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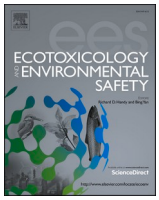

Comparative temporal response of toxicity for the neonicotinoid clothianidin and organophosphate dimethoate insecticides in two species of solitary bee (*Osmia bicornis* and *Osmia cornuta*)

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ABSTRACT

Solitary bees provide essential pollination services. Concerns for the decline of these wild bee species have led to calls for their inclusion in pesticide risk assessment. Solitary bees differ from honey bees in their physiology and ecology and this may affect how they respond to pesticide exposure. Here we investigate the life-time toxicity of two insecticides, the organophosphate dimethoate and neonicotinoid clothianidin, for two mason bee species, *Osmia bicornis* and *O. cornuta* using a toxicokinetic/toxicodynamic stochastic death model taken from Dynamic Energy Budget (DEBtox) theory. Both species showed concentration and exposure duration dependent effects for each chemical. LC_{50} values estimated from the model parameters at 48 h were $\geq$ 14 fold and 6 fold those at 480 h for dimethoate and clothianidin respectively. Survival modelling indicated greater sensitivity in *O. bicornis* than for *O. cornuta* to dimethoate, whilst for clothianidin, *O. cornuta* females but not males, were more sensitive than both sexes of *O. bicornis*. These sensitivity differences were not related to body size. Toxicokinetic and toxicodynamic traits derived from modelling indicated lower elimination rates in *O. bicornis* and higher killing rates for *O. cornuta* females for dimethoate and lower elimination rates for clothianidin in *O. cornuta* females that were related to sensitivity. This study shows the near life-time testing is possible for solitary bees and that combining adult life-time toxicity tests with toxicokinetic/toxicodynamic modelling provides a more mechanistic understanding of pesticide effects in solitary bee species.

1. Introduction

Solitary bees are an important group of wild pollinators that deliver significant pollination services for a broad range of wild and crop plants including high value crops such as orchard fruit (Garibaldi et al., 2014; Klein et al., 2007). Solitary species provide an important complementary functional role alongside managed honey bees (*Apis mellifera*) in ensuring effective pollination and fruit set (Martins et al., 2015). The current global pollinator decline in both managed bees and wild bee populations are of major concern for ecological and socio-economic reasons (Gallai et al., 2008; Zattara and Aizen, 2021). Declines have been linked to the actions of multiple stressors (Siviter et al., 2023; Vanbergen and Initiative, 2013) such as reduction in availability of floral resources, loss of habitat through agricultural intensification (Powney et al., 2019; Scheper et al., 2014), exposure to insecticides (Azpiazu et al., 2023b; Woodcock et al., 2017, 2016), and the impacts of pathogens and diseases (Godfray et al., 2014; Potts et al., 2010).

Much public and scientific debate has focussed on the negative impacts of agrochemicals on bees, especially the neonicotinoid class of insecticides which are commonly used as dressings for grain crops to protect against herbivorous insect pests. Active ingredients from the neonicotinoid class have become, and remain, the most widely used insecticides worldwide despite regional bans on some uses (Sgolastra et al., 2020; Vanbergen and Initiative, 2013). Non-target invertebrates may be exposed to these systemic chemicals via numerous routes, including direct exposure to treated plant surfaces or dusts during seed drilling, contaminated soil and water, and by ingestion of both treated plant pollen and nectar (Rortais et al., 2005). Neonicotinoids have also

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been found in wildflowers in non-crop habitats (Botías et al., 2015) raising concerns of wider, negative indirect effects on wild bees (Goulson et al., 2015; Sgolastra et al., 2020; Woodcock et al., 2016). Indeed, analyses of long-term records for 62 UK wild bee species (50 solitary) co-occurring within agricultural landscapes, suggest that exposure to neonicotinoid pesticides is significantly correlated with population declines (Woodcock et al., 2016). In addition, trends in numbers of both managed and wild species have shown marked reductions over time, although it should be noted that not all species of wild bees are thought to be declining (Powney et al., 2019).

Routine chemical toxicity testing for bees is primarily focussed on the managed honey bee *A. mellifera*. However, two species of congeneric, solitary mason bees *Osmia cornuta* and *Osmia bicornis* have been proposed as test species, most recently in the 2023 revised guidance from EFSA on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees) (European Food Safety Authority, 2013, 2023). Despite this revision of the guidance, there is still no recommended chronic toxicity test for solitary bees. Further, there is limited information on the relative sensitivities of the two *Osmia* solitary bee species that have been proposed as possible test species.

The red mason bee *O. bicornis* has a distribution range across Europe but also the Middle East and North Africa. It has increasingly been used for chemical toxicity testing (e.g. Heard et al., 2017; Martins et al., 2023; Robinson et al., 2017). The European Orchard bee *O. cornuta* (Latreille 1805) is an early spring-emerging solitary bee, found in southern and central Europe. Comparisons conducted between *Apis mellifera* and solitary species have revealed up to 25 fold differences in sensitivity (Arena and Sgolastra, 2014). Specifically for *Osmia* and *Apis* species, Heard et al. (2017) showed that *O. bicornis* can have similar, increased, or decreased sensitivities for different classes of compounds when compared to *A. mellifera.* In a comparative study of *A. mellifera* and female *O. bicornis*, species sensitivity was within a factor of 10 for only 13 of 16 chemicals tested in acute contact toxicity tests of 48 h (Uhl et al., 2019). Thus, *A. mellifera* toxicity data may only be a suitable proxy for the protection of risks for solitary bees as long as an appropriate assessment factor is applied to account for differences in sensitivity (Heard et al., 2017; Robinson et al., 2017; Uhl et al., 2018, 2016).

