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

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Abstract

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

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

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

of a.i's) 9%, insecticides 8%, molluscicides 2%;(unpublished analysis of (FERA, n.d.)).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

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

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

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

2. Material and Methods

2.1. Study species

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

The bumblebee *Bombus terrestris audax* is a more primitive eusocial species with no clear caste system. It is a common wild pollinator which is also commercially reared for pollination in closed or semi-closed cultivation situations. In the temperate zone it is generally an annual species that lives in colonies that contain *c.* 100-150 female workers during the summer. Colonies of UK native *B. t. audax* were obtained as commercially reared colonies with *c.* 30 workers (NV Biobest, Belgium). On receipt, 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, melloproteins) 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 metalloid 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

Chemical (class in brackets)	**Current usage**	**Exposure scenario**	**Mechanism of action**	**Metabolism**	**Other information**
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 *D. Melanogaster* 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°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

this method under red light, at room temperature. Each test replicate (n=4) comprised a group of 3 bees from a single colony kept together. During the experiments, both *A. mellifera* and *B. terrestris* were maintained in a constant temperature room at 25 ± 2ºC, ~60% RH, in the dark.

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

2.4. Statistical Analyses

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

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

3. Results

3.1. Toxicity of the reference toxicant

Observed sensitivity for the reference toxicant dimethoate showed very good accordance with previously published estimates for *A. mellifera*. For example the 48 h probit LC_{50} of 2.42 mg L^{-1} equated to an estimated LD_{50} of 3.39 $\times 10^{-4}$ mg/bee based on our average (±se) measured consumption rate of 69 ±4 µl/bee day^{-1} (n=25 replicate pots, 2500 bees) across the experiment. This approximates well to the upper limit of the range of the oral LD_{50} values at 24 h of 1.0 $\times 10^{-4}$ – 3.5x10^{-4} mg/bee (OECD, 1998). For *B. terrestris* our 48 h estimate of LC_{50} was >2.188 mg L^{-1} which equates to an LD_{50} > 9.21$\times 10^{-4}$ mg /bee (mean ±se consumption rate across the experiment = 421 ±20 µl/bee day^{-1} ; n=24 replicate pots, 72 bees) which is slightly below previously published estimates (24–72 h oral LD_{50} = 17 – 47$\times 10^{-4}$ mg /bee; (Ladurner et al., 2005). Overall this indicates a comparative sensitivity of bees within normal expectations.

3.2. Overall toxicity and relative species' effects

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

Figure 1. DEB Tox predictions of LC_{50} for four chemicals across all species extending past length of test to: 480 h (twice test length); 720 h (=lifetime 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) cadmium, d) tau-fluvalinate (note non- toxic to *A. mellifera*). ■= *A. mellifera*, ●= *B. terrestris*, ▲=*O. bicornis. O. bicornis* data on combined male and female, except for tau-fluvalinate (=females).

