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1 **Comparative toxicity of pesticides and environmental contaminants in bees: are honey bees a**  
2 **useful proxy for wild bee species?**

3

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15 **Running title:** Do managed honey bees predict wild bee toxicology?

16

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19

20 **Abstract**

21 Threats to wild and managed insect pollinators in Europe are cause for both ecological and socio-  
22 economic concern. Multiple anthropogenic pressures may be exacerbating pollinator declines. One  
23 key pressure is exposure to chemicals including pesticides and other contaminants. Historically the  
24 honey bee (*Apis mellifera* spp.) has been used as an ‘indicator’ species for ‘standard’ ecotoxicological  
25 testing but it has been suggested that it is not always a good proxy for other types of eusocial and  
26 solitary bees because of species differences in autecology and sensitivity to various stressors. We  
27 developed a common toxicity test system to conduct acute and chronic exposures of up to 240 hrs of  
28 similar doses of seven chemicals, targeting different metabolic pathways, on three bee species (*Apis*  
29 *mellifera* spp., *Bombus terrestris* and *Osmia bicornis*). We compared the relative sensitivity between  
30 species in terms of potency between the chemicals and the influence of exposure time on toxicity.  
31 While there were significant interspecific differences that varied through time, overall the magnitude  
32 of these differences (in terms of treatment effect ratios) was generally comparable (<2 fold) although  
33 there were some large divergences from this pattern. Our results suggest that *A. mellifera* spp. could  
34 be used as a proxy for other bee species provided a reasonable assessment factor is used to cover  
35 interspecific variation. Perhaps more importantly our results show significant and large time  
36 dependency of toxicity across all three tested species that greatly exceeds species differences (>25  
37 fold within test). These are rarely considered in standard regulatory testing but may have severe  
38 environmental consequences, especially when coupled with the likelihood of differential species  
39 exposures in the wild. These insights indicate that further work is required to understand how  
40 differences in toxicokinetics vary between species and mixtures of chemicals.

41  
42 **Keywords:** *Apis*, *Bombus*, *Osmia*, neonicotinoid, trace metal, DEBtox

43

44

## 45 **1. Introduction**

46 Concerns over reductions in global pollination services encompass both losses of managed  
47 populations of insect pollinators, chiefly the Western honey bee (*Apis mellifera* spp.)(Laurent et al.,  
48 2015; Seitz et al., 2015), and declines in wild insect pollinators such as natural bee populations  
49 (Vanbergen, 2013). Both eusocial and solitary wild bees have shown dramatic declines in range and  
50 diversity across Europe and North America over recent decades(Laurent et al., 2015; Seitz et al.,  
51 2015; Vanbergen, 2013; Williams and Osborne, 2009). These declines have serious economic as well  
52 as conservation implications. Pollination, primarily by both managed and wild insects, provides direct  
53 commercial benefits to crop production (the value of insect pollination for world agriculture has been  
54 estimated >€150 billion p.a. (Gallai et al., 2009; Lautenbach et al., 2012) and makes a key  
55 contribution to the dynamics and persistence of native plant species and communities (Fontaine et al.,  
56 2005).

57 Global threats to insect pollinators could arise from multiple environmental pressures which,  
58 singly and/or in combination may alter survival, behaviour and reproduction (Vanbergen, 2013) and  
59 in turn jeopardize the delivery of pollination services to crops and wild plants. These environmental  
60 pressures include land-use intensification, pesticides, urbanization, invasive alien species, the spread  
61 of diseases and parasites and climate change.

62 One key pressure is exposure to chemicals (Goulson et al., 2015; Scott-Dupree et al., 2009;  
63 Whitehorn et al., 2012) through contact and consumption of contaminated nectar, pollen, water and  
64 guttation fluids, or via contact during foraging or nesting (e.g. in the air with contaminated dust  
65 particles, on crops and in soil with contaminated surfaces). This includes pesticide classes routinely  
66 applied to flowering crops and pesticides and environmental contaminants that may co-occur as a  
67 result of agrochemical use and diffuse or point source pollution (Botías et al., 2015; Long and Krupke,  
68 2016; Samson-Robert et al., 2014). For example, over the last decade a median of >16 active  
69 ingredients (a.i) have been applied to an ‘representative’ UK arable field crop (proportion area treated  
70 2014 =fungicides 40%, herbicides 31%, growth regulators 11%, seed treatments (often combinations

71 of a.i's) 9%, insecticides 8%, molluscicides 2%;(unpublished analysis of (FERA, n.d.)). Analysis of  
72 honey bees and hive products in North America and Europe have shown that most managed colonies  
73 contain a suite of chemical contaminants, including insecticides, acaricides, herbicides and fungicides  
74 (Bogdanov, 2006; Johnson et al., 2013; Mullin et al., 2010). It is highly likely that other pollinator  
75 species, foraging in similar habitats to honey bees, will be exposed to the same range of chemicals  
76 (Goulson et al., 2015).

77 Although there are well established protocols for the testing of the acute toxicity of chemicals for  
78 pollinating insects this is almost exclusively focused on honey bees (OECD 1998; Medrzycki et al.,  
79 2013). This species is considered as highly sensitive to insecticides and fungicides and, although  
80 sensitivity it is generally less to herbicides, is considered a good environmental indicator of pesticide  
81 pollution. This is partly corroborated by the lower number of genes encoding xenobiotic detoxifying  
82 enzymes in the *A. mellifera* spp. genome compared with other insect species such as flies and  
83 mosquitoes (Claudianos et al., 2006). While some review studies have compared the relative  
84 sensitivity of *A. mellifera* spp. to other bees (Arena and Sgolastra, 2014; Tasei et al., 2000) and insect  
85 species (Hardstone and Scott, 2010), quantitative comparisons of differences in sensitivity, especially  
86 using the same experimental approaches are lacking (but see (Scott-Dupree et al., 2009)). In addition,  
87 most of the 'standard' tests conducted to date tend to be of short duration (48-96 hours, e.g. (OECD,  
88 1998)) with 'pulse' dosing frequently limited to topical exposures for testing contact toxicity. Policy  
89 decisions based on the assumption that honey bees are good proxies for other pollinating insects,  
90 including other bee species, have been challenged (Dicks, 2013) and there is a general consensus  
91 about a need to fully evaluate the importance of differing routes of exposure for different chemicals  
92 on non-*Apis* bee species (Carreck and Ratnieks, 2014; EFSA, 2012) over more realistic timeframes  
93 if they are to better inform environmental risk assessment and ecological understanding (Goulson et  
94 al., 2015; Rondeau et al., 2014).

95 The key question is how widely wild bees differ from honey bees in their responses to a range of  
96 chemicals that affect different metabolic pathways? In this study we developed both acute (short-

97 term; up to 96 hrs) and chronic (extended up to 240 hrs) continuous feeding exposure tests to compare  
98 and predict the long term impacts of seven different chemicals on two wild bee species (*Bombus*  
99 *terrestris audax* and *Osmia bicornis*) and managed honey bees (*A. mellifera* spp.). We focused on  
100 oral exposure since recent evidence suggests this is often the most relevant and the most conservative  
101 approach for bees (EFSA, 2012). *A priori* our null hypothesis was that there would be no interspecific  
102 difference in sensitivity over time.

