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Research article

Insecticide Susceptibility in Asian Honey Bees (*Apis cerana* (Hymenoptera: Apidae)) and Implications for Wild Honey Bees in Asia

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Abstract

To conserve local biodiversity and ensure the provision of pollination services, it is essential to understand the impact of pesticides on wild honey bees. Most studies that have investigated the effects of pesticides on honey bees have focused on the European honey bee (*Apis mellifera* (Hymenoptera: Apidae)), which is commonly domesticated worldwide. However, the Asian honey bee (*Apis cerana*) is widely distributed throughout Asia, and toxicity data are lacking for this species. This study aimed to fill this important knowledge gap. In this study, we determined the acute contact toxicity in *A. cerana* to various pesticides, including neonicotinoids, fipronil, organophosphorus, synthetic pyrethroids, carbamate, and anthranilic diamide. Based on the test duration of 48 h of contact LD_{50} tests, *A. cerana* was most sensitive to dinotefuran (0.0014 μg/bee), followed by thiamethoxam (0.0024 μg/bee) and fipronil (0.0025 μg/bee). Dinotefuran is used extensively in Asia, thereby potentially creating a substantial hazard. More generally, *A. cerana* was approximately one order of magnitude more sensitive than was *A. mellifera* to most of the pesticides evaluated. The results of our study suggest that neonicotinoid pesticides should not be considered as a single group that acts uniformly on all honey bees, and that more careful management strategies are required to conserve *A. cerana* populations than *A. mellifera*.

Key words: *Apis cerana*, neonicotinoid, pesticide toxicity, honey bee

Honey bees (*Apis* spp.) are important pollinators that provide essential pollination services to agricultural plant communities (Klein et al. 2007). Reductions in pollination services can greatly affect plant communities and wider ecosystem functions (Stanley et al. 2015). Apart from providing pollination services, honey bees provide a range of commercially important products, including honey, wax, pollen, propolis, and royal jelly (Winston 1991). Although Asian populations of managed European honey bee (*Apis mellifera*) colonies have increased (Aizen and Harder 2009), there have been recent reports of declines in honey bee populations in Europe and North America (vanEngelsdorp and Meixner 2010). Several factors may contribute to this decline, including foraging habitat loss (Dietemann et al. 2009, Goulson et al. 2015), changes in land use (Vaudo et al. 2012), parasites and disease (Fries 2010, Rosenkranz et al. 2010), and pesticide use (Decourtye and Devillers 2010, Blacquière et al. 2012, Tan et al. 2015). In addition, several other studies have raised concerns over the potential impacts of pesticides, particularly the newly introduced neonicotinoid pesticides, the use of which has increased dramatically within a short period (Decourtye et al. 2013, Godfray et al. 2014, Sanchez-Bayo 2014, Simon-Delso et al. 2015).

Neonicotinoids are a relatively new class of targeted pesticides widely used to control a broad range of agricultural pest insects (Jeschke et al. 2011). They act systemically, in that they are absorbed quickly by plants via roots or leaves and then translocated to all parts of the plant (Bonmatin et al. 2015). Therefore, they can protect the whole plant from pest insects because the entire plant, including flowers, pollen, nectar, and roots, becomes poisonous (van der Sluijs et al. 2013). Although neonicotinoids are lethal to many species of insects, they have a much lower toxicity to vertebrates than other insecticide classes (Simon-Delso et al. 2015). Regardless, they are shown to have negative impacts on some nontarget organisms (Desneux et al. 2007, Pisa et al. 2015), including pollinators (Han et al. 2010a, b; Henry et al. 2012; Whitehorn et al. 2012; Gill and Raine 2014; Kessler et al. 2015; Rundlöf et al. 2015), because neonicotinoid residues are translocated into pollen and nectar

collected by foragers (Henry et al. 2015). Their negative impacts on *A. mellifera* observed in laboratory experiments include memory loss and a decline in navigation skills (Decourtye et al. 2005, Han et al. 2010b, Blacquière et al. 2012, Henry et al. 2012). Because pesticides including neonicotinoids have multiple uses, there have many exposure pathways (van der Sluijs et al. 2013) in natural habitats.

