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Effects of Life Stage on the Sensitivity of *Folsomia candida* to Four Pesticides

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Abstract: The registration of pesticides in the European Union requires the assessment of the toxicity of active substances to soil invertebrates. The most commonly tested soil microarthropod species is *Folsomia candida* (Collembola), for which toxicity tests usually start with juveniles and determine survival and reproduction after 28 days of exposure, following Organisation for Economic Co-Operation and Development test guideline 232. Test duration may be shortened to 21 days by starting exposures with adult animals. The toxicity of chemicals can, however, vary significantly between different life stages (e.g., juveniles or adults) of the same species. In the present study, we assessed the toxicity of four active substances (cyproconazole, teflubenzuron, imidacloprid, and thiacloprid) to *F. candida* aged approximately 10 days (juveniles) and 20 days (adults) at the beginning of the tests. Tests were performed in LUFA 2.2 standard soil at 20 ± 2 °C, and effect concentration (ECx) values compared using likelihood ratio tests. The tests lasted 21 days for older springtails and 28 days for the younger ones. Life stage did affect the sensitivity of the springtails, with the survival and reproduction of younger animals being a factor of 2–6.5 more sensitive to the insecticides but not to the fungicide. For teflubenzuron and imidacloprid, the EC50 for younger springtails were 0.025 and 0.111 mg a.s. kg^{-1} $soil_{dw}$, respectively, and for adults 0.048 and 0.264 mg a.s. kg^{-1} $soil_{dw}$, respectively. For the younger animals the median lethal concentration values for teflubenzuron, imidacloprid, and thiacloprid were 0.353, 0.224, and 1.02 mg a.s. kg^{-1} $soil_{dw}$, respectively, and 0.571, 0.446, and 6.91 mg a.s. kg^{-1} $soil_{dw}$, respectively, for older animals. We discuss the implication of these differences for the risk assessment of pesticides to soil arthropods. *Environ Toxicol Chem* 2023;00:1–9. © 2023 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Survival; Reproduction; Neonicotinoids; Teflubenzuron; Cyproconazole

INTRODUCTION

Ecotoxicological testing with soil invertebrates is necessary to assess the risks posed by pesticides to the structure and functioning of soil ecosystems. For that purpose, a number of internationally accepted standard guidelines have been developed for tests that cover different soil invertebrates (e.g., earthworms, predatory mites, Collembola) and endpoints (survival, reproduction, behavior; Organisation for Economic Co-operation and Development [OECD], 2004, 2008, 2016, respectively). These guidelines ensure the reproducibility and reliability of the outcomes of the toxicity tests performed. In the European Union, the information provided by such tests is a requirement for the registration of new plant protection products (Ockleford et al., 2017). According to these regulations, for all plant protection products toxicity data have to be provided for earthworms, springtails (Collembola), and predatory mites (Ockleford et al., 2017).

The standard OECD test guideline 232 is designed to determine the toxicity of chemicals to collembolans in soil (OECD, 2016). Most studies use the parthenogenetic species *Folsomia candida*. The tests are performed with age-synchronized 10–12-day-old juvenile animals. Because the animals become adult approximately 15–17 days at 20 °C (Snider, 1973), the test includes part of the juvenile period and sexual maturation as well as part of the adult phase in which the first clutches of eggs are produced. After 28 days, when the animals are 38–40 days old, the test ends and their survival and reproductive output are assessed. In this way, this guideline covers the effects of the chemical on the survival of the animals, their sexual maturation, and their

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reproductive performance, which is reflected by the number of offspring produced.

The sensitivity of animals to chemicals in ecotoxicological tests differs with their age at the start of the test (Huang et al., 2022; Muyssen & Janssen, 2007). de Lima e Silva et al. (2021) compared different species of springtails, including sexually reproducing species for which it was difficult to distinguish males and females in the juvenile phase. For that reason, they started all tests with adult animals. To limit the maximum number of juveniles produced, de Lima e Silva et al. (2021) reduced the test duration to only 21 days. For the effects of imidacloprid on the reproduction of *F. candida*, similar median effect concentration (EC50) values were found in tests started with 10–12- and 20–23-day-old animals, suggesting no difference in sensitivity. In another study with *F. candida*, however, the avoidance response of younger animals (4–5 days old) to sodium chloride contaminated soil was significantly higher than the older animals (10–12 days old) avoidance behavior EC50 (Gainer et al., 2019). In the same study, no differences were found between the avoidance behavior of the two life stages of *F. candida* to copper contaminated soils. Although it is generally assumed that younger life stages (smaller in body size) are more sensitive than older stages across a range of environments and species (Mohammed, 2013), this seems to vary depending on the stressor tested, the endpoint assessed, and the life stages considered in the study. In studies like the one mentioned above (de Lima e Silva et al., 2021), adjustment of the age of the tested animals may be required to enable comparison of the sensitivity to chemicals of different species of Collembola with different life cycles. The same species exposed to a certain chemical stressor in different life stages (that might differ by a few days, weeks or completely different stage of development) can respond differently to the same level of that stressor. According to the limited amount of data available, this change does not seem to be consistent for chemical effects on springtails.

