's D was analysed using a linear mixed effects model implemented using the *lmer* function, and pollinator group richness and abundance were both analysed using generalised linear mixed effects models implemented using the *glmer* function, fitted with a Poisson error distribution, both from the lme4 package (Bates et al., 2007). Visit number nested within Site ID was included as a random effect to account for the repeated surveys taken from each tube-nest across the three visits. We were interested in understanding the effect of the categorical variable pesticide use level (low or high) associated with the sites on our pollinator abundance, diversity and richness measures, but

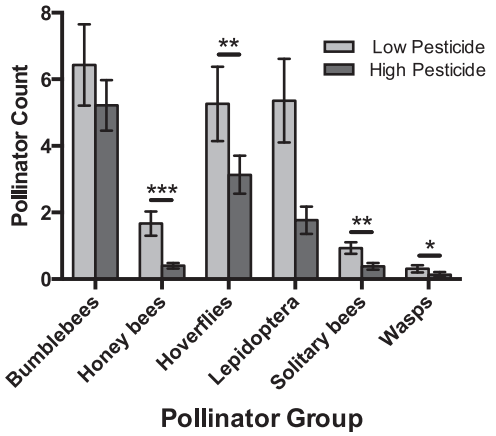

Fig. 2. Mean (± S.E.) abundance of pollinators in each pollinator group across sites associated with low (pale grey) and high (dark grey) pesticide use. Asterisks indicate statistical significance of: * = P < 0.05; ** = P < 0.01; *** = P < 0.001.

the categorical variables pollinator group (only in the abundance model) and weather (sun or overcast), and the continuous variables temperature (°C), plant species richness and wind speed (m/s) were all included as fixed effects. These analyses showed higher pollinator group richness ($\chi^2_1 = 9.60$, $P = 0.002$) and diversity ($\chi^2_1 = 6.38$, $P = 0.012$), during warmer temperatures. However, there were no effects of weather, plant diversity or wind speed in either model (Table S3). There were higher overall levels of pollinator visitations observed at sites with a higher plant diversity ($\chi^2_1 = 58.88$, $P < 0.001$). Wind, weather and temperature did not have any overall effects on pollinator abundance (Table S3). Because our model of overall pollinator abundance showed a significant interaction between pesticide use and pollinator group ($\chi^2_5 = 48.17$, $P < 0.001$), we then used the same generalised linear mixed effects model structure to assess abundance within each pollinator group (i.e. bumblebees, honey bees, hoverflies, lepidopterans, solitary bees and wasps) separately. The importance of temperature, plant diversity, wind speed, and weather for explaining the effect of pesticide use varied by pollinator group (Table S3).

For tube-nests collected from the Group A sites only, a generalised linear mixed effects model implemented using the *glmer* function fitted with a Poisson error distribution was used to analyse the effects of pesticide level on tube-nest occupancy rates (i.e. how many inner cardboard tubes contained brood, per tube-nest). We fitted tube size as a fixed factor to assess whether there were differing occupancy rates per cardboard tube size, and tested for its interaction with pesticide use level (because differences in developing brood numbers between different tube sizes might indicate differing effects of pesticide use on different species collected). To circumvent the effect of differences in larval age when assessing the effect of pesticide use level on larval weight, linear regressions were used to produce coefficients from the relationship between larval weight and the weight of the unconsumed food provisions. These coefficient values represent the Feed Conversion Efficiency (FCE) and were produced for each species recorded nesting within the tube-nests. Estimates of FCE were similar for all species and agreed with published estimates of the FCE for the solitary bee *Megachile pacifica* that are between 38.5% and 58.5% (Wightman and Rogers, 1978). The species-pooled mean FCE was 40.8% and this value was applied to all species. The remaining food of any individual larvae that still had food provisions upon collection was multiplied by the FCE and added to the larval weight to produce a projected peak larval weight. The residuals of these projected larval weights exhibited a normal distribution and were compared between low and high pesticide use sites for the two species found at both site types using a general linear mixed effect model, with species included as a fixed factor, and here we tested for its interaction with pesticide use level to again identify whether different species differed in their response to pesticide use level. In both analyses we included the plant diversity and pollinator diversity (Simpson's [1-]D) determined from the survey data as fixed effects, and the individual cardboard tube number nested within site ID was fitted as the random effect to account for the non-independence of larvae within these arrays, as they were likely to be siblings.

