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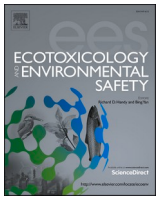

Research paper

Toxic responses of blue orchard mason bees (*Osmia lignaria*) following contact exposure to neonicotinoids, macrocyclic lactones, and pyrethroids

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ABSTRACT

Analysis of particulate matter originating from beef cattle feed yards on the High Plains of the United States has revealed occurrence of multiple pesticides believed to potentially impact non-*Apis* pollinators. Among these pesticides are those that are highly toxic to *Apis mellifera* (honey bees). However, little non-*Apis* bee species toxicity data exist; especially pertaining to beef cattle feed yard-derived pesticides. Therefore, we conducted a series of 96-h contact toxicity tests with blue orchard mason bees (*Osmia lignaria*) using three neonicotinoids, two pyrethroids, and two macrocyclic lactones. Neonicotinoids (thiamethoxam, imidacloprid, and clothianidin) were most toxic with LD_{50} values ranging from 2.88 to 26.35 ng/bee, respectively. Macrocyclic lactones (abamectin and ivermectin) were also highly toxic to *O. lignaria* with LD_{50} estimates of 5.51–32.86 ng/bee. Pyrethroids (permethrin and bifenthrin) were relatively less toxic with LD_{50} values greater than 33 ng/bee. Sensitivity ratios for each pesticide were calculated to relate *O. lignaria* LD_{50} values to existing honey bee toxicity data. All three neonicotinoids were more toxic to *O. lignaria* than *A. mellifera*, but pyrethroids and abamectin were relatively less toxic. Additionally, three of seven pesticides (43%) resulted in significantly different mass normalized LD_{50} values for male and female *O. lignaria*. These results indicate that non-*Apis* pollinators may be highly susceptible to pesticides originating from beef cattle feed yards, necessitating consideration of more stringent regulatory protections than those based on *A. mellifera* pesticide sensitivity.

1. Introduction

Managed and wild bees dominate animal pollination that is essential for 87% of angiosperm reproduction and improves yield in over 70% of crops worldwide (Grimaldi and Engel, 2005; Klein et al., 2007; Ollerton et al., 2011). It is estimated that honey bees (*Apis mellifera*) and wild bee pollination services were worth over $15 billion (USD) annually in the United States of America in the early 2000s (Losey and Vaughan, 2006). However, a report by the Center of Biological diversity asserts that 50% of native bee populations in North America are declining, and 24% are in danger of extinction (Kopec, 2017). Despite the fact that numbers of managed *A. mellifera* colonies have increased in recent years, there remains a net decrease in *A. mellifera* populations over the past 70 years (Goulson et al., 2015). Declines are likely due to a multitude of anthropogenic factors including habitat fragmentation, agricultural grazing, agrochemical use, disease, and introduction of non-native species (Kearns et al., 1998).

Sánchez-Bayo and Wyckhuys (2019) determined that the most significant contributor to insect declines is loss/change of native habitat followed by the widespread use of agrochemicals including organophosphates, pyrethroids, and neonicotinoids which are the most commonly used insecticides in agriculture (Atwood and Paisley-Jones, 2017). However, organophosphate use has declined by over 70% since the turn of the century, due to increasingly stringent regulations and the development of new insecticides (Atwood and Paisley-Jones, 2017). While organophosphate use has decreased, neonicotinoid use has increased dramatically over the past two decades, a period that coincides with global pollinator declines (Lundin et al., 2015). Neonicotinoids first became available in the early 1990's upon the introduction of imidacloprid (Blacquiere et al., 2012), and are now used on over 140 crops in 120 countries (Lundin et al., 2015). Commonly applied via foliar application sprays and as seed coatings, neonicotinoids are highly hydrophilic chemicals that result in systemic uptake and distribution throughout plants (Elbert et al., 2008). Although effective against biting and sucking pests, systemic uptake also facilitates non-target pollinating insect exposure via contaminated pollen and

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nectar (Hopwood et al., 2012; Krupke et al., 2012; Woodcock et al., 2016). Neonicotinoids cause an influx of sodium ions into insect cells by irreversibly binding to nicotinic acetylcholine receptors resulting in cell death (Casida and Durkin, 2013; Goulson, 2013; Peterson et al., 2019). Subsequently, neonicotinoids are highly toxic to bees (*A. mellifera* contact LD_{50} is 61 ng/bee for imidacloprid, 25 ng/bee for thiamethoxam, and 39 ng/bee for clothianidin), leading to an outright ban of neonicotinoids in the European Union and other countries (Iwasa et al., 2004; Sanchez-Bayo and Goka, 2014).

Similar to neonicotinoids, pyrethroid use has increased over the past twenty years (Atwood and Paisley-Jones, 2017). Pyrethroids kill chewing pests such as leafworms and bollworms on crops, control external pests on livestock, and control adult mosquito populations via fogging (Feedlot, 2011; Schleier and Peterson, 2010; Yadouleton et al., 2011). Pyrethroids are also highly toxic to non-target organisms (permethrin contact LD_{50} for *A. mellifera* is 20 ng/bee; Piccolomini et al., 2018) and fry channel catfish LC_{50} 0.62 μg/L (EPA, U.S., 2006; Jolly et al., 1978), by preventing voltage-gated sodium channels from closing, allowing continual neuron firing causing convulsions and cell death (Palmquist et al., 2012; Peterson et al., 2019). This mechanism of action and toxicity of pyrethroids has prompted the United States Environmental Protection Agency (EPA) to impose restrictions on the use of pyrethroid insecticides to mitigate unexpected exposure among non-target organisms (EPA, U.S., 2006).