To address this research gap, we investigated the differences in sensitivity of juvenile and adult life stages of *F. candida* to four pesticides. The primary goal was to assess if the adaptations suggested by previous studies, which involve starting the tests with adult springtails, could capture the most sensitive stages of the life cycle. Specifically, we focused on comparing the toxicity values for reproduction and survival obtained from reproduction tests conducted according to the standard protocol (OECD 232), which starts with juveniles aged 10–12 days, with adaptations suggested by previous studies that propose starting the tests with adult animals. The substances tested included the pure active substances of one fungicide (cyproconazole) and three insecticides (teflubenzuron, imidacloprid, and thiacloprid). These pesticides have distinct modes of action and may impact reproduction and survival of *F. candida* through direct and indirect mechanisms. Teflubenzuron disrupts hormonal regulation and inhibits chitin synthesis in arthropods (Schmid et al., 2021). Imidacloprid and thiacloprid are neonicotinoids that disturb the nervous system of insects (de Lima e Silva et al., 2021; van Gestel et al., 2017). Cyproconazole inhibits fungal sterol synthesis and is expected to mainly have indirect effects on *F. candida* (Cuco et al., 2017; Zhang et al., 2014).

It was hypothesized that younger springtails would be more sensitive than older ones. Different traits could be related to individual sensitivity. For instance, a smaller body size results in a bigger surface-to-volume ratio, increasing the surface of exposure, and body size also relates to biotransformation capacity, which may result in higher or lower detoxification of the pesticides. In addition, sexual maturation and age at first reproduction of the younger animals may be delayed, also influencing their reproductive output. It is important to note that the difference in exposure duration between the younger and older springtails does not allow the effects of life stage to be isolated from the effects of exposure duration. However, we monitored the numbers of individuals at the end of the experiments, allowing us to account for any potential relative differences in survival and reproductive output between the different life stages. The results of the present study can be used to identify the most appropriate age criteria for standard toxicity testing, facilitating the risk assessment of chemicals in Collembola.

MATERIALS AND METHODS

Test animals

Springtails of the species *Folsomia candida* (Collembola: Isotomidae) obtained from laboratory cultures maintained at the Vrije Universiteit Amsterdam were used for all the experiments. The animals were cultured on Plaster of Paris mixed with activated charcoal 10:1 (w:w). The cultures were maintained at 20 ± 2 °C, 16:8-h light:dark photoperiod, and 75% relative air humidity. The animals were fed with granulated baker's yeast (Algist Bruggeman N.V.) ad libitum, and the plaster was kept moist with demineralized water. For each substance tested, two batches of age-synchronized animals were prepared: the experiment started with 20–22-day-old animals (referred to as "adults" or "older animals") or with 10–12-day-old animals ("juveniles" or "younger animals").

Test soil

All toxicity tests used standard LUFA 2.2 standard soil (LUFA Speyer). According to the supplier, this is a sandy loam soil, having approximately 1.6% organic carbon, a maximum water holding capacity (WHC) of approximately 45%, and pH in $CaCl_2$ ranging from 5.0 to 6.0. The WHC and the pH (0.01 M $CaCl_2$) were determined following International Organization for Standardization guidelines (ISO, 2019, 2021).

Test chemicals and spiking procedure

Four pesticides were tested as pure active substances. Cyproconazole (98.24%) was purchased from MCE MedChemExpress, teflubenzuron (analytical standard) from Sigma-Aldrich, and imidacloprid and thiacloprid, both >98% purity, from Bayer Crop Science. For cyproconazole and

teflubenzuron the range of concentrations tested was prepared in pure acetone (Supporting Information, Table S1). To spike the soil, 10% of the total amount of soil required for each test concentration was mixed with the stock solution diluted to obtain the required concentration; the soil was fully saturated with the acetone solution. The jars were sealed and left overnight to equilibrate at room temperature. Subsequently, the jars were opened and incubated at room temperature to allow full evaporation of the acetone. The dry soil was then mixed with the remaining amount of dry soil, moistened with demineralized water to 50% of the WHC, and mixed again. The treatments with imidacloprid and thiacloprid were prepared in milli-Q water. The stock solutions were mixed with dry soil to reach the nominal concentrations (Supporting Information, Table S1) and at the same time reach a moisture content corresponding with 50% of its WHC. The stock solution of thiacloprid was prepared with 3% of acetone (v/v). A control with demineralized water and a solvent control with pure acetone (except for imidacloprid) were prepared following the same procedure. The choice of different solvent concentration for the different pesticides was made to ensure appropriate solubility in the stock solutions prepared and facilitate the mixing of these substances with the dry soil. All the treatments were individually prepared and then allocated to six replicate test units.