103

## 104 **2. Material and Methods**

### 105 *2.1. Study species*

106 Three bee species were used to assess the potential hazards of the selected single chemicals. The  
107 honey bee *Apis mellifera* spp. is a eusocial species that is the most frequent managed pollinator in the  
108 world. Managed colonies are typically kept in hives containing thousands of individuals (brood and  
109 adults comprising thousands of female workers, hundreds of drones and a single queen) with well-  
110 defined castes, each with specific functions within the colony. Healthy, queen-right colonies persist  
111 for several years. For this study, honey bees were obtained as nucleus hives in spring 2014, from a  
112 commercial breeder in north Oxfordshire UK, each with a queen mated naturally the previous year.  
113 Eight hives were established and were regularly inspected and maintained to ensure that they were  
114 queen-right and maintained healthy brood and adult bees. Workers foraged freely but did not visit  
115 oilseed rape (which was not flowering) during the testing period (mid to late summer during peak  
116 colony strength). No chemical disease treatments were used for 4 months prior to test trials.

117 The bumblebee *Bombus terrestris audax* is a more primitive eusocial species with no clear caste  
118 system. It is a common wild pollinator which is also commercially reared for pollination in closed or  
119 semi-closed cultivation situations. In the temperate zone it is generally an annual species that lives in  
120 colonies that contain *c.* 100-150 female workers during the summer. Colonies of UK native *B. t. audax*  
121 were obtained as commercially reared colonies with *c.* 30 workers (NV Biobest, Belgium). On receipt,  
122 colonies were fed a pure 50% w/v sucrose food source, supplemented with fresh, disease free pollen.

123 The solitary bee *Osmia bicornis* is a non-eusocial wild pollinator species that nests in cavities. It is  
124 also produced at small scales for commercial pollination (Gruber et al., 2011). The species produces  
125 single nests containing *c.* 4-8 eggs that can only be harvested for testing over the spring months. Pupae  
126 used for hatching the adult bees to be used for this study were obtained from a managed field population  
127 collected at the end of the previous year i.e. <1 year old. The overwintered *O. bicornis* pupae were  
128 obtained from German commercial stocks (Dr Schubert Plant Breeding, Germany).

129

## 130 2.2. Chemical selection

131 Chemicals were selected to reflect both current concerns about the effects of agrochemicals on  
132 pollinators and the widespread presence of other trace pollutants, such as metals, in the environment.  
133 This was balanced with mechanistic considerations to ensure that different metabolisms (e.g. by  
134 cytochrome P450s, esterases, p-glycoproteins, metalloproteins) and modes of action (e.g. neurotoxins,  
135 metabolic toxicant, reactive oxygen species production) were represented. This resulted in a list that  
136 included representatives from different insecticide, fungicide and herbicide classes, as well as a  
137 metalloloid and a toxic non-essential metal (Table 1, dimethoate, an organophosphate insecticide that  
138 is recommended as a reference toxicant for toxicity tests with honey bees, was also included in the  
139 list and used as a validation of the sensitivity of the individuals and colonies tested (OECD, 1998)).  
140 Pesticides were obtained as analytical grade pesticide standards (PESTANAL®) while cadmium and  
141 arsenic were analytical grade chemicals (all were supplied by Sigma-Aldrich®).

142 Table 1. Selected chemicals for study for bee toxicity testing to derive effects concentrations for priority chemicals

143

<b>Chemical (class in brackets)</b>	<b>Current usage</b>	<b>Exposure scenario</b>	<b>Mechanism of action</b>	<b>Metabolism</b>	<b>Other information</b>
clothianidin (Neonicotinoid insecticide)	Systemic seed treatment; oilseed rape/beet. Spray insecticide	Nectar, pollen, water	Binds to nicotinic acetylcholine receptors causing overstimulation	Cytochrome P450, such as CYP6G1 in <i>D. Melanogaster</i> so P450 inhibition could give synergism	Clothianidin is first metabolite of Thiamethoxam.
tau-fluvalinate (Pyrethroid insecticide)	Spray used on oilseed rape. In hive varroacide	Contact in field and hive products	Binds to voltage-gated sodium channels to depolarise nerves	Metabolised by CYP9Q1, CYP9Q2, and CYP9Q3 in honey bees	Low affinity for bee sodium channels mean less toxic to bees than other pyrethroids
dimethoate (Organophosphate insecticide)	Spray insecticide and reference toxicant used for bee toxicity testing	Folia exposure and drinking water if used	Cholinesterase inhibition after metabolism to the oxon-metabolite	Metabolised by CYP3A in rat to oxon-metabolite	Typical organophosphate. Water solubility allows oral exposure.
propiconazole (Fungicide)	Used widely as spray fungicide on oilseed rape	Foliar exposure during feeding on oilseed rape	Demethylation of C-14 in ergosterol biosynthesis, leading to accumulation of C-14 methyl sterols	Extensively metabolised in rat. Wide range of metabolites identified	Interacts with respiratory chain, so could affect energy metabolism
2,4-dichlorophenoxyacetic acid, (Herbicide)	common systemic herbicide used in the control of broadleaf weeds	Foliar exposure during feeding on oilseed rape	Synthetic auxin causing uncontrolled plant tissue growth	Significant species differences in clearance in mammals	Potential effects on antioxidant systems
cadmium (Metal)	None but past industrial use	Soil contact	DNA damage, oxidative stress	Metallothionein	One of most toxic metals
arsenic (Metalloid)	None but past wider pesticide use (some current)	Soil contact (especially in arable areas)	DNA damage, Epigenetic effect on DNA methylation	Metallothionein and possibly phytochelatins	Known toxicity

144



### 145 2.3. Chemical exposure

146 The same approach was used to test all species. Each species was exposed to a series of concentrations  
147 of the test chemical in sucrose solution and allowed to feed *ad libitum* for a total exposure period of  
148 10 days (240 hours). The consumption of the dosed sucrose solution was measured by weight at 48,  
149 96 and 240 hour intervals. Mortality of bees was assessed 3 times daily for the first 96 hrs of exposure  
150 and thereafter daily until 10 days. The specific test design and bee densities were modified to reflect  
151 the different habits of each species (see below). Stock solution of the test chemicals were prepared  
152 either in water (dimethoate, clothianidin, cadmium chloride, sodium arsenate) or acetone (tau  
153 fluvalinate, 2,4-Dichlorophenoxyacetic acid, propiconazole) depending on solubility characteristics.  
154 For *A. mellifera* and *B. terrestris* the stock solutions were added to a 50% w/v solution of sucrose  
155 (molecular biology grade, Sigma Chemicals) while for *O. bicornis* a 20% w/v solution was used to  
156 more closely mimic nectar concentrations (Konrad et al., 2009). Negative controls were either sucrose  
157 alone or sucrose with 1% acetone as appropriate for each chemical.