The High Plains region of the United States (Texas Panhandle, eastern New Mexico, western Kansas, Oklahoma Panhandle, Nebraska, and eastern Colorado; Crosbie et al., 2013) is an important and expansive agricultural region, and insecticide use is extensive. The High Plains is dominated by row crop agriculture (predominantly cotton, corn, and wheat) but it also supports an expansive beef cattle feeding industry (77% of all cattle on feed in the U.S.; Gollehon and Winston, 2013; NASS (National Agricultural Statistics Service), 2019). The most frequently used pesticides on beef cattle feed yards include macrocyclic lactones (ivermectin, moxidectin, eprinomectin, abamectin, and doramectin; applied on 88.6% of feed yards) which are used to control internal and external pests, and pyrethroids (e.g. bifenthrin and permethrin; applied on 24% of feed yards) which are used to control external pests and flies (Feedlot, 2011). Recently, aerial dispersal of these biocidal compounds beyond feed yard boundaries has been documented generating concern that local pollinators may be exposed to toxic concentrations of insecticides used on or near cattle feed yards (Peterson et al., 2020, 2017). An estimated 2236 kg of particulate matter emanates from average size feed yards (39,220 head of cattle) daily (Asem-Hiablie et al., 2015), which potentially contains sufficient amounts of insecticide (on a mass basis) to kill thousands of *A. mellifera* (Peterson et al., 2020). It is well documented that pyrethroids are highly toxic to native pollinators (Piccolomini et al., 2018), yet very little data pertaining to macrocyclic lactones and their impacts on pollinators exist. Macrocyclic lactones are highly toxic to dung beetle and fly communities (Finch et al., 2020; Herd, 1995), and Smith et al. (1996) determined that abamectin was highly toxic to *A. mellifera* (contact LD_{50} of 30 ng/bee). Recently Peterson et al. (2019) reported that moxidectin was highly toxic to painted lady butterfly larva (*Venessa cardui*; oral LD_{50} = 2.1 ng/g). Macrocyclic lactones are also highly toxic to other non-target organisms with larval instars because they bind to glutamate-gated chloride channel receptors causing an influx of chloride ions resulting in paralysis and death (Lumaret et al., 2012; Peterson et al., 2019; Prichard et al., 2012).

The vast majority of pollinator risk assessments are based on data derived from a single representative organism – *A. mellifera*. Recent studies have attempted to determine whether *A. mellifera* is in fact, a suitable surrogate for other non-target invertebrates, a broad collection of taxonomic groups that have exhibited significant variability in sensitivity to agrochemicals (Arena and Sgolastra, 2014). Scott-Dupree and colleagues (2009) reported that alfalfa leaf cutting bees (*Megachile rotundata*) were nearly two times more sensitive to both clothianidin and imidacloprid than *A. mellifera*. Similarly, Cresswell et al. (2014) demonstrated that bumblebees (*Bombus terrestris*) had reduced feeding and locomotor ability following dietary exposure to 100 μg/L of imidacloprid, but *A. mellifera* exhibited no noticeable effects. To address concerns about variable agrochemical sensitivity among bees, the European Food Safety Authority (EFSA) has proposed incorporating a risk assessment factor (i.e. safety factor) of 10 when extrapolating *A. mellifera* endpoints to bumblebees and solitary bees (G.O.F. EFSA, 2013). Additionally, because *A. mellifera* colonies are predominately females, males are not included in bee toxicity testing protocols (OECD, 2017, 1998, 2000). While females are generally regarded as most important in terms of reproductive output (Lipnick et al., 1995), discounting male sensitivity could result in altered sex ratios and uncertainty in insecticide risk assessments (Monzón et al., 2004; Torchio and Tepedino, 1980).

Surprisingly, there is very little toxicity data that exists pertaining to blue orchard mason bees (*Osmia lignaria*) and their relative agrochemical sensitivity compared to *A. mellifera*. Because *O. lignaria* are increasingly used as managed pollinators in agriculture in the United States (replacing *A. mellifera* pollinating services; Bosch and Kemp, 2002), it is important to understand how *O. lignaria* toxicity thresholds compare with *A. mellifera*. Therefore, our objective was to evaluate the sensitivity of male and female *O. lignaria* to agrochemicals (neonicotinoids, pyrethroids, and macrocyclic lactones) commonly detected near beef cattle feed yards in the High Plains, and to determine if the EFSA risk assessment factor of 10 would be adequate to protect *O. lignaria*.

2. Materials and methods

2.1. Acute 96H toxicity test

Eastern *O. lignaria* cocoons were acquired from Mason Bees for Sale (Deweyville, UT). Upon arrival, cocoons were kept in a Bee Safe container (Mason Bees for Sale) in the refrigerator at 4 ± 1 °C until experimentation. Acute 96 h contact toxicity tests were performed following OECD guidelines for *A. mellifera* and the International Commission for Plant-Pollinator Relationships (ICPPR) with minor modifications (Hanewald et al., 2015; OECD, 1998). To obtain sufficient sample sizes, and to ensure each test organism was of similar age, *O. lignaria* were gently removed from their cocoons and placed into a 162 mL plastic cup containing a feeding hole at the bottom (one bee per cup). Bees were fed 20% sugar water ad libitum and placed in an incubator at 24 ± 2 °C with 8:16 light to dark ratio.