Survival and reproduction tests

The toxicity tests were performed according to the OECD guideline 232 (OECD, 2016) with protocol adjustments according to de Lima e Silva et al. (2021). At the beginning of the test, 10 age-synchronized animals were placed in glass jars filled with approximately 36 g of moist test soil and some grains of dry granulated baker's yeast were spread over the soil surface. For each treatment, six replicates were prepared, one of which was used for assessing the physicochemical properties of the test soil and received no animals. The test jars were randomly placed into a 20 °C climate room with a 16:8-h light: dark cycle and 75% relative air humidity. Weekly, demineralized water was added to replenish moisture loss (assessed by weighing the test jars) and approximately 0.015 g of baker's yeast was added for food. Any visible fungi hyphae and/or plant shoots were removed with tweezers. Less food was added to the jars when there was leftover yeast to prevent excessive fungal growth. After 21 days for older springtails and 28 days for the younger ones, the tests were ended. The soil from the jars containing animals was washed with approximately 100 ml of demineralized water into a glass beaker until no soil remained in the test jars. This mixture was then carefully stirred with a spatula to allow the floatation of the surviving adults and offspring. The animals on the water surface were photographed and manually counted using ImageJ. The distinction between the surviving animals added in the beginning of the test and the produced offspring was done visually based on the obvious size differences between these two groups.

Pesticide quantification

The modified Quick Easy Cheap Effective Rugged Safe (QuEChERS) method was applied for the quantification of cyproconazole and teflubenzuron according to Jiang et al. (2018). Quantitative analysis of teflubenzuron and cyproconazole was performed using a high-performance liquid chromatography elute system coupled with an Evoq triple quadrupole mass selective detector (Bruker) using an Omega Luna 1.6 µm PS C18 (50 × 2.1 mm) column for separation. The mobile phases of Milli-Q water with 0.4 mM NH_4F and 100% acetonitrile were selected for the analysis. Teflubenzuron was analyzed in negative electrospray ionization mode and cyproconazole in positive mode with the following settings: IonSpray voltage 4500 V, cone temperature 350 °C, cone gas flow 20, probe gas flow 40, nebulizer gas flow 60 (the parameters are displayed in the units of the system). Validation studies were performed to evaluate the extraction method (see Supporting Information, Tables S2 and S3). The limits of detection (LOD) and quantification were 0.012 and 0.04 ng g^{-1} $soil_{dw}$, respectively, for teflubenzuron, and 0.009 and 0.03 ng g^{-1} $soil_{dw}$, respectively, for cyproconazole. The average (±SD, $n = 8$) extraction recoveries were 80.6% ± 2.3% for teflubenzuron and 88.4% ± 5.3% for cyproconazole. For imidacloprid and thiacloprid a composite sample from each treatment was collected at the beginning and end of the experiment and stored at −20 °C. The quantification of active substances in these samples was done by Groen Agro Control (Delfgauw, the Netherlands) following a certified protocol. The LOD for both compounds was 0.01 mg a.s. kg^{-1} $soil_{dw}$. Due to resource limitations, we were not able to analyze all the samples individually. Instead, we selected specific samples for quantification of the active ingredient in the soil (control and treatment around the ECx/lethal concentration [LCx] values estimated). The results for the quantification of the four pesticides can be found in Supporting Information, Table S4. The half-lives (DT50) of imidacloprid and thiacloprid were estimated from the measured values, assuming first-order kinetics. For cyproconazole and teflubenzuron, the DT50 were found in the literature. The equation

$$C = C_0 e^{-kt}$$

was fit to the data and DT50 was derived as $\ln(2)/k$. In this equation, C is the measured concentration in mg a.s. kg^{-1} $soil_{dw}$ at time t, C_0 is the measured concentration in mg a.s. kg^{-1} $soil_{dw}$ at time zero, and k is the degradation rate constant (day^{-1}).

Statistical analysis

All the statistical analyses were performed in the software R Ver 4.1.2. Concentration–response curves for survival and the numbers of offspring produced were fit using the package *drc* applying a general three-parameter dose–response model (Ritz & Streibig, 2005) and used to estimate EC10, EC50, LC10, and LC50 values and respective 95% confidence intervals (CIs). For cyproconazole and teflubenzuron, these calculations were

performed using measured concentrations in the soil at the end of the tests. For thiacloprid and imidacloprid nominal concentrations were converted to measured concentrations based on the average recovery of the pesticide in relation to nominal concentrations (as %) in the soil samples collected at the beginning of the experiments. The average percentage of the measured concentrations was then used to derive estimated actual concentrations, which were used to estimate LCx and Ecx values with 95% CIs using concentration–response models. To test the difference between the two life stages for each test compound, a likelihood-ratio test (LRT) was performed comparing the models for each life stage with a constrained model (constraining the models for each life stage to the same EC50, LC50 or the same slope). All the plots in this manuscript were generated with ggplot2 (Ver 3.4.2).

RESULTS

Pesticide quantification and physicochemical properties of the soil

The pH-$CaCl_2$ of the LUFA 2.2 soil used in the experiments was 5.36. The WHC_{max} of the soil was approximately 46%. The pH-$CaCl_2$ of the soils measured after the experiments ranged from 5.36 to 5.77 for all the pesticides tested, and there was no concentration-related difference in the pH values measured (Supporting Information, Table S5). The measured concentrations of active substances in soil at the end of the tests ranged between 20% and 120% of the nominal concentrations (Supporting Information, Table S4). Furthermore, no differences were observed between the measured concentrations in the soils from tests with different life stages. The DT50 values calculated for imidacloprid and thiacloprid can be found in Supporting Information, Table S4. No exposure concentration-related differences were found. The DT50 of imidacloprid varied between 51 and 87 days in the experiments that lasted 21 days. In the 28-day experiments, the DT50 was higher than 242 days. The DT50 estimated for thiacloprid varied from 7 to 10 days.