Under field conditions any differences in sensitivity of solitary bee species compared to *A. mellifera* may be further exacerbated by differences in physiology, ecology, and genetics between different solitary bees, and eusocial species (Alkassab et al., 2020; Sgolastra et al., 2019). For cavity-nesting species of solitary bee such as *Osmia* that utilise above-ground vacancies that exist naturally to build single nests, this may result in differences in exposure compared to colony living species (Chan et al., 2019; Kopit and Pitts-Singer, 2018). There are also potential differences between solitary bee sexes in exposure routes and duration of exposure. For example, as female bees collect materials such as soil and masticated plant tissue to form brood cells within the nest this may increase exposure to pesticide contaminated material (Cane et al., 2007). Actively foraging female *Osmia* spp. may also be potentially exposed through consumption of fresh pollen over the duration of their lifespan. Most bee risk assessments are focussed on females as the greatest risk to populations is the loss of potential offspring, but for solitary bees, the inclusion of males is also important; sex ratios in solitary bees are generally more even than in eusocial bees which have few males, compared to females, and thus impacts on male solitary bees should also be accounted for, in order to understand population level effects.

Current standardised laboratory toxicity tests to measure chemical effects on bees are usually conducted using female bees in acute toxicity tests run for 48 or 96 h duration and in chronic tests of 10 day duration. Clearly, in nature exposure over longer periods (i.e., individual lifetimes) is more likely. For example, long-term effects under semi-field conditions have been demonstrated with significant impact on *O. bicornis* populations, compared to *A. mellifera* (Peters et al., 2016). Short-term assays can, therefore, substantially underestimate the effects of longer duration exposure, depending on the toxicokinetics and toxicodynamics of the chemical of concern (Sánchez-bayo and Tennekes, 2020). Differences in the effects of exposure duration on sensitivity can exceed interspecific variation (Heard et al., 2017). However, whilst several studies have demonstrated the importance of extended exposures to understand such effects on sensitivity (Anderson and Harmon-Threatt, 2019; Azpiazu et al., 2019; Heard et al., 2017; Mokkapati et al., 2022; Mulvey and Cresswell, 2020; Robinson et al., 2017; Sgolastra et al., 2017; Simon-Delso et al., 2018) this is currently not common practice in laboratories that conduct toxicity tests for regulatory purposes. Thus, despite recommendations from such studies and the existing OECD guidelines (OECD, 2017) many laboratories continue to use only acute toxicity tests.

Here we tested the feasibility and robustness of extending chronic oral exposure regimes in both female and male *O. bicornis* and *O. cornuta* up to representative adult "lifetime" durations 696 h (29 days) using novel laboratory assays. The two species of bee were used to assess the effect of adult life-time exposure to two insecticides, the neonicotinoid clothianidin and the organophosphate dimethoate (commonly used as the standard toxic reference compound for toxicity testing). We hypothesized that: 1) *Osmia* species are amenable to toxicity testing over the full adult life-span; 2) interspecific differences in sensitivity exist but can be largely explained by species differences in traits, such as body size; and that, 3) toxicokinetic and toxicodynamic traits derived from models based on dynamic energy budget theory (DEBtox) and derived from long term (life-time) testing can describe the effect of temporal exposure and temporal trend in LC_{50} values, including the decline in LC_{50} values to incipient values over time for both species and sexes.

2. Methods

2.1. Test bee species

Pupae of both *Osmia* species were purchased from Dr Schubert Plant Breeding (Landsberg, Germany) and were originally collected from managed field populations. On arrival, pupae were sexed according to pupal weight as female pupae are significantly larger than males (Table 1). Pupae were maintained at 4 ± 1 °C, 65 ± 10 % relative humidity in the dark with a single pupa in each well of a 25 multi-well plastic culture plate. Prior to use in experiments in spring, cohorts of male *O. cornuta* were warmed at 23°C in the dark in an incubator for one day and females for 2 days. *Osmia cornuta* emerge early in the season so a short warming period was suitable to break diapause. For the later emerging *O. bicornis*, warming times were extended to two days for males and 3–4 days for females. The average dry body weight for each species and sex was calculated from sub-sets of the emerging bees (n = 31–55; Table 1).

Table 1
Mean fresh pupal weight and adult dry weight by sex for *Osmia bicornis* and *Osmia cornuta*.

	Pupa fresh weight (mg) ± SEM	Adult dry weight (mg) ± SEM
O. cornuta ♀	174 ± 34 (n = 44)	52.8 ± 1.6 (n = 55)
O. bicornis ♀	133 ± 13 (n = 58)	39.1 ± 1.0 (n = 34)
O. cornuta ♂	96.3 ± 23.1 (n = 32)	24.5 ± 0.8 (n = 43)
O. bicornis ♂	82.6 ± 13.8 (n = 76)	23 ± 0.8 (n = 31)
O. cornuta ♀: *O cornuta* ♂	1.8	2.16
O. bicornis ♀: *O bicornis* ♂	1.61	1.70
O. cornuta: *O. bicornis*♀	1.31	1.35
O. cornuta: *O. bicornis* ♂	1.17	1.06

2.2. Chemical selection and preparation

The two insecticides used for testing were the organophosphate insecticide dimethoate as a standard toxic reference compound and Clothianidin, a systemic neonicotinoid insecticide and of Thiamethoxam (used extensively as a seed dressing against a wide variety of pests). Both pesticides were obtained as analytical grade PESTANAL® reagents (Sigma-Aldrich® Ltd., Poole, UK). The exposure concentrations selected were based on previous LC_{50} values for *O. bicornis* generated using the method of oral exposure of Heard et al. (2017). The exposure concentrations were thus based on our previous studies with *O. bicornis* and additionally applied to *O. cornuta* for testing in the current study. In this study, bees were exposed to the chemicals in 20 % (w/v) aqueous sucrose solution (following OECD, 2017 guidance), made in autoclaved, ultrapure water using molecular grade $\geq$ 99.5 % GC quality sucrose (Sigma-Aldrich® Ltd., Poole, UK). Stock solutions of the two chemicals were initially prepared in water with 480 μg ml^{-1} for dimethoate and 20.5 μg ml^{-1} for clothianidin. These stocks were then serially diluted with 20 % (w/v) aqueous sucrose solution to give stocks at 100-fold of the desired final concentration. These stocks were then further diluted in a ratio 1:99 with 20 % w/v aqueous sucrose solution to achieve the desired exposure concentration in the food source. Final exposure concentrations in the sucrose feed were: 1.21, 2.42 and 4.83 μg ml^{-1} for dimethoate and 0.025, 0.051, 0.103, 0.205 μg ml^{-1} for clothianidin.