After *O. lignaria* were habituated for 24 h, 2 μL of treatment solution was applied to the dorsal side of their thorax. A total of 30 bees were treated per concentration, at a ratio of approximately 50:50 (male: female), with a minimum of five different concentrations (doses) per chemical (doses on 2.0 scaling factor). Fresh treatment solution was made for each chemical of interest in HPLC-grade acetone and serially diluted to appropriate concentration. Following exposure, treatment solutions were evaporated down to dryness under nitrogen and reconstituted into acetonitrile with internal standard (tris (1-chloro-2-propyl) phosphate). Samples were then analyzed via triple-quadrupole liquid chromatography-tandem mass spectrometry with electrospray ionization (Thermo TSQ Quantum Access Max, Thermo Scientific, Waltham, MA) as per Peterson et al. (2017) to confirm treatment concentrations. Quality control samples and lab blanks were included in analysis (recovery = 100 ± 10%).

A solvent control treatment group (n = 30) was included concurrent with all pesticide trials, and solvent control mortality <10% was designated as criteria for trials to be considered valid. Bee mortality was defined as complete immobility and unresponsiveness to stimulus. Bees were observed at 24, 48, 72, and 96 h after exposure, and any dead bees were weighed and their sex recorded. Upon completion of each trial (96 h), live bees were cold-anesthetized, and then weight and sex were

determined.

2.2. Statistics

All statistical analyses were performed using R (version 3.5.2; R Core Team, 2019) with RStudio for Windows (version 1.1.422; Team, 2015). Dose-response models were fitted to acute toxicity test data for each chemical using the "drc" package (Ritz et al., 2015). Male and female bee data were analyzed separately and models fit to each sex. The mselect() function of "drc" was used to determine which model function was most appropriate for data present. Two-parameter log-logistic models were used for all chemicals and both sexes. For each chemical, dose-response models of each sex were compared using the compParm() function of "drc", with slope of the model and the LD_{50} value compared. Post-hoc power analysis for two-parameter log-logistic regression was conducted using the "WebPower" package to confirm sample sizes were adequate.

Due to variation in size among individual bees and between sexes, wet mass of each organism was recorded at either time-of-death or termination of the experiment. Individual wet mass was then used to normalize the dose administered to each bee. Nominal dose was divided by wet mass to obtain ng of pesticide/μg of bee tissue normalized dose. Mass-normalized dose was used, rather than nominal when constructing dose-response models. Sensitivity ratios were calculated by dividing *A. mellifera* LD_{50} concentrations by observed LD_{50} concentrations of *O. lignaria* (Arena and Sgolastra, 2014).

3. Results and discussion

3.1. Neonicotinoids

Neonicotinoids were most toxic to *O. lignaria* among pesticides included in this study. The lowest LD_{50} estimate was for clothianidin (female LD_{50} = 4.9 ± 0.8 ng/bee, male LD_{50} = 2.9 ± 0.4 ng/bee) followed by thiamethoxam (female LD_{50} = 9.7 ± 1.5 ng/bee, male LD_{50} = 5.1 ± 0.6 ng/bee; Fig. 1). Imidacloprid was relatively less toxic to *O. lignaria* (female LD_{50} = 25.5 ± 3.5 ng/bee, male LD_{50} = 26.4 ± 3.0 ng/bee; Table 1), but would still be classified as highly toxic to bees according to the EPA (highly toxic <2000 ng/bee; USEPA, 2014). Similarly, imidacloprid appears to be the least toxic neonicotinoid to *A. mellifera* (Sanchez-Bayo and Goka, 2014). The imidacloprid contact LD_{50} for *O. bicornis* reported by Uhl et al. (2018) was twelve times higher (330 ng/bee) than the current study results (26 ng/bee), suggesting that interspecies insecticide sensitivity (even within the same genus) can vary greatly. Scott-Dupree et al. (2009) characterized the imidacloprid

Table 1
Mass normalized LD_{50} values for male and female *O. lignaria*.

Chemical	Sex	Mass Normalized LD_{50} ± SE (ng/bee) by Sex	Comparison of LD_{50} estimates by sex, p-value
Permethrin	Female	289.66 ± 39.41	0.003*
	Male	201.88 ± 32.60	
Bifenthrin	Female	74.99 ± 12.04	0.360
	Male	32.63 ± 5.84	
Abamectin	Female	32.86 ± 10.89	<0.001*
	Male	5.51 ± 1.14	
Ivermectin	Female	24.70 ± 5.85	0.211
	Male	9.14 ± 1.41	
Thiamethoxam	Female	9.65 ± 1.49	0.373
	Male	5.14 ± 0.55	
Imidacloprid	Female	25.47 ± 3.51	<0.001*
	Male	26.35 ± 2.95	
Clothianidin	Female	4.92 ± 0.83	0.120
	Male	2.88 ± 0.38	

Asterisk (*) denotes significant differences ($p < 0.05$)
Note: Differences in LD_{50} estimates by sex were evaluated by z-test using the compParm() function of the 'drc' package in R. LD50 values for permethrin, abamectin, and imidacloprid differed significantly between sexes. Combined LD50 values indicate the LD50 estimate for both sexes included in a single dose-response model, expressed as ng a.i./ bee

