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Individual and combined effects of resource and pesticide stressors on wild bees and a potential strategy to mitigate impacts

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Individual and combined effects of resource and pesticide stressors on wild bees and a potential strategy to mitigate impacts

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DISSERTATION

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Abstract

Anthropogenic environmental changes present multiple stressors that together impact biodiversity and ecosystem function. Among these, pesticide exposure and the loss of flowering plants are ubiquitous across contemporary landscapes and threaten the persistence of bee populations. In this dissertation, I explored the individual and combined effects of pesticide and floral resource stressors on bee behavior, reproduction, and population persistence, as well as a potential strategy for mitigating these impacts. I used a combination of manipulative field cage experiments and landscape studies to examine these stressors and their impacts at different scales.

Because bees often experience pesticide and resource stressors simultaneously, I first examined the potential for interactive effects of these stressors, as well as their individual impacts on wild bees. I established a fully crossed design in field cages; nesting female *Osmia lignaria*, the solitary blue orchard bee, accessed wildflowers at high or low densities, treated with or without the common insecticide, imidacloprid. In **Chapter 1**, I showed that pesticide exposure and floral resource scarcity combined additively to dramatically alter multiple vital rates, including reduced reproduction and a male-biased offspring sex ratio. In **Chapter 2**, I quantified behavioral responses in the same experiment, revealing that the resource and pesticide stressors had differential impacts with consequences for bee populations and potentially for pollination services through individual behavioral changes. Limited floral resources required bees to make fewer, longer foraging trips as well as misidentify their nests more often upon return from these trips. Bees exposed to pesticides made shorter foraging trips and did not compensate for this by

taking more trips, reducing their overall foraging activity. Pesticide exposure also interacted with age to affect antagonistic behavior.

In **Chapter 3**, I examined the carryover effects of past pesticide exposure on wild bees. Using the offspring from the previous cage experiment with known pesticide exposure backgrounds, I re-established the field cages and released bees in a crossed design with pesticide exposure or no exposure in each year. Thus, some bees experienced pesticides over two generations and others not at all. Regardless of the past exposure history, pesticides in the second year reduced reproduction. For bees that were also exposed in the past, the exposure over two years additively impaired individual performance, leading to a nearly fourfold estimated reduction in bee population growth. Furthermore, even past exposure by itself, regardless of exposure in the second year, led to a decline in offspring production.

In **Chapter 4**, I collaborated with Maj Rundlöf to investigate the potential for wildflower plantings to mitigate the negative effects of pesticide exposure in agricultural landscapes. We assessed the nesting and reproduction of *O. lignaria* and the bumble bee *Bombus vosnesenskii* in replicate agricultural landscapes, half of which contained a wildflower planting next to the nest or colony. We collected pollen from foraging bees to determine resource use and pesticide residues. The wildflower plantings were a source of pesticide exposure, especially for *O. lignaria*, but also supported *O. lignaria* nesting. The landscape-level floral resources better predicted *B. vosnesenskii* colony success, but the local flower resources mitigated the negative effects of pesticides on their reproduction.

These chapters together show that two common environmental stressors combine to negatively impact bees. They also reveal potential mechanisms underlying impacts of the stressors on reproduction and population growth. My dissertation highlights the importance of mitigating the negative effects of pesticides and floral resource limitation, especially in agricultural landscapes where the two stressors often co-occur. Finally, this work offers insight into how the stressors could be mitigated through an emerging strategy to diversity agricultural landscapes.

Contents

Chapter 1. Pesticide and resource stressors additively impair wild bee reproduction

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Abstract

Bees and other beneficial insects experience multiple stressors within agricultural landscapes that act together to impact their health and diminish their ability to deliver the ecosystem services on which human food supplies depend. Disentangling the effects of coupled stressors is a primary challenge for understanding how to promote their populations and ensure robust pollination and other ecosystem services. We used a crossed design to quantify the individual and combined effects of food resource limitation and pesticide exposure on the survival, nesting, and reproduction of the blue orchard bee *Osmia lignaria*. Nesting females in large flight cages accessed wildflowers at high or low densities, treated with or without the common insecticide, imidacloprid. Pesticides and resource limitation acted additively to dramatically reduce reproduction in free-flying bees. Our results emphasize the importance of considering multiple drivers to inform population persistence, management, and risk assessment for the long-term sustainability of food production and natural ecosystems.

Introduction

Agricultural intensification is a primary driver of global insect declines (Ollerton et al. 2014, Sánchez-Bayo and Wyckhuys 2019). This intensification has led to a loss of flowering plants and widespread pesticide use that impact pollinators and other beneficial insect populations, diminishing their ability to deliver ecosystem services critical to human food supplies (Kremen et al. 2002, Potts et al. 2010, Bommarco et al. 2012, Chagnon et al. 2015, Stanley et al. 2015a, Sánchez-Bayo and Wyckhuys 2019). Disentangling the effects of simultaneous flowering resource scarcity and pesticide exposure is a primary challenge for understanding how to mitigate ongoing pollinator declines and develop strategies for the long-term sustainability of our food systems (Goulson et al. 2015).

The effects of individual stressors on beneficial insects have been documented. For instance, limited floral resources, and resulting poor nutrition, reduces fecundity, longevity, and stress resistance (Bommarco 1998, Kim 1999, Alaux et al. 2010, Goulson et al. 2015). Pesticide exposure can directly kill beneficial insects or cause sublethal effects that reduce reproduction and impair behavior (Gill et al. 2012, Rundlöf et al. 2015, Müller 2018, Crall et al. 2018). However, there remain significant knowledge gaps on the interactive effects of combined stressors. There is evidence that stressors have additive, synergistic, and/or antagonistic interactions through physiological mechanisms (Schmehl et al. 2014, Tosi et al. 2017b), behavioral responses (Stanley and Raine 2016, Crall et al. 2018), and demographic changes (Ulbrich and Seidelmann 2001, Rundlöf et al. 2015, Dance et al. 2017; Figure 1.1), yet these remain untested through controlled, field-realistic experiments.

Understanding the interplay of these drivers is particularly important for pollinator conservation in agroecosystems, where limited floral resources and widespread pesticide use commonly co-occur and are at odds with the demands for crop pollination services (Klein et al. 2007, Garibaldi et al. 2013). Under laboratory conditions, good nutrition can improve honey bee resistance to pesticides (Schmehl et al. 2014), and combined exposure to pesticides and nutritional stress synergistically reduced survival in honey bees in the laboratory over four days (Tosi et al. 2017b). However, there has been no comparable research on wild bees in field or semi-field conditions, despite significant differences in resource acquisition and routes of exposure to toxins between species, as well as evidence for significant differences among species responses to pesticide exposure (Sgolastra et al. 2019; but see Ellis et al. 2017).

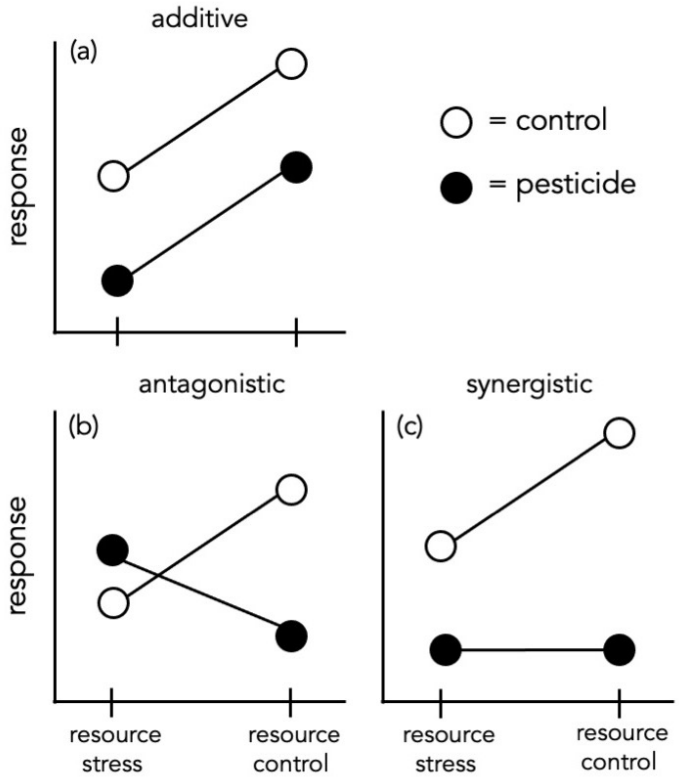

Figure 1.1. Interactions among multiple environmental stressors. Resource and pesticide stressors, for example, may have (a) additive, (b) antagonistic, and/or (c) synergistic interactions.

To address these knowledge gaps, we quantified the individual and combined effects of resource stress (limited floral resource availability) and sublethal, field-realistic insecticide exposure on the survival, nesting, and reproduction of the blue orchard bee *Osmia lignaria*. We focused on the systemic neonicotinoid insecticide imidacloprid, the most widely used neonicotinoid insecticide in the United States as of 2015 (Craddock et al. 2019). Neonicotinoids comprise nearly a quarter of the total insecticide market and pose a high risk to bees (Jeschke et al. 2011). Bees can be exposed to neonicotinoids by consuming pollen and nectar which absorb pesticides during flower development (Goulson 2013). We established nesting females in 16 flight cages using a crossed resource and pesticide design; cages contained spring wildflowers at high or low densities, treated with imidacloprid or without imidacloprid. Using cages allowed us to partition variation in pesticide exposure and resource abundance that may be correlated in real-world landscapes. Although field cages may limit additional risks bees face during long-distance foraging (e.g. predation, parasitism), they reduce variance from other environmental factors while still allowing bees to forage and nest freely. We hypothesize that resource and pesticide stressors will each directly reduce bee fitness. We expect that combined stressors will be additive or synergistic. For example, in a high-resource environment with high pesticides, the benefits conferred by increased resource availability may be negated by pesticide exposure. Alternatively, resource benefits may offset the deleterious effects of pesticide exposure.

Methods

Study system and experimental design

The blue orchard bee *Osmia lignaria* is a solitary univoltine species native to North America. It and other *Osmia* species are widely used as alternative pollinators to honey bees and/or in

combination with them in fruit orchards (Klein et al. 2007, Garibaldi et al. 2013, Rundlöf et al. 2015). Females nest above ground inside preexisting tunnels (e.g. abandoned wood boring beetle burrows in nature) but will readily nest in artificial tunnels (e.g. drilled holes, reed cane, cardboard paper tubes). Females collect pollen and nectar to mass-provision sequentially arranged brood chambers, which are separated by mud partitions. Within each chamber, a single egg is laid on or within the provision. Larvae hatch and consume the provision before spinning a cocoon and pupating. Offspring overwinter as adults and emerge the following spring. Bees for this experiment were collected in their overwintering state within nests from local sites in California (CA).

We conducted this experiment in 3 x 3 x 1.8 m flight cages at the UC Davis Bee Research Facility in North Central CA during the spring of 2018 (Figure A.1). *Osmia* will readily nest in field cages. We established a factorial design with two levels of floral resource availability (high and low) and the presence or absence of pesticide. We allocated four cages per treatment for a total of 16 cages. In each cage, we placed a wooden nesting block with 12 pre-drilled holes, 7.8 mm in diameter and 13 cm in length. We lined each hole with a translucent paper straw, which we removed and replaced as they were filled with nests. When flowers approached full bloom (late April 2018), we released six newly emerged and individually marked female and 12 male *O. lignaria* per cage. We measured the body size of all females (intertegular span; ITS) prior to release, and body size did not differ between treatments ($\chi^2 = 2.69$, $p = 0.442$). We added new bees periodically as bees died to maintain an average of four actively nesting females in each cage. To control for possible effects of timing, we balanced bee additions across treatments, and we also included release date as a covariate in models. In total, we released 121 bees across all

cages (n = 34 bees each for high-resource treatments; n = 27 and 26 bees, respectively, for low-resource pesticide and unexposed control treatments). We monitored nesting activity daily for a minimum of 20 minutes per cage by watching females take foraging trips in and out of their nests; this allowed us to associate each nest with a nesting female. We measured nesting progression daily by temporarily removing the nest straw and marking the nest progress on the outside of the straw.

Floral resource treatments

In each cage, we sowed a mix of three common wildflowers: *Phacelia tanacetifolia, Phacelia ciliata*, and *Collinsia heterophylla* (Table A.1). These flowers are known to be used by *O. lignaria* and bloom during their foraging period (Phillips and Klostermeyer 1978, Williams 2003, Lundin et al. 2017). We planted all cages with a high density of flowers in November 2017. Our goal was to create two resource levels: high, essentially not limiting to the bees, and low, which would limit resource availability during the foraging day. We based the floral availability for each resource treatment on published data on the amount of pollen per *O. lignaria* provision and per *P. tanacetifolia* flower to calculate how many flowers would be needed for each female to provision a single offspring (Phillips and Klostermeyer 1978, Williams and Thomson 2003, Williams and Tepedino 2003). Cages receiving a high-resource treatment had (mean ± SE) 2034 ± 77 flowers open at a time. We created low-resource cages by removing and covering plants to limit cages to (mean ± SE) 498 ± 27 open flowers at a time (Figure A.2). We conducted weekly flower counts to ensure that treatments were consistent across cages and made adjustments to add or remove flowers as necessary. High-resource cages contained sufficient flowers such that pollen was leftover in many of them at the end of the day; low-resource cages

were stripped of pollen by the afternoon each day (Table A.2), indicating the treatments achieved the desired goal. All cages contained high resources when we released the first cohort of bees; we established the low-resource treatment when females commenced nesting, 4-8 days after release. We released subsequent bees first into high-resource cages to facilitate nest initiation (Williams 2003, Williams and Kremen 2007). Upon nest initiation, we immediately moved them within their nests after sundown to the same location in low-resource cages of the same pesticide treatment. The move was only a few meters, and all females re-commenced foraging at the beginning of the next day; thus, we are confident that this moving had minimal impact on nesting females (Williams 2003). We provided each cage with a consistent mud source for nesting using moistened soil from each cage.

Neonicotinoid treatments

We applied a soil drench of the neonicotinoid insecticide imidacloprid (AdmirePro®, Bayer Crop Science) six weeks prior to releasing bees in cages at the maximum label rate (10.5 oz/acre; 767 mL/ha) for herbs and orchard fruit crops. Imidacloprid is the most frequently and heavily applied insecticide in California (California Department of Pesticide Regulation 2018) and the United States (Jeschke et al. 2011). AdmirePro® is the most common commercial imidacloprid product in California (California Department of Pesticide Regulation 2018). Imidacloprid has also been found in *O. lignaria* nests in agricultural landscapes (Rundlöf, Stuligross et al. 2022). To prevent lateral movement of the pesticide through the soil, we buried eight layers of 4 mm clear plastic sheeting 40 cm into the ground between treated and untreated cages. We measured pesticide exposure based on neonicotinoid residues from the pollen provisions within nests, a single male larval provision per cage, which were sent for analysis at the Metabolomics Research

Laboratory at Purdue University. Individual samples were prepared using the QuEChERS method (David et al. 2015) and analyzed using liquid chromatography triple quadrupole mass spectrometry (LC/QQQ; see Table A.3 and supplemental methods).

Offspring outcomes

Completed nests were stored in darkness at 22°C for six months, followed by four months at 6°C to overwinter. The following spring, we X-rayed all nests with brood inside before opening them. This allowed us to determine the number, sex, and condition of all offspring matched to each mother. We weighed each bee within its cocoon, visually determined the offspring sex, and measured the ITS of each female offspring.

Statistical analysis

We conducted all statistical analyses in R (version 3.4.1). To test for differences in body size of parent females between treatments, we used a Kruskal-Wallis test based on differences of ITS among bees assigned to the different experimental treatments. To test the effects of pesticide exposure and resource availability on offspring production in *O. lignaria*, as well as total nesting duration, we used a generalized linear mixed model (GLMM) with negative binomial error distribution and log link. We included pesticides (treated, not treated), resources (high, low), and date deployed in cage as fixed effects and cage as a random effect. We used a GLMM with binomial error distribution and logit link to test the difference in nesting probability, overwinter mortality, and offspring sex ratio between treatments. P-values from GLMMs were calculated using likelihood ratio tests. We tested differences in offspring body size and nest construction

rate using a linear mixed model with normal error distribution. In our analysis of offspring body size, we also included parent female ITS as a fixed effect.

Results

Resource limitation and pesticide exposure acted individually, and combined additively, to reduce bee reproductive fitness (Figure 1.2). The total impact on reproduction is a function of two processes: first, the probability of nesting, and second, the total number of offspring produced.

The probability of nesting was affected only by chronic exposure to field-realistic concentrations of imidacloprid. Female *O. lignaria* exposed to imidacloprid were 10% less likely to produce offspring, although these borderline statistical results should be interpreted with caution ($\chi^2 = 3.2$, df = 1, p = 0.074; Figure 1.2a). Resource limitation did not influence nesting probability ($\chi^2 = 0.03$, df = 1, p = 0.86).

Combined resource and pesticide stressors reduced female fecundity. Of the female *O. lignaria* that initiated nesting, those exposed to imidacloprid produced 42% fewer surviving offspring than unexposed controls (mean ± SE 19.1 ± 1.9 versus 32.7 ± 2.9, respectively; $\chi^2 = 17.59$, df = 1, $p < 0.001$; Figure 1.2b). Bees with low resources produced 26% fewer surviving offspring than bees with abundant resources (mean ± SE 22.1 ± 2.2 versus 29.8 ± 2.7, respectively; $\chi^2 = 7.17$, df = 1, p = 0.007; Figure 1.2b). Together, unstressed females produced approximately 21 more offspring on average than resource and pesticide-stressed females. Pesticide exposure and resource limitation acted additively to reduce reproduction (no significant interaction; $\chi^2 = 0.61$,

df = 1, p = 0.44; Figure 1.2b). Nearly all provisioned cells successfully developed into adults, and overwinter offspring mortality did not differ among treatments ($\chi^2 = 3.23$, df = 1, p = 0.20; Figure A.3).

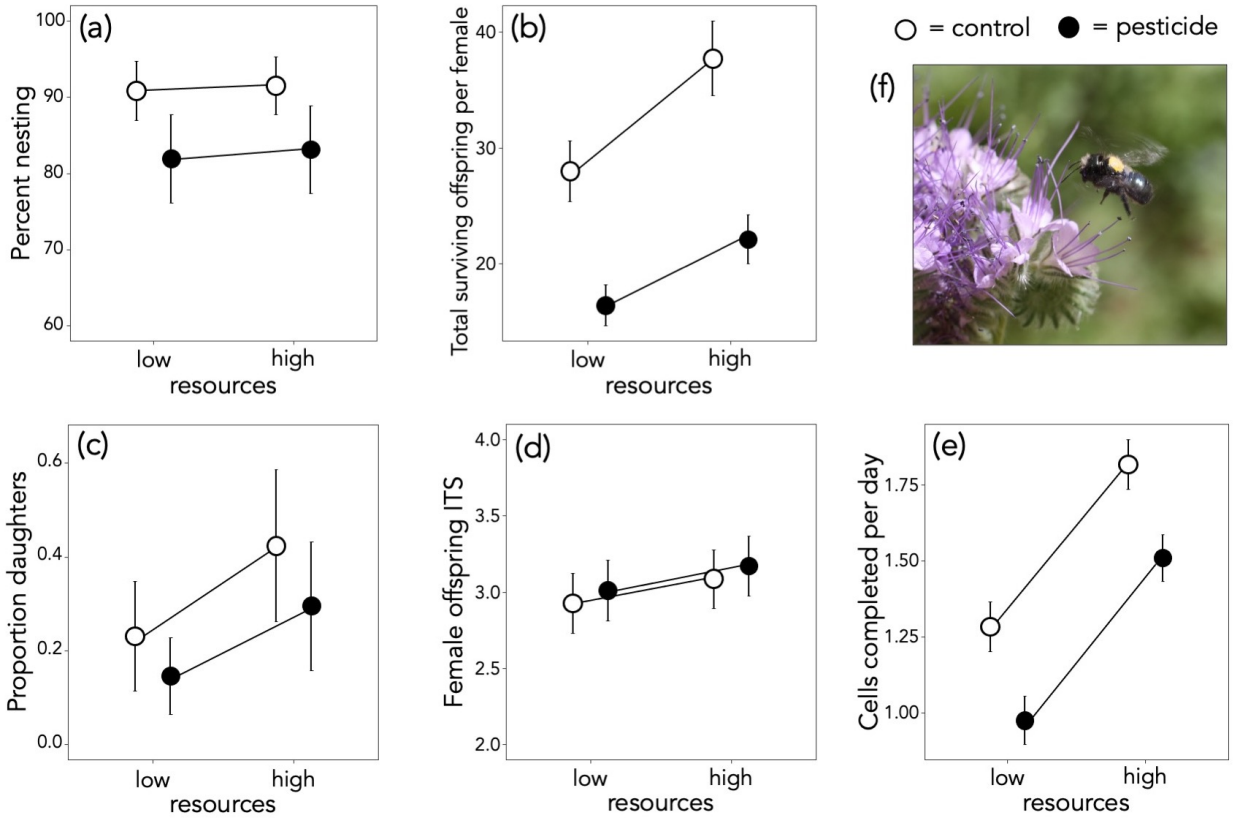

Figure 1.2. Effects of resource limitation and pesticide exposure on bee performance. (a) Percent of female *Osmia lignaria* that produced at least one offspring; (b) mean number of surviving offspring per nesting female; (c) proportion of daughters produced per nesting female; (d) ITS of female offspring; (e) mean number of cells completed per day per nesting female *O. lignaria* in 16 field cages with pesticides (black) or without pesticides (white) in high- and low-floral resource environments. Error bars show SEs; N=121. (f) Photo of a paint-marked *O. lignaria* female approaching a *Phacelia tanacetifolia* flower.

In addition to direct effects on reproduction, resource and pesticide stressors led to male-biased sex ratios, further limiting reproductive output. Pesticide exposure caused a 33% reduction in the proportion of daughters produced ($\chi^2 = 8.32$, df = 1, $p < 0.004$; Figure 1.2c). Resource limitation caused a 48% reduction in the proportion of daughters produced ($\chi^2 = 15.29$, df = 1, $p < 0.001$; Figure 1.2c). The two stressors combined additively to reduce the female:male offspring sex ratio (no significant interaction; $\chi^2 = 1.04$, df = 1, $p = 0.31$; Figure 1.2c), similar to effects on total offspring reproduction.

Surviving offspring differed by an average of 0.13 mm in body size (intertegular span; ITS) between treatments; female offspring were 5% larger in high-resource treatments ($\chi^2 = 19.92$, df = 1, $p < 0.001$) and 3% larger in pesticide treatments ($\chi^2 = 12.46$, df = 1, $p < 0.001$; Figure 1.2d). This pattern is similar for males (Figure A.4).

Bees produced fewer offspring via multiple mechanisms: changing nesting rate, onset, and duration. Stressed bees constructed nests slower and nested for fewer days than unstressed bees. Resource limitation slowed nest construction by 32% (approx. 0.5 cells/day; $\chi^2 = 23.73$, df = 1, $p < 0.001$), and pesticide exposure slowed nesting by 20% (approx. 0.3 cells/day; $\chi^2 = 11.62$, df = 1, $p < 0.001$). Again, the effects were additive (no significant interaction; $\chi^2 = 0.02$, df = 1, $p = 0.89$; Figure 1.2e). Females exposed to pesticides also spent 6.33 (28%) fewer days nesting than bees that were not exposed ($\chi^2 = 9.54$, df = 1, $p = 0.002$; Figure 1.3). Pesticide-exposed females started nesting an average of 49% later than unexposed bees, about 3.6 days ($\chi^2 = 16.54$, df = 1, $p < 0.001$; Figure 1.3). Pesticide and resource stressors acted additively on the total nesting

duration and delayed start (no significant interaction on nesting duration, $\chi^2 = 2.40$, df = 1, p = 0.12; delay $\chi^2 = 0.09$, df = 1, p = 0.76).