2.3. Toxicity assays

Continual oral exposure of male and female bees was used to examine intra- and inter-species differences in sensitivity over time, following methods of Heard et al. (2017). Following emergence, bees were placed individually into bioassay containers of the design developed by Heard et al. (2017, see for details). This system uses a modification of the "flower method" to feed individual bees for dietary exposure (Azpiazu et al., 2023a; EFSA, 2013; Ladurner et al., 2003, 2005). During the test, bees were observed to exhibit diurnal changes in behaviour of feeding early in the day and then "resting" later underneath the artificial flower that was provided in the bioassay chamber. After hatching, bees were initially held for 48 h in bioassay containers during which time females released a meconium. In preliminary tests, it was noted that approximately 20 % of bees would not feed following emergence and consequently died within a few days. Hence, only those bees that had visibly started to feed within this initial holding period (as judged by lower sucrose levels in feeders) were used in experiments. This pre-experimental procedure ensured only activity feeding individuals entered the main test and prevented excessive non-treatment dependent background mortality due to starvation.

Unlike social *Apis* bees, *Osmia* spp. do not perform trophallaxis (the mutual exchange of regurgitated liquid food between individuals) and so were housed separately for tests as detailed in Heard et al. (2017). Following the experimental design previously published by Heard et al., (2017) and Robinson et al., (2017), a total of 10 bees were tested in each treatment, which comprised 5 male and 5 female bees (with exception of clothianidin doses 0.205 μg ml^{-1} and 0.051 μg ml^{-1} where 7 male *O. bicornis* and 9 male *O. cornuta* were tested, respectively). For each species there were an increased number of 10 control male and 10 control female bees exposed to un-spiked 20 % aqueous sucrose solution only. Overall, there were a total of 185 bees tested in individual bioassay containers for a duration of 696 h. Bee mortality was assessed 3 times a day up to 96 h exposure and then daily until a maximum of 29 days by which time, the majority of bees had either died or reached senescence in the control treatment. At each time point, a bee was classified as dead when there was no obvious movement of the body or any appendage when the bee was gently probed with forceps.

2.4. Data analysis

Data were analysed to describe and understand the toxicokinetics and toxicodynamics of the two chemicals using a survival model based on stochastic death arising from DEBtox theory (Baas et al., 2018). This stochastic survival model from within the DEBtox framework, as described initially by Kooijman and Bedaux (1996), is approved by the OECD for statistical analysis of ecotoxicity data (OECD, 2006). The DEBtox based model provides a toxicokinetic and toxicodynamic description of chemical effects on survival in time and has been used previously for the interpretation of *A. mellifera*, *B. terrestris* and solitary bee mortality data (Heard et al., 2017; Hesketh et al., 2016; Robinson et al., 2017). The survival modelling approach uses a scaled one-compartment model to describe the toxicokinetics combined with a toxicodynamic hazard model based on stochastic death. Model fits to time-series survival data provides three time-independent parameters: the No Effect Concentration (NEC), a time-independent toxicological threshold below which no effects occur; the elimination rate (Ke), which describes the kinetics of the rate determining step in the time course of the observed toxic effects; and, the killing rate (Kr), which is a measure for the toxic potency of the compound (once the NEC is exceeded). An additional parameter is used to correct for background mortality in the unexposed control population.

Model fits were made using freely standardised software (code packages downloadable from DEBlab; https://bio.vu.nl/thb/deb/deblab/), using censored data-sets up to set durations to investigate how exposure time influenced model fits. By fitting DEBtox models to all available survival data i.e., all time points, treatments, and endpoints, up to the censored duration it was possible to assess how the extension of duration of exposure changes parameter estimates. It is important to note that the DEBtox approach considers all effect measurements for all treatments/concentrations, and all time points in the model so all data points collected are used in the analysis. This leads to a more robust analysis of data than using single, end of trial time point mortality data. The model allows time-independent parameter values, and thus, the fitted DEBtox parameters can subsequently be used to calculate LC_{50} values (the concentration which kills 50 % of tested bees) for any point in time. In this case, the DEB LC_{50} values were calculated for the standard test duration time of 48 and 96 h, and three extended exposure times of 240, 384, and 480 h to determine the species and sex specific patterns of mortality. A sensitivity ratio R was calculated for the two chemicals between bee species and for each sex separately at selected time points (24, 48, 96, 240, 384 and 480 h), where $R = LC_{50\ O.\ bicornis} / LC_{50\ O.\ cornuta}$ (following Arena and Sgolastra, 2014). Where this ratio gave a value of 1, then *O. cornuta* was considered to have the same sensitivity to the tested pesticide as *O. bicornis*. A ratio higher than 1 indicated greater sensitivity for *O. cornuta*, a ratio of less than 1 indicates higher sensitivity of *O. bicornis*.

3. Results

3.1. Bee weights, control survival and assay performance

Osmia cornuta were consistently larger than *O. bicornis* as both pupae and adults, with females having a larger average size than males for both species (Table 1). Survival in the control treatment was > 90 % up to the 240 h time point for both sexes of *O. bicornis* (Fig. 1, a & b) and for *O. cornuta* females (Fig. 1, d) and > 80 % for the *O. cornuta* males (Fig. 1, c). Survivorship of the control bees remained above 80 % until 408 h (17d) and only dropped to 70 % (19d) for both sexes of *O. bicornis* and *O. cornuta* females at 456 h. Males of *O. cornuta* had lower survivorship, with 40 % surviving to 456 h (19d). The levels of mortality found are consistent with the performance criteria stipulated in the OECD test 245 that control mortality should be $\leq$ 15 % at the end of the chronic 10-day feeding test. The control mortality observed over the extended exposure times was low and the low blank killing rate (Kr) for controls suggests

