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Age and Synchronization of *Daphnia magna* Affect Sensitivity to Teflubenzuron in Acute Standardized Toxicity Tests

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Abstract: The standard *Daphnia* sp. acute toxicity test for assessing the adverse effects of chemicals on aquatic invertebrates stipulates the use of neonates that are ≤24 h old (hours post release [hpr]) at the start of the exposure. However, when one is assessing acute effects of chemicals interfering with endocrine relevant-processes such as molting, both age synchronization and absolute age can influence the test outcome, because the occurrence of molting and associated mortality is highly time specific. Hence, a 24-h age synchronization window may mask the real effects of these compounds. To explore the influence of age synchronization and absolute age in standard acute toxicity tests, we exposed *D. magna* from different synchronization windows and absolute ages (≤4, 4–8, 8–12, ≤12, and ≤24 hpr at the beginning of the exposure) to 0.5–12 µg/L of the chitin synthesis inhibitor (CSI) teflubenzuron (TEF) using the Organisation for Economic Co-operation and Development test guideline 202 (*Daphnia* sp. 48 h immobilization test). Our results show significant differences in 48-h median lethal concentrations between animals with a synchronization window of ≤4 hpr (2.9 µg/L) and longer synchronization windows such as ≤12 hpr (5.1 µg/L) and ≤24 hpr (16.8 µg/L). A concurrent decreasing trend in molting median effect concentrations was observed for the same synchronization windows: ≤4 hpr (4.0 µg/L), ≤12 hpr (5.9 µg/L), and ≤24 hpr (30.0 µg/L). Together, our results show that both synchronization and absolute age are determinant factors for the sensitivity of *D. magna* to TEF. A narrow synchronization window (e.g., ≤4 hpr) may provide a more conservative estimate of TEF toxicity and should be considered when one is performing standardized toxicity tests for molting-disrupting compounds such as TEF. *Environ Toxicol Chem* 2023;42:1806–1815. © 2023 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

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INTRODUCTION

Crustaceans such as *Daphnia* sp. have specific age windows of sensitivity to different stressors within the first 24 h after being released from the brood pouch. For example, age-related differences in sensitivity were found in *Daphnia magna* after acute exposure to cadmium: neonates synchronized between 20 and 24 hours post release (hpr) were found to be seven times more sensitive than those synchronized at ≤4 hpr (Traudt et al., 2017). Similar results were obtained for potassium dichromate: the most sensitive age window was found to be 22–24 hpr (Klein, 2000). Effects of life stage on susceptibility have been reported for different *Daphnia* species in acute toxicity tests (48-h duration) assessing mortality. Neonates of *Daphnia schodleri* exposed to hexavalent chromium, for example, were up to five times more sensitive than 24-day-old adults (Arzate-Cárdenas & Martínez-Jerónimo, 2011). *Daphnia pulex* neonates (<24 h old) exposed to polystyrene nanoplastics were found to be 1.5 times more sensitive than 14-day-old adults (Liu et al., 2018). Lastly, <24-h-old *D. magna* neonates exposed to glyphosate were three times more susceptible to glyphosate than 21-day-old adults (Cuhra et al., 2013).

Another known factor influencing susceptibility in acute toxicity tests is strain differences, as has been shown for the chitin synthesis inhibitor (CSI) diflubenzuron (Kato et al., 2022), juvenile hormone analogs, and 3,4-dichloroaniline (Oda et al., 2006, 2007). In addition, different exposure media have been reported

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to influence the susceptibility of *D. magna*, especially when determining metal toxicity (Loureiro et al., 2011).