Despite significant responses of behavior and reproduction of bees exposed to pesticides, only two of eight pesticide-treated cages had detectable levels of imidacloprid in pollen provisions (Table A.3). None of the pollen provisions from untreated control cages contained detectable imidacloprid levels (Table A.3).

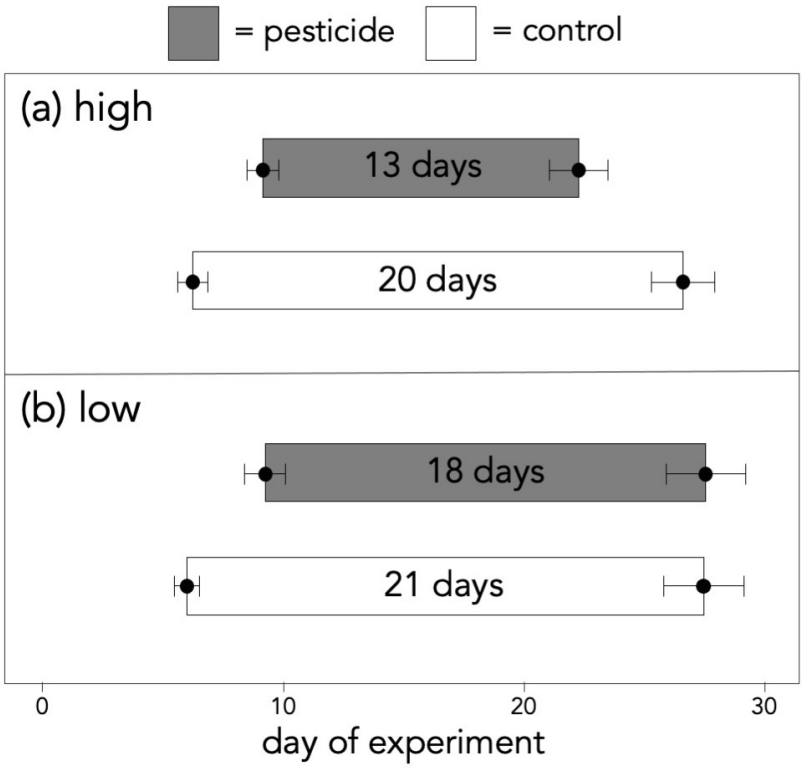

Figure 1.3. Nesting onset and duration is moderated by pesticide exposure and resource limitation. Mean (± SE) number of days between first and last offspring provisioned by each nesting female in (a) high- and (b) low-floral resource environments exposed to pesticides (gray) or unexposed controls (white). N = 121 bees.

Discussion

We show in free-foraging trials that field-realistic pesticide exposure and floral resource scarcity combine additively to dramatically reduce multiple vital rates of a solitary bee. Unlike recent predictions of worst-case scenarios of negative synergies (Sih et al. 2004, Goulson et al. 2015, Tosi et al. 2017b), effects—although substantial—were additive. The additive effects of exposure to pesticides and food limitation reduced reproduction by 57% compared to unexposed control populations. These combined stressors could dramatically impede population growth and jeopardize population persistence. Pesticide exposure had the greatest impact on offspring production and nesting activity, reducing overall reproduction 1.75 times more than food limitation. Negative effects of systemic pesticides on bee survival and reproduction are well documented but based largely on correlative (rather than experimental) field and laboratory studies (Goulson et al. 2015, Rundlöf et al. 2015, Woodcock et al. 2016).

In *Osmia*, reduced offspring production by pesticide-exposed bees resulted from a dramatically delayed onset of nesting, earlier cessation, and a substantially lower rate of offspring provisioning. *Osmia lignaria* females exposed to imidacloprid began nesting 3.6 days later and spent 5.2 fewer days nesting than unexposed control bees—eliminating nearly a week of potential nesting days. This could be due to a delay in ovary maturation, as well as decreased longevity, that together curtailed nesting at both ends (Rosenheim et al. 1996, Sgolastra et al. 2018, Anderson and Harmon-Threatt 2019).

Resource limitation also reduced the rate of offspring provisioning among nesting females, likely due to the lack of pollen and nectar available for nest provisioning (Minckley et al. 1994, Kim

1999). This reduced the rate at which bees could complete nest provisions, as well as potentially reducing overall exposure to pesticide-treated flowers.

Interestingly, pesticide exposure affected total nesting duration differently between resource treatments. Pesticides had a particularly large influence on nesting duration for bees with abundant resources, which stopped nesting two days earlier than bees in all other treatments. It is possible that resource-stressed bees nested longer to make up for a slower overall nesting rate. This seems unlikely because slower nesting was not observed in pesticide-free resource treatments. Instead, we suspect that a faster provisioning rate and associated greater number of flowers visited in the high-resource treatment increased pesticide exposure. Increased chronic exposure to pesticides reduced bee longevity despite their access to sufficient forage resources (Sgolastra et al. 2018), suggesting that bees are not rescued by more forage resources when it also exposes them to more toxins (Botías et al. 2017, Tosi et al. 2017b).

Both pesticide exposure and resource scarcity biased offspring sex ratio toward more males. Females of *O. lignaria* and most other solitary bee species are larger and are provisioned with more food than males, thus they cost more to produce (Raw 1972, Kim 1997). Pesticide exposure dramatically reduced the probability that a bee produced even a single daughter. Indeed, of all nesting females only 62% of pesticide-exposed individuals produced at least one daughter compared to 92% of unexposed individuals. This suggests sublethal effects on foraging ability whereby females shifted to produce less costly males (Rosenheim et al. 1996, Kim 1999, Rehan and Richards 2010).

The decrease in female offspring has important consequences for populations; because males rarely limit population growth, fewer female progeny will reduce the reproductive potential of subsequent generations (Werren 1987, Ulbrich and Seidelmann 2001). Combined with lower overall offspring production, as we found, it could create an extinction vortex, driving populations to decline or go extinct (Ulbrich and Seidelmann 2001, Zayed and Packer 2005, Zayed 2009). Consider, the average female in an optimal environment with abundant, pesticide-free forage resources can produce 37 offspring in her lifetime, of which approximately 10 are female (Figure 1.2b and 1.2c). Pesticide and food-stressed females produce about 16 offspring each—a difference of 57%—of which a mere 1-2 are females (Figure 1.2b and 1.2c). This difference is striking considering that even minor changes in offspring production can substantially influence population growth given solitary bees' relatively low reproductive rate (Raw 1972, Torchio 1990).

Unsurprisingly, abundant resources led to relatively larger offspring (Rosenheim et al. 1996, Peterson and Roitberg 2006). We were surprised, however, that pesticide-exposed bees also produced larger offspring than unexposed bees, albeit by a small margin (3%; 0.09 mm ITS). Pesticide-exposed bees may have allocated larger food provisions (leading to larger offspring) to compensate for fewer overall offspring, although it is unclear what fitness benefit this confers. Body size positively correlates with nesting success in some studies (Kim 1997, Rehan and Richards 2010), but not others (Johnson 1990, Alcock et al. 2006) depending on environmental conditions, and it may be mediated by other differences in addition to body size. In our study, parent female body size did not influence realized fecundity.

We applied pesticide according to label instructions; thus, it is likely that bees were exposed to field-realistic pesticide levels throughout the experiment. We were therefore surprised that only two of the eight pesticide-treated cages contained detectable levels of imidacloprid. We discuss three possibilities for this ambiguous detection: (i) bees were not exposed to pesticides in the pollen. We think this is unlikely because we found strong differences in measured outcomes between pesticide-treated and untreated control cages. (ii) The residues in the pollen degraded. We applied the pesticide six weeks prior to releasing bees in cages; degradation occurs over time, but we nonetheless found residue in multiple samples. Additionally, the half-life of imidacloprid is relatively long (in soil: 28-1250 days (Goulson 2013); in water: 30 days (Bacey 1999)). (iii) Given the small amount of pollen we sent for analysis, it is possible that low levels of pesticide residue could not be detected. Such variability in pesticide levels found in larval provisions may be a previously undocumented pattern, since samples are generally pooled for analysis, and is an important consideration for future studies.

The sublethal impacts of pesticides and resource limitation may be especially problematic in agricultural systems, which rely on robust pollinator populations. Establishing flower plantings to provide additional forage resources is a frequently implemented approach for mitigating pollinator decline (M'Gonigle et al. 2015, Scheper et al. 2015, Williams et al. 2015). We demonstrated that abundant floral resources yielded a 35% increase in bee reproduction. However, flower plantings could act as ecological traps if they also exposed bees to pesticides. This occurs via pesticide drift from agricultural crops onto nearby field margins and flower plantings (Otto et al. 2009, Botías et al. 2015). In our study, bees with unlimited pesticide-treated forage produced 21% fewer offspring than those with critically limited but pesticide-free forage,

indicating that additional resources do more harm than good if they become contaminated with pesticides (Davis et al. 1991, Otto et al. 2009, Krupke et al. 2012, Botías et al. 2015, Tosi et al. 2017b). Although we focused on pollinators, similar impacts likely apply to other beneficial insects in agriculture; parasitoids rely on nectar sources and generalist predators feed on prey found in planted field margins (Otto et al. 2009, Morandin et al. 2014).

A critical challenge facing ecologists today is predicting and understanding the effects of multiple stressors (Sala et al. 2000, Sih et al. 2004). Thus, we are encouraged that pesticide exposure and resource limitation combined additively, rather than synergistically, to affect bee health. The additive nature of the effects could enable us to make preliminary predictions about the effects of such environmental change from univariate experiments, although this must be approached with caution as chemicals are known to interact in different ways (Gill et al. 2012, Iverson et al. 2019). It is clear that insects encounter multiple stressors throughout their life cycles, each exacerbating the effects of the others. We show that pesticide exposure and resource limitation combined to additively limit bee reproduction through reduced offspring production, male-biased sex ratio, and shorter nesting duration. In addition to novel findings for understanding combined environmental stressors, our results inform practical decision-making for conservation and management of ecosystem services in agriculture. For example, they reinforce the need for caution in the placement of flower plantings intended to provide forage resources for bees to avoid them becoming traps that expose bees to potential additive negative effects of pesticides in agroecosystems.

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Chapter 2. Behavioral impacts of resource limitation and insecticide exposure reinforce negative fitness outcomes for solitary bees

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Abstract

Contemporary landscapes present numerous challenges for bees and other beneficial insects with critical functional roles in ecosystems and agriculture. Pesticide use and the loss of flowering plants are two stressors known to act together to impair fitness. Bee foraging and nesting behavior can impact pollination services and population persistence, making it critical to understand the sublethal effects of these stressors on key behaviors. We investigated the effects of insecticide exposure and floral resource limitation on the foraging and nesting behavior of the solitary blue orchard bee, *Osmia lignaria*. Bees in field cages foraged on wildflowers at high or low densities, some treated with the common insecticide, imidacloprid, in a fully crossed design. Both stressors influenced behavior, but they had differential impacts. Bees with limited food resources made fewer, but longer foraging trips and missed their nests more often. Insecticide exposure reduced bee foraging activity and also interacted with bee age to influence antagonistic behavior. Our findings point towards mechanisms underlying effects on populations and ecosystem function and reinforce the importance of studying multiple drivers to understand the consequences of anthropogenic change.

Introduction

Anthropogenic changes in contemporary landscapes involve combinations of stressors, which act together to impact diverse species. Many of these species serve key roles within functioning ecosystems (González-Varo et al. 2013, Stanton et al. 2018, Sánchez-Bayo and Wyckhuys 2019). Intensive agricultural land use, for example, reduces the amount and continuity of flower resources and increases exposure to pesticides (Matson et al. 1997, Tscharntke et al. 2005). Together, these two stressors reduce reproduction and survival in beneficial insects like bees (Tosi et al. 2017b, Stuligross and Williams 2020), potentially leading to population decline (Goulson et al. 2015, Woodcock et al. 2016, Stuligross and Williams 2021). Such stressors can also affect behaviors associated with resource acquisition and nesting (Kim 1999, Goulson et al. 2015, Siviter et al. 2021, Goulson and Nicholls 2022), thus providing a mechanistic link between the sublethal effects of these stressors and impacts on vital rates.

Most research on the effects of these stressors on bee behavior have focused on a few social taxa. Resource limitation across the landscape can influence bumble bee foraging behavior (Westphal et al. 2006, Hemberger and Gratton 2018). Neonicotinoid insecticide exposure also impairs social bee foraging efficiency (Gill et al. 2012, Gill and Raine 2014, Feltham et al. 2014), as well as olfactory learning and memory (Stanley et al. 2015b, Siviter et al. 2018, Muth et al. 2019), flight (Tosi et al. 2017a, Kenna et al. 2019), and motor function (Williamson et al. 2014). However, bee groups differ substantially in foraging and nesting behavior (Winfree et al. 2009, Sgolastra et al. 2019). We know less about how these stressors influence common behaviors associated with solitary bee nesting. For example, alternative nesting strategies, such as usurpation, can lead to aggression at the nesting site (Tepedino and Torchio 1994, Moure-

Oliveira et al. 2017). This behavioral variation could further modulate the sublethal impacts of environmental stressors on fitness beyond our understanding in social bees.

In addition to the fitness effects resulting from changes in foraging and nesting behavior, bee activity is directly linked to their functional role in ecosystems. Bee foraging at flowers positively correlates with pollination services (Goodwin et al. 2011, Mallinger et al. 2021); thus, the extent to which stressors like insecticide exposure and resource limitation impact bees' foraging activity will determine their effect on pollination. This would be especially concerning in agricultural landscapes, for which pollination is essential and where pesticide and resource stressors commonly co-occur (Koh et al. 2016).

Although the two stressors may act in different ways, many studies to date suggest that pesticide exposure and resource stress act additively (Dance et al. 2017, Stuligross and Williams 2020) or synergistically (Tosi et al. 2017b, Ingwell et al. 2021) on bee reproduction and/or survival. Genomic analyses have also found overlapping transcriptional responses to nutrition and pesticide stressors in honey bees (Schmehl et al. 2014). However, impacts on behavior are less clear (Tong et al. 2019, Ingwell et al. 2021, Wintermantel et al. 2022), and the stressors are often not studied in a way that allows for direct comparison.

To address these knowledge gaps, we investigated the individual and combined effects of food resource limitation and insecticide exposure on the nesting behavior of the solitary blue orchard bee *Osmia lignaria.* We conducted an in-field cage experiment using a crossed resource availability and pesticide design. Adult solitary bees foraged on spring wildflowers grown at

high (resource unlimited) or low densities (resource limited), treated with or without the systemic neonicotinoid insecticide imidacloprid. Imidacloprid is widely used across the United States and worldwide (Jeschke et al. 2011, Bass et al. 2015) and is the most frequently applied insecticide in California (California Department of Pesticide Regulation 2018). As systemic insecticides, neonicotinoids are taken up by plants and can be present in all plant tissues, including pollen and nectar, and pose a high risk to bees (Jeschke et al. 2011, Goulson 2013, Craddock et al. 2019).

Using a crossed cage experiment, we investigated the individual and combined effects of food resource limitation and insecticide exposure on the (1) foraging behavior, (2) nest recognition, and (3) antagonistic behavior of nesting female *O. lignaria*. Field cages provided a controlled field environment for isolating the effects of stressors on behavior while still allowing bees to forage and nest freely.

Methods

Study system and experimental design

The blue orchard bee *O. lignaria* is a solitary species native to North America. It is also widely used as an alternative pollinator to honey bees and/or in combination with them in orchard crops (Garibaldi et al. 2013). Females nest in pre-existing cavities, such as abandoned wood-boring beetle burrows in nature, but will readily nest in paper tubes or other artificial tunnels. This allows them to be collected in such trap nests (Williams and Kremen 2007) and manipulated for experiments or use in managed pollination (Boyle et al. 2020). To provision offspring, females take many foraging trips to collect pollen and nectar resources; this behavior is easily observed at nesting sites. Within their nests, females construct a linear series of brood chambers, each

containing a food provision and a single egg, separated by mud partitions. Eggs hatch into larvae, which consume the provision, spin a cocoon, pupate, and overwinter as adults; the entire life cycle takes about a year. Bees for this experiment were sourced from local populations in California.

We conducted this experiment in 3 x 3 x 1.8 m flight cages at the University of California (UC) Davis Bee Research Facility during the spring of 2018. See Stuligross & Williams (2020) for additional methodological details. In each cage, we placed a wooden nesting block with 12 predrilled holes, each lined with a translucent paper straw to facilitate nest monitoring.

We released six female and 12 male *O. lignaria* in each cage when flowers approached full bloom. We paint-marked and measured the body size of all females (intertegular span; ITS) prior to release in cages; body size did not differ between treatments ($\chi^2 = 2.69$, $p = 0.442$). We added new bees periodically as others died to maintain an average of four actively nesting females in each cage; this ensured equal competition for resources in cages. We balanced bee additions across treatments. In total, we released 121 bees among all cages (n = 34 high resource, no insecticide; n = 34 high resource, insecticide; n = 26 low resource, no insecticide; n = 27 low resource, insecticide).

Behavioral observations

We visually monitored nesting activity daily for a minimum of 20 minutes per cage by watching females take foraging trips in and out of their nests; this allowed us to associate each nest with a nesting female.

To collect additional behavioral data, we filmed bee activity at the entrance of each nest block using GoPro cameras over four weeks. Each filming interval was approximately two hours during peak foraging between 10:00 and 18:00. We balanced filming among treatments to ensure equal observation time and balance morning and afternoon intervals for a total of 97.8 hours of video collected (mean ± SD 24.46 ± 0.77 hours per treatment). We analyzed videos using BORIS software (Friard and Gamba 2016) to measure the time each bee spent performing each behavior, detailed below.

We collected data on six specific variables designed to represent the foraging and nesting behavior of *Osmia lignaria* females. We measured (1) foraging time, i.e. the time spent out of the nest; (2) the number of foraging trips, measured by a bee leaving and then returning to her nest; (3) time spent exhibiting antagonistic behavior with another bee, e.g. attempting to usurp another bee's nest or defending against a usurper; and (4) the number of antagonistic interactions. We also measured (5) nest recognition by counting the number of times a bee mistakenly entered a nest other than the one it was currently nesting in upon returning from a foraging trip (Guédot et al. 2006, 2013, Artz and Pitts-Singer 2015). We counted (6) usurpation events by identifying all instances in which a nest changed ownership from one bee to another (Tepedino and Torchio 1994). We counted this transition as a usurpation if the first owner of a nest had completed at least one nest cell and was replaced by another bee that subsequently completed at least one nest cell in the same nest. We did not always observe the usurpation taking place in the video footage, but because we visually observed nest occupancy daily to match each nest to a marked female,

we could confirm usurpation for the full duration of the study. Each of these behaviors served as a separate response variable for our analysis.

Floral resource treatments

We planted a wildflower mix in each cage, comprised of species known to be used by *O. lignaria* (Boyle et al. 2020). We established two resource levels: high, providing essentially unlimited resources for the bees, and low, which limited resource availability. We based the floral availability for each resource treatment on published data on the pollen requirements for *O. lignaria* provisions (Phillips and Klostermeyer 1978, Williams and Thomson 2003, Williams and Tepedino 2003) and conducted weekly flower counts to ensure that treatments were consistent across cages. High-resource cages contained plentiful flowers such that pollen was leftover in many of them at the end of the day; flowers in low-resource cages were stripped of pollen by the afternoon each day, indicating that the treatments achieved the desired goal. To facilitate nest initiation, we released all bees into high-resource cages and established the low-resource treatment when females commenced nesting, 4-8 days after release (Williams 2003, Williams and Kremen 2007). We also provided a consistent mud source for nesting using moistened soil from each cage. For additional details on the floral resource treatments, see Stuligross and Williams (2020).

Neonicotinoid treatments

Six weeks prior to releasing bees, we applied a soil drench of the neonicotinoid insecticide imidacloprid (AdmirePro, Bayer Crop Science) in each cage at the maximum label rate for herbs and orchard fruit crops (10.5 oz/acre; 767 ml/ha). We measured imidacloprid exposure based on

residues from the pollen provisions within nests; we sent a single male larval provision per cage for analysis using a modified QuEChERs protocol using LC/QQQ at the Purdue University Metabolomics Research Laboratory. See the supplementary information in Appendix B for analysis methods and Stuligross and Williams (2020) for additional details on the neonicotinoid treatments.

Statistical analysis

We used generalized and linear mixed model frameworks to analyze the effects of resource availability and insecticide exposure on adult *O. lignaria* nesting behavior. For all models, we initially included resource availability (high/low), insecticide exposure (yes/no), bee body size (ITS), and bee age (number of days since release in cage) as fixed effects. We removed bee body size and bee age from final models when non-significant ($p > 0.05$; Table B.1) to improve model fit (Zuur et al. 2009). We also tested interactions between resource availability x insecticide exposure, age x resource availability, and age x insecticide exposure and removed the interaction term from final models when non-significant (Table B.2). For models that included multiple observations of a single bee (i.e. all except usurpation), we also included bee identity and cage-observation date as random effects.

We fit linear models to explore the effects of predictors on bee foraging: the number of foraging trips taken and the length of each foraging trip of each observed bee. To assess the effects of predictors on the number of missed nest entries and antagonistic behavior, we fit hurdle models to account for zero-inflation in the data. First, we assessed the probability of the behavior as a binary response variable (using logit link). Then, for those individuals that performed the

behavior at least once, we assessed the number of missed nest entries or the amount of time spent engaging in antagonistic behavior using linear models assuming a Gaussian conditional distribution. The response variables for these models were log-transformed to meet normality and homogeneity of variance assumptions. For both hurdle models, we also included the number of foraging trips taken by each bee as a covariate. Finally, we fit a zero-inflated model with Poisson error distribution (log link) to explore the effects of predictors on the number of nests usurped by each individual bee.

We selected model error distributions and link functions based on residual plots and AIC, and we graphically assessed requirements of distribution and variance homogeneity for all models. We calculated p-values from mixed models using likelihood ratio tests. We conducted all analyses in R (version 3.6.3).

Results

Foraging behavior

We observed behaviors for 102 individual female *O. lignaria*, of which 93 successfully initiated nesting. Of the females that initiated nesting, those with low resources made 27% fewer foraging trips than bees with high resources ($\chi^2 = 7.27$, $p = 0.007$; Figure 2.1a), and each of those foraging trips was ~1.5 minutes (53%) longer than those taken by bees with high resources ($\chi^2 = 10.61$, $p = 0.001$; Figure 2.1b). In contrast, although insecticide exposure did not influence the number of foraging trips per bee ($\chi^2 = 0.375$, $p = 0.540$; Figure 2.1a), bees exposed to imidacloprid took shorter foraging trips than unexposed bees (about 1 minute shorter per trip (18% reduction); $\chi^2 = 4.48$, $p = 0.034$; Figure 2.1b). Bee age did not influence the number of foraging trips a bee took

per observation period (Table B.1); however, younger bees made longer foraging trips than older bees (χ^2=5.21, p=0.023; Figure 2.1b).