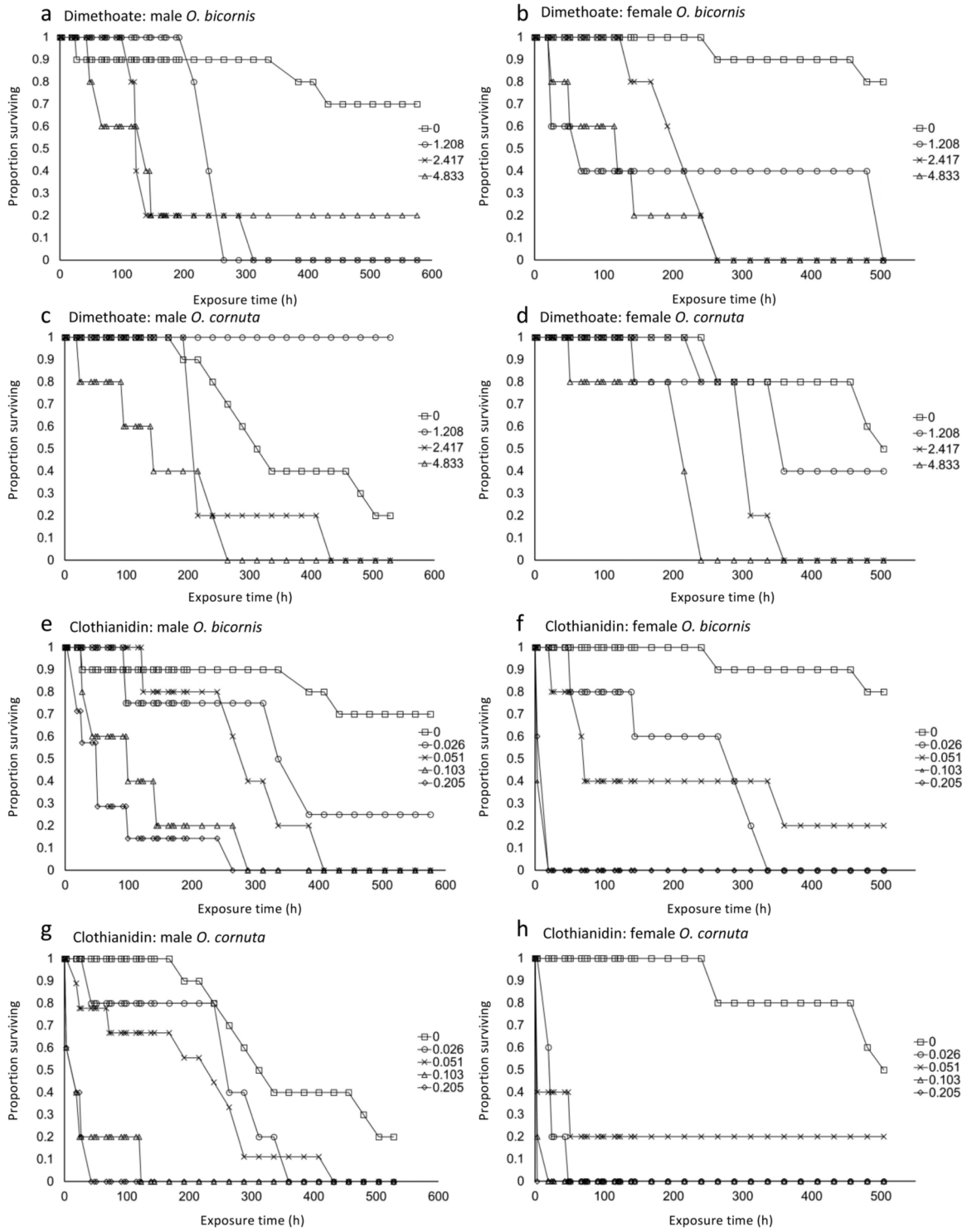

Fig. 1. Survival patterns in time given as proportion of bees surviving (n = 10 bees tested per chemical concentration) of solitary bees exposed to a series of increasing concentrations of dimethoate mg/L (a-d) and clothianidin mg/L (e-h) by continuous oral exposure up to 596 h. For dimethoate, survival by sex and species is shown as a) male *O. bicornis*; b) female *O. bicornis*; c) male *O. cornuta*; d) female *O. cornuta*. For clothianidin survival by sex and species is shown as e) male *O. bicornis*; f) female *O. bicornis*; g) male *O. cornuta*; h) female *O. cornuta*.

that this is approximately constant over time, continuing to rise slowly from the start of the experiment. The high rates of survival in both *Osmia* species and sexes, even for the extended exposure times, indicates the robustness of the bioassay method for identifying chemical impacts on survival in time. Whilst ultimately the experiment ran up to 696 h exposure, only the sub-set of survival data up to 480 h was used for data analysis, as beyond this time control survivorship dropped below 80–90 %, impacting the reliability of the DEBtox stochastic survival model fitting and providing only limited additional insight.

3.2. Species and sex specific toxicity for dimethoate over time

Exposure to dimethoate resulted in time dependent effects on survival for both species and sexes (Fig. 1, a-d). The survival models fitted

to the different sets of time-censored dimethoate time-series survival data indicated mainly only small variation in the NECs predicted across the two species and sexes. NEC values were consistently lower for *O. bicornis* (Table 2; range 0–0.17 mg/L) than *O. cornuta* (range 0.47–2.57 mg/L). Consistent with this pattern, the NEC estimated for the full dataset up to 480 h exposure indicated a lower value for each sex for *O. bicornis* (females 0.001 mg/L, males 0.02 mg/L) than for *O. cornuta* (females 0.59 mg/L, males 1.01 mg/L). Elimination rates ranged across the time points 96 h - 480 h from 0.14 to 0.0011 h^{-1}. Rates were different between the species and sexes, thus, the values found of 0.14–0.10 h^{-1} for the *O. bicornis* females were higher than those for *O. cornuta* females (0.0011–0.0014 h^{-1}), *O. cornuta* males (0.012–0.004 h^{-1}), and *O. bicornis* males (from 240 h 0.0012–0.011 h^{-1}) (Table 2). The Killing rate (Kr) ranged from 0.0015 to 0.032 mg/h and was higher for *O. cornuta* females than for males and for both sexes of *O. bicornis* (Table 2).