Effects caused by substances interfering with endocrine-relevant processes (e.g., chitin synthesis and molting) may be particularly age-dependent, because these vary substantially throughout life (e.g., molt or reproductive cycle). Chitin is an essential biopolymer and one of the main constituents of arthropod exoskeletons (Muthukrishnan et al., 2012). It protects the organism from external damages and desiccation and facilitates locomotion by providing attachment sites for muscles. Chitin is also indispensable for successful molting (Zhu et al., 2016), a periodical process for exoskeleton shedding to achieve growth and development. Molting is orchestrated by the steroid hormone 20-hydroxyecdysone and its associated receptors, ecdysone receptors (EcRs), and ultraspiracle protein in arthropods (Heming, 2018). In crustaceans such as *D. magna*, the first molt in neonates occurs at approximately 28 hpr (Mu & LeBlanc, 2004). The time interval between molt cycles increases with age and reaches a stable interval of approximately 70 h in adults (Kato et al., 2007). Both chitin synthesis (cuticle formation) and molting (ecdysis) are precisely timed processes that follow the organism's ecdysone titers and the expression of EcRs (Chang & Mykles, 2011; Kato et al., 2007). Because chitin synthesis and molting do not occur in vertebrates, these processes can be targeted for the management of unwanted arthropods (Liu et al., 2019). For example, CSIs are widely used to control unwanted arthropods in agriculture and aquaculture (Junquera et al., 2019). Therefore, the effects of CSIs on chitin synthesis and molting are highly dependent on the stage of a molt cycle and the animal's age (de Cock & Degheele, 1998; Merzendorfer, 2013).

Acute adverse effects of chemicals on aquatic invertebrates are assessed using the standard *Daphnia* sp. acute immobilization test, test guideline 202 (Organisation for Economic Co-operation and Development [OECD], 2004). This test is required in dossiers for the registration of new active ingredients by various regulatory agencies (European Commission, 2013; US Environmental Protection Agency, 2005). The guideline stipulates an exposure period of 48 h, and the use of synchronized neonatal *Daphnia* sp. that were released from the brood chamber <4 h before the start of the test. Effects of CSIs are typically observable during the progression of molting or after a complete molt cycle. In particular, these chemicals have been shown to interfere with exoskeleton formation and the molting process itself, and have also induced mortality in insects and crustaceans (Bechmann et al., 2018; Montaño-Reyes et al., 2019). The endpoints of molting and mortality are of particular interest, because they are also key events of a recent OECD-endorsed adverse outcome pathway (AOP; Schmid et al., 2022).

Due to their specific mode of action (Demaeght et al., 2014; Douris et al., 2016), the age and life stage of the test organism at the beginning of the exposure are expected to be key factors that drives the toxicity of CSIs. This can potentially influence the acute toxicity of these compounds drawn from standard regulatory toxicity tests, such as the OECD *Daphnia* acute toxicity test and the International Standards Organization (ISO) 6341:2012 toxicity test (ISO, 2012; OECD, 2004). The life stage can also affect the molecular, cellular, and phenotypical responses by CSIs, because genes and proteins involved in the molt cycle and chitin synthetic process change dramatically within a short timeframe (Kato et al., 2007; Qu & Yang, 2011; Rocha et al., 2012; Sumiya et al., 2014). Age and age synchronization may therefore be key to designing toxicity tests with relevance both to increasing mechanistic understanding and characterizing the adverse effects of CSIs.

To test these hypotheses and investigate how age and age synchronization affect the result of a standard acute toxicity tests, we used the model test species *D. magna* (water flea) and the well-studied CSI teflubenzuron (TEF) in the standardized OECD (2004) test guideline 202 (*D. magna* 48-h immobilization). We hypothesized that young daphnids that were synchronized to a narrow age window would be more susceptible to TEF, in terms of both cumulative molting frequency (number of molts during a test) and cumulative mortality.

MATERIALS AND METHODS

Culturing and toxicity tests

Five independent toxicity tests using *D. magna* from different age synchronization windows were conducted to test our hypothesis. *Daphnia magna* (DHI strain) were obtained from the Danish Hydraulic Institute (Hørsholm, Denmark) and have been continuously cultured in our laboratory for more than 5 years. *Daphnia magna* were cultured in M7 medium (pH 7.8 ± 0.2) at a density of 20 animals/800 ml of medium at a temperature of 21 ± 1 °C under a 16:8-h light:dark cycle. Culture media were renewed twice a week, and offspring were removed. Daphnids were fed daily with *Raphidocelis subcapitata* (0.1 mg/carbon/daphnid) supplemented with 200 µl of a 20-mg/ml baker's yeast suspension. Acute toxicity tests with *D. magna* were conducted according to OECD (2004) test guideline 202 with slight modifications (in terms of age and age synchronization of the test organism). All exposures were conducted using 20 individual *D. magna* neonates (five individuals for each of the four treatment replicates) of different age and synchronization windows (a total of five toxicity tests). *Daphnia magna* neonates of different age and synchronization windows (≤4 [0–4], 4–8, 8–12, ≤12 [0–12], and ≤24 hpr [0–24 hpr]) were exposed to nominal concentrations of 0.5–12 µg/L of TEF (CAS 83121-18-0, purity ≥98.0%; Sigma Aldrich) and a solvent control (0.01% dimethylsulfoxide; Sigma Aldrich) in nontreated six-well polystyrene tissue culture plates (VWR). Animals from different synchronization windows (≤4 hpr [0–4], ≤12 [0–12], and ≤24 hpr [0–24 hpr]), were collected from stock cultures after cultures were left to release neonates for 4, 12, and 24 h, respectively. Dimethylsulfoxide was used due to its ability to facilitate solubilization of lipophilic substances and its low toxicity compared with other organic solvents toward *D. magna* (Barbosa et al., 2003).