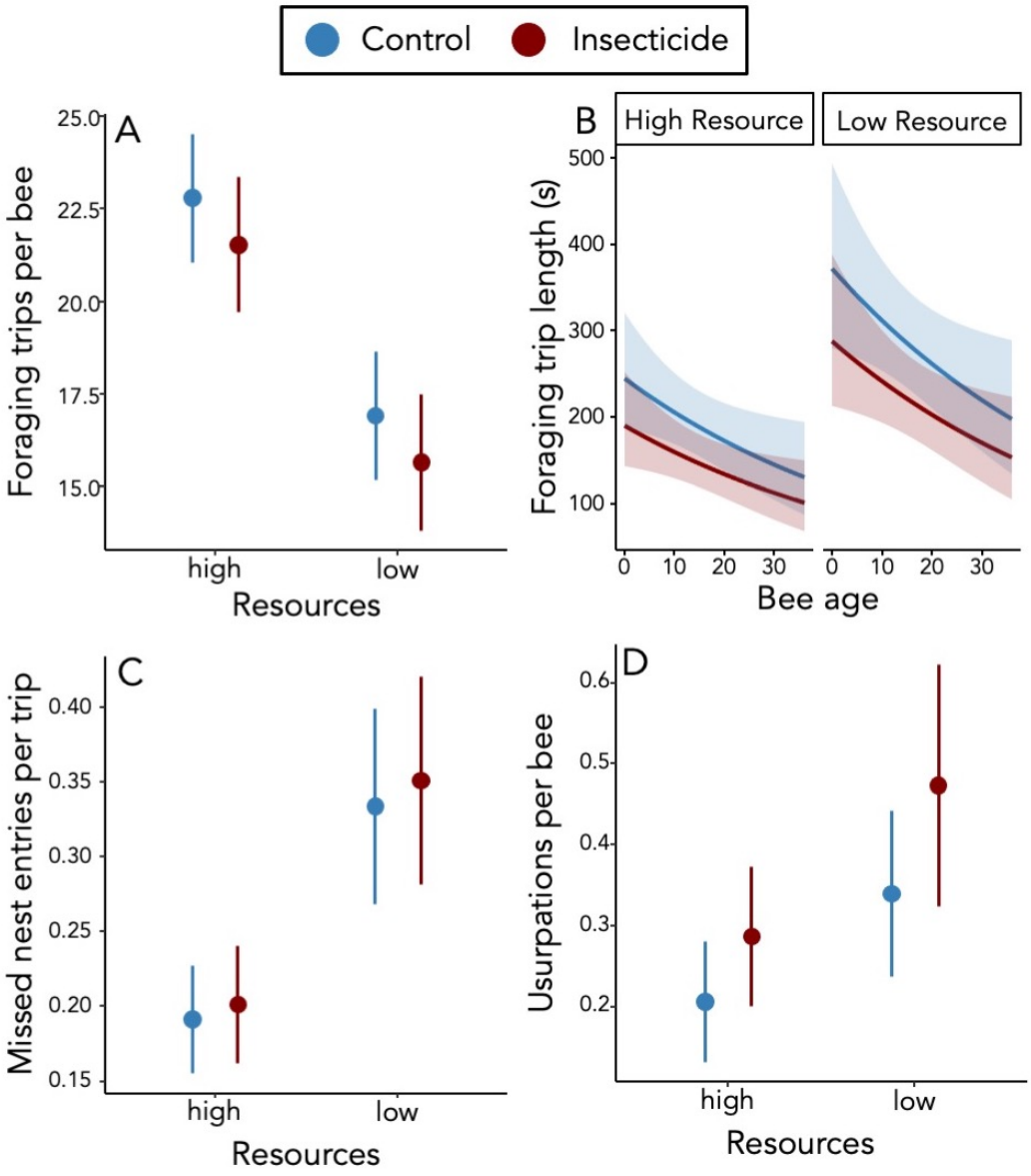

Figure 2.1. Effects of resource limitation and insecticide exposure on behavior of nesting female *Osmia lignaria* in 16 field cages with insecticides (red) or without insecticides (blue) in high- and low-floral resource environments. (A) Number of foraging trips; (B) foraging trip length in relation to bee age; (C) number of missed nest entries per foraging trip for females that missed their nests at least once; (D) number of usurpations per bee throughout the duration of the study. Model estimated means and SEs; shading in (B) indicates 95% confidence interval.

Nest recognition

Neither resource availability ($\chi^2 = 0.002$, $p = 0.960$) nor insecticide exposure ($\chi^2 = 2.15$, $p = 0.142$) influenced the probability that a *O. lignaria* females missed their nests during observation (nest recognition; Figure B.1). However, of the females that missed their nests at least once, bees with low resources missed their nests 74% more often than bees with high resources ($\chi^2 = 5.54$, $p = 0.019$; Figure 2.1c). Insecticides did not influence nest recognition ($\chi^2 = 0.051$, $p = 0.821$; Figure 2.1c). Older bees tended to miss their nests more often ($\chi^2 = 2.04$, $p = 0.153$; Figure B.2).

Antagonistic behavior

Neither resource availability ($\chi^2 = 0.208$, $p = 0.648$) nor insecticide exposure ($\chi^2 = 0.015$, $p = 0.904$; Figure B.3) significantly influenced the probability that a bee exhibited antagonistic behavior during observation. Of the bees that exhibited antagonistic behaviors, resource availability did not influence antagonism, although those with low resources spent 110% more time engaging in antagonistic behaviors than those with high resources ($\chi^2 = 2.63$, $p = 0.105$; Figure 2.2); the lack of significance could potentially be due to the high variability among low-resource bees. The effect of insecticide exposure on antagonism depended on bee age; insecticide-exposed bees spent less time engaging in antagonistic behavior with age, while unexposed control bees increased antagonism with age ($\chi^2 = 6.11$, $p = 0.013$; Figure 2.2).

During the study, there were 52 successful usurpations, performed by 40 individual bees (mean 1.3 usurpations per usurper, range 1-5). Low-resource bees usurped 65% more than high-resource bees, although this result was not statistically significant ($\chi^2 = 2.14$, $p = 0.143$; Figure 2.1d). Insecticide exposure did not influence usurpation ($\chi^2 = 0.850$, $p = 0.357$; Figure 2.1d).

Rather than treatments affecting usurpation, it appeared to be more influenced by bee body size; larger bees usurped more ($\chi^2 = 11.05$, $p < 0.001$; Figure B.4a), and smaller bees were more likely to be usurped ($\chi^2 = 4.21$, $p = 0.040$; Figure B.4b).

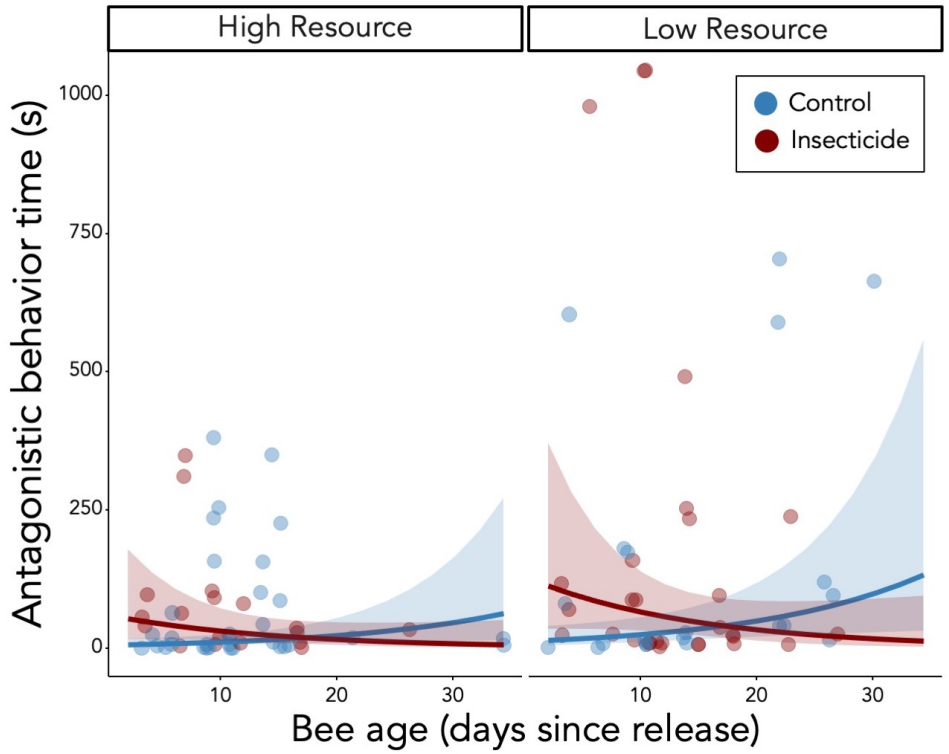

Figure 2.2. Effects of resource limitation, insecticide exposure, and age on the amount of time spent exhibiting antagonistic behavior in female *Osmia lignaria* that had at least one antagonistic interaction. Females were observed nesting in 16 field cages with insecticides (red) or without insecticides (blue) in high- and low-floral resource environments. Shading indicates 95% confidence intervals.

Discussion

Foraging and nesting are primary behaviors influencing bee reproductive success (Danforth et al. 2019). Stressors like reduced floral resources and insecticide exposure limit bee nesting and foraging, and bees often experience them together in human-altered landscapes (Goulson et al. 2015, Koh et al. 2016). As a result, understanding the combined impacts on behavior is key to supporting robust bee populations and the pollination services they provide (Goulson and Nicholls 2022). Our experiments revealed consistent sublethal impacts on foraging and nesting behavior. The observed effects may contribute to profound reductions in bee reproduction documented previously (Stuligross and Williams 2020) and have lasting implications for pollination function.

Foraging behavior

Bees with limited food resources took fewer, but longer foraging trips. This ultimately led to a reduced nesting rate and lower overall reproduction, as previously reported by Stuligross and Williams (2020) and supported here. The impacts of food resource availability on foraging behavior we observed are consistent with expectations based on optimal foraging theory (Charnov 1976, Pyke 1980). Models of central-place foragers like bees, which return to a fixed nesting location, predict that foragers will spend a longer time in a patch when resource search time increases (Charnov 1976, Pyke 1980). Because all bees in our study were restricted to identically sized field cages, the increased foraging time is likely due to extended search/collection time for resources rather than increased travel time to a resource patch. Bees in low-resource cages required more time to collect resources and, as a result, increased their foraging trip duration and reduced the number of foraging trips they could take. Field studies on

bumble bees have demonstrated the same pattern of longer foraging trips in low-resource environments (Goulson et al. 2002, Westphal et al. 2006) and relatedly, more, shorter trips during mass-flowering crop bloom (Hemberger and Gratton 2018). Although we were not able to measure the amount of pollen collected with each foraging trip in this study, our previous data for these experiments indicates that this compensation in foraging behavior was not sufficient to maintain nest provisioning rates. Resource-limited females produced 26% fewer offspring than resource-unlimited females as reported in Stuligross and Williams (2020). Future research on resource return could further fill the gap in our understanding of stress-induced changes in foraging behavior, pollen collection, and reproduction.

Imidacloprid exposure can impair bee foraging efficiency (Gill et al. 2012, Gill and Raine 2014, Feltham et al. 2014). In this case, we might have expected to see a similar effect on foraging for bees exposed to imidacloprid as we observed under resource limitation—fewer, longer foraging trips. In previous studies, bumble bees (Gill et al. 2012, Stanley et al. 2016) and honey bees (Schneider et al. 2012, Hesselbach et al. 2020) took longer foraging trips when exposed to insecticides. Other lab-based exposure trials showed that some insecticides increased bumble bee foraging trip length and increased forager recruitment, perhaps due to the decreased overall foraging efficiency in exposed colonies (Gill and Raine 2014). However, insecticide-exposed bees in our study made shorter foraging trips and did not compensate for this by taking more trips. Instead, they were less active across both measures, with their overall foraging effort reduced compared to unexposed bees.

Previous studies on neonicotinoid-exposed social honey and bumble bees measured behavioral responses in workers, which unlike female solitary bees are not solely responsible for all nesting, reproductive, and foraging efforts. These differences in sociality may underlie our contrasting results (Brittain and Potts 2011, Sgolastra et al. 2019). Insecticide exposure may differentially affect solitary bees (Brittain and Potts 2011), such as requiring females to allocate more resources towards egg-laying, that prevents them from compensating for reduced foraging efficiency in the way that social worker bees can. Solitary bees have also been reported to be more sensitive to insecticides than social bees (Devillers et al. 2003, Scott-Dupree et al. 2009, Peterson et al. 2021), although evidence is mixed and depends on the chemical, bee species, and exposure pathway (Arena and Sgolastra 2014). However, *O. lignaria* was more sensitive to imidacloprid than honey bees and bumble bees (Scott-Dupree et al. 2009, Peterson et al. 2021); this may contribute to the difference in foraging behavior observed between groups. Multiple studies report reduced overall activity resulting from insecticide exposure to solitary bees (Boff et al. 2021, Cecala and Wilson Rankin 2021; but see Ruddle et al. 2018), but more research is needed to reveal general mechanisms underlying the differential effects of insecticide exposure on solitary and social bee foraging behavior (Pyke 2022).

Nest recognition

We observed an increased rate of entries into the wrong nest hole under resource limitation, suggesting impaired nest recognition. *Osmia lignaria* females, like other bees, use both visual and olfactory cues to locate their nests (Wcislo 1992, Fauria and Campan 1998, Guédot et al. 2006, Ostwald et al. 2019). Because bees with limited floral resources make fewer foraging trips overall, and those trips are longer, they return to their nests less often. We hypothesize four ways

in which this foraging trend could explain the decreased nest recognition we observed. First, the memory of a nest location may diminish with time, thus the increased time that low-resource bees spent away from their nests could lead to reduced nest recognition. Some research indicates that bee foraging memory fades with time (Keasar et al. 1996, Raine and Chittka 2007). Second, frequent foraging trips could reinforce familiarity with the nest site and lead to fewer recognition mistakes. Bees with high floral resource availability returned to their nests approximately six times more often per observation period than those with low-resources, continually reinforcing the nest location. Other cavity-nesting solitary bees locate their nests more quickly with increasing foraging trips (Boff and Friedel 2021), and foraging memory can diminish without regular reinforcement (Keasar et al. 1996). Third, foraging female *O. lignaria* and other species regularly reapply the scent-marks within their nests, updating the olfactory cue with every foraging trip (Guédot et al. 2006, Frahnert and Seidelmann 2021). We hypothesize that increased time away from the nest, as well as fewer trips overall, could weaken scent-marks leading to decreased nest recognition ability. Fourth, regardless of foraging behavior, food stress may have a more direct negative effect on bee learning and memory. Food stress impaired olfactory learning and memory in adult honey bees (Jaumann et al. 2013, Arien et al. 2015) and solitary wasps (Kishani Farahani et al. 2021). The increased time bees spent searching for their nests could also contribute to the slower nesting rate and reproduction previously observed in these resource-limited bees (Stuligross and Williams 2020). Interestingly, although bee olfactory learning and memory (Stanley et al. 2015b, Siviter et al. 2018, Muth et al. 2019) and navigation (Fischer et al. 2014, Jin et al. 2015; but see Stanley et al. 2016) can be impaired by neonicotinoid insecticide exposure, we found no effect of imidacloprid on *O. lignaria* nest recognition.

Antagonistic behavior

Antagonistic behavior increased with age for control bees but not for those exposed to insecticides. Bee body condition deteriorates throughout the foraging season (O'Neill et al. 2015), and changes such as wing wear can reduce foraging performance (Cartar 1992, Higginson and Barnard 2004). Locomotor activity also declines with age in many insects (Ridgel and Ritzmann 2005, Overman et al. 2022), and age-related behavioral changes may be attributed to a reduction in overall energy efficiency (Overman et al. 2022). If older bees had reduced foraging and nesting capability, they could have more to gain by attempting to steal a neighbor's nest instead of collecting their own pollen and nectar for provisions. This trend of increased agitation and aggression with age has been observed in *O. lignaria* (NMW and CS, *personal observations*) and merits further study.

Insecticide exposure had the opposite effect; younger bees were more antagonistic, and antagonism decreased with age. Neonicotinoids can affect insect aggression in different ways (Barbieri et al. 2013, Pan et al. 2017), but this has been poorly studied in bees (APVMA 2001). However, our results can be explained in part by previous studies on insecticides and aging. Importantly, age is likely associated with increased insecticide exposure in our study. As a systemic insecticide, imidacloprid was present in the pollen and nectar of the flowers growing in flight cages. As a result, bees were chronically exposed, and this exposure increased with age as bees continued to forage on contaminated flowers. Furthermore, imidacloprid can have a stronger impact on older bees (Zhu et al. 2020). The result was higher antagonism in younger exposed bees relative to younger control bees and lower antagonism in older exposed bees relative to older control bees. This is consistent with the other behavioral trends in our study;

insecticide-exposed bees were generally less active, both in foraging and antagonistic behavior. This pattern could also be the result of hormesis, in which lower exposure to insecticides causes stimulation and hyperactivity, whereas higher doses have the opposite effect (Cutler and Rix 2015). Queens in imidacloprid-exposed honey bee colonies were less active than controls, and effects were generally magnified at higher doses (Wu-Smart and Spivak 2016).

A different metric of the outcome of antagonism was nest usurpation itself. Consistent with previous research on solitary bees, usurping bees were larger than usurped bees (Tepedino and Torchio 1994, Kim 1997, Bosch and Vicens 2006), likely because a larger body size can enable usurpers to out-compete usurpees for access to a nest (Fischman et al. 2017). Although neither studied stressor significantly influenced successful usurpation, the time bees spent engaging in antagonistic behavior may have contributed to the overall reduction in reproduction documented for these stressed bees (Stuligross and Williams 2020).

Conclusion

Our study reveals that exposure to insecticides and resource limitation impaired bee foraging behavior, but the two stressors had differential impacts with consequences for populations and ecosystem function. Floral resource limitation required bees to make fewer, longer foraging trips, limiting their ability to return resources to the nest. This largely confirms expectations based on previous empirical studies and foraging theory (Pyke 1980, Westphal et al. 2006). However, we know less about what to expect with insecticide stressors. Past studies on social bees have shown varying results (Gill and Raine 2014, Stanley et al. 2016), but we found depressive effects of insecticide exposure on activity, as well as age x insecticide interactions altering antagonism.

Antagonistic behavior has not been studied in this way, but our results support past research documenting a greater effect of pesticide exposure on solitary bees (leading to reduced performance; Rundlöf et al. 2015). Our results inform our understanding of the impacts of multiple stressors by providing mechanistic links between bee behaviors and fitness outcomes, including direct effects on reproductive success (Stuligross and Williams 2020) and indirect effects on the longevity and survival of bees exposed to insecticides and limited floral resources. Furthermore, the depressive effect of insecticide exposure on bee behavior has implications for ecosystem function. Bees are key pollinators of crops and wild plants, and a reduction in foraging activity will reduce bee visits to flowers, impairing pollination services. These findings reinforce the importance of preventing insecticide exposure and maintaining sufficient forage resources for beneficial insects, particularly in agroecosystems where the stressors are often found together.

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Chapter 3: Past insecticide exposure reduces bee reproduction and population growth rate

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Abstract

Pesticides are linked to global insect declines, with impacts on biodiversity and essential ecosystem services. In addition to well-documented direct impacts of pesticides at the current stage or time, potential delayed "carryover" effects from past exposure at a different life stage may augment impacts on individuals and populations. We investigated the effects of current exposure and the carryover effects of past insecticide exposure on the individual vital rates and population growth of the solitary bee, *Osmia lignaria*. Bees in flight cages freely foraged on wildflowers, some treated with the common insecticide, imidacloprid, in a fully crossed design over two years, with insecticide exposure or no exposure in each year. Insecticide exposure directly to foraging adults and via carryover effects from past exposure reduced reproduction. Repeated exposure across two years additively impaired individual performance, leading to a nearly four-fold reduction in bee population growth. Exposure to even a single insecticide application can have persistent effects on vital rates and can reduce population growth for multiple generations. Carryover effects had profound implications for population persistence and

must be considered in risk assessment, conservation, and management decisions for pollinators to mitigate the effects of insecticide exposure.

Significance Statement

Global insect declines are profoundly concerning, especially for groups like bees that provide important services to humanity. However, we do not know the extent to which recognized drivers of decline, like pesticides, may produce carryover effects that influence reproduction and population dynamics over time. We reveal that pesticide exposure, both directly to foraging bees and via carryover effects from past exposure, dramatically reduced reproduction, which reduced population growth. Carryover effects reduced bee reproduction by 20% beyond current impacts on foraging bees, exacerbating the negative impact on population growth rates. This indicates that bees may require multiple generations to recover from a single pesticide exposure; thus, carryover effects must be considered in risk assessment and conservation management.

Introduction

Global insect declines threaten biodiversity and associated ecosystem function and services (Chagnon et al. 2015, Sánchez-Bayo and Wyckhuys 2019, Wagner et al. 2021), and these dramatic declines of many populations have been linked to pesticides (Potts et al. 2010, Goulson et al. 2015, Woodcock et al. 2016, Sánchez-Bayo and Wyckhuys 2019). Substantial growth of global pesticide production (Tilman et al. 2001), as well as the toxicity of applied insecticides (Schulz et al. 2021), emphasize the necessity to understand the mechanisms and magnitude of their impacts on beneficial insects. Studies of these impacts have primarily focused on the effects of pesticide at the time of exposure (Wu-Smart and Spivak 2016, Müller 2018). However,

pesticides may substantially affect the performance of individuals and populations long after direct exposure, magnifying their consequences.

Such ecological carryover effects, in which an individual's past environment or experience impacts current performance, are well documented across taxa and different environmental stressors (O'Connor et al. 2014). For example, winter habitat quality influences bird reproduction in the following season (Norris et al. 2004, Robb et al. 2008). For organisms with complex life cycles, stress at one life stage (e.g., larvae) may carry over to affect later life stages (e.g., adults). Because many animals feed extensively as larvae, larval food resources can influence adult performance (De Block and Stoks 2005, Chelgren et al. 2006). The maternal environment also affects offspring quality (Mousseau and Fox 1998, Moore et al. 2019) and may profoundly influence the performance of subsequent generations (Mousseau and Dingle 1991, Tran et al. 2018). Although not strictly carryover effects, these too have a similar, indirect, delayed impact.

Stress associated with early life stages may be particularly pertinent for insects with complete metamorphosis from larval to adult stages, for which most feeding occurs during the larval stage (Boggs 2009). Furthermore, larvae often have limited mobility and may not be able to escape stressors as easily as adults (Sgolastra et al. 2019). For example, adult insects commonly move among microsites to moderate temperature, but developing larvae within a nest or attached to a leaf may be unable to escape sun exposure or contaminants within food provisions (Danforth et al. 2019). Carryover effects resulting from larval food environment and temperature conditions

are relatively well studied (Metcalfe and Monaghan 2001, De Block and Stoks 2005, Galarza et al. 2019), but the effects of other stressors, including pesticides, have been much less explored.

Some research indicates that larval pesticide exposure can have sublethal carryover effects on adults. For example, larval exposure to insecticides has been shown to reduce adult body size in bees (Wintermantel et al. 2018), butterflies (Olaya-Arenas et al. 2020), and beetles (Müller et al. 2019); reduce mating behaviors in adult fruit flies (Young et al. 2020); and shorten the lifespan of laboratory-reared adult honey bees (Tsvetkov et al. 2017) and solitary bees (Anderson and Harmon-Threatt 2019). However, we lack an understanding of how these effects may influence reproduction and population dynamics over time (Beckerman et al. 2002, Köhler and Triebskorn 2013).

Understanding the carryover effects of insecticides and other pesticides is particularly important for pollinators in agroecosystems, where insecticide exposure to bees may limit critical crop pollination services (Klein et al. 2007, Chagnon et al. 2015, Stanley et al. 2015a). The negative effects of insecticides to foraging bees in these landscapes are well documented; in addition to direct mortality, insecticide exposure can cause sublethal effects, including reduced reproduction and population density, impaired foraging and learning ability, and increased susceptibility to other stressors such as parasites (Rundlöf et al. 2015, Stanley and Raine 2016, Müller 2018), but carryover effects have not been examined.