Control validity criteria

The toxicity tests performed met the validity criteria of OECD test guideline 232 (OECD, 2016). Control and solvent control survival ranged from 80% to 100%, with the exception of the thiacloprid water control where survival was only 28% (Supporting Information, Table S6). Because survival was high in the acetone control, this low survival in the thiacloprid water control is considered an artefact. The average number of juveniles produced in controls was always higher than 300. The coefficient of variance criteria was met in most cases

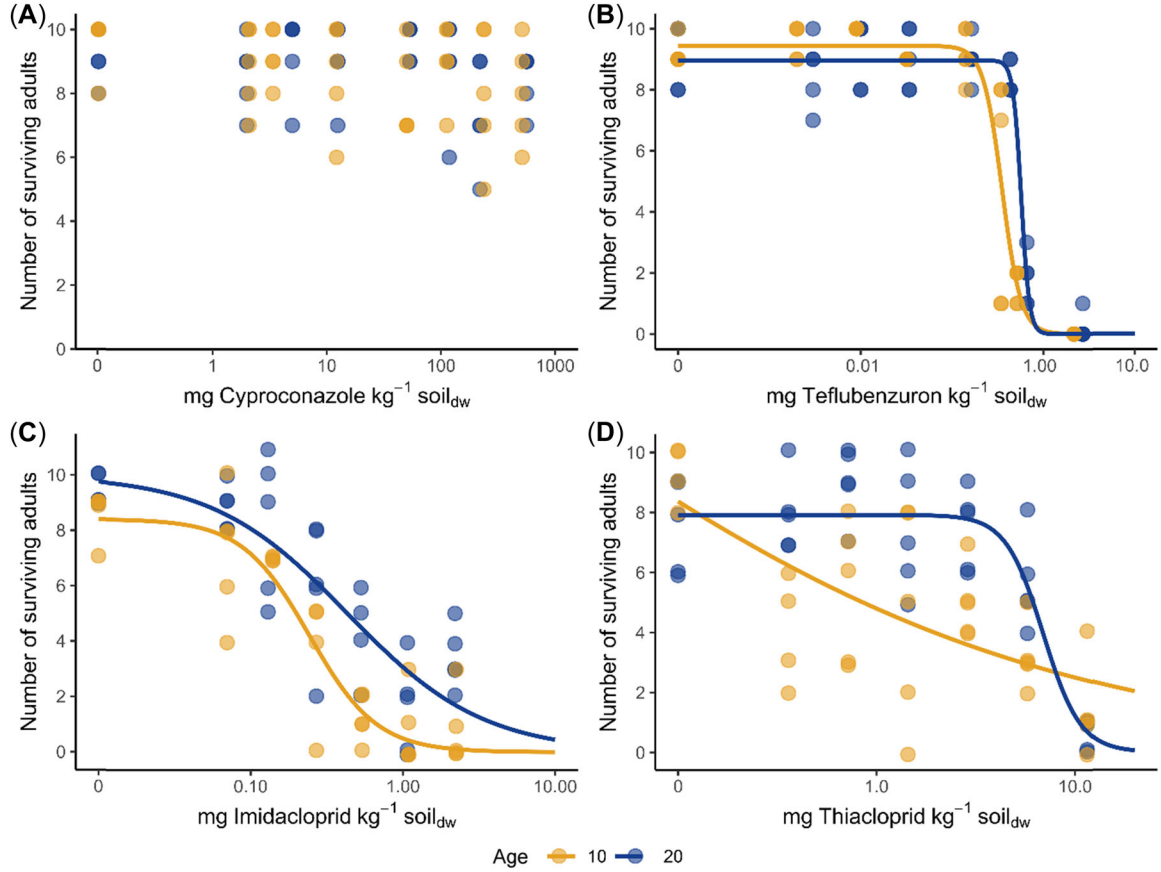

FIGURE 1: Concentration–response curves for the effect of cyproconazole (**A**), teflubenzuron (**B**), imidacloprid (**C**), and thiacloprid (**D**) on the number of surviving *Folsomia candida* aged 10–12 or 20–22 days exposed in LUFA 2.2 soil for 28 and 21 days, respectively. The individual points are the measured number of surviving animals in the replicate test jars and the curves are the fit of the logistic model to the data. dw = dry weight.