For the developing *O. bicornis* and *Megachile* spp. collected from Group B sites only, differences in the proportion of hosts testing positive for each parasite between sites of high and low pesticide use was analysed for each host species separately, using a generalised linear mixed effects model implemented using the *glmer* function fitted with a binomial error structure. Here we also included the original variables provided from the Agriland data set as co-variates (honey bee colony density, floral resource availability and habitat diversity; see Supplementary material Sections S4.2–S4.4 for details of how these variables were calculated), because this allowed us to account for how their variation may have influenced parasite prevalence across sites associated with different levels of pesticide use. We also fitted cardboard tube ID nested within Tube nest ID within Site ID as the random effect to account for shared nesting tubes influencing the likelihood of parasite detection. Finally, the weight of the developing *O. bicornis* and *Megachile* spp. were compared between sites of high and low pesticide use using a linear mixed effects model implemented using the *lmer* function, and fitted with the same parameters as above. In these analyses, more *Osmia* tested positive for *Ascosphaera* where floral resource availability was higher ($\chi^2_1 = 7.21$, $P = 0.007$), for Microsporidia where honey bee colony density was lower ($\chi^2_1 = 6.17$, $P = 0.013$), and for *Wolbachia* where habitat diversity was higher ($\chi^2_1 = 5.43$, $P = 0.02$). However, none of these variables were important in detecting parasites in *Megachile*. Again, there was no effect of any of these variables on the weight of cocoons.

3. Results

3.1. Pollinator abundance, diversity and reproductive success

Pollinator abundance ($\chi^2_1 = 19.8$, $P < 0.001$), group richness ($\chi^2_1 = 6.10$, $P = 0.014$) and Simpson's diversity Index ($\chi^2_1 = 4.36$, $P = 0.037$) were all lower across the Group A sites associated with high compared to low pesticide use (fig. 2). The abundance of honey bees ($\chi^2_1 = 21.48$, $P < 0.001$), hoverflies ($\chi^2_1 = 9.00$, $P = 0.003$), solitary bees ($\chi^2_1 = 9.53$, $P < 0.002$), and wasps ($\chi^2_1 = 6.68$, $P = 0.009$) were all lower across sites associated with high compared to low pesticide use. However, there was no difference in the abundance of bumblebees ($\chi^2_1 = 0.46$, $P = 0.496$), or lepidopterans ($\chi^2_1 = 1.82$, $P = 0.178$; Fig. 2) between sites associated with high or low pesticide use.

The average number of tubes occupied by brood within the mixed species tube-nests across Group A sites did not differ ($\chi^2_1 = 0.66$, $P = 0.418$) between sites associated with high (3.17 ± 1.03%) and low (4.04 ± 1.56%) pesticide use, and there was no effect of tube size on the occupancy of tubes ($\chi^2_1 = 8.82$, $P = 0.066$). However, there was an interaction between tube size and site pesticide use level ($\chi^2_1 = 15.05$, $P = 0.005$), which likely reflected differing species composition at sites associated with high and low pesticide use (Table 1) occupying different tube-sizes within the tube-nests. In total, 162 developing brood items from seven different species were removed from the occupied tube-nests (91 high, 71 low; Table 1). Two species were found occupying nests at sites associated with both high and low pesticide use: a potter wasp *Ancistrocerus nigricornis* (5 high, 54 low) and the red mason bee *Osmia bicornis* (3 high, 11 low). Four more species were found only at sites associated with high pesticide use: the leafcutter bees *Megachile willughbiella* (48) and *Megachile centuncularis* (16), the blue mason bee *Osmia caerulescens* (10) and one species of the solitary bee, genus *Hylaeus* (9). One more species was found at only sites associated with low pesticide use: a species of the spider-hunting wasp family *Pompilidae* (6). There was no difference in the mean projected weights of the brood

Table 1
Species occupancy of mixed-species solitary tube-nests placed in low and high pesticide sites.

