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Insecticide use in hybrid onion seed production affects pre- and post-pollination processes

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Abstract

Research on threats to pollination service in agro-ecosystems has focused primarily on the negative impacts of land use change and agricultural practices, such as insecticide use, on pollinator populations. Insecticide use could also affect the pollination process, through non-lethal impacts on pollinator attraction and post-pollination processes such as pollen viability or pollen tube growth. Hybrid onion seed (*Allium cepa* L., Alliaceae) is an important pollinator-dependent crop that has suffered yield declines in California, concurrent with increased insecticide use. Field studies suggest that insecticide use reduces pollination service in this system. We conducted a field experiment manipulating insecticide use to examine the impacts of insecticides on (1) pollinator attraction, (2) pollen/stigma interactions and (3) seed set and seed quality. Select insecticides had negative impacts on pollinator attraction and pollen-stigma interactions, with certain products dramatically reducing pollen germination and pollen tube growth. Decreased pollen germination was not associated with reduced seed set; however, reduced pollinator attraction was associated with lower seed set and seed quality, for one of the two female lines examined. Our results highlight the importance of pesticide effects on the pollination process. Over-use may lead to yield reductions through impacts on pollinator behavior and post-pollination processes. Overall, in hybrid onion seed production, moderation in insecticide use is advised when controlling onion thrips, *Thrips tabaci*, on commercial fields.

Keywords: Pollination, seed production, pesticide, *Apis mellifera*, *Allium cepa*, *Thrips tabaci*

Pollination is a key ecosystem service that increases yields for a large number of agricultural crops worldwide (Klein et al. 2007). Research on threats to pollination service in agro-ecosystems has focused primarily on the impacts of land use change and agricultural practices such as insecticide applications on pollinator populations (i.e. Kremen et al. 2004, Blacquiere et al. 2012, Klein et al. 2012, Whitehorn et al. 2012). Besides negatively impacting pollinator populations and their delivery of pollination (Brittain et al. 2010, Tuell and Isaacs 2010), insecticide use may also have non-lethal impacts that affect the pollination process pre- or post-pollen deposition. For example, pesticides might render crops unattractive to a major pollinator (Long and Morandin 2011), or negatively impact post-pollination processes such as pollen germination. Such impacts have received very little attention and given the potential for new insecticides to come into use, or for applications to increase in certain crops in response to emergent pests or diseases (i.e. Desneux et al. 2010), better understanding of these impacts is crucial.

Post-pollination impacts of pesticides could operate through pollen, stigmas or the interaction of the two. Both pollen and the stigmatic tissue may be susceptible to damage by pesticides, which could reduce pollen germination, pollen tube growth, and ovule fertilization, resulting in reduced seed set and crop yield. Research on fungicides has shown that application directly to stigmas negatively affects pollen tube growth in apple flowers and can damage the cellular structure of almond stigmas, inhibiting receptivity (Yi et al. 2003a, b, c). If insecticides have similar impacts on pollen or stigmatic tissue, they could similarly reduce seed set; yet such impacts on plant tissue have not been examined to our knowledge.

Hybrid onion seed is a small acreage, high-value crop in California's Central Valley (Voss et al. 1999) dependent on the honey bee (*Apis mellifera,* L., Hymentoptera, Apidae) for

successful pollination and seed yield. Seed yields in the region steadily declined between 2003 and 2008, despite an increase in acreage (Long and Morandin 2011). These declines coincided with a marked increase in insecticide use to control the onion thrip (*Thrips tabaci* Lindeman, Thysanoptera; Thripidae) to prevent transmission of iris yellow spot virus, a recently introduced disease (Gent et al. 2006, Long and Morandin 2011). An observational study conducted at farms in Yolo and Colusa counties in California showed that high insecticide use decreased flower visitation to onions by honey bees, with a correlated decrease in seed yield (Long and Morandin 2011). Insecticides are applied pre-bloom in this system and honey bee hives are placed in fields at high densities; thus, it is unlikely that insecticides are directly affecting pollinator numbers. Rather, some reduction in attractiveness due to pesticide residues is likely the mechanism. It is unknown whether insecticide impacts on pollen tube growth are an additional source of yield declines.

To address these questions, we conducted a replicated field experiment manipulating insecticide use to determine its effects on pollination of hybrid onion seed. We examined the impacts of insecticides on (1) pollinator attraction, and (2) post-pollination pollen/stigma interactions and (3) seed set and seed quality.

Materials and Methods

Study system.