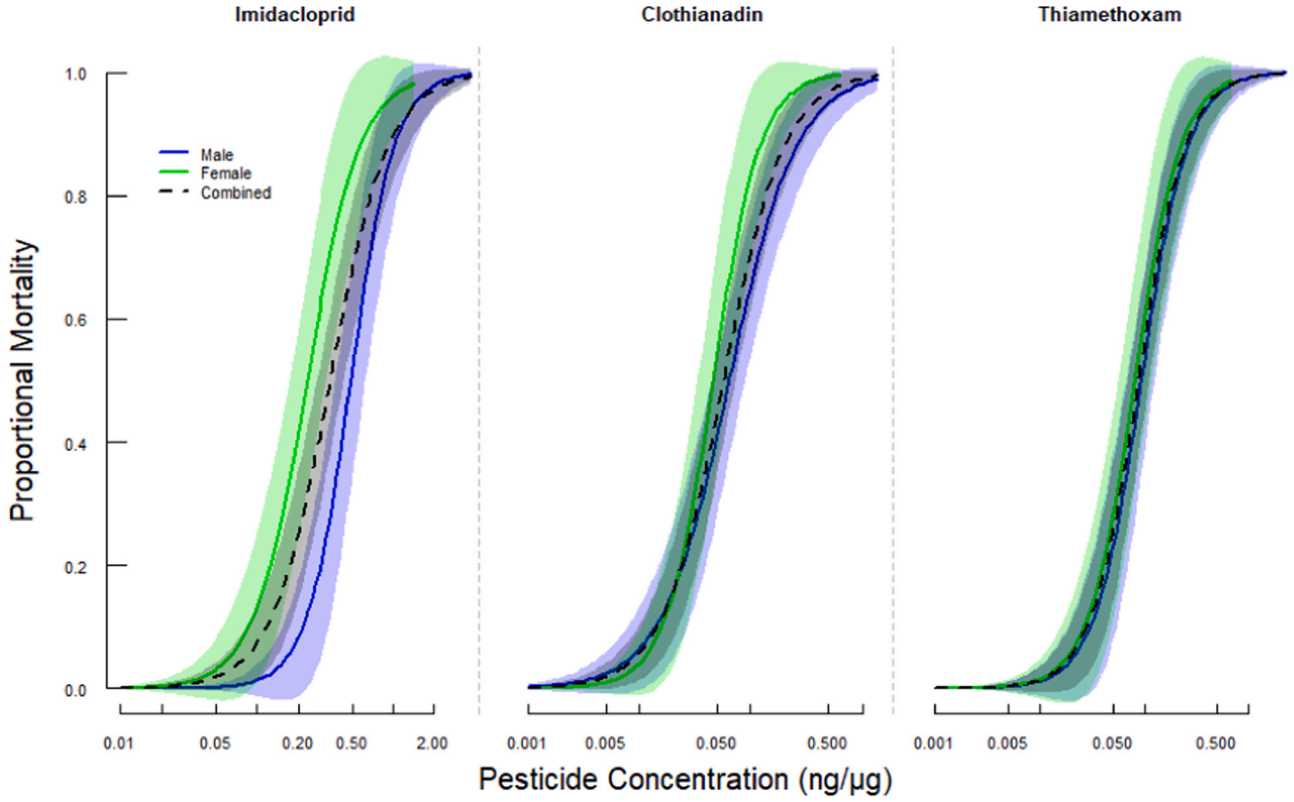

Fig. 1. Male versus female Osmia lignaria mass normalized dose-response models for 96 h contact exposure to three different neonicotinoids.

contact LC_{50} value for *O. lignaria* by extrapolating mist produced by a potter sprayer tower (by volume) and mass of individual bees (mg/L). The misting mechanism from potter sprayer towers resulted in *O. lignaria* contact LC_{50} concentrations thirty-one times lower than *A. mellifera* LC_{50} values. However, in the current study imidacloprid LD_{50} values for *O. lignaria* were approximately half previously published *A. mellifera* LD_{50} values (Table 2). Although we cannot directly compare results from Scott-Dupree et al. (2009) to those from the current study due to differences in application methods (misting versus direct application), both studies suggest that *O. lignaria* are more sensitive to imidacloprid than *A. mellifera* (Scott-Dupree et al., 2009).

Recently, to better characterize differences in sensitivity between *A. mellifera* and native bees, researchers have calculated sensitivity ratios (*A. mellifera* LD_{50} divided by LD_{50} value of alternate bee species; Arena and Sgolastra, 2014). Sensitivity ratios reflect the suitability of *A. mellifera* as a surrogate for other native bees when determining agrochemical application regulations. For example, when sensitivity ratios are >1, the alternate species is more sensitive than *A. mellifera* to the agrochemical; conversely, sensitivity ratios <1 indicate that the alternate species is less sensitive. While sensitivity ratios vary dramatically among pesticides and bee species, it appears that most (>87%) are less than 10 (Arena and Sgolastra, 2014; Uhl et al., 2018).