158 For all species assays were performed using 500ml plastic cages with a ventilated lid. For *A.*  
159 *mellifera* and *B. terrestris* dosed sucrose solutions were supplied in disposable 50 ml Luer centric  
160 syringes (Latex and silicone oil free) with the tip cut off at the syringe body to provide an approximate  
161 3 mm diameter drinking hole. For *O. bicornis*, solutions were supplied in disposable 5ml Luer centric  
162 syringes with tips cut off. To encourage feeding for *O. bicornis* the feeders had a false, yellow silk  
163 ~~false~~ petal fixed over the syringe tip and glued in place. A ring of UV paint was applied around the  
164 tip (following (Ladurner et al., 2003)) as a UV colour cue.

165 For *A. mellifera* experiments, adult worker bees were collected from frames containing young  
166 brood from four hives selected at random. Each test replicate ( $n=4$ ) comprised a group of 10 bees  
167 from a single hive kept together. To aid handling, bees were anaesthetised by cooling in  $-20^{\circ}\text{C}$  freezer  
168 for 45s and then loaded into the cages using soft forceps within an hour of collection.

169 For *B. terrestris* experiments, workers were removed directly from a minimum of 4 colonies  
170 using long forceps. Bees were not anaesthetised since they could be easily transferred to cages using

171 this method under red light, at room temperature. Each test replicate ( $n=4$ ) comprised a group of 3  
172 bees from a single colony kept together. During the experiments, both *A. mellifera* and *B. terrestris*  
173 were maintained in a constant temperature room at  $25 \pm 2^\circ\text{C}$ ,  $\sim 60\%$  RH, in the dark.

174 Prior to the experiments *O. bicornis* pupae were stored in the dark at  $4 \pm 1^\circ\text{C}$ ,  $65 \pm 10\%$  RH to  
175 restrict emergence. For each experiment a cohort of pupae were selected, by weight, to give a  
176 balanced number of male and female bees (females are generally larger than males). Pupae were  
177 warmed at  $28^\circ\text{C}$  to encourage emergence and any bees emerging within 72 hrs ( $>85\%$  of individuals)  
178 were allocated at random to treatment cages (within sex). For all experiments, 5 males and 5 females  
179 were maintained individually in replicate cages. Bees were kept individually in separate cages and  
180 housed in a controlled temperature glass house at  $22 \pm 2^\circ\text{C}$ ,  $\sim 60\%$  RH, under natural lighting  
181 conditions and photoperiod. In contrast to *A. mellifera* and *B. terrestris* these conditions were found  
182 in pre-trials to lead to more natural behaviour (i.e. increased feeding, natural diurnal patterns) than in  
183 the constant temperature room under artificial light (Heard *et al.* unpublished data). Across the tests  
184 control mortality rates for both *A. mellifera* and *B. terrestris* generally remained at low levels (c. 10%)  
185 even after 240 hrs of exposure (maximum control mortality in a single test at 240 h was 23% for *A.*  
186 *mellifera* and 33% for *B. terrestris*). *O. bicornis* demonstrated higher background mortality  
187 (combined male and female control survival across all experiments averaged 65% (range 40-80%) at  
188 48 h and 75% (range 60-90%) at 240 hr) which suggests that caution should perhaps be exercised  
189 when interpreting the data.

190

#### 191 2.4. Statistical Analyses

192 We used probit analysis of mortality data to predict species' sensitivity and the magnitude of chemical  
193 toxicity, expressed as  $\text{LC}_{50}$  values i.e. the concentration of chemical required to kill 50% of test bees  
194 at 48 h, 96 h and 240 h exposure times. For each chemical the differences between species at each  
195 time period was tested using z-tests. We also used the modelled  $\text{LC}_{50}$  values at each time period to  
196 calculate the sensitivity ratio,  $R$  between different endpoints for *A. mellifera* and each other species

197 where  $R = LC_{50\text{ Apis}} / LC_{50\text{ Bombus or Osmia}}$  (Arena and Sgolastra, 2014). A Dynamic Energy Budget model  
198 approach (DEBtox; (Kooijman, 1981; Kooijman and Bedaux. J. J. M., 1996; OECD, 2006) was used  
199 to predict the longer time course of toxic effects beyond the period of testing. These were 480 h, a  
200 time twice the length of the test; 720 h, a time approximately equivalent to the lifetime of a summer  
201 worker honey or bumblebee; and 2160 h, which is a duration approximately equivalent to the over  
202 wintering life-time of a worker honey bee. As before we expressed the results as ratios of the  $LC_{50}$   
203 calculated at each time point. The DEBtox approach uses a scaled one-compartment model to describe  
204 uptake and elimination rates and a hazard model to describe survival patterns. This leads to three  
205 time-independent parameters to describe the whole time course of the toxic effects: the No Effect  
206 Concentration (*NEC*), a time-independent toxicological threshold below which no effects are  
207 predicted to occur even after life-long exposure; the killing rate, which is a measure for the toxicity  
208 of the compound (once the *NEC* is exceeded) and the elimination rate which is a measure for the time  
209 course of the toxic effects. Although several parameters are generated, here we focus on the *NEC*, the  
210 *NEC* is the most relevant environmental DEBtox parameter and particularly important for comparing  
211 chemical potencies. Whether these effects are observed depends on the modelled toxicokinetics  
212 relative to the period of interest or observation. When chemicals are predicted to slowly build up an  
213 internal concentration, the full hazard may not be realised in a short-term laboratory test or even life-  
214 time exposure because it takes time to build up an internal concentration and therefore to exceed the  
215 internal *NEC*. Once the internal *NEC* is exceeded the survival probability of an individual starts to  
216 deviate from that of the controls. The killing rate in combination with the toxicokinetics determines  
217 how fast this process will go. With an infinitely high killing rate, once the *NEC* is exceeded death  
218 will be immediate for all individuals in the population, but with a low killing rate it takes more time  
219 before the survival probability drops to zero, given enough time the survival probability will go to  
220 zero. However, for some compounds the combination of slow kinetics with a low killing rate implies  
221 that the survival probability would not go to zero during the entire lifetime of the organism.

222

### 223 3. Results

#### 224 3.1. Toxicity of the reference toxicant

225 Observed sensitivity for the reference toxicant dimethoate showed very good accordance with  
226 previously published estimates for *A. mellifera*. For example the 48 h probit LC<sub>50</sub> of 2.42 mg L<sup>-1</sup>  
227 equated to an estimated LD<sub>50</sub> of  $3.39 \times 10^{-4}$  mg/bee based on our average ( $\pm$ se) measured consumption  
228 rate of  $69 \pm 4$   $\mu$ l/bee day<sup>-1</sup> ( $n=25$  replicate pots, 2500 bees) across the experiment. This approximates  
229 well to the upper limit of the range of the oral LD<sub>50</sub> values at 24 h of  $1.0 \times 10^{-4} - 3.5 \times 10^{-4}$  mg/bee  
230 (OECD, 1998). For *B. terrestris* our 48 h estimate of LC<sub>50</sub> was  $>2.188$  mg L<sup>-1</sup> which equates to an LD<sub>50</sub>  
231  $> 9.21 \times 10^{-4}$  mg /bee (mean  $\pm$ se consumption rate across the experiment =  $421 \pm 20$   $\mu$ l/bee day<sup>-1</sup> ;  $n=24$   
232 replicate pots, 72 bees) which is slightly below previously published estimates (24–72 h oral LD<sub>50</sub> =  
233  $17 - 47 \times 10^{-4}$  mg /bee; (Ladurner et al., 2005). Overall this indicates a comparative sensitivity of bees  
234 within normal expectations.