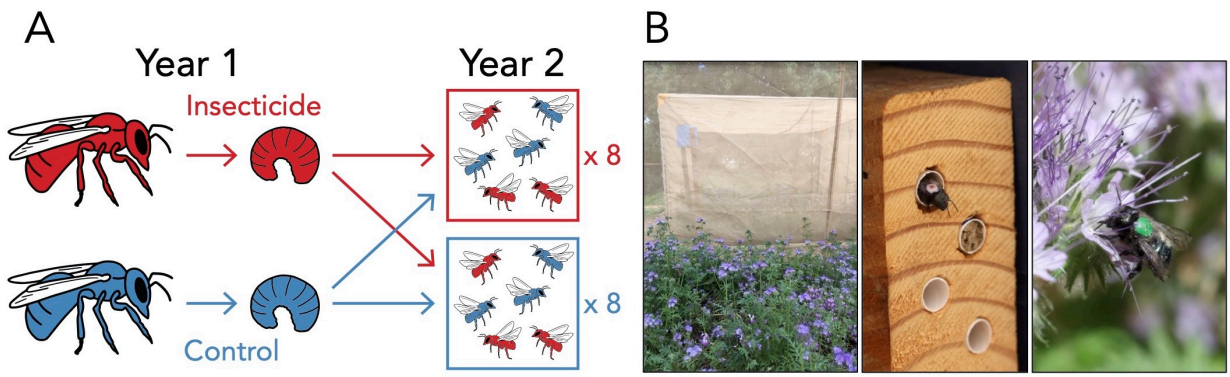

Figure 3.1. Experimental design. (A) Offspring from Stuligross and Williams (2020) were released into 16 field cages in a crossed design. Cages in year 2 were treated with (red) or without (blue) imidacloprid and contained foraging female *Osmia lignaria* with past exposure or without past exposure to imidacloprid in year 1. (B) Flight cage with abundant flower resources (left); paint-marked female *O. lignaria* emerging from a nest (center); paint-marked female foraging on a *Phacelia tanacetifolia* flower (right).

We investigated the carryover effects of insecticide exposure on the performance of the solitary bee species, *Osmia lignaria*. We conducted an in-field cage experiment in which we exposed foraging adult solitary bees to insecticides (or not) across two years. Pesticide exposure to bees is coupled between mothers and offspring because mothers mass-provision resources at the nest (Danforth et al. 2019). Adult females may be exposed to insecticides during foraging and provisioning, which leads to exposure of immature stages through pollen/nectar provisions in the nest. We used the neonicotinoid insecticide imidacloprid, a common systemic insecticide that binds to the nicotinic acetylcholine receptors (nAChRs) in the insect nervous system and poses a high risk to bees (Jeschke et al. 2011, Goulson 2013, Craddock et al. 2019). Using a crossed experiment with past (year 1) and current (year 2) imidacloprid exposure (Figure 3.1), we investigated (1) whether past insecticide exposure (earlier in the life cycle) carries over to affect adult foraging and reproduction, (2) whether current insecticide exposure to adults moderates

these effects, and (3) how exposure to insecticide across multiple years affects bee population growth rates. The crossed cage design allowed us to partition variation in insecticide exposure between the two years and identify carryover effects that may be difficult to detect when not specifically controlled for, especially in real-world landscapes. In addition, this study provides a relatively rare assessment of multiple demographic responses for solitary bees.

Results

Exposure to insecticide reduced female reproduction, both when exposure was directly to foraging adults and via carryover effects from past exposure. The effects were additive with no interactive effects of exposure between years for any measured response (Table C.1). Of the female bees that initiated nesting, those exposed to imidacloprid as adults (year 2) provisioned 30% fewer offspring than unexposed adults (mean ± SE 14.4 ± 1.5 versus 20.7 ± 1.9, respectively; $\chi^2 = 6.01$, $p = 0.01$; Figure 3.2A). Females exposed to imidacloprid in the past (year 1) provisioned 20% fewer offspring compared to individuals with no past exposure (15.6 ± 1.4 versus 19.4 ± 1.6, respectively), indicating a significant carryover effect of insecticide exposure on reproduction ($\chi^2 = 4.68$, $p = 0.03$; Figure 3.2A). Together, females exposed to imidacloprid in both years (as larvae and adults) provisioned 44% fewer offspring than females never exposed to insecticide, a difference of approximately 10 offspring.

Current exposure of adult females to insecticide (year 2) reduced their probability of nesting; adult female bees exposed to imidacloprid were 4% less likely to produce offspring ($\chi^2 = 12.65$, $p < 0.003$; Figure 3.2B). Past exposure (larvae and mothers in year 1) did not carry over to affect current nesting probability ($\chi^2 = 0.37$, $p = 0.55$; Figure 3.2B).

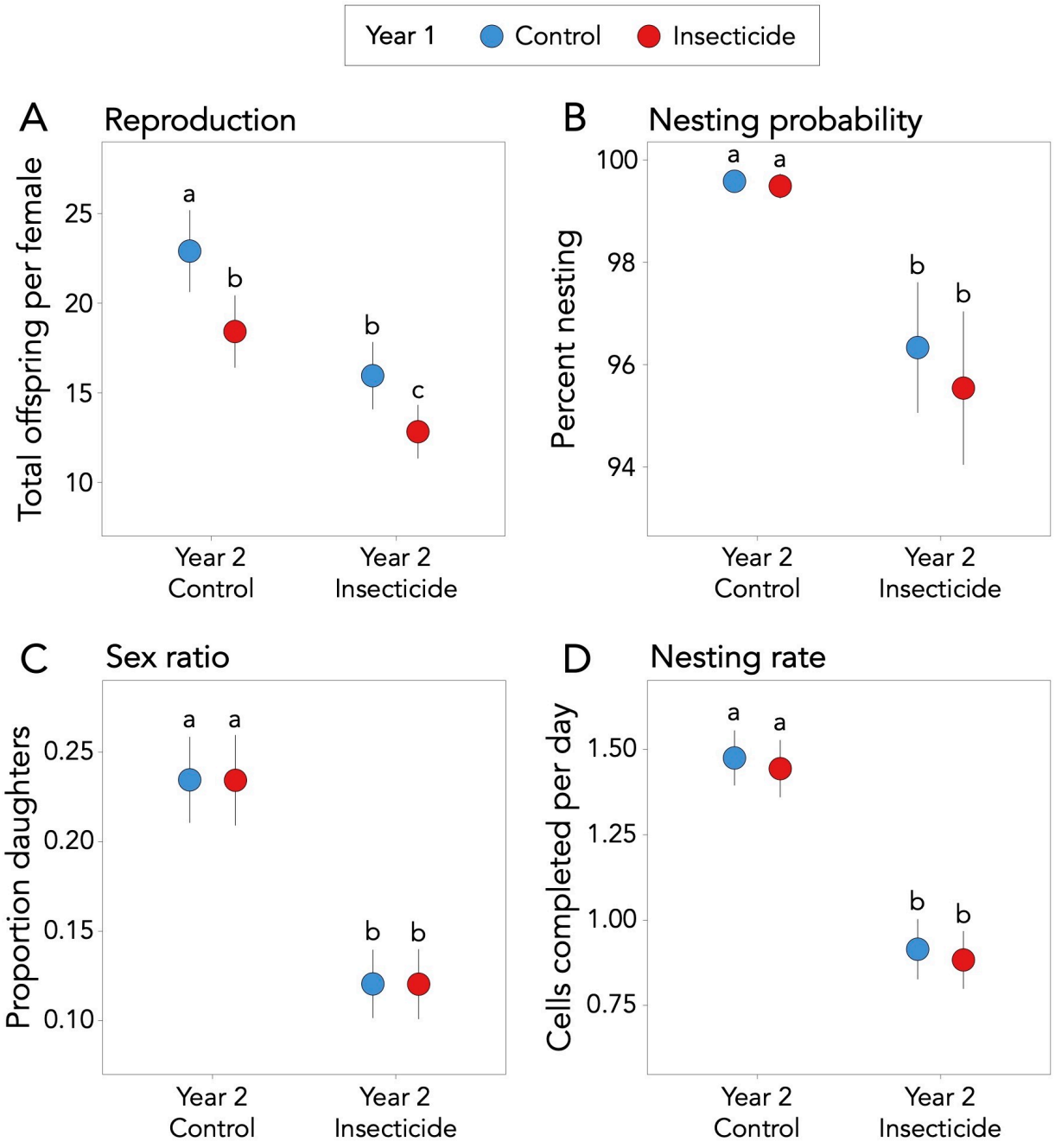

Figure 3.2. Effects of insecticide exposure on bee performance. (A) Mean number of offspring provisioned per nesting female *Osmia lignaria*; (B) percent of female bees that produced at least one offspring; (C) proportion of daughters produced per nesting female; (D) mean number of cells completed per day per nesting female bee in 16 field cages exposed to insecticide (red) or unexposed (blue) the previous year (year 1) and/or current year (year 2). Error bars are SEs; letters indicate significant differences ($p < 0.05$).

In addition to direct effects on reproduction, current exposure to insecticide increased the male-biased sex ratios in foraging adults, with a 49% reduction in the proportion of daughters provisioned by imidacloprid-exposed adult females (year 2; $\chi^2 = 13.6$, $p < 0.001$; Figure 3.2C). Past exposure (year 1) did not carry over to affect offspring sex ratio ($\chi^2 = 0.0001$, $p = 0.99$; Figure 3.2C). Overall, imidacloprid exposure reduced female offspring production by 71%—nesting mothers exposed to imidacloprid in both years provisioned an average of just 1.5 daughters each (Figure 3.2A and 3.2C).

One potential mechanism by which current (year 2) exposure reduced offspring production was nesting rate. Imidacloprid exposure to foraging adults slowed nest construction by 38% (0.56 cells/day; $\chi^2 = 17.29$, $p < 0.0001$; Figure 3.2D). Past exposure (year 1) did not carry over to affect nesting rate ($\chi^2 = 0.11$, $p = 0.74$; Figure 3.2D). Imidacloprid exposure also reduced the total number of days bees spent nesting by 2 days, although this result was not significant ($\chi^2 = 1.6$, $p = 0.45$; Figure C.1).

Carryover effects on individual offspring performance also affected population outcomes. Insecticide exposure lowered the growth rate of cage populations, regardless of the exposure timing. Cage populations exposed to imidacloprid in both the past and current year had a population growth rate ($\lambda \pm$ SE) of 1.48 ± 0.30 (Figure 3.3). This was 20% lower than exposure just in the current year (year 2; 1.85 ± 0.37), 66% lower than exposure just in the past year (year 1; 4.29 ± 0.66), and 72% lower than no exposure at all (5.35 ± 0.76; Figure 3.3). Field population estimates of offspring production from a prior study compared to unexposed cages were 67% lower in the best field environment with abundant floral resources and as much as 94% lower in

the low-resource field environment (Williams and Kremen 2007). When our corresponding insecticide effects were added on top of the field measures of offspring production to estimate population growth rates, imidacloprid exposure could convert growing populations to declining ones (Figure 3.3).

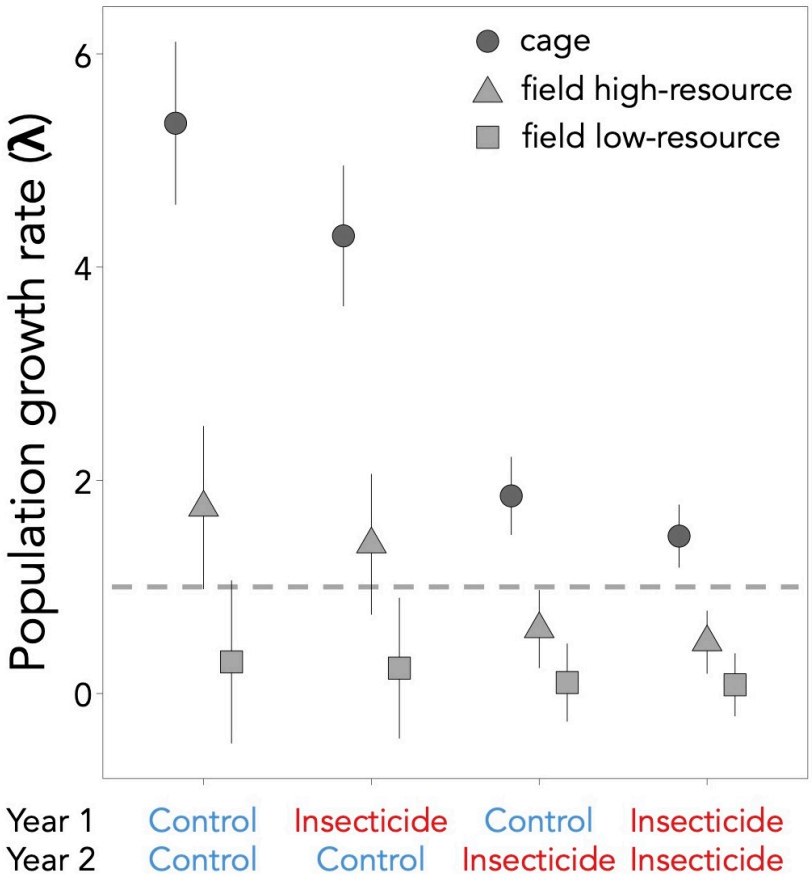

Figure 3.3. Population growth rates (±SE) for *Osmia lignaria* nesting in field cages when exposed to insecticide or unexposed in the previous year (year 1) and/or current year (year 2). Estimates are based on nesting in field cages in the present study (circles) and scaled to open field environments with high-flower resources (triangles) and low-flower resources (squares) as observed in a previous study (Williams and Kremen 2007). The horizontal dashed line marks λ = 1 (values above indicate population growth and values below decline).

Discussion

Bee populations in agricultural landscapes often experience insecticide exposure at multiple stages of the life cycle and over multiple generations (Mullin et al. 2010, Goulson et al. 2018). However, studies to date have generally examined impacts on a single life stage and within one year (but see Willis Chan and Raine 2021). The persistence of pesticide effects from one generation to the next are unknown and have important consequences, such as additive impacts on the dynamics and persistence of populations. We explore these effects to multiple life stages in the same system and on the same individual animals, allowing us to partition the current and carryover effects of chronic insecticide exposure on individual performance and populations to reveal important additive impacts of both. Bees exposed to insecticide, both as nesting adults and in the previous year as developing larvae, provisioned fewer offspring. Insecticide exposure of foraging adults reduced reproduction, reduced the proportion of female offspring produced, delayed nesting onset and cessation, and lowered the rate of nest provisioning; all confirm past research (Stuligross and Williams 2020). Insecticide exposure from the previous year had an additional negative effect, further reducing reproduction. This carryover effect had a lasting implication for population growth.

Past insecticide exposure reduced bee reproduction regardless of their current exposure as adults. Bees exposed to insecticide as larvae in the past year but not subsequently as adults nonetheless provisioned over 30% fewer offspring than control bees that were never exposed to insecticide. This indicates that even exposure to a single insecticide application could have persistent effects on vital rates and longer lasting transient effects of population dynamics (Beckerman et al. 2002). Moreover, because the impacts of insecticides appear to be additive across life stages,

repeated exposure may have profound implications for bee population persistence in many agroecosystems, where frequent exposure may lead to population decline (Woodcock et al. 2016). This is especially concerning considering the persistence of neonicotinoid insecticides in the environment long after application (Goulson 2013, Willis Chan et al. 2019), which we also found in our study (Table C.2).

Importantly, the impacts of larval exposure to imidacloprid were only expressed at the final reproductive output itself. Past insecticide exposure did not affect the subsequent probability of nest initiation, offspring sex ratio, or the rate of nest completion, but appeared only in number of offspring produced. This may be the result of minor negative impacts on each of the intermediate variables measured which add up to reduce the overall reproduction. This delayed observance of effects on later vital rate parameters is precedented in animal and plant systems (Herrera 2000, Jackson et al. 2021). A particularly striking example in plants found that daytime versus evening pollination of a flowering shrub did not influence seed mass, germination, or seedling emergence in the greenhouse (Herrera 2000). But it led only to significant reductions in seedling emergence in the field the following year (Herrera 2000).

The delayed expression of effects that we observed could help to explain the lack of carryover effects found in some past studies, which have shown no effect of larval insecticide exposure on development time or survival in bees and other insects (Nicholls et al. 2017, Olaya-Arenas et al. 2020, Strobl et al. 2021). Our finding emphasizes the importance of considering potential carryover effects of stressors and cautions against interpreting a lack of measured effects on intermediate proxies of vital rates as evidence of no impact on reproduction or population

persistence. Studies that have found no effects of larval neonicotinoid exposure are encouraging but may have missed negative effects that only become evident later.

The biology of *O. lignaria*, as well as that of other solitary bees (Danforth et al. 2019) and animals that feed their offspring (Norris et al. 2004), means that effects of past exposure could be through larval exposure to the food provision, as well as exposure of their mothers during provisioning. Offspring fitness outcomes, including changes in sex ratio and insecticide resistance in insects, have been attributed to maternal effects (Mousseau and Dingle 1991). However, it is often difficult to separate maternal effects from offspring environment or genotype (Wolf and Wade 2009). Indeed, mother bees exposed to insecticides while foraging in real-world landscapes would similarly pass the exposure onto their offspring through the pollen provisions and nesting materials (Sgolastra et al. 2019, Willis Chan et al. 2019). Our free-flying cage design allows us to study these exposure pathways and separate impacts of exposure to current adults from carryover effects. Although we cannot separate maternal from larval effects, past exposure reduced adult reproduction in our study, demonstrating carryover effects from field-realistic exposure across multiple years that impair individual performance and lower population growth.

Population growth rates for all study treatments were positive, so it is perhaps tempting to dismiss the importance of the carryover effects of insecticides on bee fitness. Growth rates from our study are based on individuals in field cages with unlimited food and nesting resources, protected from other threats such as parasites and predators that they may encounter in an open-field setting. When we applied the insecticide impacts estimated from our cages to field-realistic

growth rates, imidacloprid exposure easily converted positive growth to negative growth rates, even in landscapes with abundant floral resources (Williams and Kremen 2007). Testing this impact directly is a critical area for future research (Rundlöf et al. 2015).

The magnitude of the effects of insecticide exposure on reproduction was 55% larger when the exposure was directly to adults in the current year compared to when it was through carryover effects of past exposure as larvae. This difference may result from different pesticide sensitivity among life stages. Some studies have found that bee larvae may tolerate higher exposure to neonicotinoids than adults (Yang et al. 2012, Nicholls et al. 2017). One potential reason for this is that the expression patterns of the nAChRs in the insect nervous system change during development from the larval to adult stage (Dupuis et al. 2012). Because bees during early development have fewer structures with nAChRs in the nervous system than adults, the same exposure to imidacloprid may have a weaker effect when an individual is a larva than when it is an adult. This lowered sensitivity early in life may translate to a less dramatic carryover effect on reproduction. The effects of exposure across life stages are nonetheless additive, so both past and current exposure is more than exposure at either stage alone.

Our study reveals that past exposure to environmental stressors such as insecticides, in addition to current exposure, has profound effects, with implications for individual reproduction and population trends. Hundreds of studies have investigated insecticide effects on bees (Lundin et al. 2015, Müller 2018), but few quantify exposure across generations or to multiple life stages in the same study (Willis Chan and Raine 2021). In our study, carryover effects of past insecticide exposure were not detected until the final reproductive stage, in which their impacts indicate that

populations may take multiple generations to recover from exposure. Furthermore, repeated exposure from one year to the next can have additive effects on individuals' vital rates and, thus, a more detrimental effect on populations. Our results inform pesticide risk assessment and reinforce the importance of preventing insecticide exposure to beneficial insects in landscapes where their effects could substantially reduce population persistence. Future studies to assess multi-year insecticide exposure under field conditions will be important to understand full impacts and inform strategies to mitigate effects of potential exposure.

Materials and Methods

Study system and experimental design

The blue orchard bee *O. lignaria* is a solitary univoltine species native to North America. It and other *Osmia* species are widely used as alternative, managed pollinators to honey bees and/or in combination with them in fruit orchards (Klein et al. 2007, Rundlöf et al. 2015). Females nest above ground inside preexisting cavities (e.g., abandoned wood-boring beetle burrows or artificial paper tubes). Nests are constructed as a linear series of brood cells, which are separated by mud partitions. The entire life cycle takes about a year; females mass-provision offspring using pollen and nectar and lay a single egg on or within each provision. Larvae hatch and consume the provision before spinning a cocoon and pupating. Offspring overwinter as adults within their cocoons and emerge the following spring.

We conducted the experiment in 3 x 3 x 1.8 m flight cages at the University of California (UC) Davis Bee Research Facility during the spring of 2019. In each cage, we planted a high-density mix of three common wildflowers: *Phacelia tanacetifolia, Phacelia ciliata,* and *Collinsia*

heterophylla (Table C.3). These flowers offer high-quality nutrition for offspring, are used by *O. lignaria*, and bloom during their foraging period (Boyle et al. 2020). When flowers approached full bloom (early May 2019), we released eight newly emerged adult female and 16 male *O. lignaria* per cage to match their natural, male-biased sex ratio.

Adult bees used in the trials were sourced from different past insecticide exposure backgrounds. In the previous year (2018), we conducted an experiment using the same field cage design (Stuligross and Williams 2020). In the previous experiment, cages received the same insecticide treatments as the current study, and *O. lignaria* flying in field cages provisioned offspring in nests. Adult female *O. lignaria* in the present study were offspring from either imidacloprid-treated cages or control cages with unlimited floral resources from the past study (Stuligross and Williams 2020). Adult males were offspring only from unexposed control cages, so any effect of past imidacloprid exposure was strictly maternal. Because we sourced all bees from high-resource environments, and males were also from unexposed cages, our findings are likely conservative. The offspring from each past treatment (past exposure versus no past exposure to imidacloprid) were crossed with current imidacloprid treatments (current exposure versus no current exposure) in a reciprocal transplant to enable the differentiation of effects of the current year from those due to past exposure (Figure 3.1A). Half of the females released in each cage were randomly assigned from each of two past insecticide exposure treatments such that each cage had four females with past imidacloprid exposure and four females with no past imidacloprid exposure (Figure 3.1A). We individually marked each female to monitor nesting and distinguish between treatments (Figure 3.1B). In each cage, we placed a wooden nesting block with 12 pre-drilled holes, 7.8 mm in diameter and 13 cm in length. We lined each hole

with a translucent paper straw, which we removed and replaced as they were filled with nests. We stored all completed nests in the laboratory (Williams and Kremen 2007, Stuligross and Williams 2020). We provided a consistent mud source for nest construction throughout the trials using moistened soil from each cage.

We added new bees periodically as others died to maintain an average of five actively nesting females in each cage. To control for possible effects of timing, we balanced bee additions across treatments, and we also included the release date as a covariate in our analyses. In total, we released 161 bees among all cages (n = 40 untreated in both years, 41 untreated in year 1 and treated in year 2, 37 treated in year 1 and untreated in year 2, and 43 treated in both years). We monitored nesting activity daily for a minimum of 20 mins per cage by watching females take foraging trips in and out of their nests; this allowed us to associate each nest with a nesting female. We measured nesting progression daily by temporarily removing the nest straw and marking the nest progress on the outside of the straw (Williams and Kremen 2007).

Completed nests were stored in darkness at 22°C for six months, followed by four months at 6°C to overwinter. The following spring, we opened all nests to determine the number, sex, and condition of all offspring matched to each mother.

Neonicotinoid treatments

We applied a soil drench of the neonicotinoid insecticide imidacloprid (AdmirePro®, Bayer Crop Science) five weeks prior to releasing bees in cages at the maximum label rate (10.5 oz/acre; 767 mL/ha) for herbs and orchard fruit crops. Imidacloprid is the most frequently

applied insecticide in California (California Department of Pesticide Regulation 2018) and is widely used across the United States and worldwide (Jeschke et al. 2011, Bass et al. 2015). AdmirePro is the most common commercial imidacloprid product applied in California (California Department of Pesticide Regulation 2018). Imidacloprid has also been found in *Osmia* nests in agricultural landscapes (Centrella et al. 2020). To prevent lateral movement of imidacloprid through the soil, we buried eight layers of 4-mm clear plastic sheeting 40 cm into the ground between treated and untreated cages. We measured insecticide exposure based on imidacloprid residues from the pollen provisions within nests, a single male larval provision per cage, which were sent for analysis using a modified QuEChERS protocol (Anastassiades et al. 2003) using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis at the Cornell University Chemical Ecology Core Facility. All pollen samples from the insecticide-treated cages contained detectable levels of imidacloprid at field-realistic levels (mean ± SE 11.26 ± 2.82 parts per billion [ppb]; Table C.2). None of the pollen samples from untreated control cages contained detectable imidacloprid levels (Table C.2).