Onion, *Allium cepa* L. (Alliaceae) is a self-compatible, biennial hermaphrodite (Zomlefer 1994) grown commercially for its edible bulb and leaves in many different parts of the world (Griffiths et al. 2002). Hybrid seed, which supplies edible onion plantings, is grown in commercial fields that are pollinated primarily by honey bees (Parker 1982). Seed plants produce one or more

flowering stalks per bulb, each ending in an umbel, consisting of hundreds of florets, each of which can produce up to six seeds (Griffiths et al. 2002). Hybrid crosses are achieved by planting male fertile onion lines (the pollen donor) next to male sterile lines (female, seed producing) (Voss et al. 1999). For simplicity, we refer to them as male (male fertile) and female (male sterile) plants throughout this study. Only seeds from the female line are harvested. Seed production thus requires pollen transfer from male to female lines by insect pollinators.

Experimental Design.

The study took place at the University of California Davis vegetable crops research farm, Yolo County, California. Onion bulbs were planted on October 2010, according to standard practices (Voss et al. 1999). The experimental field was divided into 75 (6.1 x 3.8 m) plots, organized into 5 blocks of 15. Each plot had two female and two male rows planted on 30-inch (76.2 cm) beds and surrounded by a 1.5 m tilled buffer. One line of yellow onion bulbs was used for male plants (VON-095-G-122C-S2Y) and two lines for the female rows, one in blocks 1-3 (VON-108A-S3Y, “Female type A”) and another in blocks 4-5 (VON-163A-L1Y, “Female type B“). Herbicide was applied to tilled buffers in the fall and mechanical weed control was used in buffers during the spring. Late spring rains caused high levels of infection by downy mildew, which we treated with fungicides ethylene bisdithiocarbamate three weeks before observations and azoxystrobin two days before. Male plants, were infected more severely by the fungus than females. The experiment was flood irrigated twice during bloom.

Treatments.

We tested five conventional insecticides and two organic pesticides all of which are currently applied by growers in California to control thrips pests on onions (Table 1; Orloff et al. 2009), We also tested a plant growth hormone that is being considered for use in onion. We selected

two of the conventional insecticides, to conduct additional manipulations of spray number (lambda-cyhalothirn) and spray timing (methomyl; Table 1). Every plot within received a single treatment, randomly assigned within each block. All selected pesticides have different active ingredients belonging to different chemical groups and insecticide categories (IRAC 2011).

Pollinator activity.

We placed nine honey bee hives at one side of the study field on June 6th, 2011, giving a density of ~ 10 hives/acre, equivalent to that seen in commercial fields in the region. Observations of pollinator activity started when ~5 % of florets on female umbels and ~50% of florets on males were flowering (June 10th, 2011) and continued until flowering was finished 17 days later for males (June 27th, 2011) and 20 days later for females (June, 30th 2011). In total, all plots were observed six times for males and seven times for females.

We quantified pollinator activity separately in male and female rows in each plot. Visitation was observed for five minutes in a 1 m x 0.75 m quadrat approximately twice a week during peak bloom. In each scan we counted the number of visitors entering the plot and timed the duration of umbel visits. If possible we measured the time individual pollinators spent on multiple umbels. Pollinators were identified to family and morpho-groups (subdivided by honey bees, non-Apis bees, syrphid flies, other flies, beetles and other groups), and we collected samples of visitors and identified each to genus or species. However, visitation from groups besides honey bees was infrequent, thus we analyzed total flower visitors, including honey bees, then honey bee visitation only. We simultaneously recorded the number of umbels blooming in each plot. Temperatures averaged about 25^{o}-35^{o}C during the experiment and we conducted observations only on sunny/light cloud days, with light wind.

We calculated the total number of visitors in either the male or female plots during each observation period, as well as the number of honey bees separately. For time spent per umbel, we only had sufficient data on honey bees to analyze treatment effects. Where we had multiple umbel-visits for some individual honey bees, we averaged time spent per flower within a bee first, then calculated the average time for all bees across the plot for each observation period.

Pollen germination.

To isolate potential effects of insecticides on post-pollination processes acting through pollen versus stigma/style effects we used reciprocal pollen germination tests from insecticide sprayed and unsprayed plants. We bagged a large number of unsprayed umbels in untreated buffer rows, as well as 5 umbels in each of our plots, excluding the manipulations of timing and spray number. Individual receptive florets were excised from umbels, placed in water and brought into the lab for hand pollination. To test for impacts of insecticides on the style, 5 receptive styles from each treatment plot were hand-pollinated with control (untreated) pollen (n = 25 florets total). To test for effects acting through pollen, 5 control (untreated) stigmas were pollinated with pollen from each insecticide-treated plot (n = 25 florets each). Each stigma was gently brushed with pollen collected from several flowers and the pollinated floret placed in water in the lab at room temperature for 24 hours to allow pollen to germinate and pollen tubes to grow. After 24 hours, the style and part of the ovary were excised from the floret, fixed in 70% ethanol, and stored at 4°C until staining.