TABLE 1: Toxicity data for the mortality of *Folsomia candida* exposed to four different active substances in LUFA 2.2 soil

Active substance	Life stage	LC10 (95% CI)	LC50 (95% CI)	Sig	Slope (std. error)	Sig.
Cyproconazole	10	n.d.	n.d.	n.d.	0.216 (0.124)	0.43
	20	n.d.	n.d.		0.535 (0.715)	
Teflubenzuron	10	0.209 (0.146–0.272)[a]	0.353 (0.319–0.388) [a]	<0.00	4.19 (1.01)	0.01
	20	0.456 (0.373–0.539) [a]	0.571 (0.523–0.621)[a]		9.73 (2.52)	
Imidacloprid	10	0.079 (0.001–0.158[b]	0.224 (0.155–0.333)[b]	<0.05	1.95 (0.21)	0.07
	20	0.054 (−0.003–0.111)[b]	0.446 (0.248–0.672)[b]		1.02 (0.68)	
Thiacloprid	10	0.003 (−0.012–0.018)[b]	1.02 (−0.515–2.55)[b]	<0.05	0.372 (0.140)	<0.00
	20	4.29 (2.48–6.09)[b]	6.91 (5.46–8.379)[b]		4.60 (2.05)	

[a]Values were estimated based on measured concentrations of the active substances in the soil at the end of the experiment.
[b]Values were estimated from measured concentrations calculated based on the average recovery (%) at the beginning of each experiment.
Life stage stands for the age of the animals at the beginning of the test (in days). Sig stands for statistically significant differences between the median lethal concentrations (LC50s) of the two life stages for each active substance (a.s.). LC10 and LC50 are expressed in mg a.s. kg^{-1} $soil_{dw}$ and given with corresponding 95% confidence intervals (CIs).
n.d. = not determined.

(ranging from 9.4% to 27.0%), with exception of three out of four controls of the tests with thiacloprid.

Effects of survival and reproduction

Three out of the four active substances reduced springtail survival in a concentration-related manner within the range of concentrations tested (Figure 1); the exception was cyproconazole, where no significant effect on survival was seen. The LC10 and LC50 values for effects on springtail survival of the four tested substances are presented in Table 1. The LC50s for teflubenzuron, imidacloprid, and thiacloprid were significantly different ($\chi^2(1) = 44.7$, $p < 0.0001$; $\chi^2(1) = 5.17$, $p < 0.05$; $\chi^2(1) = 6.5$, $p < 0.05$; respectively) between the life stages. These differences show that 20–22-day-old springtails were significantly less sensitive in terms of LC50. The difference in LC50s was a factor of 1.6–6.5. The same difference was observed for LC10, except for thiacloprid where a higher sensitivity of the older animals was seen. The slopes differed significantly for teflubenzuron and thiacloprid ($\chi^2(1) = 6.78$, $p < 0.05$; $\chi^2(1) = 14.46$, $p < 0.01$; respectively). The difference in LC10s for imidacloprid and teflubenzuron was a factor of 0.6 and 2.2, respectively. For thiacloprid the difference was much bigger, probably because of the scatter in the data.

The exposure to the four substances resulted in concentration-related responses of springtail reproduction for both life stages tested (Figure 2). The toxicity data for the effects of the four substances on the reproduction of *F. candida* are listed in Table 2. The EC50 values for cyproconazole, teflubenzuron, and imidacloprid were significantly different for younger and older animals with $\chi^2(1) = 10.4$, $p < 0.001$, $\chi^2(1) = 4.9$, $p < 0.05$, and $\chi^2(1) = 16.2$, $p < 0.001$, respectively. The slopes of the concentration–response curves differed significantly for teflubenzuron and thiacloprid. For teflubenzuron and imidacloprid, younger animals were more sensitive (with EC10 and EC50 values being a factor of 2.0–3.0 lower), while for the fungicide the opposite was observed (EC10 and EC50 a factor of 2.0–3.0 higher). The EC50 values for the 10–12-day-old animals exposed to teflubenzuron and imidacloprid were 0.025 and 0.111 mg a.s. kg^{-1} $soil_{dw}$, respectively. For thiacloprid, age did not significantly influence springtail EC50 ($\chi^2(1) = 2.7$, $p = 0.10$).

DISCUSSION

Differences in sensitivity between life stages

The performance of younger invertebrates is expected to be more adversely affected by the exposure to chemicals than that of older life stages. In addition, the length of the exposure influences the toxicity outcomes of ecotoxicological tests. The adjustments done to the standard reproduction and survival tests performed with springtails imply reducing the time of exposure, which limits the full distinction between the effects caused by the life stage at which the animals are exposed and the time of exposure. However, a higher sensitivity of younger life stages has been observed for several species and contaminants, such as metals and pesticides. For instance, newly hatched neonates of *Daphnia magna* were more sensitive to copper than 7-day-old organisms (Muyssen & Janssen, 2007). Nevertheless, this is not always straightforward, and it varies depending on the species, chemical compound, and medium of exposure in which the toxicity parameter was assessed. For instance, in the same study by Muyssen and Janssen (2007) daphnid neonates were less or as sensitive to zinc as 7-day-old animals. Similarly, Gainer et al. (2019) showed that the avoidance by *F. candida*, *Enchytraeus crypticus*, and *Eisenia fetida* of copper, sodium chloride, and phenanthrene in soil was not consistently higher for young life stages compared with older ones. Juvenile earthworms showed less avoidance to copper-contaminated soil than adults, whilst *F. candida* with 14 and 21 days of age did not differ in their sensitivity to cadmium.