Most toxicity studies on the effects of pesticides in honey bees have focused on the most common domesticated species, *A. mellifera* (Corlett 2011). However, Arena and Sgolastra (2014) found that *A. mellifera* is slightly less sensitive to some pesticides, including neonicotinoids, carbamates, organochlorines, organophosphates, and pyrethroid, compared with the Asian honey bee (*Apis cerana*). *Apis cerana* is native to and widely distributed across southern, southeastern, and eastern Asia (Oldroyd and Nanork 2009). However, there is a lack of toxicity studies of pesticides on *A. cerana* species compared with *A. mellifera*. For instance, the Japanese honey bee (*Apis cerana japonica* Radoszkowski) is one of four subspecies of *A. cerana* (Rutter 1988) and the only native wild honey bee found in Japan, but no toxicity data of pesticides are available. Therefore, to conserve local biodiversity and ensure the provision of pollination services in Asia, it is essential to investigate the effects of the most commonly used pesticides on all wild native *A. cerana* subspecies, including *A. cerana japonica*.

The purpose of this study was to examine the susceptibility of *A. cerana japonica* to a range of commonly used insecticides, including neonicotinoids, fipronil, organophosphorus pesticides, and synthetic pyrethroids, under laboratory conditions. Our initial hypothesis was that the acute contact susceptibility of *A. cerana* does not differ from that of *A. mellifera*, and that the same management strategies used for *A. mellifera* are effective for *A. cerana*. We used this approach because, despite documented differences in sensitivity outlined above, operational assumptions are often that the same management prescriptions are appropriate for both species. Our aim was to test this implicit operational assumption.

Materials and Methods

Insecticides

We selected insecticides commonly used in many countries, including in Asia, on a wide variety of agricultural crops (Magallona 1989, Matsumura et al. 2008, Lin et al. 2013). Also, substantial volumes of the following chemicals were used in Japan in 2011 (Table 1): neonicotinoids (acetamiprid, imidacloprid, clothianidin, dinotefuran, and thiamethoxam), phenylpyrazole (fipronil), organophosphates (diazinon and fenitrothion), anthranilic acid amide (chlorantraniliprole), synthetic pyrethroid (ethofenprox), and carbamate (carbaryl). Pure forms of these chemicals were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan; see Table 1). All substances were dissolved in acetone (Wako Pure Chemicals Co., Ltd) to prepare the test solutions. Concentration were narrowed down based on known LD_{50} values for *A. mellifera*. The tested concentrations of each compound are shown in Table 1. In some cases, one or more of the control bees died. In these cases, the results for the treatment were disregarded.

Honey Bees

Apis cerana japonica individuals used in these experiments were collected as larvae from three hives at the Forestry and Forest Products Research Institute and the National Institute for Environmental Studies, Ibaraki, Japan. Sections of colonies in which larvae were pupating in cells were collected and stored at 35 °C until the larvae emerged as young adult bees. More than 10% of older (> 3-d-old) control *A. cerana japonica* died within 48 h in our experiments. Therefore, we only used newly emerged young adult bees (< 2-d-old) for the experiments to reduce the natural mortality rate and comply with the OECD test guidelines on acute toxicity testing.

Acute Contact Toxicity

The first acute toxicity test by topical application was conducted in November 2014. All other tests were conducted in June, July, and August 2015. We generated a small hole in the center of the bottom of a polypropylene cup (180 ml, 81 × 58 × 58 mm; Shingi Co., Ltd., Hiroshima, Japan) and extruded the center of a single Kimwipe (Crecia, Tokyo, Japan) through the hole, following the method of Iwasa et al. (2004). The cup was placed in a second cup containing a reservoir of a 50% aqueous sucrose syrup (Nacalai Tesque, Inc., Kyoto, Japan), and bees fed on the sucrose from the Kimwipe.