	Solitary Bee and Solitary Wasp Species						
	Ancistrocerus nigricornis	*Osmia bicornis*	*Megachile centuncularis*	*Megachile willughbiella*	*Osmia caerulescens*	*Hylaeus* sp.	*Pomilid* sp.
Low pest. n (%)	54 (91.53)	11 (78.57)	–	–	–	–	6 (100)
High pest. n (%)	5 (8.47)	3 (21.43)	16 (100)	48 (100)	10 (100)	9 (100)	–
Total n	59	14	16	48	10	9	6
Low pest. expected mass in mg (± SE)	44.89 (2.02)	57.15 (5.96)	–	–	–	–	37.51 (2.24)
High pest. expected mass in mg (± SE)	52.7 (17.34)	57.8 (7.07)	164.01 (11.82)	177.87 (16.37)	70.02 (4.36)	4.47 (0.256)	–

between sites associated with high and low pesticide use, irrespective of species (table S3).

3.2. Parasite prevalence

Host DNA was successfully extracted and amplified in 55 developing *O. bicornis* bees. Of these, 13 tested positive for *Ascosphaera,* 7 tested positive for Microsporidia, and 18 tested positive for *Wolbachia* (Fig. 3a). Overall there were more parasites detected across sites associated with low pesticide use ($\chi^2_1 = 8.57$, $P = 0.003$; Fig. 3a). The proportion of individuals testing positive differed between the three parasite types ($\chi^2_2 = 7.58$, $P = 0.02$; Fig. 3a), but there was no interaction between parasite type and site pesticide use level ($\chi^2_2 = 0.696$, $P = 0.706$; Fig. 3a). There was no difference in the weight of cocoons between sites associated with high and low pesticide use. Individual analyses of each parasite separately backed up the main result and showed more individuals testing positive for *Ascosphaera* ($\chi^2_1 = 4.35$, $P = 0.037$; Fig. 3a), Microsporidia ($\chi^2_1 = 5.85$, $P = 0.016$; Fig. 3a) and *Wolbachia* ($\chi^2_1 = 4.34$, $P = 0.037$; Fig. 3a) across sites associated with low compared to high pesticide use.

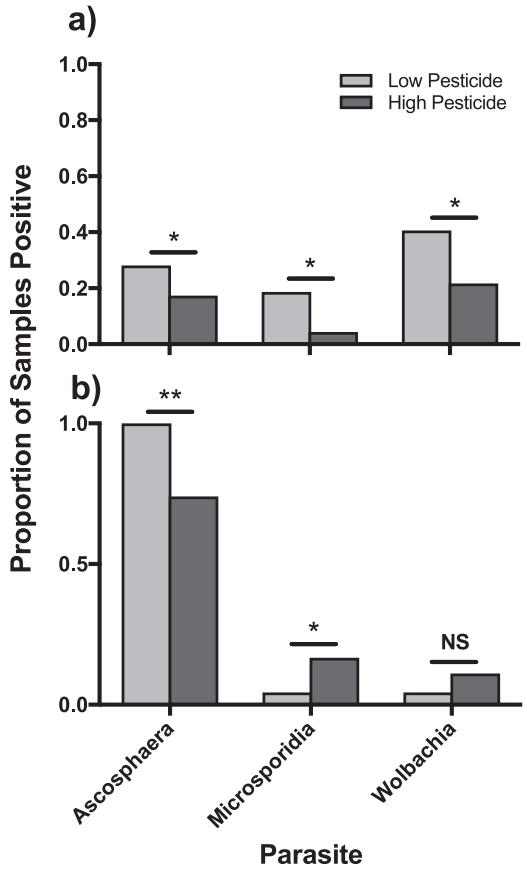

Fig. 3. The proportion of a) *O. bicornis* and b) *Megachile* solitary bees testing positive for each of the three screened parasites, across sites associated with low (pale grey) and high (dark grey) pesticide use. Asterisks indicate statistical significance of: * = $P < 0.05$; ** = $P < 0.01$.