Teflubenzuron was most toxic to springtail reproduction. This pesticide disrupts hormonal regulation and inhibits chitin synthesis in arthropods, leading to alteration in the efficiency or rate of molting, reducing growth, reproduction, and ultimately survival (Schmid et al., 2021). Campiche et al. (2006) reported an EC50 for the effects of teflubenzuron on 10–12-day-old *F. candida* of 0.05 mg a.s. kg^{-1} $soil_{dw}$, which is in the same order of magnitude as the value we obtained in our study. When the toxicity test started with 10–12-day-old

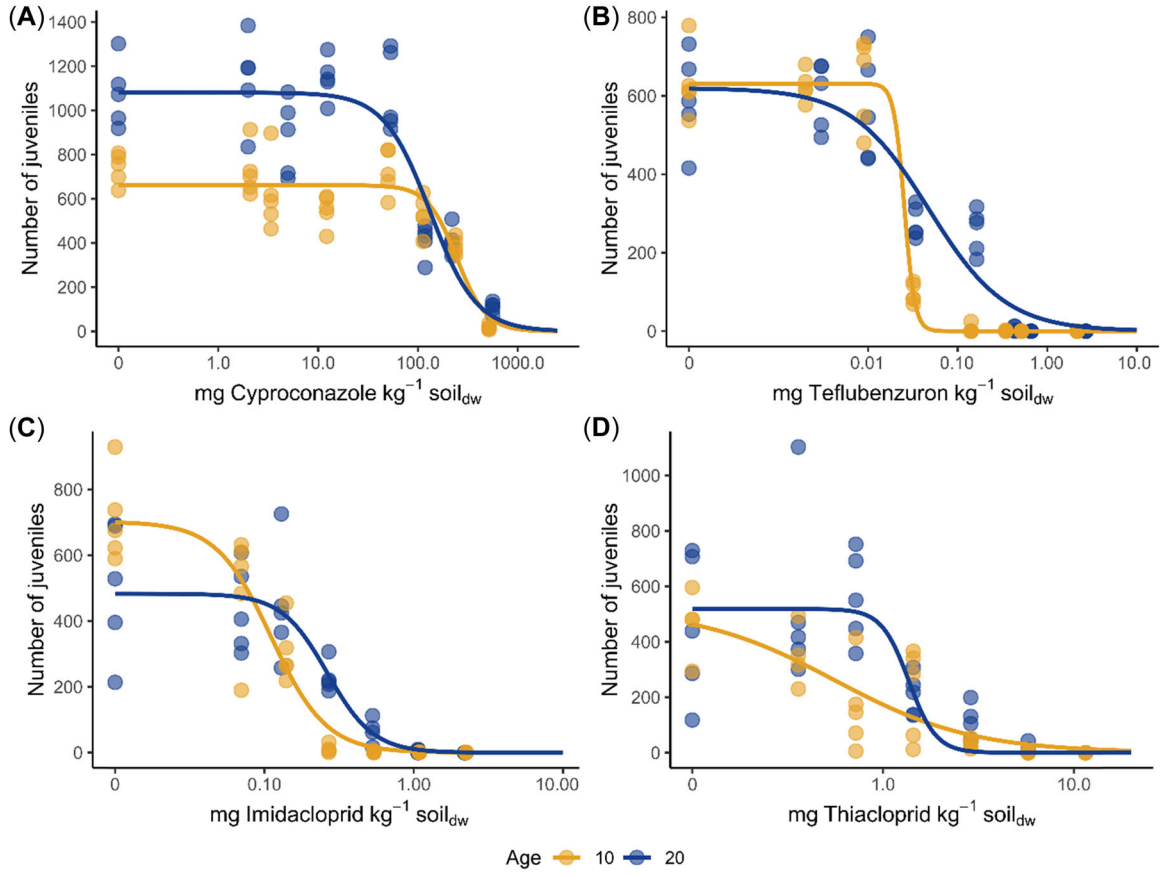

FIGURE 2: Concentration–response curves for the effect of cyproconazole (**A**), teflubenzuron (**B**), imidacloprid (**C**), and thiacloprid (**D**) on the reproduction of *Folsomia candida* aged 10–12 or 20–22 days exposed in LUFA 2.2 soil for 28 and 21 days, respectively. The individual points are the measured number of juveniles in the replicate test jars and the curves are the fit of the logistic model to the data. dw = dry weight.

animals, these individuals still had to mature and grow for approximately 1 week before reaching sexual maturity, that is, laying of the first eggs. Collembolans molt to grow and reproduce (Heming, 2018). Chitin production is essential for the composition of their cuticle and successful molting (Cohen, 2001). Teflubenzuron exposure was previously shown to reduce egg production and lower egg hatching success (Lee et al., 2019), therefore teflubenzuron may have delayed the sexual maturation of the younger animals, influencing their reproductive output. This might also explain the difference in slope of the concentration–response curve for the reproduction effect of teflubenzuron. The slope was much steeper for the juvenile animals, suggesting a more homogenous response for the juveniles compared with the adults, which may be explained by sexual maturation being the more sensitive endpoint.