To visualize pollen tubes, we followed the methods of Kho and Baer (1968), softening washed stigmas for one hour at room temperature in 1 N NaOH for 1 hour, then staining rinsed stigmas for 24 hours in 0.05% analine blue (water soluble) dissolved in 0.1 K_3PO_4. We gently squashed stigmas with a coverslip in a drop of staining solution. We then counted germinating

pollen grains and pollen tubes growing to the base of the style under a fluorescent-light microscope (Nikon E800 with wide-band UV-filter).

Seed characteristics.

We quantified seed set in each plot from a random sample of umbels tagged prior to flowering. After seeds ripened, we collected and dried tagged umbels individually then threshed seed heads and counted viable seeds. We also weighed seeds and tested a subsample for germination. To test germination, we placed twenty-five seeds from each umbel between layers of wet germination paper in petri dishes, then set in a growth chamber set at 20°C to germinate. The number of seeds with emerging roots were counted after 5, 7 and 10 days.

Statistical Analysis.

Visitation. Because male and female plants differed in phenology and disease severity, all analyses of visitation were conducted separately by gender. We examined the effects of insecticide use on three different metrics of visitation: total visitors per 5-minute observation, honey bee visitors per 5-minute observation period, and the duration of honey bee visits. The distributions for total visitors and honey bee visitors were non-normal and the relationships between response variables, date and time were frequently non-linear. Therefore, for these responses, we used general additive models with a negative binomial distribution. Poisson or quasi-Poisson distributions could not be used because of the magnitude of over-dispersion in our data (gamm, mgcv package, R-Development-Core-Team 2009, Zuur 2009, Wood 2011). Honey bee visit duration was normalized by log transforming and was analysed with a gamma distribution.

All models included fixed categorical insecticide treatment and block variables. Continuous explanatory variables were: date of observation, time of day, fungal status as mean

of two records taken for each plot, position of plots relative to the hives and number of open umbels in the plot. Because block was confounded with female type it was treated as a fixed effect. Position relative to the hives was included because hives were all placed at one end of the field, potentially creating a gradient within blocks. Date and time were initially modelled as non-linear effects using smoothing terms. When smoothing terms were not significant they were instead modelled as linear variables. In order to determine whether changes in response variables over time differed by treatment, we modelled date within treatment. If there was no variation among treatments, the within-treatment date effect was dropped. Finally we included a treatment by block interaction and a treatment by fungal disease interaction. Non-significant interactions were dropped, following the recommendations of Zuur et al. (2009), to avoid overfitting of our models.

Pollen germination. We examined how insecticide treatments impacted control pollen germination and pollen-tube growth on styles from treated plots using zero-inflated negative binomial models (hurdle, pscl package, R-Development-Core-Team 2009, Zuur 2009). Insecticide treatment and block were again used as explanatory variables. Response variables included number of germinated pollen grains on the stigma and number of pollen tubes reaching the base of the style. Zero inflated models test impacts of insecticide treatments or block first on pollen tubes as a binary variable, then test the quantitative differences among stigmas that had any pollen germinate. For pollen from treated plots germinated on control stigmas, very little pollen germinated, so these data were analyzed using simple binomial models with pollen germinated, or pollen reaching the base, as response variables and block and treatment as explanatory variables.

Seed characteristics. All seed data were normally distributed, so were analyzed with standard ANOVA (glm, stats package, R). All models included fixed categorical insecticide treatment and female type variables and their interaction, and fungus status and position as a continuous variable. We used the same models for seed weight and germination, but added seed number as a continuous explanatory variable. Because female types differed drastically in seed set, fungal status and visitation rates, where there was a significant female type by treatment interaction, we split the data by female line and re-analyzed, excluding block from the model.

Results

Visitation: Female plants.

Insecticide treatments did not significantly affect the total number of visitors to female plots (Table 2). Total visitors increased linearly with the number of open flowers and was non-linearly related to sampling time, with peak visitation at midday. Total visitation also increased non-linearly with date, saturating at later dates, and the effects of date did not vary between treatments. Honey bee visitation results mirrored those for total visitors (Table 2).

The duration of honey bee visits to female umbels was significantly shorter than controls for spirotetramat (-9.34 s, $P < 0.05$) and urea (the plant growth regulator (-0.04 s, $P < 0.01$). The change in the duration of visits over sampling dates varied among treatments. Visit duration increased over the experiment for essential oils ($F = 2.785$, $P < 0.05$), methomyl week pre-bloom ($F = 4.679$, $P < 0.001$) and 4 and 6 applications of lambda-cyhalothrin ($F = 2.943$, $P < 0.01$ and $F = 5.071$, $P < 0.001$ respectively), but it did not change in control plots ($F = 0.042$, $P = 0.92$).