Animals aged 4–8 and 8–12 hpr were collected after the stock cultures were left to release offspring for 4 h and were reared in clean M7 media for 4 and 8 h, respectively, before being exposed. The exposure time, however, was 48 h for all toxicity tests. *Daphnia magna* were held at a density of one

animal/2 ml of medium during the exposures. Molting frequency and survival were monitored at 24, 36, and 48 h post exposure, with 24 and 48 h considered the minimum observation interval in the OECD (2004) test guideline 202. We observed molting and survival after 36 h, because the effects of CSIs cannot be observed after 24 h when young animals (e.g., ≤4 hpr) are used for the exposure. Mortality was assessed by counting dead animals, and molting was assessed by counting shed exoskeletons under a stereomicroscope (Jiang et al., 2018; Song, Evenseth, et al., 2017).

Verification of exposure concentrations

Samples of exposure media (1 ml) of the solvent control and the lowest and highest concentrations were taken at the start and the end of the exposures and were frozen immediately and stored at −20 °C until analyte extraction. For extractions, 1 ml acetonitrile (ACN; Merck-Sigma Aldrich) was added to each sample, and the samples were shaken vigorously. The water was salted out by adding approximately 0.5 g of sodium chloride (Merck-Sigma Aldrich), and 500 µl of the organic phase were subsequently transferred to 2-ml liquid chromatography (LC) vials. The TEF was analyzed on a Waters Acquity ultra performance (UP)LC system connected to a Xevo TQ-S triple quadrupole mass spectrometer. Separation was achieved on a Waters BEH C8 column (2.1 × 100 × 100 mm) using gradient elution with ACN and water (containing 5.2 mM ammonium acetate). The TEF was detected in negative electrospray ionization mode with mass transitions of 379 → 196 and 379 → 339. The limit of detection was 0.01 µg/L.

Data processing and statistical analysis

Statistical treatment of data was done using GraphPad Prism Ver. 9.2 (GraphPad Software), and the R environment for statistical computing (R Development Core Team, 2011). Normality of the data was assessed by the Shapiro–Wilk test, and equal variance was assessed using Levene's test. Data that met the assumptions for parametric tests were analyzed by one-way analysis of variance followed by Dunnett's post hoc test for multiple comparisons (significant differences assumed at $p < 0.05$). Nonparametric Kruskal–Wallis tests followed by Dunn's test were used for data lacking normality or equal variance. Log-logistic concentration–response curves were fitted, and threshold values such as median lethal concentrations (LC50s) and median effect concentrations (EC50s) were determined using the R (R Development Core Team, R, 2011) package drc (Ritz et al., 2015). The 5% benchmark concentrations (BMCs) and BMC lower limits (BMCLs) were estimated using the R package bmd (Jensen et al., 2020) as a complementary effect threshold to classical no-observed-effect concentration (NOEC) and lowest-observed-effect concentration (LOEC). The BMCs are commonly suggested to be a more scientifically sound derivation method for points of departure derivation, because they consider the full set of concentration–response data and are less susceptible to the spacing of treatment groups and the intergroup variance than LOECs (EFSA Scientific Committee et al., 2022). Parameters of the concentration–response curves were compared between age synchronization groups using a z-test, implemented with the function compParm() of the package drc, as suggested by Ritz et al. (2015).