Host DNA was successfully extracted and amplified in 77 developing *Megachile* bees. Of these, 63 tested positive for *Ascosphaera,* 10 tested positive for Microsporidia, and 7 tested positive for *Wolbachia* (Fig. 3b). Overall there was no difference in the proportion of parasites detected across sites associated with high or low pesticide use ($\chi^2_1 = 0.023$, $P = 0.881$), but the proportion of individuals testing positive differed between the three parasite types ($\chi^2_2 = 120.7$, $P < 0.001$; Fig. 3b), and there was an interaction between parasite type and site pesticide use level ($\chi^2_2 = 13.79$, $P = 0.001$; Fig. 3b). Cocoons collected from sites associated with high pesticide use were heavier ($\chi^2_1 = 4.24$, $P = 0.039$). Individual analyses of each parasite separately showed again that more individuals tested positive for *Ascosphaera* across sites associated with low compared to high pesticide use ($\chi^2_1 = 12.34$, $P < 0.001$; Fig. 3b), but in contrast to the *Osmia* findings, more *Megachile* individuals tested positive for Microsporidia in across sites associated with high compared to low pesticide use ($\chi^2_1 = 3.94$, $P = 0.047$). However, there was no difference between high and low pesticide use sites ($\chi^2_1 = 0.01$, $P = 0.917$) in the prevalence of *Wolbachia.*

4. Discussion

Our pollinator surveys support mounting evidence that agricultural sites associated with higher levels of pesticide use exhibit lower pollinator abundance and pollinator group richness and diversity than those associated with lower levels of pesticide use. However, we found no evidence of any detrimental effects of nesting in sites associated with higher pesticide use on the reproductive effort in terms of brood numbers or projected larval weight of multiple solitary species of pollinator, including *O. bicornis*. Contrary to what we expected, our parasite screen of developing solitary bees revealed that the prevalence of *Ascosphaera* fungal parasites amongst both *O. bicornis* and *Megachile* spp. was lower in agricultural sites associated with higher levels of pesticide use compared to those associated with lower levels of pesticide use. In *O. bicornis* the prevalence of both Microsporidia and *Wolbachia* also followed this pattern, however a different pattern was found for *Megachile* spp. with more Microsporidia detected at sites associated with higher levels of pesticide use, and no difference in the prevalence of *Wolbachia.* Our results together suggest that when it comes to parasite prevalence, the indirect effects of pesticide use in an agricultural area, via impacts on pollinator population abundances, dynamics and vectoring (i.e. ecological effects on disease transmission), may be more important than the direct detrimental effects of rearing offspring in areas of high pesticide use, highlighting an important interaction that may be contributing to pollinator declines.

Our results corroborate similar studies that have found a negative relationship between pesticide use and pollinator abundance, richness

and diversity (Alston et al., 2007; Brittain et al., 2010b; Biesmeijer, 2012; Rundlöf et al., 2015). The interaction between the level of pesticide use and pollinator group abundance suggests that the pollinator groups we assessed were affected differently by the level of pesticide use associated with an agricultural area. Honey bees, solitary bees, hoverflies and wasps were more abundant in sites associated with lower pesticide compared to higher pesticide use, whereas the abundance of bumblebees and lepidopterans was affected not by the level of pesticide use associated with the area, but instead by the weather and local plant species diversity at the time of the survey. Although our site selection protocol, and inclusion of abiotic and landscape variables in our analyses aimed to limit site bias in our data collection, it remains important to emphasise that areas of low pesticide application rate are still likely to differ in a variety of aspects to areas of high pesticide application rate, despite our site selection protocol and use of landscape variables in our analyses to attempt to control for this effect, our results must be interpreted within this context. For example, factors that farmers must consider before deciding on a growing system, such as water content in root zones, soil type and microclimate may differ, which will affect the structure and abundance of (particularly floral resources in) semi-natural habitats at a local scale, all of which will influence both the crop that farmers choose to grow (and in turn the type and level of pesticide applied), and how attractive the area is to different types of pollinator. This, in part, is reflected in the pattern of how our sampling sites fell across the Cambridgeshire and East Anglia area, with some spatial clustering of higher or lower pesticide use areas across the landscape. Despite this caveat, we believe our findings show an important and underappreciated aspect the drivers of pollinator decline that requires further attention.