Statistical analysis

We used a generalized and linear mixed model (GLMM) framework to analyze the effects of past and current insecticide exposure on adult *O. lignaria* performance. To assess the effects of insecticide exposure on *O. lignaria* nesting probability, we fit GLMMs with binomial error distribution and logit link. We included past insecticide exposure (year 1 insecticide and year 1 control), current insecticide exposure (year 2 insecticide and year 2 control), and date released in a cage as fixed effects and cage as a random effect. Insecticide exposure did not interact between year 1 and year 2 for any response variable (Table C.1), so we removed the interaction term from

final models. We fit a GLMM with negative binomial error distribution (to account for overdispersion) and log link to assess effects of insecticide exposure on total offspring production and a binomial GLMM to assess insecticide effects on offspring sex ratio (proportion female). We assessed differences in nest construction rate (cells per day) and total nesting days using LMMs with normal error distribution. We calculated p-values of fixed effects in mixed models using likelihood ratio tests. To determine the population growth rate, we multiplied three vital rates: nesting probability x total offspring x proportion female offspring. We only considered females because they are the demographically important sex; male bees do not contribute to the next-generation offspring production. We calculated standard errors for population growth rate means using the delta method (Williams et al. 2002). To explore the relative impact of insecticide exposure on field populations, we calculated differences in reproduction between our cage study and published field data from high- and low-floral resource landscapes (Williams and Kremen 2007) to generate scaling factors that we applied to our cage λ values. We incorporated the same error structure as the cage data and scaled the values according to the effect sizes observed in the cage treatments. We conducted all analyses in R (version 3.6.3).

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Chapter 4. Flower plantings support wild bee reproduction and may also mitigate pesticide exposure effects

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Abstract

1. Sustainable agriculture relies on pollinators, and wild bees benefit yield of multiple crops. However, the combined exposure to pesticides and loss of flower resources, driven by agricultural intensification, contribute to declining diversity and abundance of many bee taxa. Flower plantings along the margins of agricultural fields offer diverse food resources not directly treated with pesticides.
2. To investigate the potential of flower plantings to mitigate bee pesticide exposure effects and support bee reproduction, we selected replicated sites in intensively farmed landscapes where half contained flower plantings. We assessed solitary bee *Osmia*

lignaria and bumble bee *Bombus vosnesenskii* nesting and reproduction throughout the season in these landscapes. We also quantified local and landscape flower resources and used bee-collected pollen to determine forage resource use and pesticide exposure and risk.

3. Flower plantings, and their local flower resources, increased *O. lignaria* nesting probability. *Bombus vosnesenskii* reproduction was more strongly related to landscape than local flower resources.
4. Bees at sites with and without flower plantings experienced similar pesticide risk, and the local flowers, alongside flowers in the landscape, were sources of pesticide exposure particularly for *O. lignaria*. However, local flower resources mitigated negative pesticide effects on *B. vosnesenskii* reproduction.
5. *Synthesis and applications*. Bees in agricultural landscapes are threatened by pesticide exposure and loss of flower resources through agricultural intensification. Therefore, finding solutions to mitigate negative effects of pesticide use and flower deficiency is urgent. Our findings point towards flower plantings as a potential solution to support bee populations by mitigating pesticide exposure effects and providing key forage. Further investigation of the balance between forage benefits and added pesticide risk is needed to reveal contexts where net benefits occur.

1. Introduction

Agriculture dominates global land use, and intensification has led to increased yield but also habitat loss and degradation (Matson et al. 1997, Tscharntke et al. 2012). Pesticide use is a ubiquitous part of intensive agricultural production that is widely implemented to promote crop

production through pest control (Oerke and Dehne 2004, Cooper and Dobson 2007), Even if pesticides provide efficient crop protection, their use comes with undesirable consequences for farmland biodiversity and the environment (Geiger et al. 2010, Beketov et al. 2013). Beneficial organisms in agricultural landscapes usually encounter pesticides as mixtures varying spatiotemporally (Henry et al. 2014, Topping et al. 2015, Sponsler et al. 2019), with ecological context influencing both exposure and impact (Park et al. 2015, Stuligross and Williams 2020, Klaus et al. 2021). Despite growing understanding of general pesticide impacts for a few model insect taxa (Siviter et al. 2021), little is known about how responses vary among beneficial insects and what actions may mitigate impacts (Rortais et al. 2017).

For bees and other flower-visiting insects, combined exposure to pesticides and loss of flower resources, driven by agricultural intensification, contribute to their declines (Goulson et al. 2015, Siviter et al. 2021). Differences among bee life-history traits, such as sociality, foraging behaviour, and nesting, result in substantial differences in resource acquisition and routes of exposure between groups (Winfree et al. 2009, Sgolastra et al. 2019); thus, efforts to mitigate stressors may affect bee groups differently.

Agri-environmental interventions have the potential to mitigate impacts of agricultural management on biodiversity and promote ecological intensification of agricultural production (Bommarco et al. 2013). Hedgerows and flower plantings on agricultural field margins are implemented to provide food and nesting resources for many bee species (Garibaldi et al. 2014) and can increase local bee abundance and richness (M'Gonigle et al. 2015, Scheper et al. 2015, Williams et al. 2015). However, high abundance and richness of bees in flower plantings may

not necessarily translate into reproduction benefits (but see Klatt et al. 2020), and there is uncertainty in how flower plantings influence bee populations (Lowe et al. 2021).

Flower resource scarcity and pesticide exposure can limit reproduction for both solitary and social bees (Holzschuh et al. 2013, Rundlöf et al. 2014, 2015, Crone and Williams 2016, Stuligross and Williams 2020), and the two have been linked to population declines (Goulson et al. 2015, Woodcock et al. 2016). Flower plantings offer diverse food resources that are not directly treated with pesticides, so they may reduce bee pesticide exposure and mitigate its effects through the benefit of abundant clean forage. For example, wild bees in landscapes with a large proportion of natural areas were less affected by pesticide use (Park et al. 2015). Furthermore, providing alternative forage resources to pesticide-treated crop flowers eliminated negative pesticide effect on bees in cages (Klaus et al. 2021, Ingwell et al. 2021). However, flower plantings in agricultural settings could also act as ecological traps if the flowers become contaminated with pesticides (Botías et al. 2015, Mogren and Lundgren 2016).

To address the potential of flower plantings to mitigate bee pesticide exposure and effects and support bee reproduction, we established replicated sites where half contained flower plantings. We used sentinel bees to assess nesting and reproduction, quantified local and landscape flower availability, and used bee-collected pollen to quantify bee pesticide exposure and forage resource use. We asked the following questions:

1) To what extent do bees use flower plantings?
2) Do flower plantings promote bee reproduction?

3) Do flower plantings modify bee pesticide exposure and effects on reproduction?

We studied two native bee species, the solitary bee *Osmia lignaria* and the social bumble bee *Bombus vosnesenskii*. Because these species differ in life-history traits including sociality, body size, foraging range and season, and diet breadth, we expect divergent responses to flower plantings and pesticide exposure.

2. Materials and methods

2.1 Study system

We selected 15 sites along a gradient in landscape contexts in Colusa, Solano, and Yolo Counties, California, USA (Figure 4.1). At seven sites, multi-year wildflower plantings were established to support pollinators. Flowering species native to California had been planted in areas covering 630-3,610 m^2, dictated by uncropped space between fields on the farms (average 1,690 m^2; see Table D1.1 and D1.2 for details). The flower planting sites were paired with control sites within 1.5-16 km lacking flower plantings but otherwise in similar landscapes (Table D2.1). Sites were all conventionally managed and selected to standardize for proximity to riparian or other semi-natural habitat (0-30 m), span the gradient of semi-natural and agricultural land, and maintain at least 1 km geographical separation. The 1 km landscapes surrounding sites, covering the majority of the study species' foraging range, consisted of 2%-63% semi-natural habitat, 37%-91% agricultural land, predominantly almond orchards, and <1%-44% other land uses (Table D2.1). Land use was identified through in-field inspection, complemented with satellite images (Google Maps in 2017) for non-accessible areas, and digitized and analysed in QGIS (version 3.10.0-A Coruña; QGIS Development Team 2021).

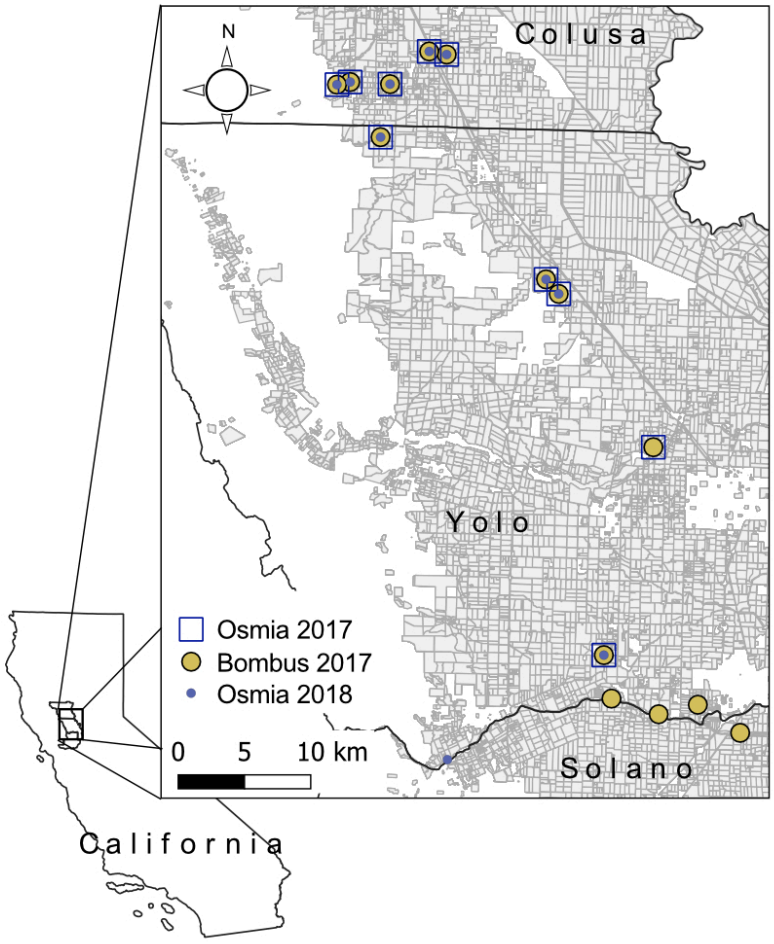

Figure 4.1. Location of the study sites in north Central California with sentinel *Osmia lignaria* nests in 2017 (squares) and 2018 (dots) and *Bombus vosnesenskii* colonies in 2017 (circles). Gray shaded areas are agricultural fields.

2.2 Study bee species

Our study species, *O. lignaria* and *B. vosnesenskii*, are native to the region, have an annual life cycle, nest in pre-existing cavities, and are managed for crop pollination. *Osmia lignaria* is solitary, nests in early spring (March-June) for ~3-6 weeks, and mass-provisions offspring. *Bombus vosnesenskii* is social, with mated queens emerging in spring (March-April) to search for nesting sites and establish colonies that grow until mid/late summer when they produce new queens and males. *Osmia lignaria* is smaller than *B. vosnesenskii* (mean ± SD intertegular span for *O. lignaria* females: 3.1 ± 0.15 mm (Stuligross and Williams 2020); *B. vosnesenskii* queens: 6.73 ± 0.31 mm (Mola et al. 2021); *B. vosnesenskii* workers: 3.65 ± 0.26 mm (Mola et al. 2020)) and can be assumed to have a smaller foraging range based on smaller body size (Greenleaf et al. 2007).

2.3 Osmia lignaria trap nests

At ten sites adjacent to almond orchards (Figure 4.1), we placed two wooden nesting blocks with holes lined with paper straws. We placed 12 female and 24 male *O. lignaria* cocoons in the nesting blocks at each site on 28-29 March 2017 and 24-25 April 2018. In 2017, we also released a cohort of 5 females and 10 males on 20 April. Bees were collected while overwintering within nests from wild-trapped Utah populations, reared in California for several generations in 2017 and from wild-trapped California populations in 2018.

We monitored nesting weekly by temporarily removing straws and marking nest progression on the straw (Williams and Kremen 2007). We sampled pollen from recently completed nests by cutting open the edge of the straw, sampling the pollen provision and then returning the straw to

its hole. Previous research found no impact of such sampling on offspring survival (Williams and Kremen 2007).

At the end of the season, we collected all nests from the field; nests were stored in darkness at 22°C for six months, followed by four months at 6°C to overwinter. The following spring, we x-rayed all nests to determine survival.

2.4 Bombus vosnesenskii colonies

Two small *B. vosnesenskii* colonies, containing a queen and 17-25 workers (mean ± SD 20.7 ± 3.0) and brood in all stages, were placed at each site (Figure 4.1) 18 April-10 May 2017 (see Malfi et al. 2022 for details). The four colonies at paired flower planting and control sites were placed on the same day, with random allocation of colonies. Colonies were raised from wild queens caught near Monterey, California during March 2017 (Permit #SC-13698; for details see Williams et al. 2012, Malfi et al. 2019, 2022). No other permits or licenses were needed for the study.

Every 10 days, colonies were weighed (Malfi et al. 2022) and pollen was collected from foragers returning to the colonies during 1 hour between 09:00-15:00, with observations alternating between morning and afternoon among sites with and without flower plantings. If fewer than 10 pollen foragers had been caught at the end of the hour, pollen collection continued until 10 pollen-carrying bees had been caught or the total collection time reached 2 hours. Corbicular pollen loads were non-lethally removed using forceps.

Colonies were removed from the field when they had lost weight on two consecutive visits, 12 June-4 August, 50-85 days after field placement. Colonies were terminated by freezing (-20 ºC) and dissected to estimate reproduction in the form of new queens. Queen cocoons were separated from worker and male cocoons by their larger size (Williams et al. 2012, Rundlöf et al. 2015).

2.5 Flower resources

Flower resources available to the bees were estimated at two spatial scales: (a) in the 200 m radius surrounding the nests, including the flower planting and hereafter referred to as local flower resources, and (b) in the 1 km radius landscape surrounding the bees, hereafter referred to as landscape flower resources. Local flower resources were estimated using abundance scores for each flowering species in five bins (1-10, 11-100, 101-1,000, 1,001-10,000, and >10,000 flowers). Landscape floral resources were estimated by combining the monthly average flower density on each land use type (see Williams et al. 2012 for details) and the area of the land use types in each study landscape. Floral resources at both scales were filtered to only include species used by *O. lignaria* and *B. vosnesenskii* during their respective foraging periods (Appendix D1) and standardized to represent flowers per m^2.

2.6 Pollen identification

We collected pollen samples randomly from 15 *O. lignaria* brood cells with available pollen and prepared a microscope slide for each. We collected 1,267 *B. vosnesenskii* pollen loads and after sorting them by colour, colony, and sampling round, prepared 594 microscope slides for all unique combinations of these three sorting metrics. See Appendix D1 for details.

We used light microscopy and a pollen reference collection containing flowering plants from the study region (Williams and Kremen 2007) to identify pollen to plant species or genera for planted flowers and regionally common crops (Appendix D1), with the remaining pollen classified as having other plant species origin.

2.7 Pesticide residues and risk index

Pollen from *O. lignaria* provisions were pooled into one sample per site per year. Pollen from *B. vosnesenskii* was pooled per site and two consecutive visits (e.g., pollen collected at 10 and 20 days past colony field placement). Pooling allowed for a sufficient sample for pesticide residue analysis and reduced the cost. Pooled samples weighed ~75 mg (14-106 mg) for *O. lignaria* and ~393 mg (6-771 mg) for *B. vosnesenskii*.

Pollen samples were prepared using a modified QuEChERS method (David et al. 2015) and analysed using LC-MSMS at the Metabolomics Research Laboratory, Purdue University (see Appendix D3). Samples were screened for 52 substances, selected based on use in the counties 2013-2015, high toxicity to bees and/or often detected in bee-related materials in prior North American studies (Table D3.1).

Based on the identified pesticide residues, we calculated a site pesticide risk index following (Sánchez-Bayo and Goka 2014) to capture the combined hazard and exposure level to multiple substances (i) at a site over each bee species' foraging season:

$$\text{Site pesticide risk index} = \sum_{i-1}^{n} \frac{residues\ in\ pollen\ (i)\ ,\ \ ng/g}{average\ toxicity\ oral\ and\ contact\ (i),\ \ ug/bee}$$

2.8 Statistical analyses

We used a generalized and linear model ([G]LM) framework to conduct statistical analyses in R (version 3.6.3; R Core Team 2020) and SAS (version 9.4; SAS Institute Inc.). For analysis of reproduction, we selected model error distributions and link functions after evaluating Poisson, quasi-Poisson, negative binomial, zero-inflated Poisson, zero-inflated negative binomial, hurdle Poisson, and hurdle negative binomial distributions based on residual plots, AICc (Akaike's information criteria for small samples), and the goodness-of-fit chi-squared test for overdispersion. We explored correlation among predictors prior to analyses (Table D2.2) and graphically assessed requirements of distribution and variance homogeneity for all models. We included additional terms to account for variance heterogeneity and overdispersion where necessary (Zuur et al. 2009). P-values from mixed models were calculated using likelihood ratio based on chi-square or F tests. Because of limited number of sites and low bee reproductive success, we chose to consider reproductive effects $p < 0.10$.

To explore how local and landscape flower resources varied with presence of flower plantings, we fit a GLM with gamma error distribution and log link for *O. lignaria*-filtered flowers and a linear model for *B. vosnesenskii*-filtered flowers. *Osmia lignaria* models also included year as a covariate.

To determine if *O. lignaria* reproduction was influenced by local and landscape flower resources, we fit a manual two-step hurdle model to account for zero-inflation in the data. First, we assessed nesting probability as a binary response variable (using a logit link; $n = 20$) predicted by site type (flower planting, control), year, and landscape flower resources. For sites

with nesting, we then assessed the total number of *O. lignaria* offspring provisioned as the response variable, predicted by site type, year, and landscape flower resources with a negative binomial error distribution and logit link (n = 9). To assess the effect of local flower resources on *O. lignaria* reproduction, we replaced site type with local flower abundance in each model, keeping the same structure and distribution.

We used a GLM with negative binomial error distribution and log link to assess the influence of pesticide risk on *O. lignaria* offspring production at sites with nesting. We included year, pesticide risk, and an interaction between local flower resources and pesticide risk as fixed effects (n = 9). We compared the *O. lignaria* pesticide risk between sites with flower plantings and control sites in 2017 using a linear model. Pesticide risk could not be compared between flower and control sites in 2018 because there was no nesting at control sites in 2018, thus no pollen from which to determine pesticide residues. We assessed the difference in overwinter mortality among site types with a binomial GLM, including year as a covariate (n = 9).

We used a GLM with a quasi-Poisson error distribution and log link to explore *B. vosnesenskii* queen production in relation to site type, landscape flower resources, pesticide risk, and the interaction between site type and pesticide risk. Like for *O. lignaria*, we evaluated the influence of local flower resources by including this instead of site type. We removed the interaction term when non-significant ($p > 0.05$). To explore *B. vosnesenskii* pollen collection, the proportion of flower planting and crop pollen was related to sample time (1-3) and site type using GLMs with binomial error distribution and a logit link.

3. Results

3.1 Osmia lignaria reproduction

Osmia lignaria was nearly six times more likely to nest at sites with flower plantings than control sites ($\chi^2 = 3.50$, $p = 0.061$; Figure 4.2a). At the nine sites with nesting, bees provisioned 260% more offspring at sites with flower plantings than control sites, but this was not significant ($\chi^2 = 1.36$, $p = 0.24$; Figure 4.2b).

Nesting probability increased with local flower resources ($\chi^2 = 12.74$, $p < 0.0010$; Figure 4.3a) but was unaffected by landscape flower resources ($\chi^2 = 2.25$, $p = 0.13$; Figure 4.3b). Offspring production was not influenced by pesticide risk ($\chi^2 = 0.08$, $p = 0.78$; Figure 4.4a), local flower resources ($\chi^2 = 0.28$, $p = 0.60$) or their interaction ($\chi^2 = 0.40$, $p = 0.53$). Most provisioned cells successfully developed into adults (88 ± 0.16%, mean ± SD), and overwinter mortality was similar between flower planting and control sites ($\chi^2 = 1.43$, $p = 0.23$).

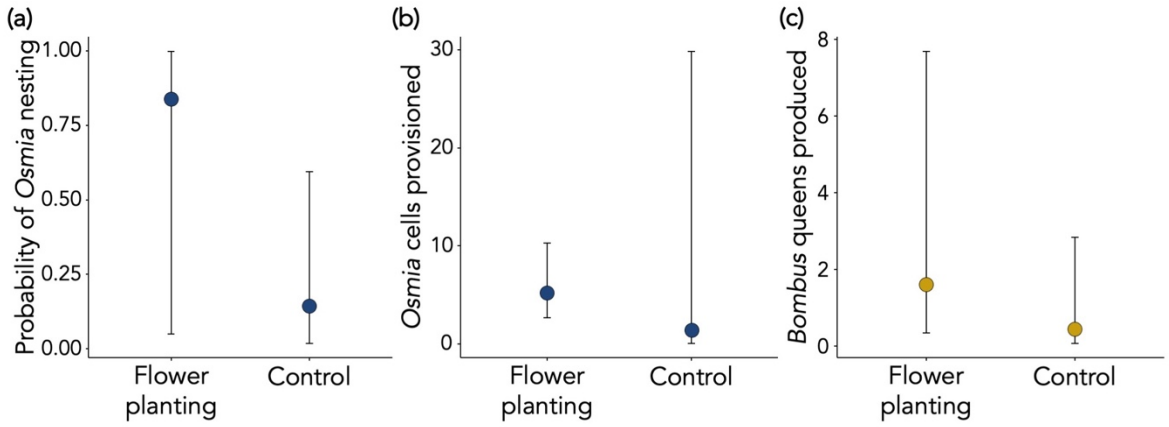

Figure 4.2. (a) Probability of *Osmia lignaria* nesting, (b) total *O. lignaria* cells provisioned, and (c) *Bombus vosnesenskii* queen production at flower planting and control sites. Error bars show 95% confidence intervals.