Both species showed similar temporal responses to dimethoate exposure when effects on survival are expressed as a time series of LC_{50} values calculated from the fitted model parameters (Fig. 2a). Modelled LC_{50} values for the censored time-series datasets for 96 h ranged from 6.7 to 11.3 mg/L between the species and sexes (lowest LC_{50} value of 6.7 mg/L for *O. bicornis* male, highest LC_{50} value of 11.3 mg/L for *O. cornuta* female) and for the 480 h censored dataset from 0.5 to 1.8 mg/L (lowest LC_{50} value of 0.5 mg/L *O. bicornis* male, highest LC_{50} value of 1.8 mg/L for *O. cornuta* female; Table 2). The 96 h LC_{50} values for female and male *O. bicornis* of 7.0 and 6.7 mg/L were consistent (within 2 fold) of our previously reported LC_{50} values for *O. bicornis* (sexes combined) of 3.68 mg/L indicating the repeatability of the assay method and consistency of the responses to similar exposure concentrations used in previous toxicity studies with *O. bicornis* (Heard et al., 2017; Robinson et al., 2017). The LC_{50} values were highly time dependent with the values at 96 h being 3–4 fold lower than those at 48 h (Table 2; Fig. 2a) and values after 480 h being $\geq$ 12 fold lower. By 240 h, the LC_{50} values approached the incipient value calculated for the full exposure duration of 480 h, being within a factor of two of this final value for both sexes of *O. cornuta* and *O. bicornis* females and a factor of three for *O. bicornis* males. The LC_{50} values for *O. bicornis* were always lower than those for *O. cornuta* at any given time point, independent of sex. The magnitude of this difference increased for censored data for longer exposure times (48 h: R = 0.79: *O. bicornis* female / *O. cornuta*

Table 2
DEBtox parameter values and calculated LC_{50}s for model fits for the effects of dimethoate and clothianidin on survival over time for *Osmia cornuta* and *Osmia bicornis*. Fitted DEBtox parameters were used to calculate LC_{50} values for standard test duration of time of 48 and 96 h and three extended exposure times of 240, 384 and 480 h to determine species and sex specific patterns of mortality.

Bee species	Chemical	Time (h)	Blank killing rate[a] (h^{-1})	No Effect Concentration[b] (mg/L)	Elimination rate[c] (h^{-1})	Killing rate[d] (mg/h)	LC_{50} (mg/L)
O. bicornis ♀	Dimethoate	48	-	-	-	-	24.1
		96	0.0003	0	0.14	0.0015	7.0
		240	0.0003	0	0.11	0.0019	1.7
		384	0.0003	0	0.10	0.0021	0.9
		480	0.0004	0.001	0.10	0.0019	0.7
O. bicornis ♂	Dimethoate	48	-	-	-	-	24.6
		96	-	-	-	-	6.7
		240	0.0003	0.12	0.0012	0.032	1.4
		384	0.0005	0.17	0.0053	0.0054	0.7
		480	0.0007	0.02	0.011	0.0027	0.5
O. cornuta ♀	Dimethoate	48	0.0007	1	0.001	0.03	30.4
		96	0.0004	1.1	0.0011	0.03	11.3
		240	0.0004	0.47	0.0011	0.03	3.6
		384	0.0009	0.58	0.0014	0.03	2.2
		480	0.0011	0.59	0.0014	0.03	1.8
O. cornuta ♂	Dimethoate	48	-	-	-	-	25.1
		96	0.0005	2.57	0.012	0.010	9.2
		240	0.0006	1.06	0.015	0.003	3.1
		384	0.0012	0.87	0.004	0.011	2.1
		480	0.0012	1.01	0.004	0.011	1.7
Bee species	Chemical	Time (h)	Blank killing rate[a] (h^{-1})	No Effect Concentration[b] (mg/L)	Elimination rate[c] (h^{-1})	Killing rate[d] (mg/h)	LC_{50} (mg/L)
O. bicornis ♀	Clothianidin	48	0.0005	0.05	2	3.01E−06	0.0549
		96	0.0007	0.048	2	2.95E−06	0.0365
		240	0.0006	0.05	2	3.01E−06	0.0256
		384	0.0012	0.05	2	3.01E−06	0.0228
		480	0.0012	0.051	2	3.01E−06	0.0219
O. bicornis ♂	Clothianidin	48	0.0012	0.051	0.18	1.08E−07	0.257
		96	0.0011	0.058	0.20	1.02E−07	0.133
		240	0.0004	0.047	0.22	6.25E−08	0.064
		384	0.0006	0.006	0.14	7.38E−08	0.0478
		480	0.0007	0.008	0.12	6.82E−08	0.0423
O. cornuta ♀	Clothianidin	48	0.0012	0.013	2	2.50E−06	0.0199
		96	0.0012	0.011	2	1.99E−06	0.0181
		240	0.0012	0.016	2	4.26E−06	0.0171
		384	0.0006	0.016	2	4.26E−06	0.0168
		480	0.0009	0.016	2	4.26E−06	0.0167
O. cornuta ♂	Clothianidin	48	0.0017	0.044	2	5.34E−07	0.0917
		96	0.0017	0.046	2	4.66E−07	0.0567
		240	0.0017	0.049	2	4.94E−07	0.0358
		384	0.0017	0.045	2	4.77E−07	0.0306
		480	0.0017	0.045	2	4.71E−07	0.0289

- indicates that a 50 % effect was not reached at a time point so no value
[a] the blank killing rate is a measure of the rate of background mortality in a population not subject to exposure
[b] the No Effect Concentration (NEC) is a time-independent toxicological threshold below which no effects occur even over infinite exposure time
[c] the elimination rate is a rate parameter determining when the equilibrium between internal and external concentration is reached in time; Ke was fixed at value of 2
[d] the killing rate is the toxic potency of the compound (once the NEC is exceeded) expressed in relation to the environmental concentration and time