RESULTS AND DISCUSSION

Exposure verification confirmed that the lowest concentrations (0.5 µg/L) were close to the nominal concentration (0.53 ± 0.22 µg/L; Supporting Information, Table S1). Unexpectedly, the measured concentrations of the highest exposure group (12 µg/L) was approximately three times higher than expected in all experiments. However, because the exposure levels of the highest exposure group in all five experiments were in the same range (36.7 ± 5.8 µg/L), results from all five experiments were considered comparable, and hence the core message of the present study remains unaffected by this discrepancy. Nevertheless, this discrepancy, occurring in the highest exposure group, indicates a systematic error that could have been introduced during stock solution preparation, sample extraction, or instrumental analysis. The exposure levels of TEF were considered stable during the 48-h exposure period because no significant differences in exposure concentrations were identified between start and end of the study. On average, TEF concentrations decreased by 4.7% and 4.8% in the lowest and highest concentrations, respectively. The overall exposure quality met the test validity criteria defined in OECD (2004) test guideline 202.

We observed significant age- and age synchronization-dependent changes in survival during the 48-h test. Animals that were synchronized to ≤4 hpr were two times more susceptible to TEF compared with the 8–12-hpr group, and six times more susceptible than the ≤24-hpr group after 48 h of exposure (Table 1 and Figure 1). After 36 and 48 h of exposure, only partial concentration–response relationships could be obtained for the ≤24-hpr group (Figure 2). Animals from this group were significantly more tolerant to TEF compared with the other groups (Figure 1; Supporting Information, Table S2). The partial concentration–response curves might indicate an underestimation of 48-h TEF toxicity to *D. magna* synchronized within the wide age window (i.e., ≤24 hpr) that is recommended by the OECD protocol. On the contrary, a narrower age synchronization window may provide a substantially more conservative and protective estimation of the toxicity of TEF. This was evidenced by the higher susceptibility and existence of complete concentration–response curves for all synchronization windows shorter than 12 hpr compared with longer synchronization windows (Figure 2 and Supporting Information, Table S3).

We observed similar patterns of age- and age synchronization-dependent effects on molting as those seen for mortality in juvenile *D. magna*. Although molting is currently not included as a standard endpoint in OECD (2004) test guideline 202, it can easily be incorporated into the test guideline 202 protocol for endocrine disruption-related assessment by

TABLE 1: Summarized effect concentrations (μg/L) for molting and survival after 24, 36, and 48 h of exposure to teflubenzuron of *Daphnia magna* for different synchronization and age windows