Bees were anesthetized with carbon dioxide and immediately transferred to a cup. Each cup contained 10 bees. The bees were treated with 1 µl of the appropriate dose of pesticide per bee on the dorsal surface of the abdomen, following the standard acute contact toxicity test procedure for *A. mellifera* (OECD 1998). A previous study (Smirle et al. 1984) found no significant differences in *A. mellifera* worker longevity between acetone-treated ($N = 60$) and untreated controls ($N = 60$; t-test, $P > 0.05$). This study was sufficiently powerful to detect significant differences between the treatment and control. In our experiments, the control group was treated with 1 µl of pure acetone (MacKenzie and Winston 1989). After treatment, the polypropylene cup was covered with a nylon mesh sheet fastened with a rubber band and kept in the dark, photoperiod of 0:24 (L:D) h, in a temperature-controlled chamber at 25.1 ± 0.2 °C and 67 ± 6.6% RH. Mortality was recorded at 24 and 48 h after treatment. Moribund bees that were unable to walk or fly were not considered dead in this study (Laurino et al. 2013, Stanley et al. 2015). This procedure was repeated at least three times for each treatment, but in some cases, where one or more of the control cages had mortality above 10%, the data were not used for the further analysis.

Data Analysis

The pooled data from the separate tests conducted with each chemical were subjected to a probit analysis using PriProbit ver. 1.63 (Sakuma 1998). The SAS equivalent method assuming normal function distribution was used to calculate confidence intervals, assuming a binomial distribution and an all-or-nothing response parameter. The unit of estimated LD_{50} values of this study was µg/bee. Mean body weight of *A. cerana* is 0.075 g (Thompson 2016).

Reference LD_{50} values for the test insecticides in *A. mellifera* at 24 and 48 h were obtained from the ECOTOX (U.S. Environment Protection Agency [http://cfpub.epa.gov/ecotox/, accessed 10 December 2015]) and AgriTox databases (Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environment et du Travail in France [http://www.agritox.anses.fr/index.php, accessed 10 December 2015]), as well as from the literature (Sanchez-Bayo and Goka 2014). The unit of obtained values of LD_{50} for *A. mellifera* from these databases was µg/bee. The mean body weight of *A. mellifera* was 0.1 g (Thompson 2016). The LD_{50} values of the target pesticides in *A. mellifera* and *A. cerana japonica* were compared using the Wilcoxon signed-rank test in R ver. 3.1.2 for Windows (R Core Team 2014).

Table 1. The target pesticides evaluated in this study

Class	Pesticide	Purity (%)	Tested concentrations (μg/bee)	Annual shipment (t)
Neonicotinoid	Acetamiprid	98	0, 0.000061, 0.00025, 0.00099, 0.00198, 0.00395, 0.0079, 0.016, 0.032, 0.063, 0.13, 0.25, 0.51, 2.023, 8.09, 32.36	48.1
	Imidacloprid	98	0, 0.000024, 0.000049, 0.000098, 0.000195, 0.00039, 0.00078, 0.0016, 0.011, 0.045, 0.18, 0.72, 2.88	68.1
	Clothianidin	99	0, 0.00017, 0.00034, 0.00069, 0.0014, 0.0028, 0.0055, 0.011, 0.022, 0.044	60.7
	Dinotefuran	99	0, 0.0000073, 0.000015, 0.000029, 0.000059, 0.00012, 0.00023, 0.00047, 0.00094, 0.0019, 0.0038, 0.0058, 0.0075, 0.023, 0.092, 0.368, 1.47	155.9
	Thiamethoxam	99	0, 0.000047, 0.000094, 0.000375, 0.00075, 0.0015, 0.003, 0.006, 0.012, 0.024	38.5
Phenylpyrazole	Fipronil	98	0, 0.000094, 0.00019, 0.00038, 0.00075, 0.0015, 0.003, 0.006, 0.012, 0.024, 0.048, 0.096, 0.192, 0.384	34.9
Organophosphorus	Diazinon	98	0, 0.000078, 0.00016, 0.00031, 0.00063, 0.0013, 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, 0.32, 1.28, 5.12	352.5
	Fenitrothion	98	0, 0.000078, 0.00016, 0.00031, 0.00063, 0.0013, 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.64, 2.56, 10.24	464.2
Anthranilic diamide	Chlorantraniliprole	98	0, 0.00049, 0.00098, 0.00195, 0.0039, 0.0078, 0.016, 0.031, 0.062, 0.125, 0.25, 0.5, 1.0, 2.0	22.2
Synthetic pyrethroid	Ethofenprox	98	0, 0.0000078, 0.000031, 0.000125, 0.0005, 0.001, 0.002, 0.004, 0.008, 0.016, 0.031, 0.062	106.1
Carbamate	Carbaryl	99	0, 0.0014, 0.0028, 0.0056, 0.011, 0.023, 0.045, 0.09, 0.18	63.4

Annual shipment data were delivered from Webkis-plus database, National Institute for Environmental Studies, Japan (http://db-out.nies.go.jp/kis-plus/index_3.html, accessed 30 November 2015).