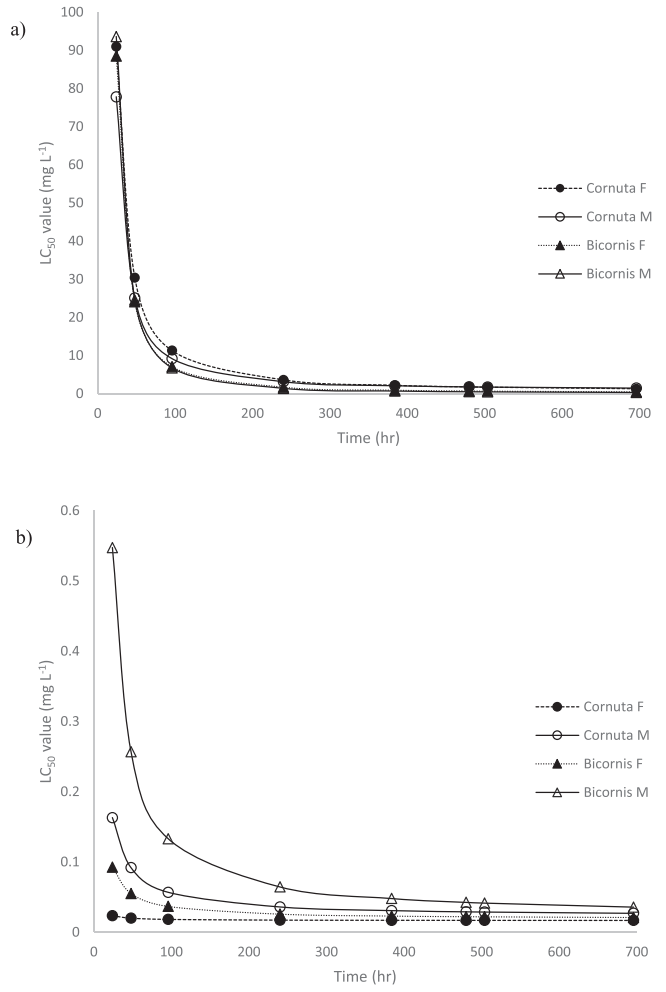

Fig. 2. DEBtox calculated LC_{50} values over time for *Osmia cornuta* and *Osmia bicornis* adult bees exposed orally to a) dimethoate and b) clothianidin. Calculated values are shown for key time points of 24, 48, 96, 240, 384, 480, 504 and 696 h.

female, R = 0.98: *O. bicornis* male / *O. cornuta* male; 480 h: R= 0.28: *O. bicornis* male / *O. cornuta* male, 480 h: R = 0.39: *O. bicornis* female / *O. cornuta* female). The finding of both lower NECs and associated LC_{50} values indicated a consistently greater sensitivity for *O. bicornis* compared to *O. cornuta* for dimethoate.

3.3. Species and sex specific toxicity for clothianidin over time

Exposure to clothianidin also resulted in a time dependent effect on survival (Fig. 1, e-h). Clothianidin was at least an order of magnitude more toxic than dimethoate in both species and for both sexes, as indicated by both the lower NECs (0.006–0.058 mg/L) and survival module fit derived LC_{50} values (Table 2; Fig. 2b). The patterns of change of the LC_{50} values in time indicated a greater divergence for the effects of clothianidin between species and sexes than for dimethoate (Fig. 2b). NEC values indicated a higher sensitivity for *O. cornuta* females (range 0.011–0.016 mg/L) than for *O. cornuta* males (range 0.044–0.049 mg/L) or for both females (range 0.048–0.051 mg/L) and males of *O. bicornis* (0.047–0.051 mg/L, with the exception of two lower NEC for the *O. bicornis* males calculated using censored data for 384 and 480 h exposure) (Table 2). Elimination rates estimated from the survival module DEBtox fits for the censored time series data for clothianidin were higher than for dimethoate for all species and sexes (clothianidin 0.12–2 h^{-1}; dimethoate 0.001–0.14 h^{-1}) indicating a more rapid potential metabolism for the neonicotinoid compared to the organophosphate. The killing rate values for clothianidin were lower than those for dimethoate for both *O. cornuta* and *O. bicornis*, indicating that once the NEC is exceeded, mortality occurred at a slower rate for clothianidin than for dimethoate.

At 48 h, the DEB survival module estimated LC_{50} values were lower in *O. cornuta* than for *O. bicornis* and were higher for males than females of both species (Table 2, Fig. 2b). Greatest differences in sensitivity were predicted for shorter duration exposures (Fig. 3). Thus at 96 h, ratios values for sex and species O. *bicornis* / *O. cornuta* comparisons are R= 2.35 males and R= 2.02 females, while these values are R= 1.46 males and R= 1.31 females after 480 h exposure.

Model predicted LC_{50} values for the two sexes converged similarly over time for the two species. Thus, after 48 h exposure R= 0.207 for *O. cornuta* females / *O. cornuta* males and R= 0.214 for *O. bicornis* female / *O. bicornis* males and after 240 h R= 0.578 for *O. cornuta* females / *O. cornuta* males and R= 0.518 for *O. bicornis* females / *O. bicornis* males. The calculated LC_{50} values for *O. bicornis* females at 96 h and 240 h using DEB analysis (0.0365 and 0.0256 mg/L) are similar to those reported for LC_{50} values for *O. bicornis* both sexes combined calculated using probit analysis by Heard et al. (2017) (0.031 and 0.029 mg/L respectively. The exception was for the male *O. bicornis* where current values of 0.133 mg/L at 96 h were four fold higher than those previously reported and there was also a two-fold difference at 240 h. These relatively small-scale differences indicate high assay repeatability, although the extent of agreement is dependent on the sex of the exposed bees.

4. Discussion

For the first time our study demonstrates a standardised, and robust novel test method that enables accurate quantification of chronic chemical exposure effects for two *Osmia* solitary bee species over the near full adult life span under laboratory conditions. This greatly extends previous reported results and allows a fuller exploration of time course toxicology effects on important functional species, with robust interpretation including through DEBtox survival modelling.