Visitation: Male plants.

There was a significant treatment effect for total visitors to male plants. Specifically, plots treated with essential oils, or with lambda-cyclohathrin six times were visited significantly less

than the controls (estimate = -2.609, $P < 0.05$ and estimate = -3.679, $P < 0.01$ respectively; Fig. 1). Furthermore, visitation increased with the number of open flowers, and increased non-linearly over time. There was a significant negative effect of fungal infection on visitation to male umbels, which was more pronounced in plots treated six times with lambda-cyclohathrin (significant treatment x fungus interaction). The pattern for honey bees was qualitatively similar. No factor affected honey bee visit duration in male plots.

Pollen germination.

Pollen germination and tube growth were affected only through styles on treated female plants (Table 3), not via impacts on pollen from treated plants (statistics not shown: all $P > 0.05$). Untreated pollen had a lower probability of germinating on the stigmas of flowers from plants treated with methomyl (binomial model; Table 3B) and fewer grains germinated on stigmas from plants treated with acetamiprid and spinetoram (count model; Table 3; Figs. 2A &B). Fewer pollen tubes reached the base of the style of flowers in plots treated with acetamiprid, spirotetramat, methomyl and lambda-cyhalothrin compared to controls. There were marginally significant, but notable effects of methomyl on the probability of tubes reaching the base of the style (Fig. 2C; Table 3). Curiously, flowers from plots treated with methomyl had higher numbers of pollen tubes reaching the base of the style than controls - but this was driven by only one stigma out of 25 that had high numbers of pollen tubes - the rest had zero (Fig. 2D). Females of type B had significantly fewer pollen tubes reaching the base of the style overall.

Seed characteristics.

Seed set and weight both showed significant effects of pesticide treatment and but these differed by female type (Table 4). Seed characteristics of female type A were not affected, those of female type B were. For females of type B, seed set was significantly lower than the control for

3x lambda-cyhalothrin plots, and marginally significantly lower for lambda-cyhalothrin 4x and 6x plots (Table 5). Conversely, seed set was higher than control in spirotoram treated plots (Fig. 3A, Table 5). Seed weight for females of type B was significantly lower than the control for plots treated with methomyl at 2 and 5 weeks and for plots treated with essential oils and with lambda-cyhalothrin 6x (Fig. 3B, Table 5).

For seed germination, again there were significant treatment-by-female type interactions for 5, 7 and 10 days germination tests (all $p < 0.001$; Supp. Table S1). There was a significant effect of treatment for females of type A and type B at 5 days ($F = 1.914$, $P < 0.05$; $F = 3.44$, $P < 0.001$ respectively). Seeds from females sprayed with methomyl 5 and 8 weeks before flowering showed higher seed germination than the controls. Conversely, seeds of females of type B treated with urea, spirotetramat, essential oils, and lambda-cyhalothrin four or six times all had higher germination than the controls (Fig. 3C, Supp. Table S2). At 7 days, the pattern was qualitatively similar - except that the significant effect of lambda-cyhalothrin six times for type B disappeared (Supp. Table S2). At 10 days, most significant treatment effects disappeared, with the exception of the positive effect of methomyl on female A, and urea on female B (Supp. Table S2).

Discussion

Our experimental approach confirmed our hypothesis that insecticide use can impact both pollinator visitation and on post-pollination processes; however, those effects depend upon how frequently chemicals are applied and the specific type used. The highest spray rates (lambda-cyhalothrin six times) had overall negative effects on visitation to males, supporting the observation that excessive insecticide use was negatively affecting honey bee visitation in commercial fields in 2009 (Long and Morandin 2011). Essential oils reduced visitation to males

as well. However, no treatment affected visitation to females, which differs from previous finding where visitation to males and females were similar (Long and Morandin 2011). Certain insecticides also changed honey bee behavior on female flowers, some by reducing visit duration throughout the experiment, others by only reducing visit duration early in the experiment, an effect that appeared to degrade over time.

Interestingly, the specific products that affected pollinator behavior were not always those considered the most toxic to pollinators. Several are traditional insecticides, whereas essential oils are an organic certified biopesticide (http://www.omri.org/), while urea (Bioforge) has no insecticidal activity. Yet all had potentially negative impacts on pollinator behavior that seems to translate into reduced seed set. The negative impact of urea on visitation is surprising because it is a plant growth regulator, not an insecticide. Possibly, it changes floral rewards and thus impacts the time bees spend on a flower. Overall, these patterns suggest that insecticides may have a general repellent effect that is not dependent on toxicity. This indicates that growers cannot necessarily simply replace one product with one of lower overall toxicity to avoid negative effects on bee behavior – rather, reductions in overall spray number may be necessary.