Despite the visitation surveys revealing differences in pollinator abundance between agricultural sites associated with differing levels of pesticide use, no differences were found in the occupancy rate of the mixed-species tube-nests, which suggests that the level of pesticide residue on nearby crops and wildflowers has little impact on nest site selection, at least for the species we recorded occupying the tube-nests. However, the differences in species composition between agricultural sites associated with differing levels of pesticide use could suggest more subtle effects of pesticide use in the local area on nest site preference. For example, *O. bicornis* were more prevalent at sites associated with low pesticide use and *Megachile* spp. were only found at sites associated with high pesticide use when sampled using the mixed-species tube nests. Of the species collected nesting within the tube-nests at sites associated with both high and low pesticide use (*A. nigricornis* and *O. bicornis*), there were no differences in their mean projected peak larval weights. Indeed, the *Megachile* spp. cocoons collected using the single size tube-nests (for parasite screening at the Group B sites) were heavier in sites associated with high compared to low pesticide use. This is in line with previous studies that propose there are no significant sub-lethal effects of pollen contamination by pesticides at field-realistic doses on the development of solitary bees (Abbott et al., 2008; Nicholls et al., 2017). Other studies have reached similar conclusions for bumblebees (Franklin et al., 2004; Woodcock et al., 2017) and honey bees (Cutler and Scott-Dupree, 2007; Cutler et al., 2014). There is also some evidence to suggest that the use of pesticides on farms can have a positive effect on reproductive success in solitary bees. For example, Williams and Kremen (2007) found that *O. lignaria* produced and provided for more offspring on farms using pesticides compared to farms not using pesticides, as long as they had access to floral resources from semi-natural habitats. The use of some pesticides could therefore be affecting population dynamics in subtler ways by influencing nest site preference and provisioning rates. For example, if pollen availability is higher due to fewer pests or competitors, that might have a beneficial effect on the reproductive success of pollen foragers, particularly species such as *Megachile* spp. that use leaf material to line their brood cells. However, brood weight is not the only viable indicator of stable and healthy development. It is therefore important that the effect of field-realistic levels of pesticide use on larval or pupal mortality, or other factors such as pupal head width and development time, is investigated across multiple taxa in response to multiple pesticides to understand whether these effects occur through direct toxicity or via more complex behavioural pathways.

We found evidence that the prevalence of *Ascosphaera* fungal parasites amongst both *O. bicornis* and *Megachile* spp. was lower in agricultural sites associated with higher pesticide use compared to those associated with lower pesticide use. Hosts and vectors of *Ascosphaera* include honey bees, hoverflies, solitary bees and wasps (Evison et al., 2012; Wynns et al., 2013). Considering that our surveys showed that the abundance of all these groups were lower across agricultural sites associated with higher pesticide use, this suggests that such sites support more limited vectoring opportunities for some parasites and pathogens. The prevalence of Microsporidia and *Wolbachia* also followed this pattern in *O. bicornis*, but interestingly the pattern was not the same for the *Megachile* spp. with higher prevalence of Microsporidia across sites associated with higher pesticide use, and no difference in prevalence of *Wolbachia*. This suggests that the biology of the host, rather than these parasites may be more important in influencing their vectoring patterns. Microsporidia can cause nosemosis, a form of dysentery, in their hosts, and sub-lethal exposure to neonicotinoids increases the susceptibility of honey bees to the microsporidion *Nosema ceranae* (Pettis et al., 2012, 2013; Wu et al., 2012) and causes increased mortality in individuals already infected with *N. ceranae* (Vidau et al., 2011). Ladas (1970) found a similar interaction between the presence of *N. ceranae* spores in honey bees and the insecticide dichlorodiphenyltrichloroethane (DDT). This might explain the higher prevalence of Microsporidia in *Megachile* spp. in sites associated with higher pesticide use; they will be foraging for leaf material to line their nests, which is more likely to be contaminated with Microsporidia spores. Even if honey bee abundance is lower in areas associated with higher pesticide use, a higher potential for horizontal transmission due to a change in disease pathology would negate the lower vectoring potential as a result of there being fewer hosts. Similarly, *Wolbachia* is thought to primarily transmit vertically (Werren, 1997), however recent evidence suggests common horizontal transmission routes in Lepidoptera (Ahmed et al., 2016). Our surveys suggested that Lepidoptera abundance was not influenced by the level of pesticide use associated with the area, again suggesting that the higher incidence of *Wolbachia* in Megachile cocoons (relative to *Osmia*) from higher pesticide use sites could be due to transmission via leaf foraging. All three of the parasites screened for in this study have been found in bumblebees (Evison et al., 2012; Blaker et al., 2014), which were the most commonly observed pollinator group during our surveys and are likely to be acting as important hosts and/or vectors of many parasites (Graystock et al., 2015a). Co-infection by these parasites is known to exacerbate disease outbreaks in honey bees (Hedtke et al., 2011) and co-infection by other parasites can cause increased virulence effects in bumblebees (Graystock et al., 2015b). Despite the evidence that pesticides also compound disease virulence in some pollinators (Vidau et al., 2011; Pettis et al., 2012, 2013; Wu et al., 2012) the complex interaction between co-infection, pesticide effects on virulence, and host mortality influencing vectoring opportunity is vastly underappreciated, particularly in wild pollinators.