A critical step in the test, is to exclude non-feeding, predominantly

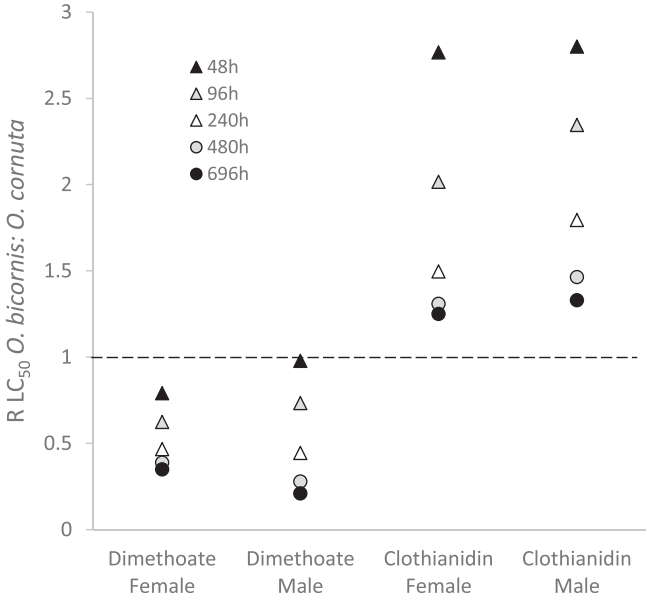

Fig. 3. Distribution of ratios of DEBtox LC_{50} values for *Osmia cornuta* and *Osmia bicornis* adult bees (*O. cornuta*: *O. bicornis*), at different time points post-exposure orally to clothianidin and dimethoate. Data are given as ratios of the LC_{50} values. A ratio of 1 (dashed line) indicates that *O. cornuta* has the same sensitivity as *O. bicornis* to a chemical whilst values > 1 indicate higher sensitivity of *O. cornuta* compared to *O. bicornis* and ratios < 1 indicate that *O. bicornis* has higher sensitivity than *O. cornuta*.

female, bees from experiments (after initially providing food for two days), in addition to supplying food throughout the duration of exposure. We demonstrate that tests are extended beyond the suggested chronic testing period of 240 h, with < 20 % control mortality occurring up to 480 h (20 days) of exposure (survival in controls <10 % in all species and sexes, except *O. cornuta* males which had <20 % survival at 240 h). This is within the mean lifespan recorded for *Osmia* female bees of 16–30 days (Azpiazu et al., 2023a and references therein; Bosch and Vicens, 2006). Generally, control mortality in experiments with solitary bees has been recorded in the region of 25 %, which is currently considered an 'acceptable' level (e.g., Mokkapati et al., 2022). Guidance for laboratory chronic 10 day feeding exposure tests in *Apis mellifera* (OECD, 2017) includes validity criteria that requires mortality of ≤ 15 % in control bees. Other laboratory feeding tests using the 'petal method' have recorded control mortality in *O. bicornis* female bees as low 15 % after 10 days exposure (Azpiazu et al., 2023a), but by 20 days in these tests, the control mortality was > 60 % in large cages and > 80 % in small cages. Our bioassay method, thus, significantly improves upon existing methods. By extending exposure time beyond the current 48 h and 96 h, we demonstrate an improved indication of inherent sensitivity in solitary bees under laboratory conditions, as indicated by the NEC parameter within the toxicokinetic and toxicodynamic survival model, and by conventional assessment by determining near incipient LC_{50} values.

The time dependence of observed toxicity, as described by the LC_{50} statistic from survival model parameters, has been recognised in studies with other species and for different endpoints (He et al., 2017; Jager and Kooijman, 2009). Indeed, a reduction in LCx values over time is recognised as a general principle in ecotoxicology (Kooijman and Bedaux, 1996; Sprague, 1969) and in this respect, our study is consistent with previous work with honeybees *A. mellifera* and solitary bees (Heard et al., 2017; Hesketh et al., 2016; Simon-Delso et al., 2018). The fact that LCx values are exposure time dependent has been identified as a possible reason to censure the use of such values for risk assessment (Jager, 2011). The expectation is that LCx values could be replaced, or at least augmented, by time independent values such as the NEC that are derived from the full time-series effect data using toxicokinetic/toxicodynamic modelling, such as the DEBtox survival modelling approach used here. A key property of the NEC is that it is derived using all the data points across the course of the experiment. Thus, use of the NEC value avoids comparative sensitivity being described based on the concentration response data for a single time point e.g., acute mortality at an end point of 48 h or 96 h. This use of all data means that NEC values are often more robust. In this study, LC_{50} values at 48 h suggest that *O. bicornis* females may be up to 5 fold more sensitive to clothianidin than males. However, the NEC values derived from the full dataset indicate *O. bicornis* males and females actually have similar inherent sensitivity. Thus, any differences suggested in sensitivity for males and females seen at any individual time-point (i.e. 48 h), is a consequence of differences in the time course development of the effects over the exposure.

In field and under glasshouse conditions, adult *Osmia* species have a short lifespan of 16–30 days (Bosch and Vicens, 2006; Mokkapati et al., 2022), during which they may be exposed to pesticide contaminated materials. This may be through ingestion, for example, of contaminated nectar and pollen whilst feeding or provisioning a nest, or through contact including on plant surfaces, contaminated plant material when cutting or masticating leaves or via soil collected for nest building. Through such activities, adult solitary bees may potentially undergo prolonged, chronic exposure to topical foliar and systemic pesticides. Hazard assessments for pesticides should be designed to assess the effects of these realistic continuous exposure scenarios, recognising the potential for individuals to be exposed to pesticides through pollen and nectar in addition to contact exposure over the adult life-span (Kopit and Pitts-Singer, 2018; EFSA, 2012). Currently, there are no standard test methods available for chronic toxicity testing the effects of prolonged exposure in solitary bees. Here we clearly demonstrate the extent to which short-term toxicity tests underestimate longer term effects; for example, the effects of exposure to dimethoate when expressed as the conventional LC_{50} approached 50 fold decrease from short- (48 h) to chronic (408 h) exposure and clothianidin showed a 6 fold decrease over the same time frame. On this basis of the time dependence of toxicity, we suggest that pesticides toxicity testing should be extended beyond the current 48 h tests to at least 10 days to reliably capture a clear picture of the cause of toxicity.