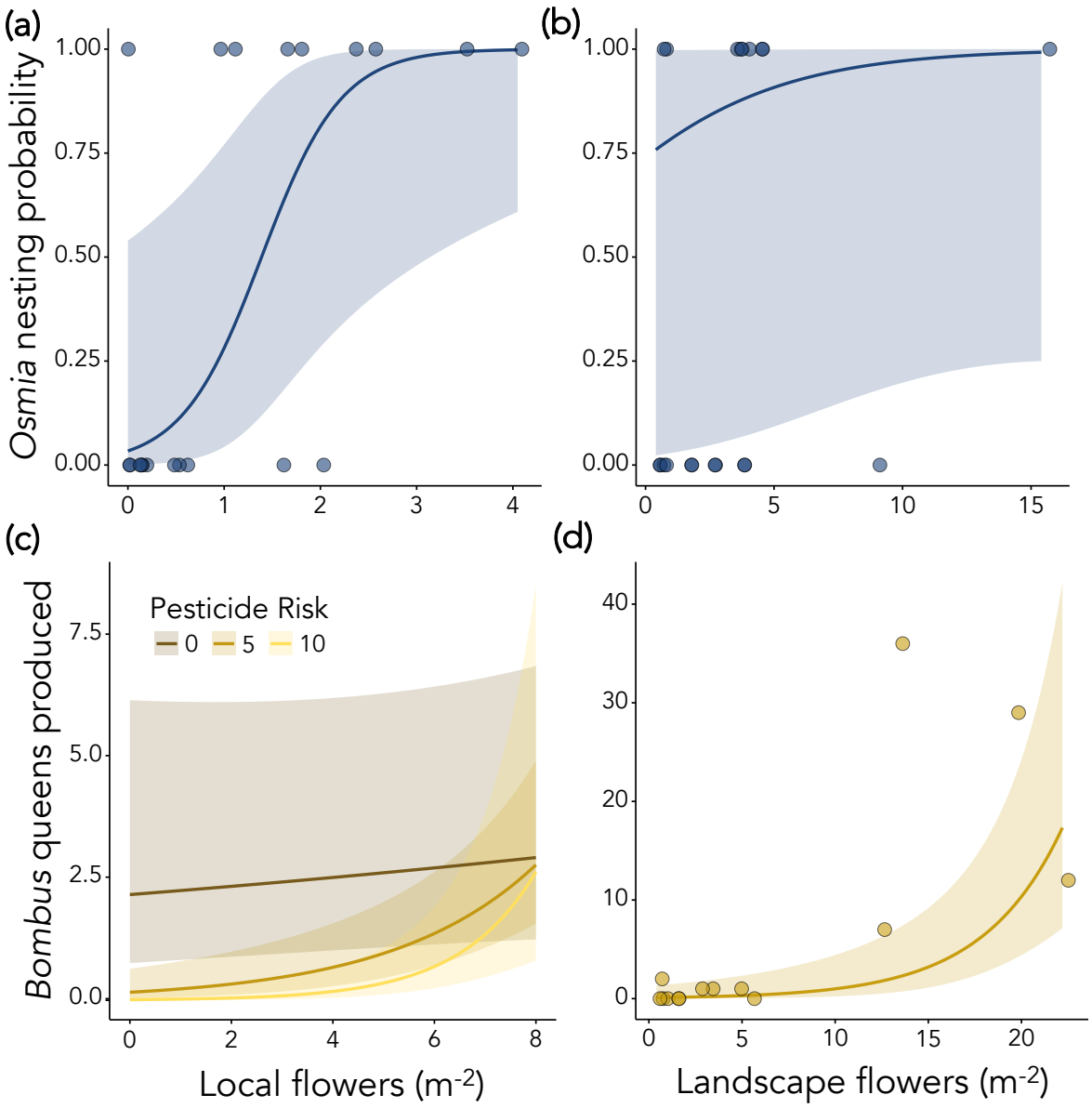

Figure 4.3. Probability of *Osmia lignaria* nesting per site in relation to (a) local and (b) landscape flower resources (flowers/m^2). *Bombus vosnesenskii* queen production by (c) local flower resources in interaction with pesticide risk (see also Figure D3.1) and (d) landscape flower resources. Shading indicates 95% confidence intervals.

3.2 Bombus vosnesenskii reproduction

Bombus vosnesenskii queen production was 266% higher at sites with flower plantings than control sites ($\chi^2 = 3.55$, $p = 0.060$; Figure 4.2c), increased with landscape flower resources ($\chi^2 = 28.97$, $p < 0.0010$; Figure 4.3d), and decreased with pesticide risk ($\chi^2 = 5.75$, $p = 0.017$; Figure 4.4b). Flower planting presence did not modify pesticide risk impact on queen production ($\chi^2 = 1.24$, $p = 0.26$). However, local flower resources interacted with pesticide risk for queen production ($\chi^2 = 8.19$, $p = 0.0042$; Figure 4.3c and Figure D.3.1). With rising pesticide risk, the local flower resource availability became increasingly beneficial for *B. vosnesenskii* queen production.

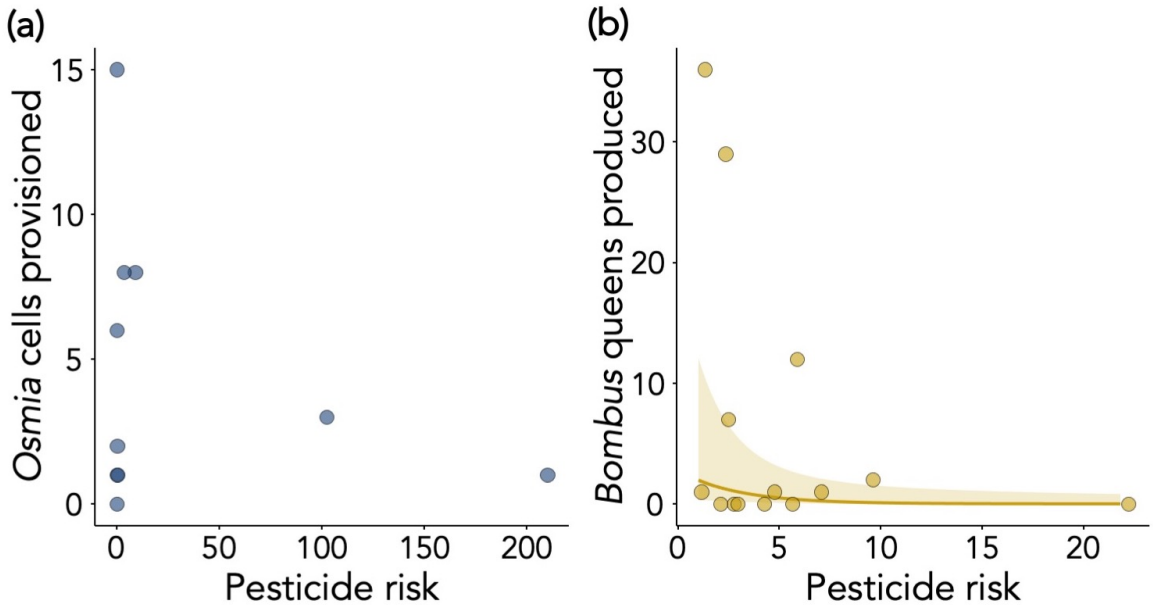

Figure 4.4. Total number of (a) *Osmia lignaria* cells provisioned and (b) *Bombus vosnesenskii* queens produced in relation to pesticide risk. Shading indicates 95% confidence interval.

3.3 Flower resources

Flower resources available locally to *O. lignaria* and *B. vosnesenskii* were higher at flower planting sites compared to control sites ($\chi^2 = 13.49$, $p < 0.0010$ and $\chi^2 = 11.14$, $p = 0.011$, respectively; Figure D1.1a) but did not differ at the landscape scale (*O. lignaria*: $\chi^2 = 0.31$, $p = 0.58$; *B. vosnesenskii*: $\chi^2 = 1.29$, $p = 0.29$; Figure D1.1b). Flower resources at the two scales were not correlated (Table D2.2).

3.4 Pollen

Osmia lignaria primarily collected pollen from sown flowers at flower planting sites (mostly *Collinsia heterophylla*) and from trees at control sites (*Cercis* and *Quercus*; Table 4.1). *B. vosnesenskii* collected a higher proportion of pollen from the sown species, mostly *Eschscholzia californica* and *Lupinus densiflorus* (Table D1.2), at flower planting sites compared to control sites ($F_{1,36} = 5.62$, $p = 0.023$; Table 4.1), with similar proportions over time ($F_{2,24} = 1.03$, $p = 0.37$; Figure D1.2). The proportion of collected crop pollen ($F_{1,11} = 1.22$, $p = 0.29$) and other pollen ($F_{1,11} = 0.02$, $p = 0.90$) did not differ between sites with and without flower plantings (Table 4.1) but varied over time (crop: $F_{2,26} = 5.06$, $p = 0.014$; other: $F_{2,27} = 3.66$, $p = 0.039$; Figure D1.2). The primary crop pollen source for *B. vosnesenskii* was tomato (*Solanum lycopersicum*; Table D1.2). Site type and time period did not interact for the proportion of pollen collected (flower planting: $F_{2,23} = 0.35$, $p = 0.71$; crop: $F_{2,24} = 0.82$, $p = 0.45$; other: $F_{2,25} = 1.13$, $p = 0.34$; Figure D1.2).

Table 4.1. Proportion of flower planting, crop, and other pollen collected by *Osmia lignaria* (means and ranges based on raw data) and *Bombus vosnesenskii* (model estimated means and 95% confidence intervals) at flower planting and control sites.

	Flower planting pollen	Crop pollen	Other pollen
Osmia lignaria			
Flower planting sites	0.88 (0.50-1.00)	0	0.13 (0-0.50)
Control sites	0	0	1.00
Bombus vosnesenskii			
Flower planting sites	0.33 (0.26-0.40)	0.06 (0.02-0.15)	0.61 (0.47-0.74)
Control sites	0.20 (0.14-0.28)	0.11 (0.04-0.27)	0.63 (0.48-0.75)

3.5 Pesticides in pollen

We detected 16 of the 52 screened substances (Table D3.2). Both bee species were exposed to a pesticide mixture, predominantly insecticides and fungicides, but different insecticides presented the highest risks: beta-cyfluthrin and spinetoram for *O. lignaria* and bifenthrin and carbaryl for *B. vosnesenskii* (Table D3.3). Pesticide risk to *O. lignaria* was similar between flower planting and control sites ($\chi^2 = 2.00$, $p = 0.16$; Figure D3.2). *Bombus vosnesenskii* pesticide risk was not related to presence of flower plantings ($F_{1,12} = 0.33$, $p = 0.58$; Figure D3.2) anytime during the season (planting x period $F_{2,24} = 0.41$, $p = 0.67$), but the risk was lower earlier in the season (May) than later (June-July; $F_{2,24} = 5.31$, $p = 0.012$; Figure D3.3).

4. Discussion

Flower plantings can attract and support bees in intensively used agricultural landscapes by providing food resources and nesting habitat (Garibaldi et al. 2014, Williams et al. 2015). These same plantings have complex relationships with agricultural inputs, potentially serving as routes of pesticide exposure (Botías et al. 2015, Mogren and Lundgren 2016) and/or mitigating the negative effects of pesticide exposure (Klaus et al. 2021, Ingwell et al. 2021). We found support for both scenarios, but they differed among taxa in ways consistent with their life-history traits. Flower plantings supported both *O. lignaria* and *B. vosnesenskii* reproduction, particularly at sites with more abundant resources. However, *O. lignaria* relied heavily on the added local flower resources whereas landscape-level flower resources were more important for *B. vosnesenskii*. *Bombus vosnesenskii* reproduction also decreased with increasing pesticide risk, but flower plantings ameliorated this impact. We confirm that flower plantings can add food resources locally to support bee reproduction and mitigate negative effects of pesticide exposure on reproduction for some bee species.

4.1 Local and landscape flower resources

Both bee species' reproduction increased with increasing flower resources, but the predictive scale differed between the two, with *O. lignaria* responding to local flower resources and *B. vosnesenskii* responding more strongly to landscape flower resources. Generally, landscape-level flower resources benefit bees (Williams and Kremen 2007, Williams et al. 2012, Holzschuh et al. 2013, Rundlöf et al. 2014), but the relevant scale varies among species in relation to traits including body size, sociality, and diet breadth. *Osmia lignaria* has a smaller body size and foraging range compared to *B. vosnesenskii* and responds to landscape context at smaller spatial

scales (cf. Steffan-Dewenter et al. 2002). Additionally, although both species are pollen generalists, *O. lignaria* exhibits a narrower diet breadth than *B. vosnesenskii* (Williams and Kremen 2007, Jha et al. 2013). *Osmia lignaria* exclusively collected pollen from sown species at flower planting sites and tree pollen at control sites. However, no *O. lignaria* provisioned any offspring at control sites in 2018 when bees were released later in the season, perhaps due to missing tree bloom and thus lacking this preferred pollen source. This pattern confirms earlier work in the region that found *O. lignaria* preference for pollen of native wildflowers and trees, even in agricultural landscapes (Williams and Kremen 2007). *Bombus vosnesenskii* also collected pollen from sown species to a greater extent at sites with flower plantings. However, contrary to *O. lignaria*, *B. vosnesenskii* also collected pollen extensively from non-sown plants, including crops, at most sites. This preference for non-crop pollen and extensive collection of *E. californica* pollen is consistent with reported *B. vosnesenskii* pollen use in the region (Jha et al. 2013). Although flower plantings only weakly promoted *B. vosnesenskii* reproduction, abundant local flower resources mitigated the negative reproductive effects of pesticide exposure.

4.2 Pesticide exposure and impacts on reproduction

The reproductive consequences of pesticide exposure were more evident for *B. vosnesenskii*, for which queen production decreased with increasing pesticide risk. The pattern was similar for *O. lignaria*, which showed low reproductive success at the two sites with the highest pesticide risk, despite a lack of statistical significance likely due to high control site variability. Rather than a mismatch between pesticide risk and reproduction between the two species, we suspect the difference lies in a limitation of pollen provision-derived residue data. Lack of nesting and pollen provisioning precludes assessment of pesticide-related risk. Field studies have verified that

pesticide exposure from agricultural use can limit reproduction in other *Osmia* and *Bombus* species (Rundlöf et al. 2015, Woodcock et al. 2017). Neither species performed well in our study; *O. lignaria* did not replace itself at any site, and *B. vosnesenskii* reproduction was below regional averages (Crone and Williams 2016). Our study sites likely had higher pesticide use and lower flower resource availability than past study landscapes (Williams and Kremen 2007, Crone and Williams 2016), and both factors combine to influence reproduction. At low pesticide risk, bees had the potential to produce offspring, but reproduction was variable due to other factors. Pesticide risk instead appeared to set an upper limit to reproduction regardless of other enabling factors, resulting in low reproduction with little variance at higher pesticide risk. Recent studies show that forage availability and pesticide exposure can additively affect reproduction in *O. lignaria* (Stuligross and Williams 2020) and that alternative forage resources can reduce and thereby mitigate exposure consequences (Klaus et al. 2021). In our study, although the estimated pesticide risk was similar between sites with and without flower plantings, abundant local flowers mitigated the negative effects of pesticide exposure at sites with higher pesticide risk, promoting increased *B. vosnesenskii* reproduction. This points towards flower plantings as a potential tool to mitigate pesticide effects on bees and a solution to ameliorate the negative effects of agricultural intensification with concomitant loss of flowers and exposure to pesticides. However, because the influence differed between species, further studies should verify the generality and underlying mechanisms.

4.3 Phenology of pesticide risk and flower resources

In agricultural landscapes, flower resources and pesticide use change over space and time (Larsen et al. 2020). Thus, flower phenology influences bee activity and expected pesticide

exposure (Sponsler et al. 2019). *Bombus vosnesenskii* and *O. lignaria* have overlapping but distinct activity periods, with *O. lignaria* nesting earlier in spring for just a few weeks and *B. vosnesenskii* nesting later and flying into the summer. The identified high-risk pesticides have a wide range of uses in the study region during these periods. During *O. lignaria*'s activity period (April-May), beta-cyfluthrin is applied primarily to orchard trees, including almond (California Department of Pesticide Regulation 2017). Spinetoram is occasionally applied to a few crops in April but is heavily applied to walnut, tomato, and almond in May (California Department of Pesticide Regulation 2017). This suggests that exposure to *O. lignaria* likely came from drift to flower plantings and trees from applications in adjacent almond orchards. During *B. vosnesenskii*'s activity period (April-August), bifenthrin is mostly applied to almond, as well as other crops including tomato, melon, and squash (California Department of Pesticide Regulation 2017). Carbaryl is also applied to many crops during these months, especially tomato and almond (California Department of Pesticide Regulation 2017). About 10% of the *B. vosnesenskii* pollen came from crop plants, with tomato being the dominant source. This points to tomato as both a favoured forage plant and a source of pesticide exposure. However, most pollen came from non-crop sources, so it is likely that both non-crop and crop sources contribute to pesticide risk.

Osmia lignaria experienced a more variable pesticide risk, including the highest risk, of the two species, despite more limited data, whereas *B. vosnesenskii* experienced a more consistently higher average risk. This could be related to *O. lignaria*'s smaller foraging range and period, narrower pollen diet, and resulting reliance on the flower plantings adjacent to crops receiving high-risk pesticides, while *B. vosnesenskii* integrated resources over a larger area and longer

season using proportionally less pollen from species in the flower plantings (although more crop pollen). Non-crop pollen can be a substantial source of pesticide exposure and risk to bees (McArt et al. 2017b), but so can pollen from specific crops (Böhme et al. 2018). Regardless of the source of pesticide exposure, the flower plantings provided a net benefit to both bee species.

4.4 Implications for agricultural management and bee conservation

The creation of small habitat patches offers a strategy to mitigate correlated threats to beneficial insects from simplification and intensified use of agricultural landscapes. Our results suggest that such action in the form of flower plantings offers net positive outcomes for bees, but impacts are likely to vary among species, locations, and pesticide use practices. Our results indicate that both flower resource availability and pesticide use reductions are critical tools for supporting bee populations in agricultural landscapes. The value of the flower plantings—particularly those that provided abundant resources—to *O. lignaria* suggests that smaller flower plantings can benefit bee species with limited spatial and temporal foraging. The weaker influence of the flower plantings and stronger influence of landscape flower resources for *B. vosnesenskii* reproduction points towards the need for landscape-level conservation planning for wider-foraging bees. It also highlights the potential benefit of habitat at greater distances from nesting sites and thus flexibility in habitat arrangement in the landscape.

Although placement of flower plantings adjacent to crops offers potential to capture pollination benefits (Blaauw and Isaacs 2014, Albrecht et al. 2020, Lowe et al. 2021), it can lead to unintentional pesticide exposure (Mogren and Lundgren 2016), as observed for *O. lignaria* via pollen from sown species. Optimizing planting placement to support pollination services but

simultaneously reducing the probability of pesticide drift requires understanding of bee activity patterns in relation to habitats and spatiotemporal pesticide distribution. Coordination among neighbouring growers to take a landscape perspective that includes integrated pest and pollinator management (IPPM) strategies is an important approach to magnify habitat benefits (Lundin et al. 2021). Nevertheless, even when crops were adjacent to flower plantings, the benefits of the added flowers outweighed the pesticide impacts. As such, wildflower plantings could be a key part of IPPM strategies, providing net benefits where there is more limited flexibility to change or reduce pesticide-related risk.

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Appendix

A. Supplementary material, Chapter 1

Supplementary figures

Figure A.1. Photographs of experimental setup. (A) Exterior of flight cages; (B) Inside of a flight cage with a high-resource flower resource treatment; (C) Inside of a flight cage with a low-resource flower resource treatment. Here you can also see the wooden nest block installed in the corner of the cage.

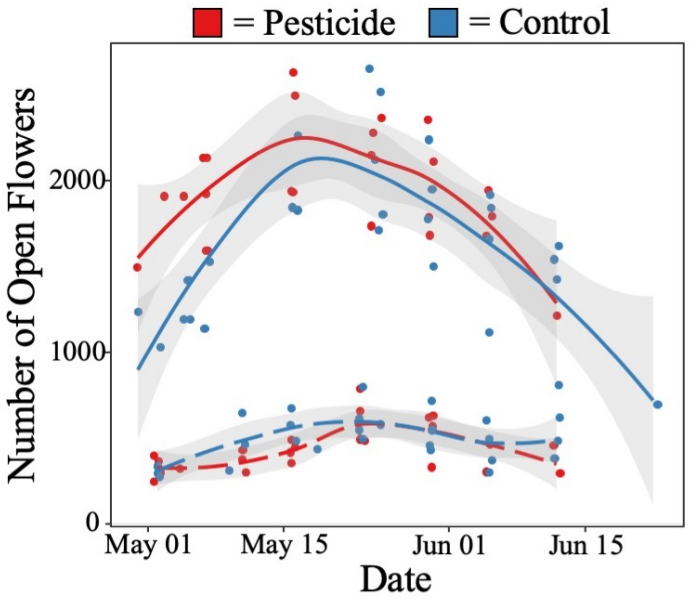

Figure A.2. Number of open flowers in pesticide treated (red) and untreated (blue) cages with high (solid line) and low (dashed line) floral resource treatments.

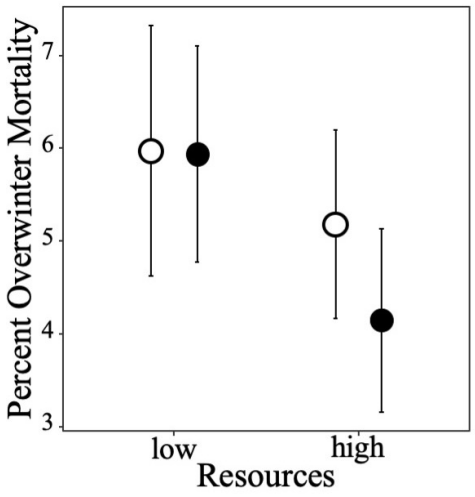

Figure A.3. Percent overwinter mortality of *Osmia lignaria* offspring with pesticides (black) or without pesticides (white) in high and low floral resource environments. Error bars show SEs.

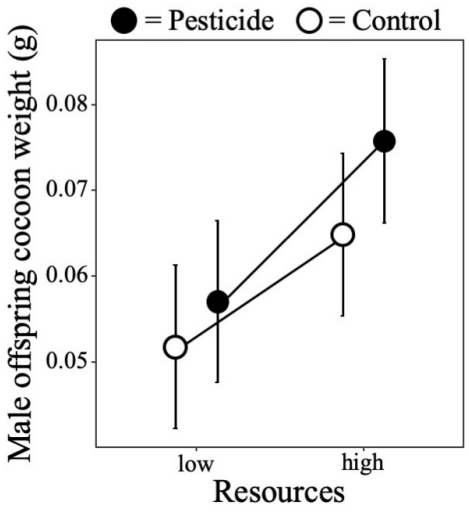

Figure A.4. Mean (± SE) cocoon weight (g) of male offspring produced in cages with pesticides (black) or without pesticides (white) in high and low floral resource environments. Male offspring weighed 29% more in high-resource treatments ($\chi^2 = 21.28$, $p < 0.001$) and 14% more in pesticide treatments ($\chi^2 = 16.8$, $p < 0.001$).

Supplementary tables

Table A.1. Flower seed mix planted in field cages. Amount of seed sown in each cage was calculated based on the number of live seeds per gram in each seed lot to achieve a target number of live seeds per m^2. *Phacelia* spp. seeds were sourced from Pacific Coast Seed in Tracy, CA. *Collinsia* seed was sourced from Hedgerow Farms in Winters, CA.

Plant Species	Target Live Seeds per m^2	Grams sown per cage
Phacelia tanacetifolia	121	0.80
Phacelia ciliata	75	0.50
Collinsia heterophylla	97	0.87

Table A.2. Mean number of flowers containing pollen at the end of the day.

Pesticide treatment	Resource treatment	Mean number of flowers open with pollen	SE
Not treated	High	618.45	25.74
Not treated	Low	0.30	0.04
Treated	High	1126.95	31.93
Treated	Low	0.83	0.06

Table A.3. Imidacloprid concentrations in *Osmia lignaria* nest provisions. The limit of detection (LOD) is 0.03-0.1 ng g^{-1} per sample. The limit of quantification (LOQ) is 1 ng g^{-1} per sample.

Cage	Imidacloprid treatment	Imidacloprid residue level (ng g^{-1})
1	Treated	Below LOD
2	Treated	Between LOD and LOQ; 3.94
3	Treated	17.87
4	Treated	Below LOD
5	Treated	Below LOD
6	Treated	Below LOD
7	Treated	Below LOD
8	Treated	Below LOD
9	Not treated	Below LOD
10	Not treated	Below LOD
11	Not treated	Below LOD
12	Not treated	Below LOD
13	Not treated	Below LOD
14	Not treated	Below LOD
15	Not treated	Below LOD
16	Not treated	Below LOD

We are confident that the pesticide levels we established in study treatments represent field-realistic exposure. Two samples from our study contained detectable levels of imidacloprid (3.94 and 17.87 ppb). This is well within the range of imidacloprid levels quantified in honey bee-collected pollen from other studies (range: <0.1-912 ppb; Blacquière et al. 2012). In North America, a broad sample of pollen from honey bee hives detected an average of 39 ± 19 ppb imidacloprid in pollen (Mullin et al. 2010). Pollen collected from *O. lignaria* nests in Davis, CA in 2017 contained 13.81 ppb imidacloprid (Rundlöf, Stuligross et al. 2022). The pollen was from wildflowers planted adjacent to an almond orchard near the bee nesting site, confirming that pesticide drift onto flower plantings poses a real risk to bees in working CA landscapes.

Supplementary methods: Pesticide residue analysis

Sampling pollen from nests

Upon nest completion, we collected nest straws and immediately carried them into the lab where they were stored in complete darkness. Nests were not exposed to light for extended periods, likely less than five minutes in total—only during transport from field cages into the lab and during pollen sampling. We sampled pollen upon nest collection from each cage during the third week of the study. We placed pollen into a single microcentrifuge tube per cage and stored in darkness at -80°C. We shipped samples overnight to the Purdue Metabolomics Research Laboratory on dry ice.