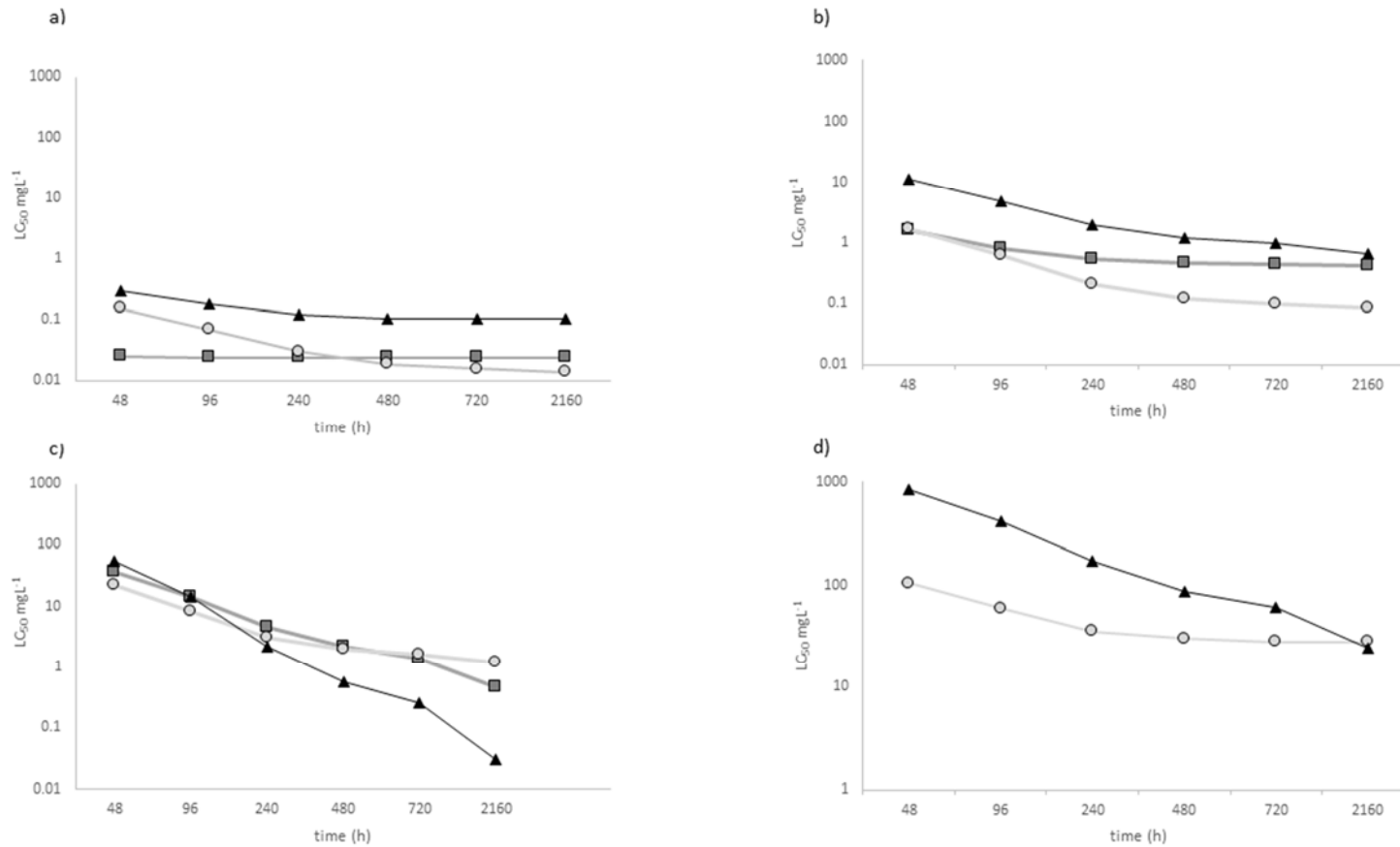
235

#### 236 3.2. Overall toxicity and relative species' effects

237 Across the range of tested chemicals, sensitivity spanned several orders of magnitude both within and  
238 between time points. For example, the LC<sub>50</sub> for the most toxic chemical, clothianidin was an order of  
239 magnitude lower than that of dimethoate indicating the higher potency of the neonicotinoid compared  
240 to the organophosphate (Figure 1). Overall the oral toxicity of the seven chemicals showed a broadly  
241 consistent ranking across the three bee species (Table 2; pairwise Pearson's  $r$  (*Apis: Bombus*) = 1,  $p < 0.001$ ,  
242 Pearson's  $r$  (*Apis: Osmia*) = 0.999,  $p < 0.001$ ). After 240 hours exposure the order from most to least toxic  
243 was: clothianidin > dimethoate > cadmium > arsenic > tau-fluvalinate > 2,4-D > propiconazole. There  
244 was some variation in the strength of concentration dependent effects between species, with  
245 significant differences in LC<sub>50</sub> at different time points (up to 240 h) for dimethoate, cadmium and  
246 tau-fluvalinate (Table 2). However the majority of interspecific effect sizes for LC<sub>50</sub>s from same time  
247 intervals were not significantly different. When expressed as the treatment effect ratio,  $R$ , 83% of  
248 tests across the three time points showed a less than 2-fold difference in predicted LC<sub>50</sub> ( $R_{\text{median}} = 1.05$ ;