	Molting														
	24 h					36 h					48 h				
Synchronization/ age (hpr)	EC50 (±95% CI)	Slope (95% CI)	BMC (BMCL)	NOEC	LOEC	EC50 (±95% CI)	Slope (95% CI)	BMC (BMCL)	NOEC	LOEC	EC50 (±95% CI)	Slope (95% CI)	BMC (BMCL)	NOEC	LOEC
≤4	—	—	—	—	—	2.2 (0.1–4.2)	0.9 (0.1–1.8)	0.1 (–0.2)	4	8	4.0 (3.8–4.2)	10.7 (0.0–146.1)	3.0 (–6.63)	4	8
4–8	—	—	—	12	—	6.5 (5.2–7.9)	4.8 (1.8–7.8)	3.5 (1.9)	12	—	5.8 (4.8–6.8)	3.8 (2.2–5.4)	2.7 (1.7)	4	8
8–12	—	—	—	12	—	8.2 (7.4–9.0)	6.6 (0.9–14.2)	5.3 (3.0)	8	12	8.0 (7.4–8.5)	6.7 (1.5–11.8)	5.1 (3.4)	4	8
≤12	2.6 (0.0–3.0)	0.5 (0.1–0.1)	—	4	8	5.7 (4.4–7.0)	4.1 (0.7–7.4)	2.8 (1.3)	4	8	5.9 (5.0–6.7)	5.7 (3.3–8.1)	3.5 (2.6)	4	8
≤24	—	—	—	12	—	25.6* (3.8–47.3)		5.1 (2.2)	12	–	30.0* (6.1–53.8)	1.4 (0.5–2.4)	3.9 (1.5)	8	12
	Survival														
	24 h					36 h					48 h				
Synchronization/ age (hpr)	EC50 (±95% CI)	Slope (95% CI)	BMC (BMCL)	NOEC	LOEC	EC50 (±95% CI)	Slope (95% CI)	BMC (BMCL)	NOEC	LOEC	EC50 (±95% CI)	Slope (95% CI)	BMC (BMCL)	NOEC	LOEC
≤4	—	—	—	—	—	10.0 (7.9–12.1)*	1.6 (1.0–2.1)	1.6 (0.8)	4	8	2.9 (2.4–3.4)	3.6 (1.9–5.3)	1.3 (0.8)	4	8
4–8	13.7* (0.0–631.5)	1.2 (0.2–2.2)	16.8 (5.9)	12	—	5.7 (5.2–6.2)	6.0 (4.3–7.8)	3.5 (3.0)	4	8	4.5 (3.7–5.3)	9.0 (0.0–21.7)	3.3 (2.3)	4	8
8–12	15.2* (10.9–19.4)	2.4 (0.9–4.0)	4.5 (2.5)	8	12	6.6 (5.8–7.4)	7.0 (2.5–11.6)	4.4 (3.0)	4	8	5.7 (5.2–6.1)	6.2 (4.7–7.7)	3.5 (3.1)	4	8
≤12	11.5* (8.6–)	2.2 (0.8–3.7)	3.1 (1.1)	4	8	7.3 (6.0–8.5)	2.5 (1.5–3.7)	2.3 (1.4)	4	8	5.1 (4.4–5.7)	4.5 (4.5–6.5)	2.6 (1.9)	4	8
≤24	31.6* (0.0–70.6)	2.4 (0.3–4.9)	2.5 (1.0)	12	—	22.2* (9.9–34.4)	1.4 (0.6–2.1)	2.5 (1.0)	4	8	16.8* (10.3–23.4)*	1.4 (0.8–2.1)	2.1 (0.8)	4	8

Asterisks indicate predicted LC50s and EC50s when the maximal response observed was <50%. Missing values indicate that the data did not allow the calculation of the respective effect concentrations.
BMC = benchmark concentration; BMCL = benchmark concentration lower confidence limit; EC50 = median effective concentration; LC50 = median lethal concentration; LOEC = lowest observed effect concentration; NOEC = no observed effect concentration; 95% CI = 95% confidence interval.