Our data provide interesting insight into the results of Long and Morandin (2011) from commercial seed fields. First, insecticide use in our experiment had less dramatic impacts on visitation than was seen in their study. This may be in part because their study included higher spray levels (>8 application) than ours (maximum 6 applications). Furthermore, in order to identify specific chemicals that repel pollinators, we treated each plot with a single product. In commercial production fields growers apply a mixture of different classes of insecticides, and rarely use the same one more than once. Complex combinations of pesticides may have synergistic repellency that we did not see in our experimental data. However, in the field, the

diversity of insecticides applied is highly correlated with the number of applications (S. Gillespie, unpublished data), meaning teasing apart this relationship will require additional experiments.

Several pesticide treatments had strong negative effects on pollen germination and pollen tube growth. This is surprising, given that treatments were applied pre-bloom, and thus did not directly contact the stigmatic surface, unlike in previous studies documenting fungicidal impacts on pollen tubes (Yi et al. 2003a, c). Rather, our insecticidal sprays occurred when umbels were in the pre bud and bud stage. The products that significantly reduced germination all appear to have either systemic or translaminar effects, meaning that they are designed to penetrate plant tissues either locally in the case of translaminar movement, or throughout the plant in the case of systemic insecticides. Thus they may penetrate the stigmatic tissue and cause cellular damage, even as it is developing in the bud stage. Little is known about the potential for pesticides with translaminar movement or systemic effects to have impacts on developing flowers. Both the mechanisms and implications of these results need further investigation.

Though insecticide treatments reduced visitation and pollen tube growth, the seed set results suggest that impacts on visitation were ultimately more important for seed set. Treatments that dramatically reduced pollen tubes had no impact on seed set (i.e. methomyl), or showed even higher seed set than controls (spinetoram - female B only). This suggests that consistent pollinator visitation can overcome pollen tube impacts. Conversely, essential oils or lambda-cyhalothrin treatments reduced visitation to males, and reduced seed set and seed weight from females. This suggests that negative effects of pesticides on visitation to males had negative effects on ultimate seed set, while negative impacts on pollen tube growth did not translate into such changes. However, our data still raises concerns about the possibility of synergistic negative

effects between pesticides. If a grower applies one product that reduces visitation and another that reduces pollen germination this could lead to particularly dramatic seed set reductions.

The negative effect on seed yield acting through visitation to male lines is intriguing - no treatment changed visitor number to females; however, we have evidence that visitation impacts in males changed ultimate seed set. For hybrid seed production, movement between male and female rows is essential for seed set (Free 1993), thus a reduction in visits to pollen producing flowers can reduce seed set in female rows. Our results highlights the need to investigate male and female function for understanding pollination processes.

Finally, it is important to note that any negative effects on seed set were only evident for one of our two female types – female type B. Given that female type B had low establishment, lower visitation, fewer pollen tubes, and greater disease severity compared to female A, it seems that plant stress, or other varietal difference such as vigor, compound the negative effects of insecticides on pollination service, leading to negative impacts on seed set or seed quality.

Insecticide use seems to have positive effects on the rate of seed germination. More seeds from treated plants germinated within 5 days; however, this effect was only maintained over ten days for two insecticides, each on a different female type (Supp. Table S1). In one case, the positive effect was from urea, the plant growth regulator meant to stimulate plant growth. It seems likely that this could lead to maternal effects on seed germination. In the other case it was methomyl sprayed 5 weeks pre bloom, but no other methomyl treatment. Given that differences disappeared rapidly for most treatments it seems as though treatments may simply accelerate seed germination relative to the control. The inconsistency in these patterns makes it difficult to conclude that a strong effect exists; however, clearly more investigation is needed.

Overall our results show that insecticides can negatively affect multiple stages of the pollination process. However, many factors, such as varietal differences, will determine whether this translates into negative impacts on seed yield. Our results highlight the importance of considering the indirect effects of pesticides on the pollination/fertilization process. Careful timing and rates of spray applications may minimize impacts on pollinator health, over-use might reduce seed yield.

Supplement: Detailed statistical tables for seed germination

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Tables

Table 1. Description of insecticide treatments.