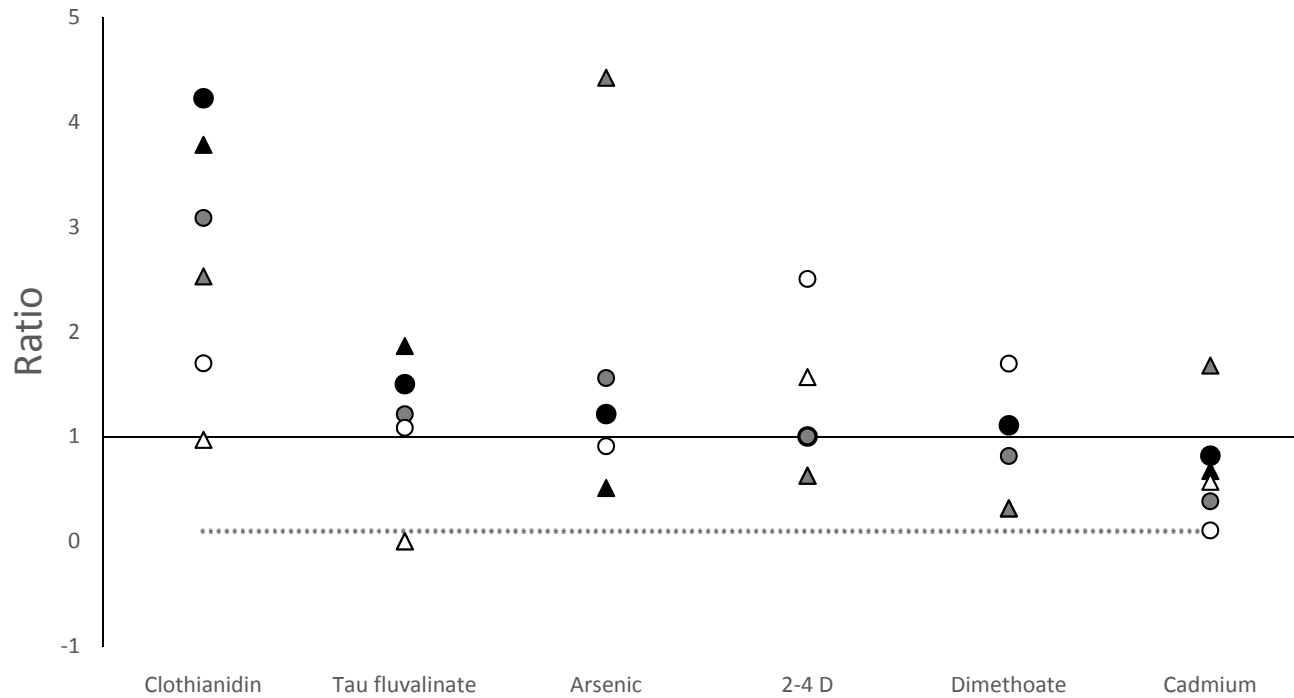
249 Figure 2), but did exceed 10 for both species exposed to cadmium for 240h (and for two clear outliers  
250 for tau-fluvalinate in *O. bicornis* at 96h  $R=66.75$  and 240h  $R=-22.98$ ). Overall *A. mellifera* showed a  
251 higher sensitivity to chemicals ( $R < 1$ ) in 40% of the comparisons across time (Figure 2).

252 Figure 1. DEB Tox predictions of LC<sub>50</sub> for four chemicals across all species extending past length of test to: 480 h (twice test length); 720 h (=lifetime  
 253 of a summer worker *A. mellifera* or bumblebee) and 2160 h (=life-time over wintering of a worker *A. mellifera*). a) clothianidin, b) dimethoate, c)  
 254 cadmium, d) tau-fluvalinate (note non- toxic to *A. mellifera*). ■ = *A. mellifera*, ● = *B. terrestris*, ▲ = *O. bicornis*. *O. bicornis* data on combined male and  
 255 female, except for tau-fluvalinate (=females).



257 Figure 2. Distribution of the sensitivity ratios of bee species ( $\circ = A. mellifera: B. terrestris$ ,  $\Delta = A. mellifera: O. bicornis$ ) for the  $LC_{50}$  for each chemical  
 258 at different time points (*black* = 48h, *grey* = 96h, *open* = 240h) ordered by median values for each chemical. A ratio of 1 (solid line) indicates that the  
 259 comparator species has the same sensitivity to pesticide as *A. mellifera*, values >1 indicate higher sensitivity of the comparator species. The dotted line  
 260 represents the 10-fold difference when the sensitivity ratio <1. Note two values have been excluded for *O. bicornis* tau-fluvalinate exposure: negative  
 261 value for 240h and large outlier for the *A. mellifera:O. bicornis* ratio at 96h (66.8). Note that where accurate estimates of the relevant dose were  
 262 calculated as 'greater than' exceedance values we have used that value +0.01 to allow plotting.

263



264

265 Table 2. Toxicity of six chemicals to all species (chemicals ordered by mean 240h LC<sub>50</sub> values, low to high) : Probit estimates of oral LC<sub>50</sub> values (mgL<sup>-1</sup>) with SE in parentheses. Values could not be calculated for some tau-fluvalinate, 2,4-D and propiconazole assays as mortality levels were insufficient  
 266 to establish any dose-response relationship. †negative value calculated for *O. bicornis* at this time point was similar using logistic binary regression,  
 267 clearly as this value spans 0 i.e. a very low dose, retained here for illustration and z test. \**P*≤0.05, \*\**P*≤0.01, \*\*\* *P*≤0.001  
 268

Chemical	Time (h)	LC <sub>50</sub> mg L <sup>-1</sup> (S.E.)			z score		
		<i>A. mellifera</i>	<i>B. terrestris</i>	<i>O. bicornis</i>	<i>A. mellifera</i> vs <i>B. terrestris</i>	<i>A. mellifera</i> vs <i>O. bicornis</i>	<i>O. bicornis</i> vs <i>B. terrestris</i>
clothianidin	48	0.158 (0.035)	0.037 (0.008)	0.042 (0.014)	3.95	2.87	39.47
clothianidin	96	0.079 (0.011)	0.025 (0.004)	0.031 (0.011)	3.82	1.78	29.1
clothianidin	240	0.028 (0.005)	0.016 (0.003)	0.029 (0.011)	0.05	-1.01	27.52
dimethoate	48	2.42 (0.24)	>2.188	7.73 (1.052)	-	<b>-4.92***</b>	
dimethoate	96	1.16 (0.11)	1.43 (0.18)	3.68 (0.554)	-0.6	<b>-4.47***</b>	1.24
dimethoate	240	0.62 (0.079)	0.36 (0.056)	-	1.01	-	-
Cadmium	48	18.36 (4.73)	22.47 (3.17)	27.38 (18.72)	-0.81	-0.47	26.2
Cadmium	96	3.70 (4.19)	9.68 (1.32)	2.21 (2.44)	-1.38	0.31	-1.28
Cadmium	240	0.57 (1.41)	5.50 (1.035)	1.003 (0.33)	<b>-2.83**</b>	0.56	<b>-4.07***</b>
Arsenic	48	25.68 (1.76)	21.15 (393.71)	50.5 (27.92)	0.23	-0.89	50.44
Arsenic	96	13.56 (0.80)	8.71 (1.57)	3.07 (2.02)	3.27	4.84	-0.34
Arsenic	240	4.03 (0.37)	4.44 (0.73)	-	-0.45	-	-
tau-fluvalinate	48	>67.08	>44.72	36.023 (17.23)	-	-	-
tau-fluvalinate	96	>67.08	55.34 (11.31)	1.005 (14.98)	-	-	<b>-1.94*</b>
tau-fluvalinate	240	>67.08	61.96 (27.68)	-2.35 (7.83) †	-	-	<b>-4.52***</b>
2,4-D	48	>900	>900	>1437.5	-	-	-
2,4-D	96	>900	>900	>1437.5	-	-	-
2,4-D	240	>900	>900	>1437.5	-	-	-
propiconazole	48	-	>300	-	-	-	-
propiconazole	96	-	>300	-	-	-	-
propiconazole	240	-	>300	-	-	-	-