Our results highlight the complex nature of the interactions between diverse stressors on pollinator health, however they do not resolve targets for action. Agricultural sites associated with higher pesticide use appeared to support a reduced abundance of some pollinator groups, which may result in reduced or altered vectoring opportunities for parasites of those pollinators. Laboratory studies that show increased virulence of parasites, and higher mortality of hosts after pesticide exposure (Alaux et al., 2010; Aufauvre et al., 2012; Pettis et al., 2012; Wu et al., 2012) do not determine how pesticide exposure influences the biological relationship between virulence and transmission. The level of pesticide use in an area is likely to act as an indicator of how

agricultural land use practises in general are augmenting disease associations in wild pollinators. But understanding the precise mechanisms involved in how pesticide use influences natural parasite transmission routes requires field (or semi-field) studies that incorporate natural foraging by pollinators. If direct exposure to pesticides increases the susceptibility to parasites, a consequent higher mortality will lead to reductions in detectible infections as fewer bees survive to provision their nests. Again, how this influences parasite virulence in wild populations, and the subsequent impact on the number of foundresses surviving to provision nests is unknown. Our results do not allow us to separate out these effects; because our methods relied on collecting pollinators healthy enough to fly and provision a nest, the results are therefore skewed towards collecting either benign infections or more resistant hosts; virulent infections would remove hosts from the sampling pool. The results of our farmer questionnaires (Table S1.1.2) showed that fungicides and organophosphates were only applied in the high pesticide sites, and neonicotinoids were more frequently applied, which again introduced an element of bias in our data collection because the effects of pesticide exposure on parasite virulence and transmission may differ between functional types of chemicals. For example, fungicides may directly kill fungal pathogens such as *Ascosphaera* and Microsporidia present on forage (Parker, 1984). Pesticides can also target different life stages of insects and the application of larval-targeted pesticides to adults may produce skewed results of lethal and sub-lethal effects (Cutler and Scott-Dupree, 2007); such as the fungicide Captan, which was previously thought to be relatively harmless to honey bees but has been found to have lethal effects on larvae at the recommended field dose (Mussen et al., 2004). In addition, the toxicity of some agrochemicals varies with body size, surface-area-to-volume ratio and mass-specific metabolic rate, so larger bees such as bumblebees will be affected differently to smaller bees such as *Hylaetus* (Valdovinos-Nunez et al., 2009). The mounting evidence that pesticides and fungicides may affect pollinators of different sizes and life-stages differently underlines the importance of acquiring data regarding dissimilarities in risk factors for pollinator groups to better inform policy makers about the impact of pesticides and parasites on non-*Apis* pollinators. The mechanisms behind the patterns found in this study and others urgently require more attention, particularly with regards to understanding how the synergistic effects of multiple agrochemical use and multiple parasite infections play out in the field via large-scale surveys, on both managed and wild non-*Apis* pollinators.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.agee.2018.02.002.

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