Imidacloprid also was highly toxic to *F. candida*, as also shown by other studies, reporting EC50 values for reproduction ranging from 0.44 to 0.82 mg a.s. kg^{-1} soil$_{dw,}$ (Mabubu et al., 2017; van Gestel et al., 2017). This neonicotinoid disturbs the nervous system of insects by binding to nicotinic acetylcholine receptors. The same mechanism may affect *F. candida*. Huang et al. (2021)

TABLE 2: Toxicity data for the reproduction of *Folsomia candida* exposed to four different active substances in LUFA 2.2 soil

Active substance	Life stage	EC10 (95% CI)	EC50 (95% CI)	Sig	Slope (std. error)	Sig.
Cyproconazole	10	117 (29.2–204)[a]	245 (176–315) [a]	<0.00	2.95 (1.18)	0.23
	20	41.1 (23.4–58.9)[a]	133 (105–161)[a]		1.88 (0.331)	
Teflubenzuron	10	0.018 (−0.029–0.066)[a]	0.025 (−0.004–0.054)[a]	<0.05	6.92 (0.161)	<0.00
	20	0.006 (0.002–0.009) [a]	0.048 (0.029–0.068) [a]		1.02 (0.119)	
Imidacloprid	10	0.044 (0.025–0.067)[b]	0.111 (0.085–0.138)[b]	<0.00	2.37 (0.491)	0.67
	20	0.121 (0.032–0.211)[b]	0.264 (0.191–0.337)		2.82 (1.116)	
Thiacloprid	10	0.125 (−0.089–0.339)[b]	0.689 (0.142–1.24)[b]	0.10	1.287 (0.463)	0.04
	20	0.949 (−0.791–2.69)[b]	1.36 (1.11–1.61)[b]		6.055 (14.8)	

[a]Values were estimated based on measured concentrations of the active substances in the soil at the end of the experiment.
[b]Values were estimated from measured concentrations calculated based on the average recovery (%) at the beginning of each experiment.
Life stage stands for the age of the animals at the beginning of the test (in days). Sig stands for statistically significant differences between the median effect concentrations (EC50s) of the two life stages for each active substance (a.s.). EC10 and EC50 are expressed in mg a.s.kg^{-1} soil$_{dw}$ and given with corresponding 95% confidence intervals (CIs).

found that imidacloprid is rapidly metabolized into imidacloprid-olefin (IMI-ole) by the mayfly *Cloeon dipterum*. In this species, IMI-ole showed similar toxicity to imidacloprid but persisted longer in the body. Although this biotransformation mechanism has not been described in *F. candida*, other studies have shown that IMI-ole contributes to the toxicity and delayed effect of imidacloprid in other species of freshwater arthropods and bees (Huang et al., 2022; Suchail et al., 2001). In addition, Bakker et al. (2022) showed that neonicotinoids can disrupt detoxification by inhibition of cytochrome P450 enzymes in *F. candida*. The present study shows that younger life stages are more sensitive to imidacloprid. This complements earlier studies that showed age- and size-dependent differences in the sensitivity of the freshwater arthropods *Asellus aquaticus* and *Gammarus pulex* to imidacloprid (Huang et al., 2021). Thiacloprid, although having a similar mechanism of action to imidacloprid, was less toxic to the same parameters (higher EC50 and LC50 values), which is consistent with observations in other studies (de Lima e Silva et al., 2021; van Gestel et al., 2017). This is most likely related to the low DT50 of approximately 8 days of this compound. At the end of the exposure, on average only 4–16% of the initial spiking concentrations were recovered. The concentrations of the pesticides measured in the test soil show some variation and recoveries also were considerably lower at the end of the test in some cases, suggesting considerable degradation. However, the DT50 values reported in the literature for cyproconazole and teflubenzuron are much higher than the length of the experiments (Supporting Information, Table S4), therefore discrepancies between measured and nominal concentrations of these pesticides might be related the spiking procedure. The recoveries and degradation patterns for each pesticide, however, did not seem to be much different between the tests with the two life stages (Supporting Information, Table S4), which shows that the discrepancies in the recovery of cyproconazole and teflubenzuron do not influence the conclusions of the present study regarding the effects of life stage and length of the tests. The lack of a significant difference in survival between younger and older animals exposed to thiacloprid can be attributed to experimental factors rather than just differences in toxicity and DT50 compared with imidacloprid. Specifically, the coefficient of variation criteria for the number of surviving adults exceeded acceptable limits in the water controls for both life stages and the solvent controls in the test involving older animals, therefore caution should be exercised when interpreting the data for thiacloprid.

The alteration of the molting process caused by teflubenzuron and the disruption of the nervous system caused by imidacloprid and thiacloprid could negatively impact resource acquisition, either by reducing access to food or changing the feeding rate of the animal. This effect should be more significant for 10–12-day-old springtails due to their smaller body size and lower energy reserves. The lower surface-to-volume ratio (Janssens et al., 2021) and differences in uptake and biotransformation capacity (Huang et al., 2022) should also contribute to the lower capacity to cope with the insecticides. In fact, Huang et al. (2022) showed that the uptake rate of imidacloprid decreased with size of *G. pulex* and the metabolite IMI-ole was 8.4 times more toxic than imidacloprid to adult females of *A. aquaticus* compared with juveniles. The longer exposure duration (4 weeks compared with 3 weeks) for juveniles compared with adult springtails may have also contributed to their higher sensitivity. These reasons all might explain the higher sensitivity of the younger animals to the three insecticides in terms of both reproduction and survival.