270 3.3. *Variation in time course effects*

271 The time dependencies of LC<sub>50</sub>s across chemicals were found to be greater in magnitude than between  
272 species i.e. LC<sub>50</sub>s calculated at 48 h were up to 25 times higher than values calculated at 240 h (see  
273 table 2). Across species the median values for this time point showed the strongest temporal effect  
274 for cadmium, arsenic and dimethoate (3.9-6.4 fold difference), an intermediate change for  
275 clothianidin (1-5) and low change for tau-fluvalinate and propiconazole (0.7-1). Cases with a strong  
276 time dependence are associated with slow kinetics, reflected in low elimination rates and lower killing  
277 rates (caused by the toxicodynamics), both of which will increase the time between initial exposure  
278 and ultimate effect. When longer term predictions of LC<sub>50</sub>s for lifespan durations were estimated from  
279 DEBtox parameters they approached the NEC, meaning that the ratios calculated from these values  
280 were often larger compared to those calculated using shorter-term LC<sub>50</sub>s.

281

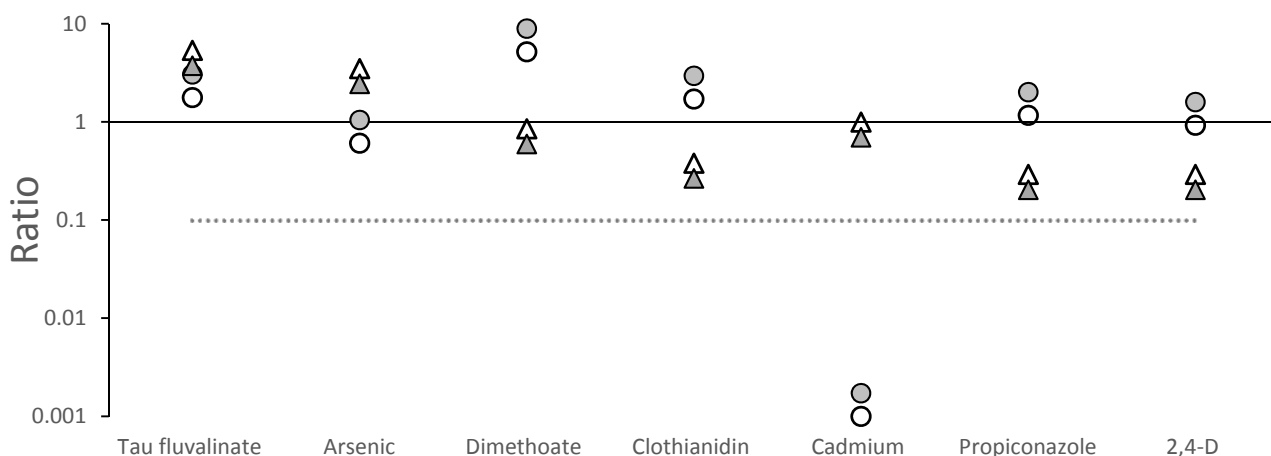
282 3.4. *DEBtox derived no effect concentrations (NEC) and body weight scaling*

283 DEBtox models to predict the NEC for each compound did not converge in all cases e.g.  
284 propiconazole or 2,4-D. For propiconazole there were few effects on survival of *A. mellifera* and *B.*  
285 *terrestris*, even at the top concentration of 300 mg ml<sup>-1</sup> after 240 h exposure, thus no DEB (or LC<sub>50</sub>)  
286 parameters could be calculated. Similarly there were no effects of 2,4-D on bumblebees; since the top  
287 concentration tested (900 mg ml<sup>-1</sup>) represents the maximum water solubility for this herbicide, we  
288 would predict no risk from exposure through feeding in the field by oral exposure via water. Despite  
289 this lack of convergence we have used these maximum estimates (+0.01) for cross species  
290 comparative purposes in order to plot *A. mellifera*: *B. terrestris* and *A. mellifera*: *O. bicornis* NEC  
291 ratios for all chemicals (Table 3). On calculation of the species NEC ratios, most (86%) were found  
292 to be less than two (Figure 3). Although of a similar range, the values for *A. mellifera*: *B. terrestris*  
293 and *A. mellifera*: *O. bicornis* were not significantly correlated ( $P>0.05$ ). This difference appears to  
294 be driven by an increased relative sensitivity of *O. bicornis* to tau-fluvalinate and arsenic with NEC  
295 ratios to *A. mellifera* of 5.4 and 3.5 respectively and a relative decrease in sensitivity of *B. terrestris*

296 to cadmium (Figure 3). We also corrected these NEC ratios for differences in body weight, which  
 297 span an order of magnitude (*B. terrestris*=170mg ± 2.5 n = 582, *A. mellifera* 100mg± 3.5 n= 582, *O.*  
 298 *bicornis* = 69mg± 2.7 n=500). Although this adjustment did not alter the overall order of NEC ratios  
 299 (Figure 3), the difference between the individual and body weight adjusted slopes was significant for  
 300 *A. mellifera*: *B. terrestris* (one sample  $t(7) = -9.2, p < 0.001$ ) suggesting that accounting for body weight  
 301 significantly increased the estimate of sensitivity for *B. terrestris* per unit mass. For *O. bicornis*  
 302 controlling for body weight lowered estimates of sensitivity relative to *A. mellifera*, but there was no  
 303 significant difference between slopes ( $p > 0.05$ ).

304

305 Figure 3. The NEC ratio of bee species ( $\circ = A. mellifera$ : *B. terrestris*,  $\Delta = A. mellifera$ : *O. bicornis*)  
 306 calculated for individuals (unfilled symbols) or per unit mass i.e. corrected for differences in body  
 307 weight (filled symbols). A ratio of 1 (solid line) indicates that the comparator species has the same  
 308 sensitivity to pesticide as *A. mellifera*, values  $>1$  indicate higher sensitivity of species *s* than *A.*  
 309 *mellifera*. The dotted line represents a 10-fold difference when the sensitivity ratio  $<1$ . Note that  
 310 where accurate estimates of the relevant dose were calculated as ‘greater than’ exceedance values we  
 311 have used that value +0.01 to allow plotting.