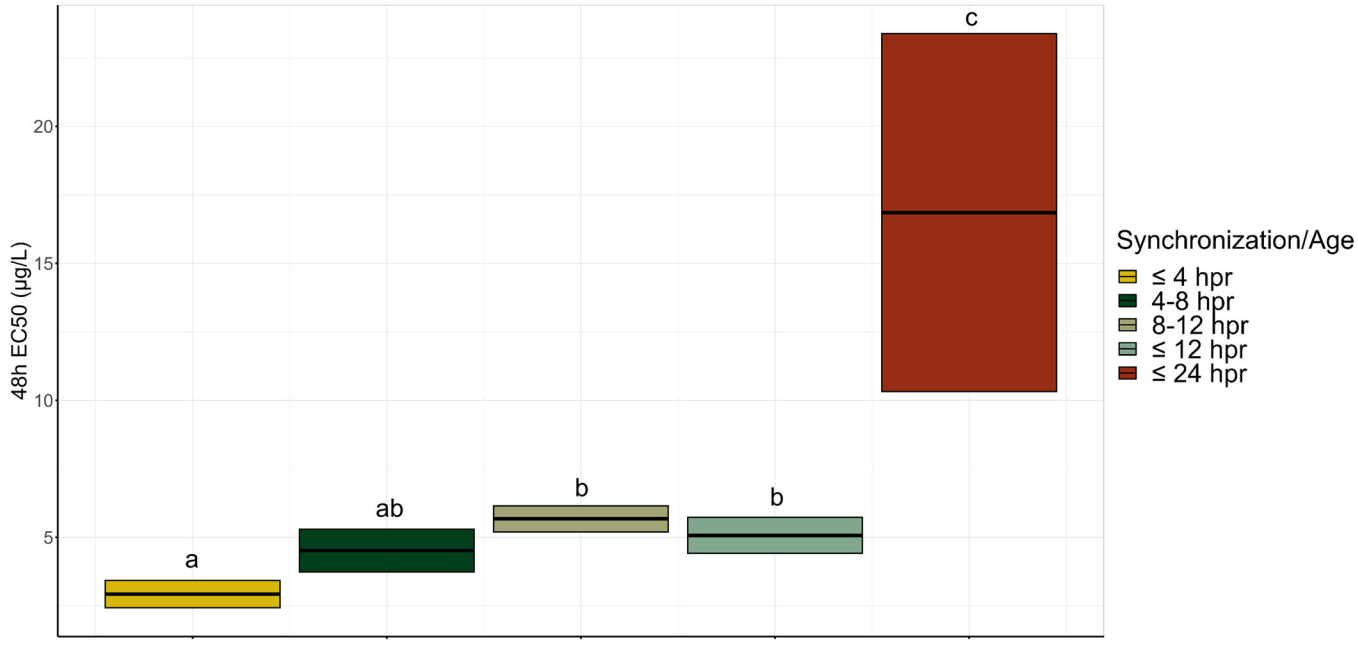

FIGURE 1: Comparison of 48-h median lethal concentrations (LC50s) between different age windows. Bold lines in the middle of the boxes represent LC50 values, and top and bottom of the boxes represent the lower and upper level of the 95% confidence intervals, respectively. Different letters above the boxes indicate different significance groups ($p \leq 0.05$). EC50 = median effective concentration; hpr = hours post release.

counting the shed exoskeletons at the bottom of the test vessel (Jiang et al., 2018; Song, Evenseth, et al., 2017). Unsuccessful molting was observed as daphnids being unable to completely shed their old cuticle (Figure 3). We observed a trend of animals being stuck completely in their old exoskeleton when they were exposed to high concentrations of TEF (≤8 µg/L).

We observed a concentration-dependent reduction in molting frequency after 48 h of exposure to TEF across all age and synchronization windows, with a significant reduction at 4 µg/L, except for the ≤24-hpr group (Figure 4 and Supporting Information, Table S3). The reason for the discrepancy in that group may likely be that for such wide age synchronization