The exposure to the fungicide cyproconazole resulted in significant inhibition of reproduction, despite the lack of significant effects on survival. The high EC50 values obtained are in accordance with the low toxicity reported in other studies with *F. candida* (i.e., no effects on reproduction or survival at concentrations up to 70 mg a.s. kg^{-1} $soil_{dw}$; Gomes et al., 2021). Cyproconazole is a triazole fungicide that inhibits the synthesis of sterol, a component of the fungal cell wall (Zhang et al., 2014). This mechanism of action is not related to arthropods, but similar fungicides were shown to impair molecular processes in arthropods (e.g., inhibition of mitochondrial respiration; Syromyatnikov et al., 2017). Despite the lack of mortality of *F. candida* at the higher concentrations of cyproconazole, we observed lower rates of consumption of the yeast, Although the amount of food added to the jars was not explicitly standardized, it was consistently provided under the same conditions, ensuring uniformity in terms of quantity and timing. Consequently, qualitative assessments of yeast consumption were made based on visible differences in the remaining yeast on the soil surface. It should be noted that no quantitative measurements were performed. However, the relative differences in yeast consumption between samples were discernible. Cyproconazole could decrease the quality of the food (Cuco et al., 2017), decreasing its consumption by the springtails. In addition, indirect effects of cyproconazole through changes in the microbial community of the soil (Zhang et al., 2014) or the microbiome of the animals should not be disregarded. Conversely to what was observed for the insecticides, adult springtails were more sensitive to cyproconazole in terms of reproduction. This difference might be related to the mode of action of the pesticide, although there is no evidence in the literature relating the mode of action of cyproconazole and possible differences in sensitivity between adults and juveniles. Future studies with this or similar compounds should explore the mechanisms behind the effects of cyproconazole on *F. candida* and take these observations into consideration.

Implications of selecting different life stages

The current environmental risk assessment of pesticides to in-soil organisms aims to protect biodiversity and ecosystem functioning (Ockleford et al., 2017). Collembolans contribute to the provision of ecosystem services by their interaction with the soil microbial community and their role in the degradation of organic matter. The OECD test guideline 232 was designed to cover the most relevant life stages of springtails tested, with survival and reproduction as the endpoints (OECD, 2016). In this way, the test covers the effects on maturation by including

parts of the juvenile phase (hatching, juvenile growth, maturation) as well as effects on reproduction and survival. Despite the recommendations of the OECD test guideline, some recently published studies started exposures with 20–23-day-old springtails and with a duration of 21 days as an alternative for a better comparison with other species with different life cycles (e.g., de Lima e Silva et al., 2021). The implications of these differences in methodology for the risk assessment of pesticides are not known. In the present study we proved that different life stages of *F. candida* respond differently in terms of their reproduction and survival to pesticides. Initially, it was hypothesized that younger springtails would be more sensitive than older ones. This was confirmed for the three insecticides tested (teflubenzuron, imidacloprid, and thiacloprid), which resulted in significantly lower EC50 values for effect on the reproduction of 10–12-day-old animals. For survival, the same was observed for teflubenzuron and imidacloprid, but not for thiacloprid (LRT, $p > 0.05$). We showed that changes in the age of the animals tested and length of the exposure result in significant changes in sensitivity, generally by a factor of 2.0–3.0 but in some cases even larger differences in EC50 values were seen. Performing reproduction and survival tests with *F. candida* according to OECD guideline 232 ensures a level of protection for this species because it encompasses the most sensitive life stages of these organisms. Our findings suggest that alterations in the age of the tested animals and the duration of exposure can lead to significant variations in sensitivity, indicating that employing older animals may underestimate the risk of pesticides. However, this was not observed in the case of cyproconazole, which exhibited effects only at very high concentrations. Nonetheless, our study supports the current utilization of OECD guideline 232 as a reliable and safe method for assessing the effects of pesticides on *F. candida*, and careful consideration is needed when interpreting results obtained from older organisms or longer exposure periods.

CONCLUSIONS

The life stage at the start of the exposures influenced the toxicity of cyproconazole, teflubenzuron, imidacloprid, and thiacloprid to *F. candida*. This effect was partially dependent on the compound tested. For cyproconazole the reproduction was more sensitive for older than for younger springtails, while for teflubenzuron, imidacloprid, and thiacloprid younger springtails were more sensitive in terms of their reproduction and survival. This indicates that for insecticides, the OECD 232 guideline for the reproduction toxicity test with Collembola covers the most sensitive life stages of *F. candida*. Studies in which adjustments to these guidelines are proposed should take into consideration that the life stage of *F. candida* at the start of the test (i.e., choice between juvenile or sexually mature stages) and the length of exposure may significantly influence the outcomes of the test. Further studies are needed to assess differences in sensitivity of different life stages of *F. candida* for chemicals having different modes of action.

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Data Availability Statement—The raw data used in the present study, along with the R scripts for analysis, are available on Zenodo, an open-access research data repository. The dataset and R scripts can be accessed at https://zenodo.org/record/7974083.

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