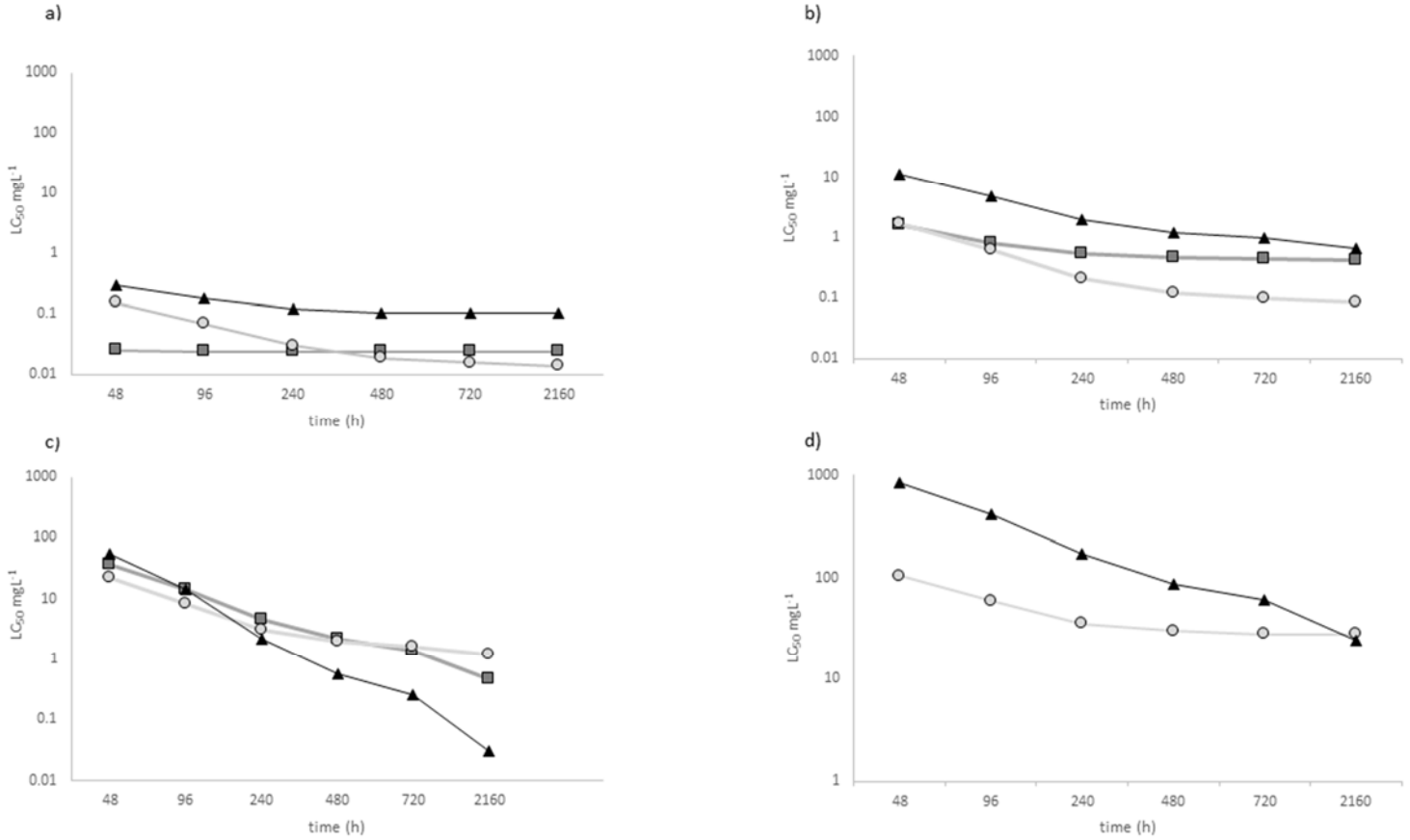


Figure 2. Distribution of the sensitivity ratios of bee species (○= *A. mellifera: B. terrestris*, Δ =*A. mellifera: O. bicornis*) for the LC_{50} for each chemical at different time points (*black = 48h, grey =96h, open =240h*) ordered by median values for each chemical. A ratio of 1 (solid line) indicates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