Active Ingredient/manipulation	Commercial product	Chemical class	Penetration[a]	Application time[b]
No spray	Control	N/A	N/A	N/A
urea & potassium hydroxide	Bioforge	carbamide	N	8, 6 & 4
essential oils (cottonseed, clove, garlic)	Pest Out + Oroboost	hydrocarbons, terpenes, phenylpropanes	N	8, 6, 4 & 2
azadirachtin and neem	Aza-Direct	tetranortriterpen-toids	T	8, 6, 4 & 2
spirotetramat	Movento	keto-enoles	B	8, 6 & 4
acetamiprid	Assail 30 SG	neonicotinoids	B	8, 6, 4 & 2
methomyl	Lannate SP	carbamates	T	8, 6, 4 & 2
spinetoram	Radiant SC	spinosyn	B	8, 6 & 4
lambda - cyhalothrin	Warrior II	pyrethroids	N	8 & 4

Table 1 continued

Active Ingredient/manipulation	Application time°
methomyl, **2** weeks before bloom	2
methomyl, **5** weeks before bloom	5
methomyl, **8** weeks before bloom	8
3 x lambda - cyhalothrin	8, 7 & 4
6 x lambda - cyhalothrin	10, 8, 7, 5, 3 & 2

a. Weeks before bloom, start of blooming corresponds to start of observations.

b. N: None, S: Systemic, T: Translaminar, B: Both

Table 2: Effects of insecticide treatments on pollinator visitation and behavior in female and male plots.

	Females			Males	
Factor	Total visitors	Honey bee visitors	Time per flower	Total visitors	Honey bee Visitors
treatment	0.954	0.861	1.968*	2.144 *	1.424
Block	1.797	2.608 *	1.814	6.035 ***	5.464 ***
Open	5.646 *	3.912 *	-	36.426 ***	17.539 ***
Position	2.818 •	2.766 •	-	0.681	0.285
Fungus	0.586	1.738	-	8.218 ***	7.455 ***
Time (s)	14.79 ***	21.68 ***	-	-	-
Date (s)	34.65 ***	33.71 ***	-	8.61***	9.555 ***
treatment x fungus	-	-	-	2.092 *	1.413
treatment x block	1.291 •	1.324 •	1.415*	-	-

General linear models. All Values are F-values. • $P < 0.1$ * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3: Impacts of treatments on control pollen germination and pollen tube growth on treated stigmas.

(A)		Pollen germination at tip			Pollen tubes to base		
Statistic	Factor	Estimate	z-value	*P*	Estimate	z-value	*P*
	Acetamiprid	-1.183957	-2.089	0.036667 *	-1.19531	-1.994	0.04314*
	Azadirachtin/neem	-0.161479	-0.290	0.771520	0.42483	1.047	0.2951
	Urea/KOH	0.366859	0.734	0.463157	0.33419	0.907	0.36425
Count	Methomyl	1.505670	1.362	0.173352	1.42596	2.601	0.00928**
model	Spirotetramat	-1.036313	-1.493	0.135361	-2.20097	-2.086	0.03700*
coeffecients	Essential oils	-0.062584	-0.092	0.926865	0.03052	0.070	0.94455
	Spinetoram	-1.516855	-2.250	0.024473 *	-0.75771	-1.133	0.25739
	Lambda-cyhalothrin	-0.501725	-0.873	0.382582	-1.20363	-2.225	0.02608*
	Female B	0.039945	0.123	0.902076	-0.65212	-2.338	0.01941*

Table 3 continued

(B)		Pollen germination at tip			Pollen tubes to base		
Statistic	Factor	Estimate	z-value	*P*	Estimate	z-value	*P*
Zero hurdle model coefficients (binomial w logitlink)	Acetamiprid	0.0518	0.801	0.423	-0.085	-1.151	0.250
	Azadirachtin/neem	0.0329	0.496	0.620	-0.043	-0.609	0.542
	Urea/KOH	1.009	1.479	0.139	-0.015	-0.218	0.828
	Methomyl	-2.389	-2.109	0.035*	-2.172	-1.910	0.056•
	Spirotetramat	-0.848	-1.226	0.220	-1.249	-1.591	0.115
	Essential oils	-0.933	-1.272	0.203	-0.717	-0.969	0.333
	Spinetoram	-1.360	-0.203	0.839	-1.067	-1.348	0.178
	Lambda-cyhalothrin	-5.236×10^{-16}	7.97×10^{-16}	1.000	-2.98×10^{-15}	-4.43×10^{-15}	1.000
	Female B	-0.0077	-0.223	0.824	0.124	0.329	0.742

Results of zero inflated negative binomial analysis. Coefficients represent difference relative to control for insecticide treatments, and relative to Female type A for female effect (n=250). • $P < 0.1$ * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 4: Effects of insecticides on seed set.