The magnitude of variations in species sensitivity, which at longer timescales (240 h or longer) differ no greater than four-fold, are largely consistent with results of a previous comparative study of the toxicity of 6 chemicals, including dimethoate and clothianidin to three bee species namely *A. mellifera*, *B. terrestris* and *O. bicornis* (Heard et al., 2017). In a meta-analysis of the comparative sensitivity of other bee species to *A. mellifera*, Arena and Sgolastra (2014) found high variability of sensitivity among bee species (R from 0.001 to 2085.7), although in 95 % of cases the sensitivity ratio was < 10. In a comparative study of *A. mellifera* and *O. bicornis* in acute toxicity tests (48 h exposure), Uhl et al. (2019) demonstrated *O. bicornis* was less sensitive than *A. mellifera* and reported sensitivity ratios in the range < 0.1–18.0, noting that an endpoint assessment factor of 10 achieved a protective level for 87 % of all evaluated chemicals. The differences found in our study in sensitivity for *O. bicornis* and *O. cornuta* for dimethoate and clothianidin lie well within this range. Within the range of variation seen, time consistent differences in the order of comparative sensitivity to the two chemicals between the two species were found. For dimethoate, there was consistently a greater sensitivity for *O. bicornis* compared to *O. cornuta*, with lower NEC and modelled LC_{50} values up to 480 h exposure. In contrast, clothianidin was more toxic overall than dimethoate and there was a greater divergence of effects. Female *O. cornuta* were consistently more sensitive than male *O. cornuta* and compared to both sexes of *O. bicornis*. The chemical specific nature of comparative sensitivity indicates that simple trait differences, such as the greater body size of *O. cornuta* than *O. bicornis* cannot alone explain differences in sensitivity. Our study contributes further evidence to that already in the literature noting that toxicity responses and detoxification processes differ significantly between different bee families (Arena and Sgolastra, 2014; Phan et al., 2020).

For more detailed analysis of the underlying causes of sensitivity, valuable insights can be drawn from the toxicokinetic and toxicodynamic traits derived from the DEBtox model fits. For dimethoate for example, the lower elimination rates found for *O. bicornis* compared to *O. cornuta* indicate a potentially lower rate of compound metabolism in *O. bicornis* which in turn may cause higher and more sustained internal exposure leading to greater effects (Ashauer et al., 2016). Physiological measurements relating to toxicokinetic traits such as assays for metabolising enzymes (e.g., cytochrome P450 activity) or uptake and elimination can be used to quantify these traits (Cedergreen et al., 2017; Rubach et al., 2010; Van Den Berg et al., 2019). For clothianidin, the killing rate for *O. cornuta* females exceeded that for any other group of tested bees. It may be important to understand how the target receptor is expressed in female *O. cornuta* and how the interaction of the insecticide with the receptor translates to effects within the adverse outcome pathways for insecticides (LaLone et al., 2017). Individual species traits linked to different toxicokinetic and toxicodynamic traits are likely to lead to differential species-specific sensitivity to chemicals (Li et al., 2020; Short et al., 2021).

Recent work on neonicotinoid and other pesticide effects on bees have highlighted that direct effects on survival only provide a partial picture of the consequences of exposure in the field. The impacts of insecticides on fitness can further be realised through effects on other population relevant traits including egg production, larval development, and pupal survival. Further, it has been widely reported that insecticides acting on the nervous system of bees can affect traits related to learning and navigation that are critical to maintain colony structure and dynamics among eusocial species (Gill et al., 2012; Klein et al., 2017; Smith

et al., 2016). Solitary mason bees such as *Osmia* sp. also display complex behaviours relating to nest building and provisioning. During this time female bees navigate to and from nest locations to provision supplies for the laid eggs and soil to separate the brood cells. Further studies to investigate effects of insecticide exposure beyond measurements of survival are, therefore, relevant, and important for informing the protection of solitary bees.

A number of models exist that integrate effects on multiple traits to predict chemical impacts of different aspects of colony performance in eusocial bees (Becher et al., 2014). These models incorporate chemical effects not only on life-cycle parameters, but also on behavioural traits (e.g., provisioning) relevant to colony performance. To develop similar predictive ecotoxicological models for solitary bees will require not just the measurement of effects on relevant key vital rates, but also different model structures that capture their solitary rather than social nature. Full DEBtox models have a history of value in assessing chemical impacts on multiple demographic traits (Jager and Zimmer, 2012). When coupled with individual based models, the models developed from DEBtox theory have the potential to make a significant contribution to understanding how chemicals impact solitary bee populations over realistic exposure times. DEBtox model and more broadly TK-TD models for survival allow for a better comparison of species sensitivity. Lifespan based adult tests can provide important parameterisation information for such models, that in future should be augmented by measurement for reproductive and behavioural effects.

The method described here, demonstrates valid control survival, and has the potential for further development to establish a robust testing approach for assessing risks to solitary bees from plant protection products. We recommend this method as a significant improvement over current chronic assay tests, especially due to its ability to exclude female bees that fail to feed during testing and the extended exposure duration. Indeed, here we demonstrated that it is feasible to run tests for longer (480 hr) exposures. Importantly, the shift in relative sensitivity over time indicates that a short-term test for clothianidin would erroneously identify *O. cornuta* as substantively more sensitive than *O. bicornis*, when in fact the two species have rather similar sensitivities after extended exposure. On this basis we can recommend that chronic tests could be extended to 20 days. However, given the average lifespan of *O. bicornis* is in the region of 19 days (Mokkapati et al., 2022), exposure over this time would likely impact on survival of control bees in any tests.

In conclusion, our studies provide valuable first evidence of chronic toxicity data for the near life span of two species of solitary bees under laboratory conditions, supporting their inclusion in the recently revised EFSA guidance for risk assessment on bees (EFSA, 2023). We present a repeatable, robust chronic toxicity test assay method with valid control survival which may potentially be developed further. We propose that this method is a significant improvement on current chronic assay tests, particularly through the experimental process of removing female bees that fail to feed from cohorts tested and through the extended exposure duration of 240 h or potentially even to near full lifespan. Our results do not suggest one species of solitary *Osmia* bee species as inherently more sensitive or amenable for testing than the other. Indeed both species proved suitable for chronic testing to understand the impact of plant protection products on solitary bees over extended exposure times.

CRediT authorship contribution statement

Spurgeon David J: Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Heard Matthew S:** Writing – review & editing, Funding acquisition, Formal analysis, Conceptualization. **Lahive Elma:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Robinson Alexander G:** Writing – review & editing, Investigation. **Hesketh Helen:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Baas Jan:** Writing – review & editing, Writing – original draft, Formal analysis.

Declaration of Competing Interest

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

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Data availability

Data will be made available on request.

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