312

313

314

315 Table 3 DEBtox *NEC* parameters calculated for each species for all chemicals. Values= maximum  
316 exposure level +0.01 for propiconazole and 2,4-D (see text for details)

Compound	<i>Apis mellifera</i> spp. (µg/l)	<i>Bombus terrestris</i> (µg/l)	<i>Osmia bicornis</i> (µg/l)
cadmium	0.001	1	0.001
chlothianidin	0.024	0.014	0.063
dimethoate	0.41	0.079	0.48
Arsenic	4.2	6.9	1.2
tau fluvalinate	67	37.8	12.5
propiconazole	292	250.0	1000
2,4-D	833	900	2850

317

#### 318 4. Discussion

319 There is wide variation in the life history, demographic, behavioral, morphological and physiological  
320 traits of bees but relatively few species have been compared systematically in ecotoxicological studies  
321 (Arena and Sgolastra, 2014; Hardstone and Scott, 2010). Our test species varied in both sociality  
322 (complex eusociality = *A. mellifera* vs. primitively eusocial = *B. terrestris* vs. solitary = *O. bicornis*),  
323 feeding behaviour (tropholaxis = *A. mellifera* vs. individual) and mean body size (69 -170mg). While  
324 it has been suggested that the different modality of feeding between social and solitary bees makes  
325 comparison among species more difficult for oral toxicity tests (Ladurner et al., 2003) our methods  
326 promoted good feeding and control survival rates across extended time periods. This meant we were  
327 able to make comparisons over durations that exceeded 'standard' regulatory exposures by 144 hours.  
328 Incorporating survival in time data for longer-term exposures into DEBtox models and linking the  
329 effect to physiological efficacy is an important step forward to understanding the holistic implications  
330 of different toxicological effects on pollinators.

331 Although we observed some variation in species sensitivity, within exposure tests there was  
332 generally a less than 2-fold difference in observed 240 h LC<sub>50</sub> between species. The sensitivity ratio  
333 (*R*) median value for LC<sub>50</sub> across the seven types of chemicals up to 240h was 1.05 suggesting relative  
334 equivalence between species across the tests for a range of different compounds. This is a higher  
335 value than found in a recent meta-analyses of both chronic and acute effects across a wider number

336 of bee species and compounds (Arena and Sgolastra, 2014). This study, based on generally short term  
337 effects across a wide range of compounds and test systems, estimated the median sensitivity ratio ( $R$ )  
338 to be 0.57 (with a range from 0.001 to 2085.7) indicating that in most cases the sensitivity of *A.*  
339 *mellifera* was higher than other bee species. Arena and Sgolastra (Arena and Sgolastra, 2014) also  
340 found that the median estimate of the sensitivity ratio for acute oral LD<sub>50</sub> was lower than this ( $R=0.39$ ,  
341 97% of cases  $<10$ ). Our comparisons were over a longer time period and for four of the seven  
342 chemicals there was a clear decrease in  $R$  through time.

343 Comparing the sensitivity ratio of the tested chemicals, the neonicotinoid clothianidin showed  
344 that the two other bee species were more sensitive than *A. mellifera* at 48h ( $R_{Osmia}=3.8$ ,  $R_{Bombus}=4.3$ )  
345 and 96h ( $R_{Osmia}=2.5$ ,  $R_{Bombus}=3.1$ ) although less so after 240h exposure, when *B. terrestris* was only  
346 1.7 times more sensitive and *O. bicornis* equally sensitive to *A. mellifera* (although by this time the  
347 control survival rates (60%) for *O. bicornis* were sub-optimal). Scott-Dupree *et al.* (Scott-Dupree *et*  
348 *al.*, 2009) also compared 48h toxicity of clothianidin across three non-*A. mellifera* bee species (*B.*  
349 *impatiens*, *Megachile rotundata* and *O. lignaria*) following topical application. Although this route  
350 of exposure is not directly comparable with our longer oral toxicity test approach, similar sensitivity  
351 ratios could be calculated. For example, when the 48h LC<sub>50</sub> (expressed as percentage of solution,  
352 w:v,) for each species (Scott-Dupree *et al.*, 2009) is compared with *A. mellifera* data from (Bailey *et*  
353 *al.*, 2005), that used the same exposure protocol, it suggests that *B. impatiens* was more tolerant than  
354 *A. mellifera* to clothianidin ( $R_{Bombus}=0.5$ ) which contradicts the results of this study on *B. terrestris*  
355 ( $R_{Bombus}=4.2$ ), while the solitary bees were more sensitive ( $R_{Osmia}=2$ ,  $R_{Megachile}=2.5$ ) which confirms  
356 the *O. bicornis* results from this study.

357 Across the three tested insecticides, the median values for  $R$  for species comparisons were  
358 comparable with values (in brackets) calculated by (Arena and Sgolastra, 2014); 2.8 (vs 1.06) for  
359 neonicotinoids, 0.8 (vs 0.5) for organophosphates and 1.4 (vs 0.33) for pyrethroid. Overall this points  
360 to a relatively consistent magnitudes of difference in species sensitivity *in short to medium* term tests  
361 of adult mortality.

362 A problem when comparing species sensitivity based on toxicity test results is that effect  
363 concentrations may be given for different exposure times. Thus, if values (e.g. LC<sub>50</sub>s) for different  
364 exposure time are directly compared, the observed difference may result both from temporal changes  
365 in effects, as well as inherent difference in species sensitivity. In contrast, as a time invariant  
366 parameter, the DEBtox *NEC* can be used to compare the predicted threshold of sensitivity for the  
367 three tested species. For the insecticides dimethoate and clothianidin, the *NEC* values for the three  
368 species were broadly comparable indicating similar sensitivity. For the pyrethroid tau-fluvalinate, the  
369 *NECs* indicate greater differences in sensitivity than for the other two insecticides with *O. bicornis*  
370 showing a >5-fold greater sensitivity than *A. mellifera*. This insecticide has been widely used to  
371 control *Varroa* mites in *A. mellifera* colonies because it has reportedly less impact relative to other  
372 pyrethroids due to detoxification by P450 enzymes and carboxylesterase (Johnson et al., 2013). It is  
373 also applied as a contact insecticide to control cabbage seed weevil, aphids and cabbage stem flea  
374 beetle in flowering crops like oilseed rape. A number of eusocial and solitary wild bee species  
375 frequently visit such crops (Woodcock et al., 2013) and are likely to be exposed to this compound. In  
376 addition it has been shown to interact with other compounds including fungicides which can increase  
377 its toxicity 2000-fold (Johnson et al., 2013). The differences in sensitivity we observed across species  
378 could be an important consideration for the risk assessment of this chemical.

379 The metals also showed wide variation in species predicted *NECs*. For cadmium, although  
380 the difference in sensitivity ratio for *A. mellifera*: *B. terrestris* was  $\ll 1$ , in reality all species showed  
381 low *NEC* values (for *A. mellifera* and *O. bicornis* the *NEC* was effectively zero). For arsenic the  
382 variation in sensitivity was driven primarily by the relatively low sensitivity for *B. terrestris* and  
383 increased sensitivity of *O. bicornis*. While there have been few studies on the effects of heavy metal  
384 pollution on wild bee communities, it has been shown that cadmium, lead and zinc were increasingly  
385 expressed in pollen collected by *O. bicornis* across an industrial contamination gradient (Moroń et  
386 al., 2012). For cadmium this increased from a background of 0.8-1.3 mg kg<sup>-1</sup> to 6.7-9.3 mg kg<sup>-1</sup> and

387 overall this was highly correlated with a 7.5 fold decrease in species richness and 4 fold decrease in  
388 the abundance of bees, especially solitary species. Clearly *A. mellifera* showed similar sensitivities.