Arena and Sgolastra (2014) reported that neonicotinoids produced the highest bee sensitivity ratios among all pesticide classes included in their analysis (carbamates, neonicotinoids, organochlorines, organophosphates, and pyrethroids). The two most commonly used neonicotinoids had sensitivity ratios of 0.96 (imidacloprid) and 1.14 (thiamethoxam) suggesting that *A. mellifera* may indeed be a reliable surrogate for solitary bees exposed to neonicotinoids (Arena and Sgolastra, 2014; Wood and Goulson, 2017). In contrast, the mean neonicotinoid sensitivity ratio in this study was 4.31. Further, the highest sensitivity ratio for all agrochemicals examined in this study was for clothianidin (7.96; Table 2). Sensitivity ratios for thiamethoxam and imidacloprid were 2.6 and 2.4, respectively, which are greater than any of the 16 sensitivity ratios referenced in Arena and Sgolastra (2014). Thus, *O. lignaria* appear to be more sensitive to neonicotinoids than many other solitary bees, but a risk assessment factor of 10 (as proposed by the EFSA) from *A. mellifera* LD_{50} values would be adequate to protect *O. lignaria* exposed to neonicotinoids.

Additionally, to more appropriately illustrate risk to *A. mellifera* from environmental residues, Sanchez-Bayo and Goka (2014) created a hazard quotient using the frequency of pesticide detections in the environment, *A. mellifera* contact LD_{50} values, and concentrations observed in different environmental matrices. Thus, scientists and regulators are able to characterize risk and relate agrochemical concentrations to *A. mellifera* toxicity values. Neonicotinoid (thiamethoxam, imidacloprid, and clothianidin) residues in pollen (mean concentration of 12–35 ng/g from USA, France, Poland, and Spain) were determined to pose a significant threat to *A. mellifera* and potentially have detrimental impacts on *A. mellifera* populations. Taking into consideration the sensitivity ratios determined in this study (described above), *O. lignaria* are therefore likely impacted to a greater extent than *A. mellifera* and may be at significant risk from neonicotinoid residues in wildflower pollen across the landscape.

Table 2
Contact LD_{50} values for *A. mellifera* and *O. lignaria* (females) and corresponding sensitivity ratio (R).

Chemical	*A. mellifera* (ng/bee)[a]	*O. lignaria* (ng/bee)	R
Bifenthrin	15	75.0	0.20
Permethrin	63	289.7	0.22
Abamectin	30	32.9	0.91
Ivermectin	–	24.7	–
Clothianidin	39	4.9	7.96
Imidacloprid	61	25.5	2.39
Thiamethoxam	25	9.65	2.59

Note: no contact LD_{50} data available for *A. mellifera and ivermectin (–)*
[a] = LD_{50} concentrations from Pesticide Manual (2009) (Tomlin, 2009) and ECOTOX databases (Agency, U.S.E.P., 2001)

3.2. Macrocyclic lactones

Two macrocyclic lactones included in this study, abamectin and ivermectin, yielded similar LD_{50} estimates of 32.9 ± 10.9 ng/bee and 24.7 ± 5.9 ng/bee for females, along with 5.5 ± 1.1 ng/bee and 9.1 ± 1.4 ng/bee for males, respectively (Fig. 2). To our knowledge, this is the first study to quantify contact LD_{50} concentrations for ivermectin in a bee species. The abamectin contact LD_{50} value for *A. mellifera* was 30 ng/bee, while *A. mellifera* sensitivity to ivermectin was not found in the open literature. Therefore, the sensitivity ratio for abamectin was 0.91, but could not be calculated for ivermectin (Table 2).

Macrocyclic lactones have not heretofore been considered significant threats to pollinators since they are not sprayed on crops and were thought to primarily occur in soil/manure mixtures generated by livestock (Finch et al., 2020). This has limited the scope of non-target organism toxicity testing to dung beetles such as *Aphodiine* and invertebrates in the order *Diptera* (Finch et al., 2020; Lumaret et al., 2012). Nonetheless, the recent discovery of aerially transported particulate matter containing macrocyclic lactones indicates that a wider array of non-target organisms may be at risk (Peterson et al., 2020). An estimated 572,764.5 kg of ivermectin is aerially dispersed via particulate matter into the environment from beef cattle feed yards on the High Plains daily (Peterson et al., 2020). Macrocyclic lactones (abamectin and moxidectin) have also been detected and quantified on wildflowers located near feed yard boundaries (Peterson et al., 2017). Applying the hazard quotient methodology (described above) from Sanchez-Bayo and Goka (2014) for concentrations of abamectin on wildflowers (26.1 ng/g; Peterson et al., 2017) near beef cattle feed yards and LD_{50} values determined in this study, macrocyclic lactones likely pose a significant risk to *O. lignaria*. While this is an imprecise metric and oversimplified, it highlights the relative toxicity macrocyclic lactones may pose to pollinators. Data from this study therefore suggests that pollinators near beef cattle feed yards may be exposed to lethal concentrations of endectocides via particulate matter and/or via contact with contaminated flowers.