		Seed set			Seed weight		
Statistic	Factor	Combined	Female A	Female B	Combined	Female A	Female B
F-value	treatment	1.302	0.826	2.953***	2.340**	0.788	2.180*
	female type	3.773•	-	-	0.259	-	-
	seeds	-	-	-	9.649**	4.880*	2.936•
	position	3.724•	2.756•	1.808	0.778	0.306	1.768
	fungus	0.305	3.072•	0.526	0.208	5.838*	0.064
	treatment x female type	1.965*	-	-	1.793*	-	-
	treatment x fungus	1.176	-	-	2.576**	-	-

• $P < 0.1$ * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 5: Effects of insecticides on seed characteristics.

	Mean Seed number(±SE)		Seed weight (µg±SE)	
Treatment	Female A	Female B	Female A	Female B
control	293.03(±27.21)	199.10(±42.41)	3.89(±0.09)	3.78(±0.09)
acetamiprid	343.42(±44.85)	172.27(±40.26)	3.74(±0.10)	3.57(±0.13)
azadirachtin/neem	414.00(±43.68)	235.07(±65.55)	4.23(±0.16)	3.61(±0.13)
Urea and KOH	359.18(±43.18)	148.50(±31.69)	3.78(±0.14)	3.53(±0.13)
methomyl	419.26(±38.85)	114.80(±33.49)	4.01(±0.15)	3.64(±0.16)
spirotetramat	463.57(±66.24)	166.21(±33.07)	3.86(±0.16)	3.85(±0.14)
essential oils	372.26(±43.26)	135.23(±55.25)	3.72(±0.14)	3.09(±0.14)***
spinetoram	409.95(±50.06)*	332.20(±56.99)	3.69(±0.13)	3.77(±0.17)
lambda-cyhalothrin 3x	291.23(±59.04)*	82.93(±20.32)	3.74(±0.13)	3.62(±0.12)
lambda-cyhalothrin 4x	350.04(±44.23) •	90.00(±41.36)	3.92(±0.11)	3.44(±0.16)
lambda-cyhalothrin 6x	338.73(±33.64) •	87.80(±29.68)	4.01(±0.11)	3.23(±0.15)**
methomyl 2 week	366.17(±54.75) •	329.57(±93.87)	3.93(±0.18)	3.48(±0.18)*
methomyl 5 weeks	362.08(±42.47)	188.57(±55.20)	3.65(±0.12)	3.42(±0.14)*
methomyl 8 weeks	391.04(±43.24) •	297.07(±59.94)	4.00(±0.15)	3.70(±0.13)

Stars represent significant differences in treatments relative to control for insecticide treatments, from ANOVA analysis. • $P < 0.1$ * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure Captions

Figure 1: Average number of visitors to male flowers treated with varying numbers of applications of lambda cyhalothrin. Diamonds represent the mean, whereas horizontal bars show the median.

Figure 2: Insecticide use had significant impacts on the germination and growth of control pollen tubes on treated stigmas. Asterisks show treatments that were statistically different from the control in zero inflated negative binomial models, which simultaneously ask whether treatments differ in the likelihood of pollen germinating and in the number of pollen grains germinating. Thus, for germinating pollen grains, (A) shows how treatments differ in the probability of pollen germinating while (B) shows that, for stigmas with germinating pollen grains treatments differed in the number of germinating grains. For pollen tubes to the base (C) treatments differed in probability of tubes reaching the base and for (D) those stigmas with any pollen tubes to the base (thus excluding zeros), treatments also differed in the number of tubes to the base. Note that for Methomyl, the one stigma with any pollen tubes to the base had significantly more than the control. In bar plots (A and C), bars represent a proportion of 25 stigmas sampled. In boxplots (B and D), diamonds represent the mean, whereas horizontal bars show the median. Zeros are excluded from B and D, as the analysis only tests whether there is an impact on the number of pollen tubes where there was a least one germinated grain.

Figure 3: Pesticide effects on seed characteristics for female type B only. (A) Average seed set (± SE), (B) Average seed weight (µg ± SE), and (C) Average seed germination (proportion out of 25 germinated per umbel ± SE). Stars indicate significance relative to the control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 1

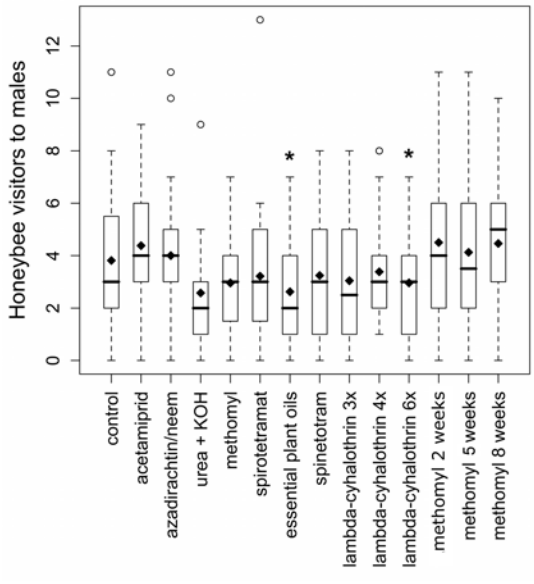

Honeybee visitors to males
0
2
4
6
8
10
12
control
acetamiprid
azadirachtin/neem
urea + KOH
methomyl
spirotetramat
essential plant oils
spinetotram
lambda-cyhalothrin 3x
lambda-cyhalothrin 4x
lambda-cyhalothrin 6x
methomyl 2 weeks
methomyl 5 weeks
methomyl 8 weeks