Pesticide residue analysis protocol used by Purdue Metabolomics Research Laboratory

The protocol is based on David et al. (2015)

- 500uL Acetonitrile and 400uL ddH2O is added to each pollen sample
- Add 10uL of 1ng/uL internal standard (d4-Imidacloprid)
- Add 200mg magnesium sulfate and 50mg sodium acetate
- Shake and vortex for 10min; centrifuge at 13000rpm for 10min
- Transfer supernatant to Agilent QuEChERS Dispersive Kit (cat.no. 5982-5421) containing 50mg PSA, 50mg GCB, 50mg C18EC, and 150mg MgSO4
- Shake and vortex for 10min; centrifuge at 13000rpm for 10min
- Transfer and save supernatant to a clean tube
- Extract the QuEChERS Dispersive Kit solid phase again with 400uL acetonitrile/toluene (3:1)
- Shake and vortex for 10min; centrifuge at 13000rpm for 10min
- Transfer and combine the supernatant with the previous saved extract
- The samples are dried using a rotary evaporation device at room temperature
- Reconstitute the dried samples with 120uL 50% acetonitrile in ddH2O prior to LC-MSMS analysis

The samples were quantified with an Agilent 6460 Triple Quadrupole (QQQ) (Santa Clara, CA, USA) using Liquid Chromatography tandem Mass Spectrometry (LC-MSMS). An Agilent 1200 Rapid Resolution liquid chromatography (LC) system coupled to an Agilent 6460 series QQQ mass spectrometer (MS) was used to analyze pesticides in each sample. A Waters XBridge Phenyl 2.1 x 100 mm, 3.5 µm column was used for LC separation (Waters Corporation, Milford, MA). The buffers were (A) water + 5 mM ammonium acetate + 0.1 % formic acid and (B) acetonitrile (90%) + 5 mM ammonium acetate (10%) + 0.1% formic acid. The linear LC gradient was as follows: time 0 min, 5 % B; time 2 min, 5 % B; time 8 min, 100 % B; time 12 min, 100 % B; time 12.1 min, 5 % B; time 17 min, 5 % B. The flow rate was 0.3 mL/min. Multiple reaction monitoring was used for MS analysis. The data were acquired in positive electrospray ionization (ESI) mode. Precursor ions of d4-Imidacloprid and Imidacloprid had respective molecular weights of 260 and 256 g/mol with product ions of 179 and 175 g/mol. For both ions, the dwell was set at 50 msec, fragmentor voltage at 70, collision energy at 10V, cell accelerator voltage at 1, with positive polarity. The jet stream ESI interface had a gas temperature of 330°C, gas flow rate of 10 L/min, nebulizer pressure of 35 psi, sheath gas temperature of 250°C, sheath gas flow rate of 7 L/min, capillary voltage of 4000 V in positive mode, and nozzle voltage of 1000 V. The ΔEMV voltage was 300. All data were analyzed with Agilent Masshunter Quantitative Analysis (Version B.06.00).

B. Supplementary material, Chapter 2

Supplementary figures

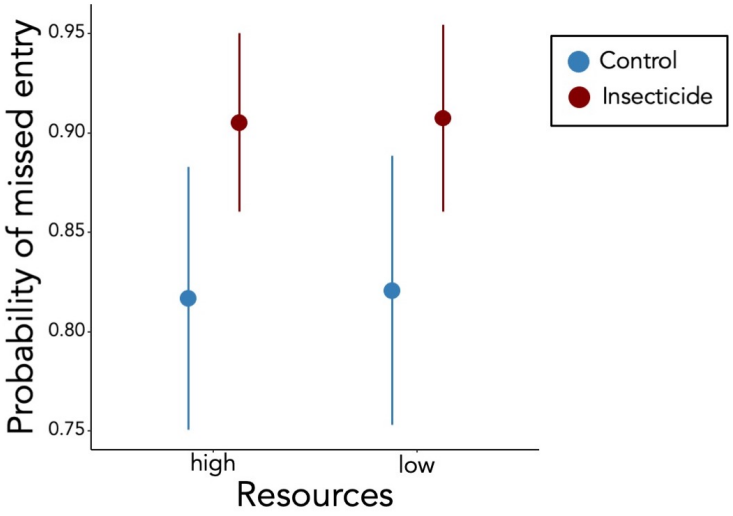

Figure B.1. Effects of resource limitation and insecticide exposure on the probability of female *Osmia lignaria* missed nest entry during observation in 16 field cages with insecticides (red) or without insecticides (blue) in high- and low-floral resource environments. Model estimated means and SEs.

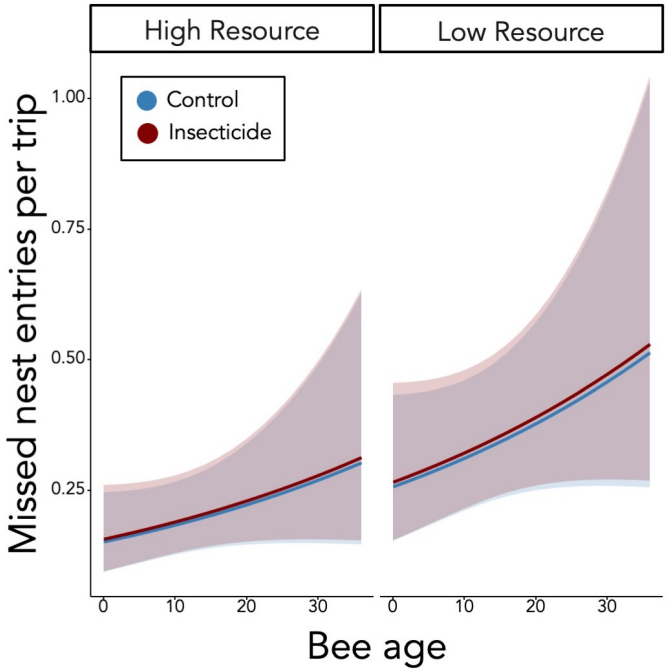

Figure B.2. Number of missed nest entries per foraging trip in relation to bee age (number of days since release in cage) in high- and low-resource environments and with (red) or without (blue) insecticide exposure. Shading indicates 95% confidence intervals.

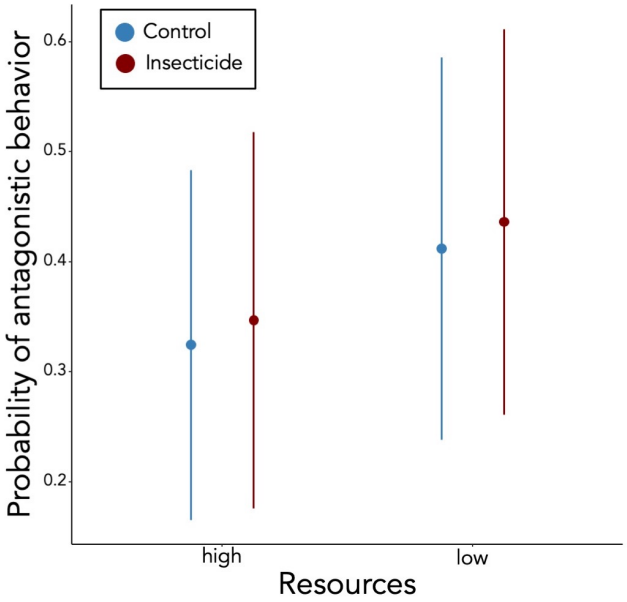

Figure B.3. Effects of resource limitation and insecticide exposure on the probability of female *Osmia lignaria* engaging in antagonistic behavior during observation in 16 field cages with insecticides (red) or without insecticides (blue) in high- and low-floral resource environments. Model estimated means and SEs.

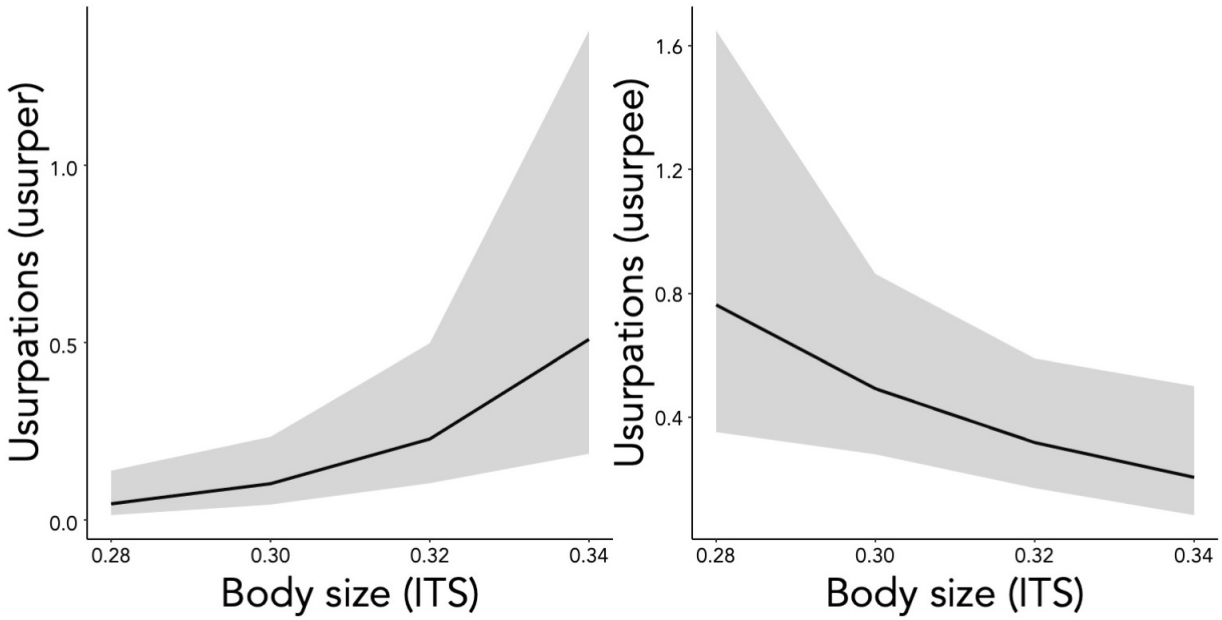

Figure B.4. (a) Number of usurpations and (b) number of times usurped per individual bee in relation to bee body size. Shading indicates 95% confidence intervals.

Supplementary tables

Table B.1. Results of mixed model testing the effect of (a) body size (ITS) and (b) bee age on measured responses. Significance was estimated using a likelihood ratio test on nested models with and without the term included. When significant, results are reported in the main manuscript.

Response	**ITS**		**Bee age**	
	χ^2	**P-value**	**χ^2**	**P-value**
Total trips	0.652	0.420	0.174	0.677
Foraging trip length	0.017	0.897	*Main manuscript*	
Prob. of missed nest entry	1.10	0.295	0.833	0.361
Number of missed nest entries	0.444	0.505	2.04	0.153
Prob. of antagonistic behavior	2.30	0.130	1.37	0.242
Antagonistic behavior time	0.013	0.910	*Main manuscript*	
Number of usurpations	*Main manuscript*		*NA*	

Table B.2. Results of mixed model testing the interactions between (a) resource availability and insecticide exposure, (b) bee age and insecticide exposure, and (c) bee age and resource availability on measured responses. Significance was estimated using a likelihood ratio test on nested models with and without the term included. When significant, results are reported in the main manuscript.

Response	**Resource-insecticide interaction**		**Age-insecticide interaction**		**Age-resource interaction**	
	χ^2	**P-value**	**χ^2**	**P-value**	**χ^2**	**P-value**
Total trips	0.338	0.561	0.180	0.914	1.68	0.432
Foraging trip length	0.528	0.468	1.23	0.268	0.936	0.333
Prob. of missed nest entry	0.048	0.827	0.835	0.659	1.22	0.543
Number of missed nest entries	0.339	0.560	2.17	0.338	2.12	0.346
Prob. of antagonistic behavior	2.78	0.095	2.52	0.283	1.54	0.463
Antagonistic behavior time	0.819	0.365	*Main manuscript*		1.36	0.507
Number of usurpations	0.401	0.526	*NA*		*NA*	

C. Supplementary material, Chapter 3

Supplementary figures

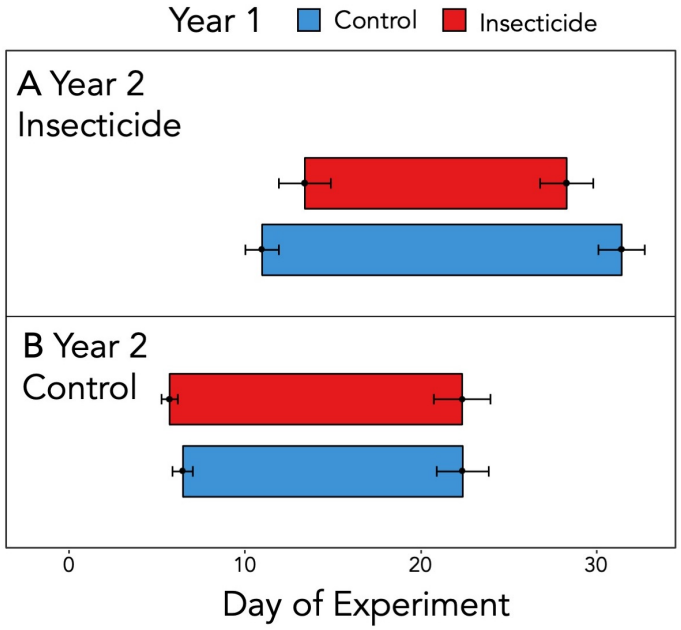

Figure C.1. Nesting onset and duration. Mean (± SE) number of days between the first and last offspring provisioned by each nesting female exposed to insecticide (red) or unexposed (blue) in the previous year (year 1) and (A) exposed to insecticides or (B) unexposed in year 2.

Supplementary tables

Table C.1. Results of generalized and linear mixed model testing the interaction between insecticide exposure in year 1 and insecticide exposure in year 2 on measured responses. Significance of interactions were estimated using a likelihood ratio test on nested models with and without the interaction term.

Response	χ^2	**P-value**
Total offspring	2.18	0.140
Percent nesting	1.67	0.196
Proportion daughters	0.05	0.816
Cells per day	0.21	0.646
Total nesting days	1.82	0.178

Table C.2. Imidacloprid concentrations in *Osmia lignaria* nest provisions. The limit of detection (LOD) is 0.07 ng per sample. The limit of quanitification (LOQ) is 0.21 ng per sample.

Cage	Imidacloprid treatment	Imidacloprid residue level (ng g^{-1})
1	Treated	5.35
2	Treated	4.83
3	Treated	7.33
4	Treated	7.39
5	Treated	28.84
6	Treated	16.31
7	Treated	9.00
8	Treated	11.01
9	Not treated	Not detected
10	Not treated	Not detected
11	Not treated	Not detected
12	Not treated	Not detected
13	Not treated	Not detected
14	Not treated	Not detected
15	Not treated	Not detected
16	Not treated	Not detected

We are confident that the pesticide levels we established in study treatments represent field-realistic exposure. The residue levels are well within the range of imidacloprid levels quantified in honey bee-collected pollen from other studies (range: <0.1-912 ppb; Blacquière et al. 2012). In North America, a broad sample of pollen from honey bee hives detected an average of 39 ± 19 ppb imidacloprid in pollen (Mullin et al. 2010).

Table C.3. Flower seed mix planted in field cages. Amount of seed sown in each cage was calculated based on the number of live seeds per gram in each seed lot to achieve a target number of live seeds per m^2. *Phacelia* spp. seeds were sourced from Pacific Coast Seed in Tracy, CA. *Collinsia* seed was sourced from Hedgerow Farms in Winters, CA.

Plant Species	Target Live Seeds per m^2	Grams sown per cage
Phacelia tanacetifolia	121	0.80
Phacelia ciliata	75	0.50
Collinsia heterophylla	97	0.87

D. Supplementary material, Chapter 4

APPENDIX D1. Flower plantings, flower resources, and pollen identification

Supplementary figures

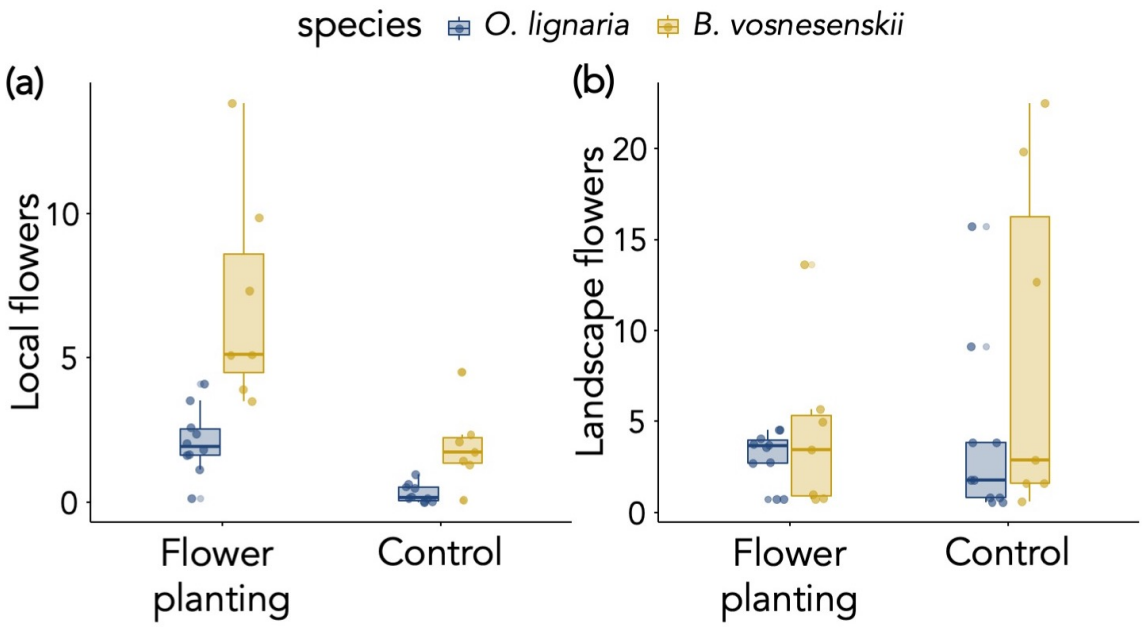

Figure D1.1 (a) Local and (b) landscape flower resources (flowers per m^2) available to *Osmia lignaria* (blue) and *Bombus vosnesenskii* (yellow) at flower planting and control sites. Box plots indicate the median, the 25th and 75th percentiles (box edges), the range (whiskers), and outliers (small dots).

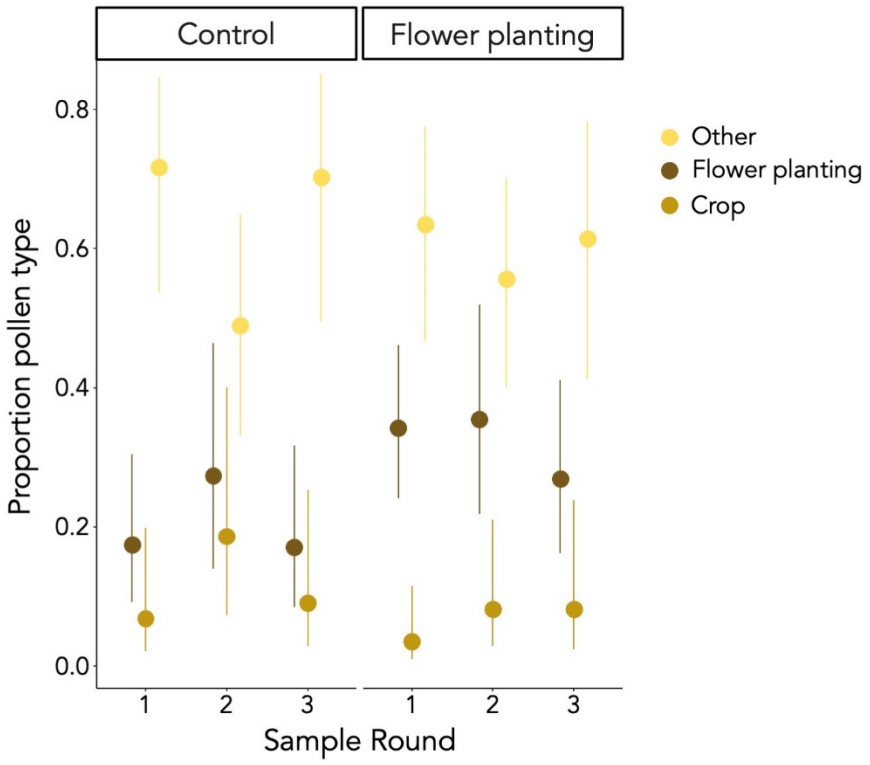

Figure D1.2 Proportion flower planting (brown), crop (mustard), and other (yellow) pollen collected by *Bombus vosnesenskii* at flower planting and control sites over three sampling rounds. Model estimated means and 95% confidence intervals.

Supplementary tables

Table D1.1 Flower planting details on planting sizes, planting year, and planted flower species.

Site	Planting size (m^2)	Planting year	Species planted
ABE	632	2015	*Acmispon glaber, Clarkia unguiculata, Clarkia williamsonii, Collinsia heterophylla, Eschscholzia californica, Grindelia squarrosa, Helianthus bolanderi, Lupinus formosus, Lupinus microcarpus, Lupinus succulentus, Monardella villosa, Nemophila menziesii, Phacelia californica, Phacelia campanularia, Phacelia ciliata, Scrophularia californica, Sphaeralcea ambigua, Trichostema lanceolatum, Trifolium fucatum, Trifolium gracilentum*
GAL	991	2015	Same as above
FAH	3612	2015	Same as above
LON	1029	2015	Same as above
GIL	1497	2016	Same as above
RUS	3070	2015	*Clarkia williamsonii, Eschscholzia californica, Grindelia camporum, Lupinus densiflorus, Lupinus formosus, Lupinus succulentus, Monardella villosa, Phacelia californica, Phacelia ciliata, Phacelia tanacetifolia, Trichostema lanceolatum, Trifolium fucatum*
BEE	972	2015	*Achillea millefolium, Amsinckia intermedia, Antirrhinum cornutum, Asclepias eriocarpa, Asclepias fascicularis, Calandrinia menziesii, Camissoniopsis cheiranthifolia, Clarkia purpurea, Clarkia unguiculata, Clarkia williamsonii, Collinsia heterophylla, Eriogonum fasciculatum, Eriophyllum lanatum, Eschscholzia californica, Fagopyrum esculentum, Gilia capitata, Grindelia camporum, Helianthus annuus, Helianthus bolanderi, Helianthus californicus, Heterotheca grandiflora, Lasthenia fremontii, Lasthenia glabrata, Limnanthes alba, Lupinus formosus, Lupinus microcarpus densiflorus, Lupinus succulentus, Madia elegans, Malacothrix saxatilis, Monardella villosa, Nemophila maculata, Nemophila menziesii, Oenothera elata, Phacelia californica, Phacelia campanularia, Phacelia ciliata, Phacelia tanacetifolia, Salvia columbariae, Scrophularia californica, Sphaeralcea ambigua, Trichostema lanceolatum, Trifolium fucatum, Trifolium gracilentum*

Table D1.2 Plant species available to (flower resources) and used by (pollen) *Bombus vosnesenskii* across the 14 study sites. Flower resources: Flower plantings, mass-flowering crops, and the crop species are presented as the percent of the *Bombus vosnesenskii* foraging area (within 1 km from the colonies) and the flower planting species as the percent of the local flower abundance (within 200 m from the colonies). Pollen: Percent of the pollen collected by *Bombus vosnesenskii* from flower planting species, crop species, and other species.