3.3. Pyrethroids

Of the two pyrethroids included in this study, bifenthrin (female LD_{50} = 75.0 ± 12.0 ng/bee, male LD_{50} = 32.6 ± 5.8 ng/bee; Fig. 3) was more toxic than permethrin (female LD_{50} = 289.7 ± 39.4 ng/bee, male LD_{50} = 201.9 ± 32.6 ng/bee; Table 1). Similar to *A. mellifera, O. lignaria* (females) were five times more sensitive to bifenthrin than permethrin (4-fold difference in *A. mellifera*; Table 2). Additionally, *O. lignaria* (males and females) were three times less sensitive to permethrin and bifenthrin than *A. mellifera* (Table 2). Other studies have also characterized *Osmia* species as less sensitive than *A. mellifera* (Arena and Sgolastra, 2014; Biddinger et al., 2013). For example, mean sensitivity ratios calculated for horned-face bees (*Osmia cornifrons*) ranged from 0.1 to 0.5 for pyrethroids (Arena and Sgolastra, 2014; Biddinger et al., 2013). Whereas the mean sensitivity ratio for pyrethroids in this study was 0.21. Despite reduced sensitivity of *Osmia* to pyrethroids relative to *A. mellifera*, they are nonetheless considered highly toxic to bees (Pilling and Jepson, 1993; USEPA, 2014).

Pyrethroid application to row crops and use in mosquito control programs are well characterized (Feedlot, 2011; Schleier and Peterson, 2010; Yadouleton et al., 2011), however recent data suggest that pyrethroids also enter the environment from feed yards in a manner similar to macrocyclic lactones (Peterson et al., 2020). Pyrethroids were the most frequently detected analytes and were quantified at the highest concentrations (192.1 ± 117.3 ng/g) in particulate matter samples collected on the Southern High Plains (Peterson et al., 2020). Assuming

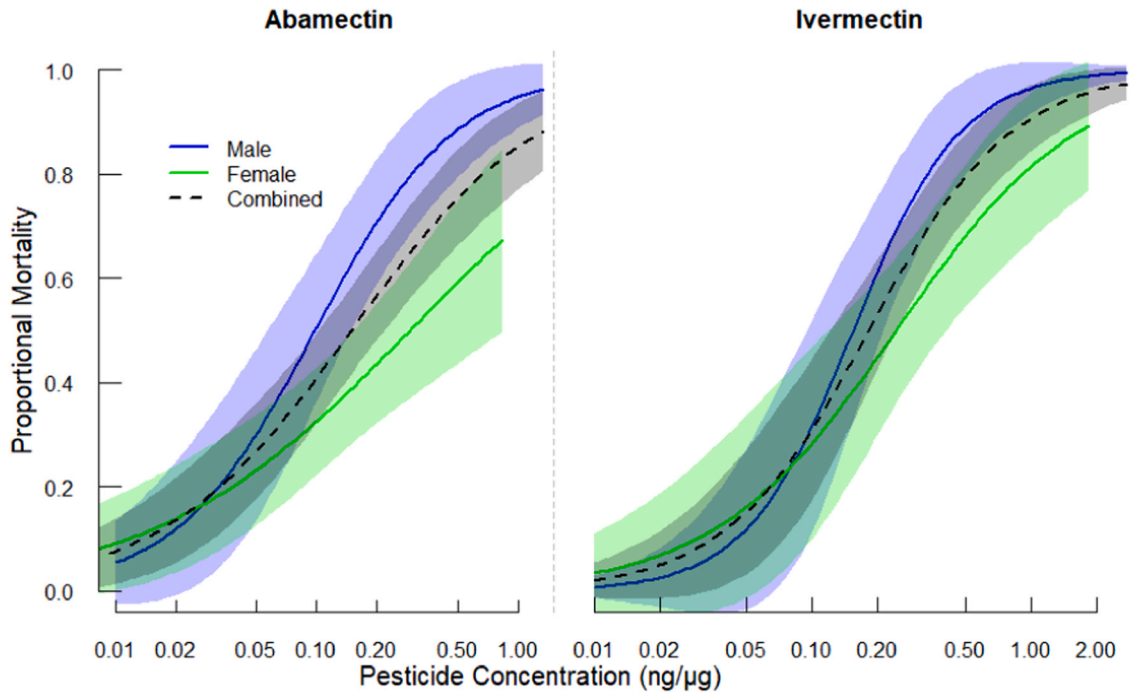

Fig. 2. Male versus female Osmia lignaria mass normalized dose-response models for 96 h contact exposure to two macrocyclic lactones.