Figure 2

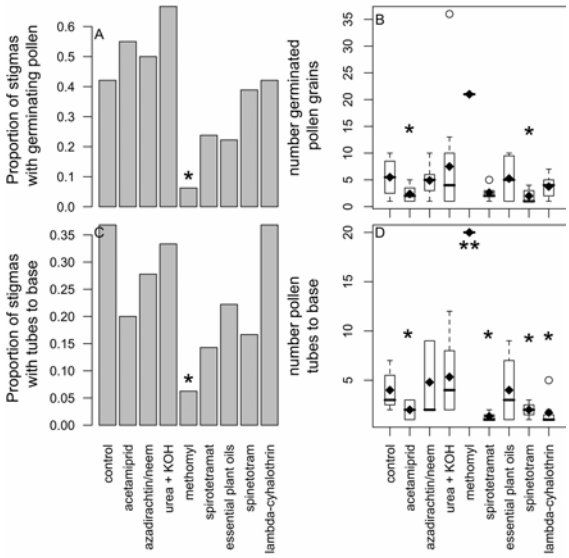
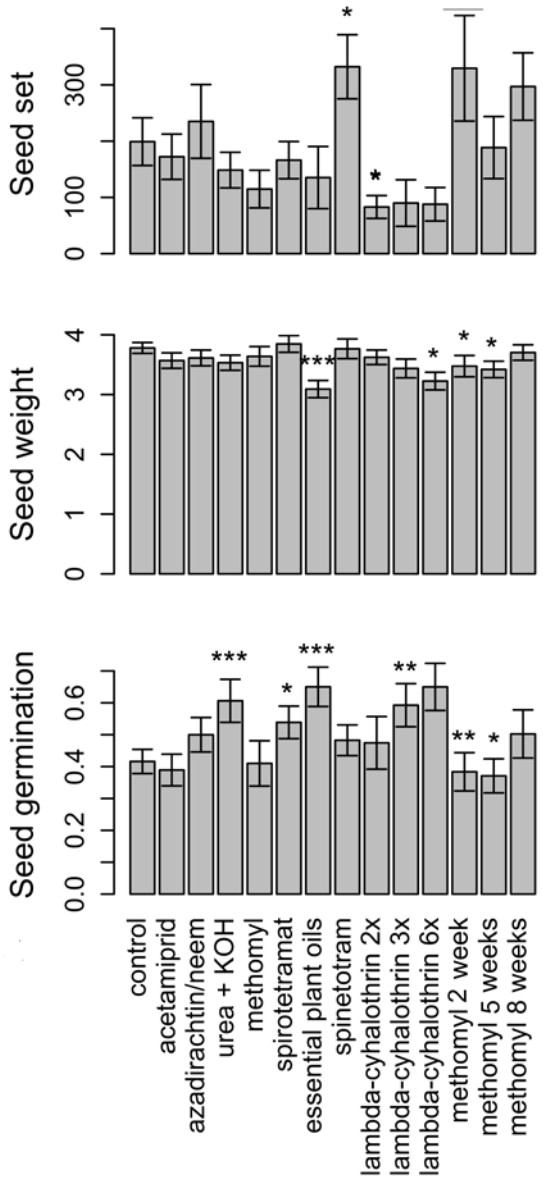

A
Proportion of stigmas with germinating pollen
B
number germinated pollen grains
C
Proportion of stigmas with tubes to base
D
number pollen tubes to base
control
acetamiprid
azadirachtin/neem
urea + KOH
methomyl
spirotetramat
essential plant oils
spinetoram
lambda-cyhalothrin

Figure 3


Seed set
0
100
300
*
*
Seed weight
0
1
2
3
4
***
*
*
*
Seed germination
0.0
0.2
0.4
0.6
***
*
***
**
**
*
control
acetamiprid
azadirachtin/neem
urea + KOH
methomyl
spirotetramat
essential plant oils
spinetotram
lambda-cyhalothrin 2x
lambda-cyhalothrin 3x
lambda-cyhalothrin 6x
methomyl 2 week
methomyl 5 weeks
methomyl 8 weeks