	Flower resources (%)	**Pollen (%)**
Flower plantings	**0.023**	**24.4**
Clarkia unguiculata	5.7	0.0020
Collinsia heterophylla	3.1	1.9
Eschscholzia californica	3.1	14
Clarkia williamsonii	2.4	0.019
Phacelia californica	1.7	1.5
Grindelia camporum	1.5	
Helianthus bolanderi	0.52	0.046
Nemophila menziesii	0.21	
Lupinus densiflorus	0.11	9.6
Sphaeralcea ambigua	0.062	
Trichostema lanceolatum	0.053	
Phacelia spp.	0.042	0.10
Lupinus succulentus	0.012	
Phacelia ciliata	0.0059	
Trifolium fucatum	0.00095	
Mass-flowering crops	**9.7**	**10.4**
Solanum lycopersicum	2.9	8.9
Helianthus annuus	2.7	0.63
Carthamus tinctorius	0.99	0.61
Cucurbita spp.	0.57	

Vicia faba	0.28	0.081
Medicago sativa		0.26
Solanum spp.		1.6
Other		**64.5**

Plant species filtered for each bee species

List includes plant species that are both used by *Osmia* or *Bombus* and also occurred in the local (within 200 from the bee nests) flower resource data during the time period in which the bee species was active in the field.

<u>*Osmia* filter:</u>

Amsinckia menziesii, *Brassica* spp., *Ceanothus oliganthus, Cercis occidentalis, Collinsia* spp., *Eriophyllum lanatum, Fragaria* spp., *Heliantheae* spp., *Helianthus* spp., *Lepidium latifolium, Lupinus* spp., *Nemophila* spp., *Phacelia* spp., *Quercus* spp., *Rubus discolor, Saxifraga california, Sonchus oleraceus, Toxicodendron diversilobum, Triteleia laxa*

<u>*Bombus* filter:</u>

Adenostemma fasciculatus, *Amsinckia menziesii*, *Asclepias* spp., *Baccharis salicifolia*, *Brassica* spp., *Calandrinia ciliata*, *Carduus pycnocephalus*, *Centaurea solstitialis*, *Cephalanthus occidentalis*, *Collinsia heterophylla*, *Convolvulus arvensis*, *Eriodictyon californicum*, *Erodium botrys*, *Eschscholzia californica*, *Fragaria* spp., *Grindelia camporum*, *Helianthus annuus*, *Heliotropium* spp., *Heteromeles arbutifolia*, *Lavandula* spp., *Lotus scoparius*, *Lupinus* spp., *Marrubium vulgare*, *Medicago polymorpha*, *Phacelia* spp., *Plantago* spp., *Raphanus sativus*, *Rosa californica*, *Rubus* spp., *Sambucus* spp., *Scrophularia californica*, *Silybum marianum*, *Solanum* spp., *Sonchus oleraceae*, *Trichostema lanceolatum*, *Trifolium hirtum*, *Vicia villosa*

Pollen identification methods

We collected pollen from *Osmia lignaria* larval provisions (15 samples), prepared a microscopy slide for each sample, and assessed on average 194 pollen grains per slide (range 150-201 grains) to determine plant origin. We collected 1267 *Bombus vosnesenskii* pollen loads and sorted them by colour, colony, and sampling round under natural light conditions. Pollen for each unique combination of colour, colony and sampling round was used to prepare microscopy slides, resulting in 594 slides. For loads with multiple colours (in total 154 loads with two colours, 12.1%, and 15 loads with three colours, 1.2%), we estimated the proportion of each colour and subsampled separately if the colour constituted >5% of the pollen load. Plant origin was based on assessing on average 208 pollen grains per slide (range 75-803 grains, excluding one slide where the majority of grains were damaged and only 5 grains could be assessed). Identified samples were used to extrapolate to the remaining samples of the same colour, colony and sample round based on proportion of the grains identified to species or genera and the weight of pollen loads.

We prepared microscopy slides by taking a small amount of pollen from each sample and containing the pollen in basic fuchsin stained gel, following Kearns and Inouye (1993). We identified pollen to plant species or genera using light microscopy for the flowers that were planted at the majority of the flower planting sites (*Clarkia unguiculata, Clarkia williamsonii, Collinsia heterophylla, Eschscholzia californica, Grindelia camporum, Helianthus bolanderi, Lupinus densiflorus, Lupinus succulentus, Nemophila menziesii, Phacelia californica, Phacelia ciliata, Phacelia* spp.*, Sphaeralcea ambigua, Trifolium fucatum, Trifolium gracilentum, Trichostema lanceolatum*) and regionally common crops (*Carthamus tinctorius, Cucurbita spp., Helianthus annuus, Medicago sativa, Solanum lycopersicum, Solanum* spp.*, Vicia faba, Zea mays*).

Appendix D2. Study system, land use, and predictor correlations

Supplementary tables

Table D2.1 Land use composition (%) similarity (model estimated mean (95% confidence limits)) of the landscapes (1 km radius) surrounding bee nests with and without flower plantings.

	Flower planting sites	Control sites	F_{df}	P
Semi-natural habitat (oak savannah, riparian, oak woodland, forbs plantings, hedgerows)	23 (11-40)	15 (6-32)	$0.77_{1,12}$	0.40
Agricultural land (orchard, annual crops, pasture, vineyard)[1]	62 (49-74)	71 (59-81)	$1.36_{1,12}$	0.27
Other land uses (developed, water, bare, fallow)	14 (7-29)	14 (6-28)	$0.01_{1,12}$	0.91

[1]The agricultural land consisted of 70% orchards with almond, walnut, fruit or olive, with almond dominating (83% of the orchard land), 5% pasture, 4% tomato, 4% sunflower, 4% wheat, 3% alfalfa, 2% corn, 1% safflower, <1% each of squash, bean, vineyard, hay and strawberry and 5% of mixed or unidentified crops.

Table D2.2 Correlation (Pearson's r) among predictors.

	Landscape flowers	Pesticide risk	Year
Osmia lignaria			
Local flowers	-0.20	0.35	-0.19
Landscape flowers		-0.14	-0.10
Year	-0.10	-0.05	
Bombus vosnesenskii			
Local flowers	0.024	-0.17	
Landscape flowers		-0.23	

Appendix D3. Pesticide residues

Supplementary figures

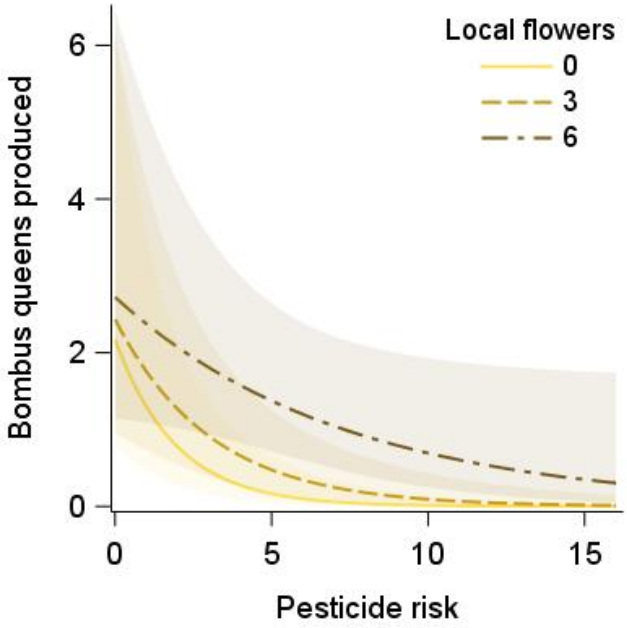

Figure D3.1 *Bombus vosnesenskii* queen production in relation to pesticide risk at different levels of local flower resources (flowers/m^2). Model estimated means and 95% confidence intervals at a fixed landscape flower resource availability of 6.6 flowers/m^2.

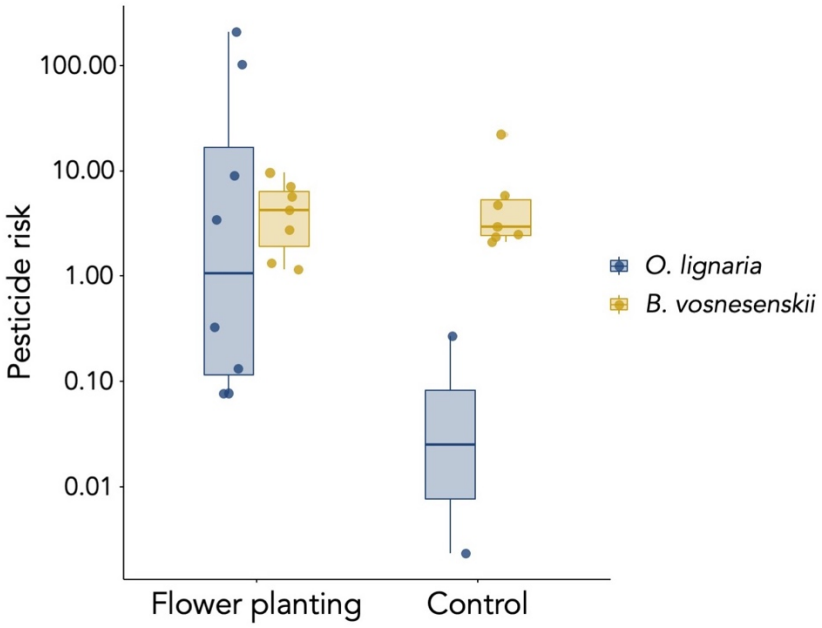

Figure D3.2 Pesticide risk for *Osmia lignaria* (blue) and *Bombus vosnesenskii* (yellow) at flower planting and control sites based on residues quantified in pollen collected by bees. Box plots indicate the median, the 25th and 75th percentiles (box edges), and the range (whiskers).

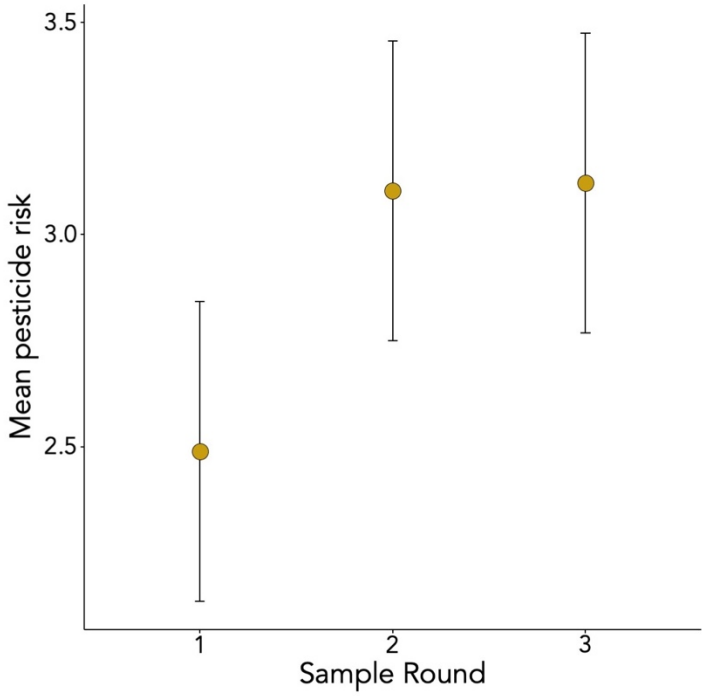

Figure D3.3 Pesticide risk (model estimated means and 95% confidence intervals) to *Bombus vosnesenskii* over three sampling rounds.

Supplementary tables

Table D3.1 Fifty-two substances were selected based on availability of developed assays by the Metabolomics Research Laboratory at Purdue University, use (amount applied and area treated) in the study area during three years (2013-2015) prior to the study, relatively high toxicity to bees or often detected in bee related materials in North American pesticide residue studies. Table reports chemicals selected for quantification and their type (H - herbicide, F - fungicide, I - insecticide, M - metabolite), contact and oral LD_{50} (48 h) for *Apis mellifera* based on the Pesticide Properties Database (PPDB, http://sitem.herts.ac.uk/aeru/ppdb/), listed as bee toxic by the US Environmental Protection Agency (EPA), amount applied and area treated during the years 2013-2015 in the 1 km radius landscapes surrounding the bee nests in 2017 based on California Pesticide Use Registry (PUR, https://www.cdpr.ca.gov/docs/pur/purmain.htm), and comments on the reasons for selecting the chemicals. Chemicals in bold were detected in pollen collected by the bees (see Table D3.2).

Chemical name	Contact LD_{50} (ug/bee)	Oral LD_{50} (ug/bee)	EPA bee toxic	Amount 2013 (lbs)	Area 2013 (acres)	Amount 2014 (lbs)	Area 2014 (acres)	Amount 2015 (lbs)	Area 2015 (acres)	Selection comments
2,4-D (H)	>100	94		394	428	536	660	466	519	High use.
Acetamiprid (I)	8.09	14.53	1	23	230	530	647	466	519	Relatively high toxicity to Apis, used in the area.
Acetamiprid-d3 (M)						50	465	16	147	Metabolite of acetamiprid.
Allethrin (I)	**>3.4**		**1**					**<1**	**3**	**Toxic to Apis, used in the area only 2015.**
Atrazine (H)	>100	>100						6	10	Used in the area, high frequency in Long and Krupke (2016)
Azoxystro-bin (F)	**>200**	**>25**		**440**	**2940**	**503**	**3532**	**436**	**3133**	**High use and relatively high oral toxicity to Apis, high frequency in Long and Krupke (2016).**
Beta-cyfluthrin (I)	**0.001**	**0.05**	**1**	**6**	**630**	**1**	**252**	**3**	**773**	**High toxicity to Apis, used in the area.**
Bifenthrin (I)	**0.015**	**0.1**	**1**	**407**	**3353**	**843**	**7161**	**625**	**5671**	**High use and toxic.**

Captan (F)	>200	>100						1105	939	High use, high residue load in Sánchez-Bayo and Goka (2014).
Carbaryl (I)	**0.14**	**0.21**	**1**	**375**	**828**	**514**	**1217**	**721**	**1451**	**High use and toxic, high residue load in Sánchez-Bayo and Goka (2014).**
Chlorotha-lonil (F)	>40	>40		5674	2081	7647	3090	7499	2980	Large amount used, identified in McArt et al. (2017a) as correlated to bee decline and high residue load in Sánchez-Bayo and Goka (2014).
Chlorpyrifos (I)	0.059	0.25	1	191	236	321	309	1129	835	High use and toxic, high risk in Sánchez-Bayo and Goka (2014).
Clothianidin (I)	0.044	0.004	1	19	295	24	349	24	364	High toxicity to Apis, used in the area, high risk in Sánchez-Bayo and Goka (2014).
Clothianidin-d3 (M)										Metabolite of clothianidin.
Coumaphos (I)										High frequency in Long and Krupke (2016), high residue load and risk in Sánchez-Bayo and Goka (2014).
Cyhalothrin (I)		0.027								High risk in Sánchez-Bayo and Goka (2014) in combination with other pesticides.
Cypermethrin (I)	0.023	0.172	1							High toxicity to Apis, used in the area, high risk in Sánchez-Bayo and Goka (2014).
Deltamethrin (I)	0.001 5	0.079	1							High toxicity to Apis.
Diazinon (I)	0.13	0.09	1					1	1	High toxicity to Apis, used in the area, high

										frequency in Long and Krupke (2016).
Dimethoate (I)	**0.1**	**0.1**	**1**	**333**	**921**	**491**	**974**	**321**	**849**	**Relatively high use and toxic.**
Dinotefuran (I)	0.023		1					6	2143	High toxicity to Apis and relatively large area treated, high risk in Sánchez-Bayo and Goka (2014).
Endosulfan sulfate (I)	>7.81	>15.6	1							High residue load in Sánchez-Bayo and Goka (2014).
Esfenvalerate (I)	0.06	0.21	1	22	415	29	549	81	1036	High toxicity to Apis, used in the area.
Fenbucon-azole (F)	>5.5	>5.2	1	147	1517	83	864	85	879	Relatively high oral toxicity to Apis, used in the area.
Fenpropath-rin (I)	>0.05		1					84	270	High toxicity to Apis, used in the area 2015 only, high residue load in Sánchez-Bayo and Goka (2014).
Fipronil (I)	0.0059	0.0042	1							High contact toxic to Apis and possibly not in PUR due to seed treatment use.
Flumioxazin	>200	>229.1		66	250	48	283	139	776	Used in the area.
Imidaclo-prid (I)	**0.081**	**0.0037**	**1**	**126**	**1597**	**115**	**1855**	**200**	**3583**	**High toxicity to Apis, used in the area, high risk in Sánchez-Bayo and Goka (2014).**
Imidacloprid-d4 (M)										Metabolite of imidacloprid.
Indoxacarb (I)	0.08	0.232	1	115	1570	59	719	44	627	Relatively high toxicity to Apis, used in the area.
Lambda-cyhalothrin (I)	0.038	0.91	1	135	4257	94	3119	101	3392	High toxicity to Apis, used in the area, high frequency in Long and Krupke (2016).

Malathion (I)	**0.16**	**0.4**	**1**	**302**	**260**					**High oral toxicity to Apis, used in the area only 2013.**
Methomyl (I)	0.16	0.28	1			82	91			High toxicity to Apis, used in the area only 2014.
Methoxy-fenozide (I)	**>100**	**>2000**		**306**	**1307**	**507**	**2013**	**946**	**3719**	**High use.**
Metolachlor (H)	**>110**	**110**		**779**	**442**	**539**	**293**	**844**	**536**	**High use, high frequency in Long and Krupke (2016).**
Nitenpyram (I)										Probably high toxicity to Apis, but no documented use in the area.
Oryzalin (H)	40.8	32		548	299	242	262	1432	10456	Large area treated and relatively high toxicity to Apis.
Permethrin (I)	0.024	0.13	1	6	93	2	6	39	156	High toxicity to Apis, used in the area.
Phenothrin (I)			1							High frequency and quantity in Long and Krupke (2016).
Phosmet (I)	0.22	0.37	1							High residue load and high risk in Sánchez-Bayo and Goka (2014).
Prallethrin (I)	0.026		1							High frequency and quantity in Long and Krupke (2016).
Propargite, NH4adduct (I)	**47.9**	**>100**				**56**	**25**			**Used in the area only 2014.**
Propicon-azole (F)	**>100**	**>100**		**689**	**4335**	**519**	**2853**	**597**	**3173**	**High use, high frequency in Long and Krupke (2016), high risk in Sánchez-Bayo and Goka (2014).**
Pyraclostro-bin (F)	**>100**	**>110**		**515**	**5015**	**582**	**5337**	**600**	**5846**	**High use, high frequency in Long and Krupke (2016).**

Spinetoram-major (I)	**0.024**	**0.14**	**1**	**24**	**320**	**12**	**174**	**248**	**3764**	**High toxicity to Apis and relatively high use.**
Spinosad-major (I)	0.0036	0.057	1	27	746	40	753	90	1228	High toxicity to Apis, used in the area.
Tetramethrin (I)			1							Listed as bee toxic by US EPA.
Tau-fluvalinate (I)	12	12.6	1			3	17	0	0	High toxicity to Apis, used in the area, high residue load in Sánchez-Bayo and Goka (2014).
Thiacloprid (I)	38.82	17.32								Relatively high toxicity to Apis, but no documented use in the area, high residue load and high risk (in combination with propiconazole) in Sánchez-Bayo and Goka (2014).
Thiametho-xam (I)	**0.024**	**0.005**	**1**	**4**	**148**	**1**	**40**	**3**	**80**	**High toxicity to Apis, used in the area, high frequency in Long and Krupke (2016), high residue load and high risk in Sánchez-Bayo and Goka (2014).**
Thiametho-xam-d3 (M)										Metabolite of thiamethoxam.
Trifloxy-strobin (F)	**>100**	**>110**		**78**	**898**	**169**	**2048**	**202**	**2215**	**Large area treated, used in the area, high frequency in Long and Krupke (2016).**

Table D3.2 Detected chemicals and type (H - herbicide, F - fungicide, I - insecticide) in pollen collected by *Osmia lignaria* (10 samples) and *Bombus vosnesenskii* (42 samples). Number of samples containing the chemical, mean and range of residues (ng g^{-1}), mean LD_{50} for *Apis mellifera* averaged over oral and contact (ug bee^{-1}) based on the Pesticide Properties Database (PPDB, http://sitem.herts.ac.uk/aeru/ppdb/), and the limits of detection (LOD) and quantification (LOQ) (ng g^{-1}) as determined by the Metabolomics Research Laboratory at Purdue University.

Chemical name (type)	***Osmia* detections**	***Bombus* detections**	**Mean *Osmia* residues (range)**	**Mean *Bombus* residues (range)**	**LOD**	**LOQ**
allethrin (I)	0	13	<LOD	243 (28-732)	0.3	1
azoxystrobin (F)	10	41	277 (5-1740)	124 (2-1022)	0.03-0.1	0.1
beta-cyfluthrin (I)	2	0	3980 (2607-5352)	<LOD	30-50	100
bifenthrin (I)	0	37	<LOD	102 (10-1164)	0.3	1
carbaryl (I)	5	41	16 (3-44)	31 (2-531)	0.03	0.1-1
dimethoate (I)	0	4	<LOD	1.5 (1-3)	0.006	0.02
imidacloprid (I)	1	0	14 (14)	<LOD	0.3	1
malathion (I)	2	0	18 (15-21)	<LOD	0.3	1
methoxyfenozide (I)	10	41	525 (4-4813)	563 (31-13431)	0.02-0.06	0.2
metolachlor (H)	10	34	3 (0.5-22)	19 (1-130)	0.01-0.03	0.1
propargite (I)	0	3	<LOD	61 (1-177)	0.03	0.1
propiconazole (F)	6	40	23 (8-73)	493 (24-11365)	0.1-0.3	1
pyraclostrobin (F)	5	40	73 (31-148)	192 (3-4518)	0.1	1
spinetoram (I)	2	0	500 (279-721)	<LOD	1	10
thiamethoxam (I)	2	0	2 (1.8-2.4)	<LOD	0.1	1
trifloxystrobin (F)	7	34	11 (3-28)	14 (0.6-223)	0.03	0.1-1

Table D3.3 Detected pesticides and type (H - herbicide, F - fungicide, I - insecticide) in pollen collected by *Osmia lignaria* (10 samples) and *Bombus vosnesenskii* (42 samples). Frequency of chemical detection (%), 90th percentile of residue levels detected (ng/g; Auteri et al. 2017), mean LD_{50} for *Apis mellifera* averaged over oral and contact, and the substance risk index (Substance risk index = ((frequency of detection [%] x 90th percentile residue level [ng/g]) / LD50 [µg /bee])/1000 (based on Sánchez-Bayo and Goka 2014)).

	Frequency (%)	**90th percentile residue level (ng/g)**	**Mean LD_{50} (ug/bee)**	**Substance risk index**
Osmia lignaria				
beta-cyfluthrin (I)	20	5080	0.0310	2570
spinetoram (I)	20	677	0.0820	122
carbaryl (I)	50	31.9	0.175	4.55
imidacloprid (I)	10	13.8	0.0424	3.26
thiamethoxam (I)	20	2.30	0.0145	2.89
malathion (I)	20	20.7	0.280	1.28
methoxyfenozide (I)	100	705	100	0.525
azoxystrobin (F)	100	590	113	0.246
pyraclostrobin (F)	50	117	105	0.0347
propiconazole (F)	60	46.3	100	0.0139
trifloxystrobin (F)	70	20.4	105	0.00723
metolachlor (H)	100	3.70	110	0.00290
Bombus vosnesenskii				
bifenthrin (I)	88	181	0.0575	278
carbaryl (I)	98	48.0	0.175	26.8
allethrin (I)	31	469	3.40	4.27
methoxyfenozide (I)	98	548	100	0.535
propiconazole (F)	95	549	100	0.523
azoxystrobin (F)	98	403	113	0.350

dimethoate (I)	10	3.00	0.100	0.286
pyraclostrobin (F)	95	246	105	0.223
metolachlor (H)	81	35.7	110	0.0263
trifloxystrobin (F)	81	22.3	105	0.0172
propargite (I)	7	177	74.0	0.0171

Pesticide quantification methods

For the pesticide residue quantification protocol, see Appendix A.

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