Results

The acute LD_{50} values for contact toxicity at 48 h of the 11 tested pesticides in *A. cerana japonica* are shown in Table 2. The most toxic pesticides were dinotefuran (LD_{50}, 0.0014 μg/bee), thiamethoxam (0.0024 μg/bee), and fipronil (0.0025 μg/bee), followed by clothianidin (0.0034 μg/bee), imidacloprid (0.0036 μg/bee), and ethofenprox (0.0048 μg/bee). Organophosphorus (diazinon and fenitrothion), carbaryl, and chlorantraniliprole were about one order of magnitude less toxic than the previous six compounds. Acetamiprid exhibited the lowest toxicity (0.278 μg/bee) by a large difference. The 95% confidence intervals were found to be wider for acetamiprid and fenitrothion, two of the pesticides for which 95% confidence levels could be determined.

The acute LD_{50} contact toxicities at 24 h in *A. cerana japonica* of the 11 tested pesticides are shown in Table 3. The most toxic pesticides were thiamethoxam (0.003 μg/bee) and clothianidin (0.004 μg/bee), followed by ethofenprox (0.007 μg/bee) and imidacloprid (0.008 μg/bee). The 95% confidence intervals were wider for acetamiprid, fenitrothion, and dinotefuran. While the most toxic pesticides differed between the 24- and 48-h tests, acetamiprid exhibited the lowest toxicity (0.22 μg/bee) after 24 h, similar to that after 48 h.

We compared the LD_{50} values of the 11 pesticides for *A. cerana japonica* with those for *A. mellifera* (Tables 2 and 3). Imidacloprid, clothianidin, dinotefuran, thiamethoxam, fipronil, and ethofenprox exhibited the greatest similarities in LD_{50} values between *A. cerana japonica* and *A. mellifera*. The LD_{50} 95% confidence interval of fenitrothion was greater than those of diazinon, fenitrothion, carbaryl, and acetamiprid. When the Wilcoxon signed-rank test by analyzing LD_{50} values of *A. cerana japonica* for each test compound corresponding to those of *A. mellifera* as data sets was carried out, the result indicated that the susceptibility of *A. cerana japonica* to pesticides is significantly higher than that of *A. mellifera* (Wilcoxon signed-rank test, $P < 0.001$).

Discussion

By comparing our results on *A. cerana* with those of previous studies on *A. mellifera*, we found that the majority of the LD_{50} values of the test insecticides were lower for *A. cerana* (Tables 2 and 3). A ratio of LD_{50}s renders an average factor of 11.2 ± 9.96 (mean ± SD) between two honey bee species, meaning that *A. cerana* is about one order of magnitude more sensitive than *A. mellifera*. Studies on other bee species (Arena and Sgolastra 2014) have observed similar differences in responses, indicating that other *A. cerana* subspecies are also slightly more sensitive to pesticides compared with *A. mellifera*. The other study (Thompson 2016) reported the sensitivity between *A. mellifera* and other bees, including *A. cerana*, to pesticides varies by the factor of 5 approximately after correcting for the body size, but our data agree more with the study done by Arena and Sgolastra (2014). More generally, closely related insects may respond very differently to the same chemicals (Suchail et al. 2000, Laurino et al. 2013); therefore, toxicity tests performed on different species of wild honey bees are essential, as it is not always applicable to assume that *A. mellifera* toxicity levels apply to other bee species. Arena and Sgolastra (2014) found that the differences in pesticide sensitivities between two honey bee species were difficult to explain. *Apis cerana* (body length: 10–13 mm, body weight: 60–90 mg; Sasaki 1999) has a smaller body size than that of *A. mellifera* (12–14 mm, 70–120 mg), which together with a relatively larger surface area-to-volume ratio may contribute to the greater sensitivity of *A. cerana* to pesticides. Other studies have observed similar relationships between bee body size and sensitivity to toxicants (Johansen 1972, Devillers et al. 2003). Conversely, Helson et al. (1994) did not detect a relationship between body size and the relative susceptibility of insects to toxicants, including *A. mellifera*.