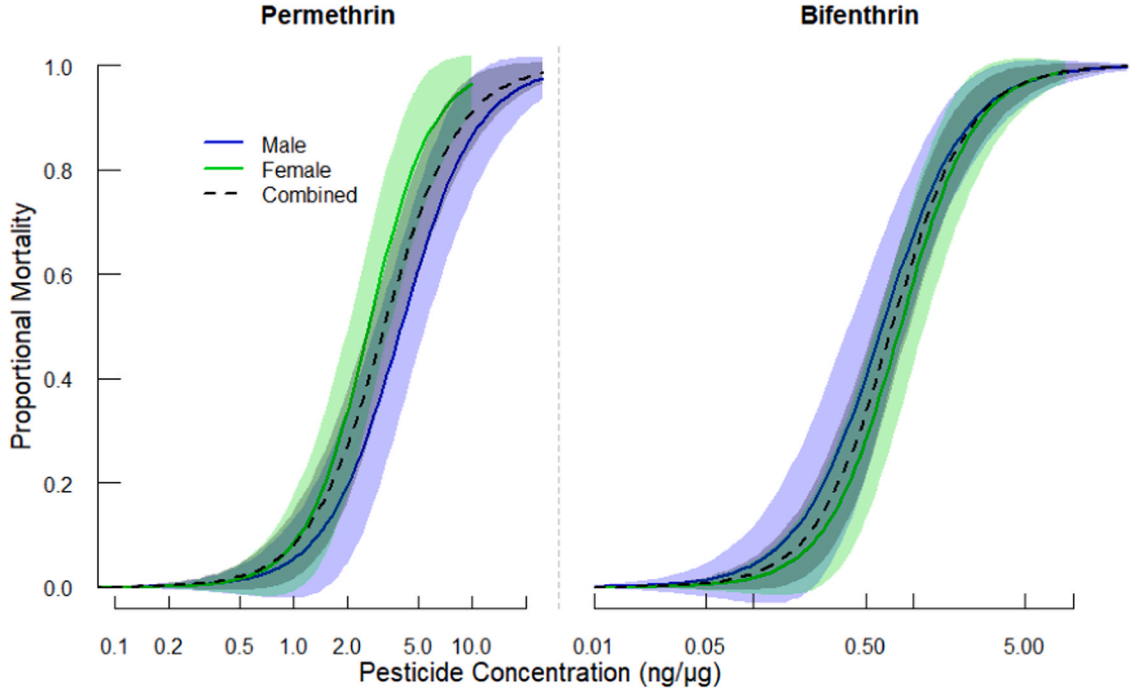

Fig. 3. Male versus female Osmia lignaria mass normalized dose-response models for 96 h contact exposure to two pyrethroids.

pyrethroid concentrations are bioavailable in particulate matter; *O. lignaria* may be exposed to toxic levels and be at significant risk from aerial deposition of particulate matter from feed yards. Whereas pyrethroid concentrations quantified in pollen of wildflowers would pose relatively little risk to *O. lignaria* since they are less sensitive than *A. mellifera* and pyrethroid residues are rather low (<13 ng/g; Sanchez-Bayo and Goka, 2014). Therefore, pollinators inhabiting areas near feed yards may be exposed to unexpectedly toxic concentrations of pyrethroids from aerial deposition, but likely not pollen residues from row crop agriculture.

3.4. Differences in pesticide toxicity between sexes

OECD dictates the use of female worker bees (*A. mellifera* and *B. terrestris*) for bee toxicity testing, however, there are no such guidelines for solitary bees (OECD, 2017, 1998, 2000). Both *A. mellifera* and *B. terrestris* are colony nesting species with a single queen surrounded by female workers and very few male bee drones (<10% of total colony individuals; Page and Metcalf, 1984). Moreover, pesticide regulations are based on effects at the colony level (not individual bees); males are often neglected because they represent a relatively small percentage of

the overall colony population and are sacrificed each fall to ensure the colony (female workers and queen) has enough honey stores to survive the winter (Regulation, E.U., 2009). However, colony-level metrics are not appropriate for most bees, since a majority (>85%) are solitary nesting species. Solitary nesting species typically exhibit a 2:1 male: female sex ratio (Monzón et al., 2004; Torchio and Tepedino, 1980), thus males are predominant among non-colony nesting bees. Therefore, when assessing potential agrochemical impacts on solitary nesting bee populations, sex-specific toxicity data should be included since males account for roughly 66% of all solitary bees (Strobl et al., 2019).

Male and female bee (*O. lignaria*) cocoons are nearly identical, and reliable screening methods for identifying and hatching only females are not well developed or easily incorporated into toxicity testing protocols. Consequently, large numbers of male bees are discarded when OECD guidelines for toxicity tests are followed. Excluding males from toxicity tests may also result in unrealistic LD_{50} estimates because male insects are oftentimes more susceptible to agrochemicals than females (Medrzycki et al., 2013). For example, male alfalfa leaf cutting bees (*Megachile rotundata*) were approximately ten times more sensitive to Captan (phthalimide fungicide) than females (reduced survival rate for male = 2.445 g/L, females = 24.45 g/L; Huntzinger et al., 2008).

To examine differences in agrochemical sensitivity between sexes, both male and female *O. lignaria* were included in toxicity tests conducted in the current study (females 48% and males 52%). Significant differences in male versus female LD_{50} estimates were observed for three of seven (43%) agrochemicals. One agrochemical from each chemical class (permethrin, abamectin, and imidacloprid) yielded significantly different LD_{50} values for male and female *O. lignaria* (all p-values <0.003). Alternatively, thiamethoxam, clothianidin, bifenthrin, and ivermectin LD_{50} estimates were not significantly different between male and female bees (all p-values > 0.12).

Huntzinger et al. (2008) determined that males were equally or more sensitive than females following exposure to fungicides. However, in this study, we observed males to be more sensitive than females to some chemicals but less sensitive to others. Differences between male and female sensitivity may be attributed to sex-specific differences in metabolic function and detoxifying enzymes such as esterase and glutathione s-transferase which may deteriorate more quickly in male bees than females (Frohlich, 1990; Huntzinger et al., 2008) decreasing xenobiotic transformation and/or excretion. *Megachilinae* females also have a higher metabolic capacity which may promote more rapid xenobiotic metabolism and greater ability to tolerate insecticide exposures (Guirguis and Brindley, 1975; Huntzinger et al., 2008). However, males were not uniformly more sensitive than females in this study signifying that other behavioral, metabolic, or enzymatic factors may influence insecticide sensitivity differences between male and female *O. lignaria*. Although sample sizes were relatively small, post-hoc analysis determined that statistical power for dose-response regression analysis was 0.75 (≥0.8 is ideal when fitting logistic regression models; Demidenko, 2007; Hallahan and Rosenthal, 1996). Additionally, two-parameter log-logistic regressions were significant (all p-values < 0.003); therefore we believe that our study design and sample sizes provided acceptable power to detect significant differences between male and female *O. lignaria* sensitivities to agrochemicals.