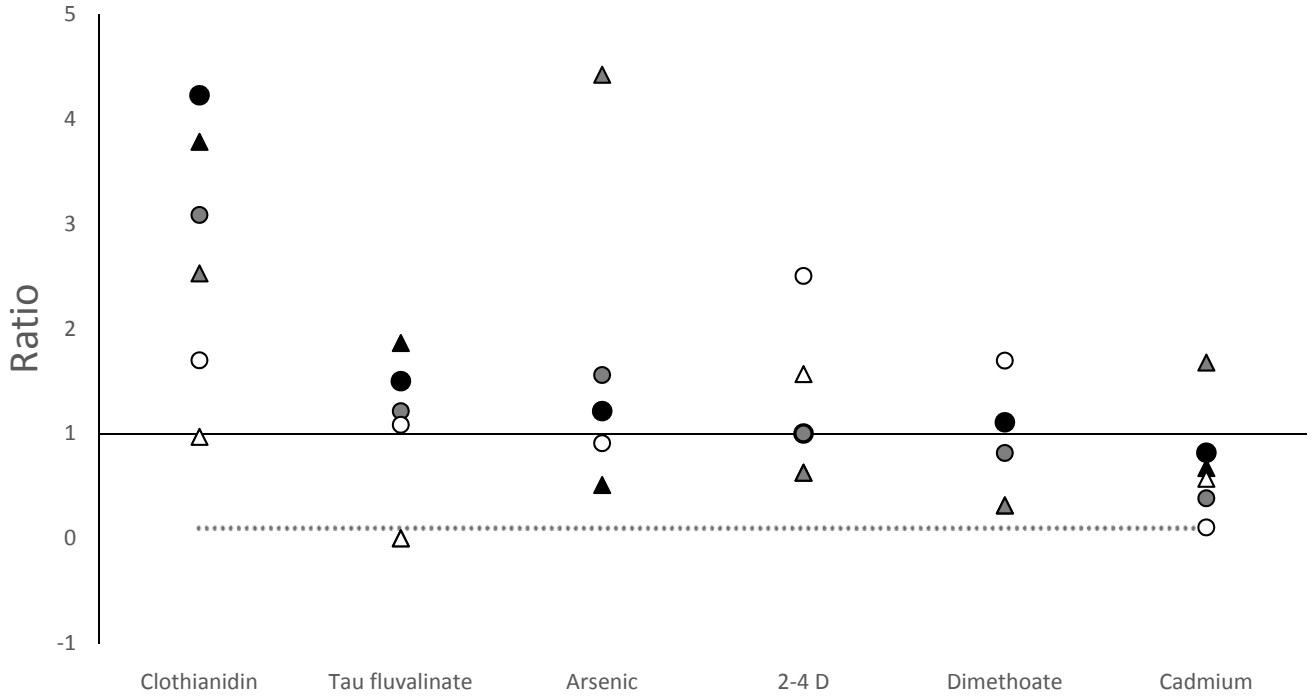


Table 2. Toxicity of six chemicals to all species (chemicals ordered by mean 240h LC_{50} values, low to high) : Probit estimates of oral LC_{50} values (mgL^{-1}) with SE in parentheses. Values could not be calculated for some tau-fluvalinate, 2,4-D and propiconazole assays as mortality levels were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

Chemical	**Time (h)**	**LC_{50} mg L^{-1} (S.E.)**			***z* score**		
		A. mellifera	***B. terrestris***	***O. bicornis***	***A. mellifera vs B. terrestris***	***A. mellifera vs O. bicornis***	***O. bicornis vs B. terrestris***
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)	-	**-4.92*** **	
dimethoate	96	1.16 (0.11)	1.43 (0.18)	3.68 (0.554)	-0.6	**-4.47*** **	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)	**-2.83****	0.56	**-4.07*** **
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)	-	-	**-1.94***
tau-fluvalinate	240	>67.08	61.96 (27.68)	-2.35 (7.83) †	-	-	**-4.52*** **
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	-	-	-	-

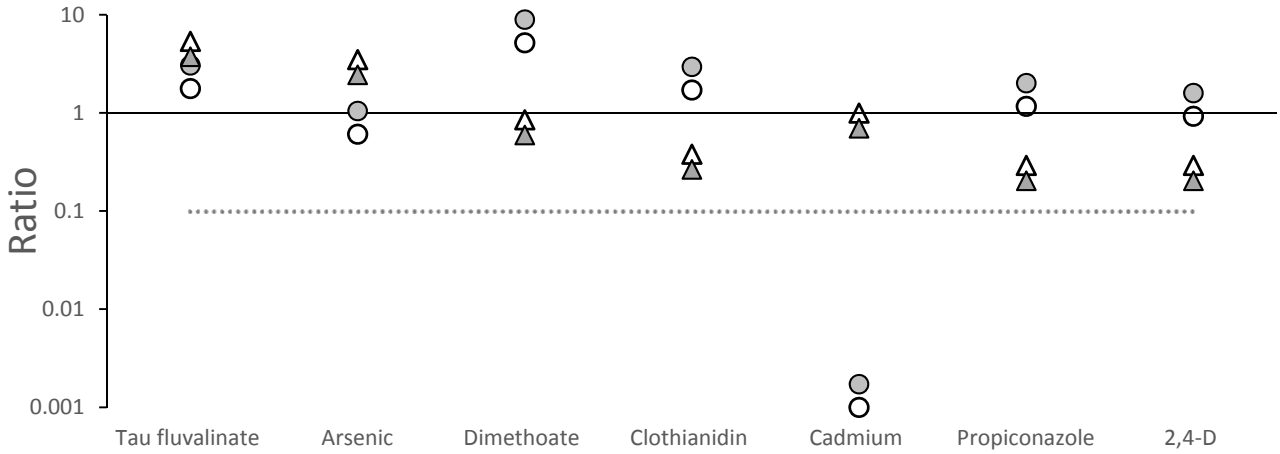
3.3. Variation in time course effects

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

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

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



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

Compound	***Apis mellifera* spp. (µg/l)**	***Bombus terrestris* (µg/l)**	***Osmia bicornis* (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_{50} between species. The sensitivity ratio
333 (*R*) median value for LC_{50} 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

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

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

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

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

The metals also showed wide variation in species predicted NECs. For cadmium, although the difference in sensitivity ratio for *A. mellifera*: *B. terrestris* was <<1, in reality all species showed low NEC values (for *A. mellifera* and *O. bicornis* the NEC was effectively zero). For arsenic the variation in sensitivity was driven primarily by the relatively low sensitivity for *B. terrestris* and increased sensitivity of *O. bicornis*. While there have been few studies on the effects of heavy metal pollution on wild bee communities, it has been shown that cadmium, lead and zinc were increasingly expressed in pollen collected by *O. bicornis* across an industrial contamination gradient (Moroń et al., 2012). For cadmium this increased from a background of 0.8-1.3 mg kg^{-1} to 6.7-9.3 mg kg^{-1} 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_{50}s) 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 *Api*s 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

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

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

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

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

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Author Contributions

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

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