389 Some studies have suggested that the sensitivity of different bee species is inversely  
390 proportional to mean body weight (Devillers et al., 2003) while others have found no effect (Helson  
391 et al., 1994). In our study accounting for differences in body weight did not alter the overall patterns  
392 of NEC ratios, but for *B. terrestris* did significantly alter the slope of sensitivity ratio with *A. mellifera*.  
393 Although these differences were relatively small, it does suggest that there can be clear differences  
394 between species that are not solely accounted for by body weight differences. Other studies have  
395 suggested this may be linked to differences in physiology (e.g. haemolymph pH), metabolism (e.g.  
396 *A. mellifera* have a lower number of detoxifying cytochrome P450 genes, (Claudianos et al., 2006),  
397 volume to surface area ratios, sociality and feeding behaviours or pre-adapted diet choice (Arena and  
398 Sgolastra, 2014; Cresswell et al., 2012).

399 The comparative time dependent (e.g. LC<sub>50s</sub>) and absolute (e.g. NEC) indicators of relative  
400 sensitivity we observed across species may not be consistent in the wild where differential exposure  
401 probability needs to be considered alongside species' sensitivities (Brittain and Potts, 2011).  
402 Laboratory assessment of direct toxicity is only one measure of potential impact, and mortality may  
403 differ greatly under natural conditions where diet selection, rates of pollen and nectar consumption,  
404 storage and processing can vary widely among bee species (Falk and Lewington, n.d.). Other oral and  
405 non-oral routes of exposure are also likely, such as contact with soil contaminants in ground nesting  
406 species or nesting material in surface and aerial nesting species. In addition the impact on species  
407 survival is likely to vary with species traits. Whereas *Apis* species have colonies (and queens) that  
408 live for years, solitary bee and *B. terrestris* species often exhibit multivoltinism; if reproductives of  
409 these species are exposed to pesticides or other contaminants during key lifecycle phases e.g. nest  
410 establishment, the impacts on reproductive capacity (and thus population persistence) can be severe.  
411 These differences among bee species (both in exposure routes and in sensitivity) and potential for  
412 interactions between different factors highlight the need to take a more holistic approach to risk

413 assessment than current prevailing standards (i.e. lab-based, short-term, lethal effects on model  
414 species) require, especially if the results are to be used to predict impacts on populations, communities  
415 and ecosystems and set meaningful environmental protection goals (Food and Authority, 2014;  
416 Sanchez-Bayo and Goka, 2014).

417 In addition to species effects there was a wide range of time dependence in toxicity for the  
418 seven selected chemicals. A key insight from this is that it represents a summary of the extent to  
419 which the results of short-term toxicity tests can underestimate longer term effects. Indeed these  
420 temporal effects were much greater than interspecific differences. At present, regulatory guidelines  
421 primarily assess the survival of adult honey bees after a short exposure to pesticides, typically up to  
422 four days, i.e. 96 h (OECD, 1998). Regulatory standards based on these tests thus emphasize a toxic  
423 threshold that does not include any time dependence. While some authors have stressed the  
424 importance of longer duration toxicity tests (Decourtye et al., 2013) there have been no systematic  
425 longer-term experimental studies comparing across bee species. Our data clearly suggest that, across  
426 a range of compounds and species, this assumption of non-time dependence is not realistic, an insight  
427 established in other ecotoxicological studies (Heckmann et al., 2010). The ratios of values measured  
428 for experimental exposures between 48 and 240 h showed up to 25 fold differences while longer term  
429 DEBtox predictions (up to total average lifespan) revealed ratios that exceeded several orders of  
430 magnitude. Recently Rondeau *et al* (Rondeau et al., 2014) explored time dependence of the  
431 neonicotinoid imidacloprid on *A. mellifera* using published data to plot time-to-lethal-effect. They  
432 used a temporal power-law to fit curves to these data and found that for *A. mellifera* LD<sub>50</sub> values after  
433 time  $t$  scaled from  $t^{1.6}$  to  $t^5$ . When we calculated the time dependence from our *A. mellifera* data for  
434 exposure to the neonicotinoid clothianidin up to 240h it was  $t^{0.93}$  ( $R^2=0.99$ ) suggesting simple  
435 accumulation to a toxic threshold directly proportional to time. In contrast using data predictions from  
436 the DEBtox models we found stronger time dependence for *Bombus* ( $t^{1.3}$ ,  $R^2=0.92$ ) and *Osmia* ( $t^{2.7}$ ,  
437  $R^2=0.82$ ) that are highly comparable with the approach and conclusions of Rondeau *et al.* (Rondeau  
438 et al., 2014).

439 Other compounds clearly showed greater predicted time dependence because of slow  
440 elimination kinetics e.g. cadmium and arsenic. In this respect there are a number of advantages of  
441 using a DEBtox approach for analysis of toxicity test data. The DEB approach uses all of the available  
442 information in the analysis of the time course effects of a hazard which includes all endpoints,  
443 treatments, and all time points. The resulting time-independent parameters like the NEC, allow for  
444 educated extrapolation to untested situations. In contrast, summary statistics like the LC<sub>50</sub> derived  
445 from more descriptive dose-response analyses can clearly vary greatly between exposure times. This  
446 fact is disguised because exposure time are often standardised in regulatory protocols (Baas et al.,  
447 2010; Jager, 2011). Clearly the ecotoxicological consequences of delayed toxicity are potentially  
448 profound and as such, deriving simple toxic thresholds from such short term acute LC<sub>50</sub>s to define  
449 safe residual levels could severely underestimate risks to organisms. Protection from longer-term  
450 exposure effects for such chemicals may require protection levels greater than those currently applied.

451 Overall, our results suggest that the current approach of using *A. mellifera* as a surrogate bee  
452 test species in environmental risk assessment may be sufficient for a number of compounds when  
453 considering direct oral toxicity on survival as long as an assessment factor (e.g. of >10) is applied to  
454 LC<sub>50</sub> endpoints. However, for some compounds there are clear exceptions and care must be taken if  
455 these estimates are to be used to predict environmental hazard. Of potentially more environmental  
456 importance is the need to assess and include the delayed toxicity effects resulting from extended  
457 continuous exposure for different compounds within risk assessments. The use of DEBtox models  
458 and calculation of time independent parameters from extended lab assays offers great potential to  
459 overcome the intrinsic difficulties of predicting the environmental hazard that arise from the  
460 assumptions associated with more standard descriptive statistics.

461

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470

471

472 **Author Contributions**

473 DJS, MSH, and CS conceived the original study in consultation with AR and J-LD. All authors  
474 designed the experiments; HH, EL, AR, DS and MSH carried out the experiments; HH, JB, DJS and  
475 MSH analysed the data; MSH, HH and DJS prepared the manuscript; all authors edited the manuscript  
476

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