Females (vertebrates and invertebrates) are often utilized in toxicology studies due to their clear and direct influence on reproductive output (Lipnick et al., 1995; Medrzycki et al., 2013). While female *O. lignaria* only lay a finite number of eggs (Brittain and Potts, 2011), males are also vitally important contributors to solitary nesting bee reproduction, and may contribute more to pollination serves than females (Brittain and Potts, 2011; Monzón et al., 2004). Male *O. lignaria* travel greater distances than females to mate resulting in long-distance pollen flow (Brittain and Potts, 2011; Monzón et al., 2004). Greater distances between flower visits by male *O. lignaria* facilitate wildflower fitness due to out-crossing, and overall health of the local environment (Brittain and Potts, 2011; Monzón et al., 2004). However, increased foraging distances by male *O. lignaria* may result in greater exposure to agrochemicals from multiple sources compared to females, even though both sexes frequent relatively equal number of wildflowers (Sampson et al., 2004).

Sex-linked differences in agrochemical sensitivity could manifest as skewed sex ratios leading to decreased reproductive output or genetic diversity bottlenecks (Luikart et al., 1998). Loss of genetic diversity can have detrimental population-level effects due to reduced heterozygosity (Soro et al., 2017) and subsequent reduced ability to adapt and evolve in response to stressors in the environment including disease, natural disasters, or agrochemicals (Furlan et al., 2012). Thus exclusion of males from solitary nesting bee toxicity studies, and promulgation of regulatory policies based solely on female-derived toxicity data may add significant uncertainty to risk assessments involving *O. lignaria* and other solitary nesting bees.

3.5. Existing bee toxicity test protocols

Established standardized honey bee protocols (e.g., OECD Test No. 214) are included in many studies (Hoang et al., 2011; Ignasiak and Maxwell, 2017; Peterson et al., 2019) to help predict the effects of pesticides to other non-target invertebrates (e.g., butterflies and moth). However, there remain questions regarding the suitability of *A. mellifera* model as a reliable surrogate for other important pollinators including the many species of mason bees. Uhl and colleagues (2016) detail concerns regarding differences in bee size and sex-dependent responses to dimethoate. Specifically, larger bees were less sensitive than smaller bees (e.g., *A. mellifera* vs. male *O. bicornis*). Other potential differences identified in solitary versus colony nesting bees that may influence sensitivity to pesticides included maturation of the cuticle and antioxidant enzyme levels relative to age at the time of testing (Uhl et al., 2016).

The current study was based on existing honey bee protocols and adapted to account for differences in physiology – primarily differences in individual bee mass due to sexual dimorphism. These important life-history and physiological traits differ among pollinator taxa and should be given due consideration when utilizing existing pollinator toxicity testing protocols and in development and adoption of non-*Apis* protocols. Standardization of non-*Apis* specific pollinator protocols (e.g. mason bee protocols) will enable relevant comparison of toxicity data among species while maintaining robust study designs that account for species-specific variation. This, in turn, will allow for more comprehensive risk assessments, more effective pesticide regulation, and in turn, pollinator conservation.

4. Conclusion

Macrocyclic lactones were highly toxic to *O. lignaria* in this study with LD_{50} values similar (6–33 ng/bee) to neonicotinoids (3–26 ng/bee), suggesting that agrochemical concentrations detected in particulate matter from beef cattle feed yards and on surrounding wildflowers may result in bee mortality. *O. lignaria* were more sensitive than *A. mellifera* to neonicotinoids; clothianidin was the most toxic agrochemical included in this study (LD_{50} value of 4.9 ± 0.8 ng/bee for female *O. lignaria* and 2.9 ± 0.4 ng/bee for males). Further, male *O. lignaria* sensitivity was significantly different from females for three (permethrin, abamectin, and imidacloprid) of the seven agrochemicals, indicating that exclusion of males from bee toxicity tests may not be appropriate. Failure to consider male bee sensitivity in toxicity tests and subsequent regulatory decisions could result in alteration of sex ratios, reduced reproduction, and loss of ecological services (i.e. pollination).

Sensitivity ratios (0.20–7.96) determined in this study were consistently below the risk assessment factor of 10 as proposed by the EFSA. Neonicotinoids were the only chemical class that yielded sensitivity ratios >1, while *O. lignaria* were less sensitive than *A. mellifera* to macrocyclic lactones and pyrethroids with sensitivity ratios <1. In

comparison to other *Megachilidae*, it appears that *O. lignaria* may be more sensitive to commonly encountered agrochemicals. Nonetheless, these results support the EFSA risk assessment factor of 10 which would provide adequate protection for *O. lignaria* when extrapolating regulatory guidelines from *A. mellifera* toxicity endpoints from commonly encountered agrochemicals on the High Plains.

CRediT authorship contribution statement

Eric M. Peterson: Conceptualization, Methodology, Investigation, Writing - original draft, Validation. **Frank B. Green:** Investigation, Formal analysis, Validation. **Philip N. Smith:** Supervision, Writing - review & editing, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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