Another possible explanation for the difference in sensitivity among species is the number of genes encoding antioxidant enzymes (Claudianoes et al. 2006, Berenbaum and Johnson 2015). However, a similar number of genes encoding antioxidant enzymes have been

Table 2. Acute (48 h) contact toxicity of pesticides in *Apis cerana japonica* with LD_{50} values (μg/bee) of *Apis mellifera* as reference value

Class	Chemicals	Slope ± SE	Intercept ± SE	LD_{50} (μg/bee)		Goodness of fit test			LD_{50}[c] (μg/bee)
				Estimate	95% CI	df	Likelihood ratio χ^2	*P*	*A. mellifera*
Neonicotinoid	Acetamiprid	0.942 ± 0.247	0.523 ± 0.256	0.278	0.060–1.041	19	59.24	<0.001	8.09
	Imidacloprid	2.09 ± 0.47	5.098 ± 1.23	0.0036	0.0018–0.0077	15	11.31	0.73	0.06
	Clothianidin	8.00 ± 1.97	19.71 ± 4.85	0.0034	0.0029–0.005	13	8.43	0.81	0.042
	Dinotefuran	0.91 ± 0.30	2.60 ± 0.86	0.0014	0.0001–0.001	17	83.53	<0.001	0.041
	Thiamethoxam	3.47 ± 0.66	9.08 ± 1.68	0.0024	0.0018–0.0031	17	25.45	0.085	0.035
Phenylpyrazole	Fipronil	3.96 ± 0.94	10.31 ± 2.45	0.0025	0.0017–0.0036	18	30.95	0.029	0.0065
Organophosphorus	Diazinon[a]	23.93 ± 3828.0	39.5 ± 6503.7	0.022	–	20	27.43	0.12	0.37
	Fenitrothion	2.52 ± 0.80	2.92 ± 0.85	0.069	0.03–0.10	23	37.16	0.03	0.26
Anthranilic diamide	Chlorantraniliprole[b]	2.66 ± 0.82	4.67 ± 1.41	0.018	0.007–0.03	16	36.3	0.003	–
Synthetic pyrethroid	Ethofenprox	1.09 ± 17.2	2.52 ± 13.34	0.0048	–	20	45.46	<0.001	0.015
Carbamate	Carbaryl[a]	22.6 ± 9160.8	31.1 ± 12337	0.042	–	9	20.6	0.015	0.63

The 95% confidence interval was not calculated for diazinon, ethofenprox, and carbaryl treatment, because the index of significance for potency estimation exceeded 0.5.

[a] The model is uncertain, as SE of slope is too large.

[b] Finney Equivalent was used to calculate the CI.

[c] LD_{50} values of *A. mellifera* were obtained from the ECOTOX (http://cfpub.epa.gov/ecotox/) and AgriTox databases (http://www.agritox.anses.fr/index.php, accessed 10 December 2015).

Table 3. Acute (24 h) contact toxicity of pesticides in *Apis cerana japonica* with LD_{50} values (μg/bee) of *Apis mellifera* as reference value

Class	Chemicals	Slope ± SE	Intercept ± SE	LD_{50} (μg/bee)		Goodness of fit test			LD_{50}[a] (μg/bee)
				Estimate	95% CI	df	Likelihood ratio χ^2	*P*	*A. mellifera*
Neonicotinoid	Acetamiprid	0.91 ± 0.18	0.6 ± 0.19	0.22	0.081–0.53	26	68.67	<0.001	7.07
	Imidacloprid	1.23 ± 0.32	2.57 ± 0.70	0.008	0.004–0.018	52	126.8	<0.001	0.014
	Clothianidin	5.30 ± 1.24	12.68 ± 2.97	0.004	0.0032–0.005	21	19.49	0.57	0.022
	Dinotefuran	1.88 ± 0.65	3.09 ± 1.20	0.023	0.01–0.10	25	101.4	<0.001	0.075
	Thiamethoxam	3.33 ± 0.84	8.21 ± 2.05	0.003	0.002–0.005	43	166.3	<0.001	0.027
Phenylpyrazole	Fipronil	1.63 ± 0.39	2.94 ± 0.76	0.016	0.008–0.037	52	178.55	<0.001	0.0103
Organophosphorus	Diazinon	4.52 ± 0.94	6.56 ± 1.39	0.035	0.028–0.045	27	29.8	0.32	0.13
	Fenitrothion	2.94 ± 0.84	3.12 ± 0.88	0.087	0.056–0.12	43	70.89	0.005	0.34
Anthranilic diamide	Chlorantraniliprole	1.23 ± 0.24	2.2 ± 0.44	0.016	0.0073–0.033	25	50.47	0.002	–
Synthetic pyrethroid	Ethofenprox	4.12 ± 0.98	8.84 ± 2.07	0.007	0.0052–0.009	34	64.68	0.001	–
Carbamate	Carbaryl	5.77 ± 1.33	7.91 ± 1.75	0.043	0.033–0.051	21	24.6	0.26	0.48

[a] LD_{50} values of *A. mellifera* were obtained from the ECOTOX (http://cfpub.epa.gov/ecotox/) and AgriTox databases (http://www.agritox.anses.fr/index.php, accessed 10 December 2015).

identified in *A. cerana* as in *A. mellifera* (Shi et al. 2013, Yan et al. 2013, Wang et al. 2014, Park et al. 2015). Therefore, more research is needed to fully explain the difference in sensitivity between these two species.

Most of the neonicotinoid LD_{50} values were lower by one order of magnitude than those of other classes of pesticides such as organophosphorus and carbamate in *A. cerana* (Table 2). Stanley et al. (2015) compared the percent mortality of various insecticides against their application rates to crops in India, and showed that organophosphorus induced the most mortalities in both *A. cerana* and *A. mellifera*, as it causes 100% mortality of the bees if the bees were flying across the field being sprayed or get spray drift onto their bodies. Moreover, because of the high annual use of other pesticides such as organophosphorus (Table 1), the relative risk of pesticide poisoning in the field cannot be based only on the susceptibilities determined in the laboratory, but also on the amounts actually applied.

Some neonicotinoid pesticides, such as acetamiprid, appear to exhibit relatively low toxicity to both *A. mellifera* and *A. cerana* (Stanley et al. 2015). A previous study reported that neonicotinoid pesticides containing nitro groups exhibited greater toxicity in *A. mellifera* than did those containing cyano substitutions, as cyano-neonicotinoids such as acetamiprid can be metabolized in bees to produce innocuous degradation compounds, whereas the other neonicotinoids produce toxic metabolites in bees (Iwasa et al. 2004). This suggests that different neonicotinoid pesticides do not act uniformly on all insects. Instead, the effects of compounds of each chemical class on nontarget organisms should be investigated separately.

According to a previous study that measured insecticide concentrations in dead honey bees sampled from hives near rice paddy fields in Japan (Kimura et al. 2014), the average clothianidin, dinotefuran, and etofenprox concentrations were 0.00032, 0.00017, and 0.00055 μg/bee, respectively, and the average worker bee weight

was <0.1 g. Based on these results, the LD_{50} values of several of the chemicals used in this study were one order of magnitude greater than environmentally relevant concentrations. Other studies have found similar gaps between laboratory experiment and field test results (Godfray et al. 2014, Henry et al. 2015). The LD_{50} value is only indicative of the reaction and relative sensitivity of an organism to a chemical. Therefore, semi-field or field exposure studies under normal agricultural conditions are required to fully understand the adverse effects of pesticides in all honey bee species (Stanley et al. 2015). However, studies on *A. mellifera* have established that LD_{50} measurements, similar to those conducted in this study, provide a reasonable estimate of the relative ecological risks for exposing *A. cerana* to contaminants over longer periods.

Our results showed that *A. cerana* was more sensitive than *A. mellifera* by a factor of 8 to 14 times. Our toxicity bioassay data provide strong *prima facie* evidence that the broader ecological effects of some of these contaminants are more severe in *A. cerana* than those in *A. mellifera*. This implies that pesticide management strategies in East Asian agricultural systems should be more site-specific than those in other global regions, and these strategies should include application rate limits, more careful discrimination of specific uses for the different pesticides, and more thorough testing of their effects on important nontarget species.

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