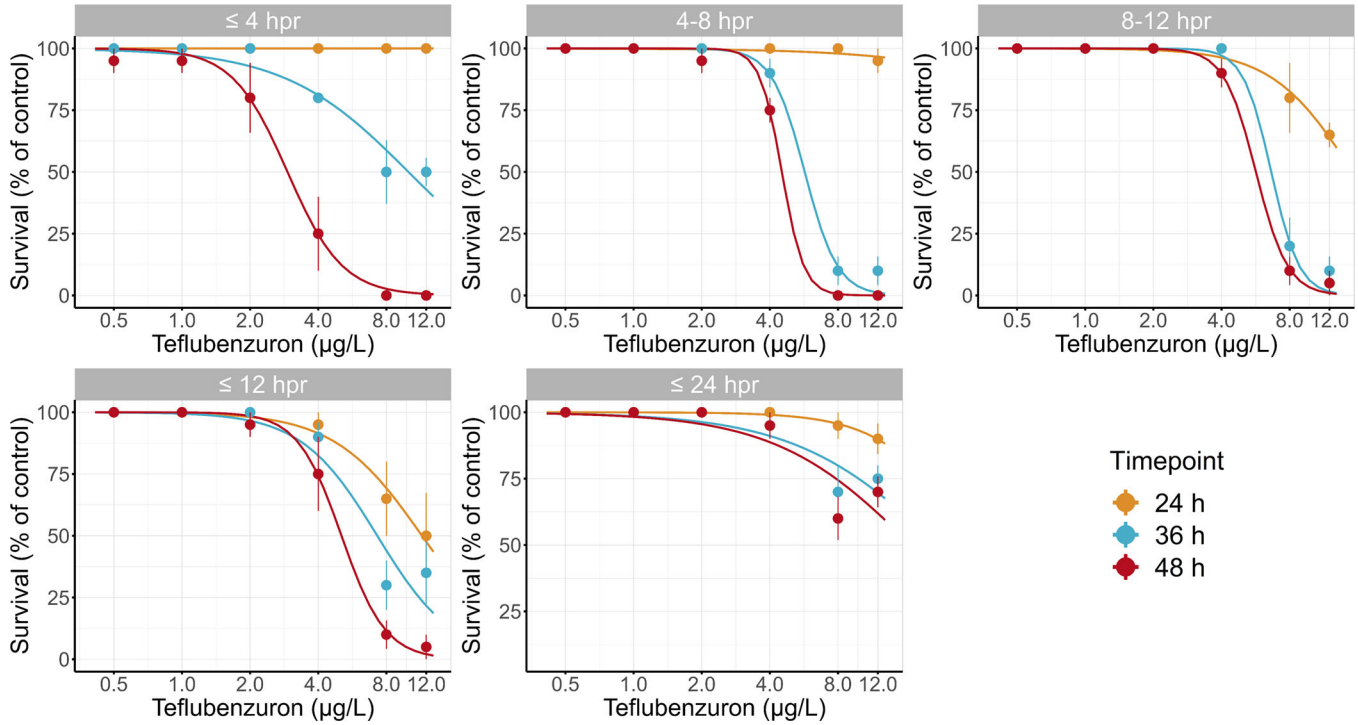

FIGURE 2: Reduction in survival of neonatal *Daphnis magna* of different synchronization and age groups (hours post release [hpr]) after 24, 36, and 48 h of exposure to teflubenzuron (TEF); ≤4, 4–8, 8–12, ≤12, and ≤24 hpr. Data are presented as mean ± SEM with fitted concentration–response curves. Observations were made after 24 h (orange line), 36 h (blue line), and 48 h (red line) of exposure to TEF.

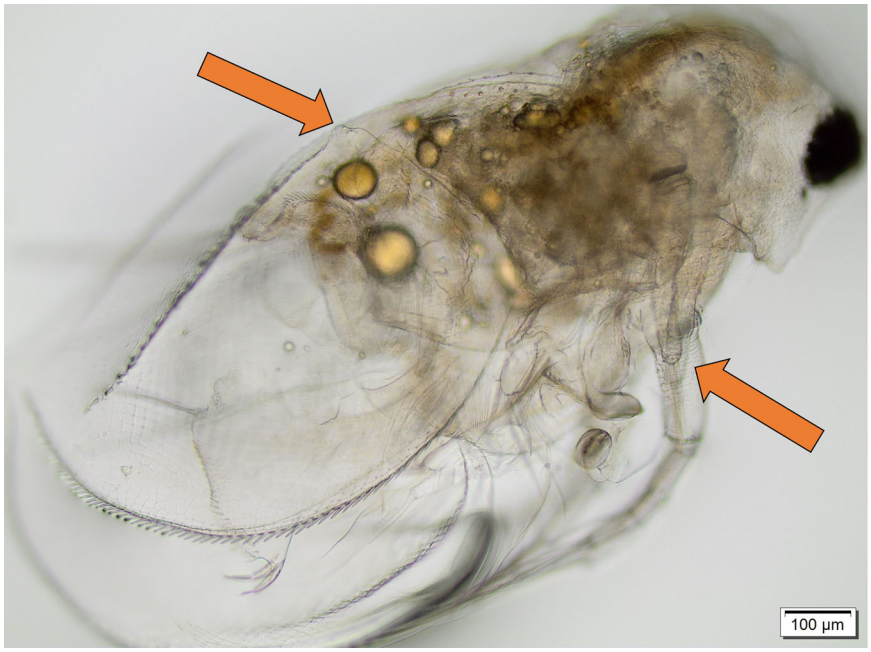

FIGURE 3: Neonatal *Daphnia magna* (≤4 hours post release synchronized) partially stuck in its old exoskeleton after 48 h of exposure to 12 µg/L teflubenzuron. The left arrow points to the breaking line of the old exoskeleton, and the right arrow shows the second antenna, which is stuck in the old exoskeleton.

window, some daphnids had already molted prior to sufficient bioaccumulation of TEF, making the overall response pattern of the test population inconsistent. The molting frequency also varied considerably in the ≤4-hpr group after 36 h of exposure (Figure 4) and in all remaining experiments after 24 h of exposure (Supporting Information, Figure S1), thus indicating that molting had not occurred in all animals at these specific time points. No difference in EC50 values for molting was found between 36 and 48 h of exposure, except in the ≤4-hpr group, indicating that most animals likely completed the first molt cycle within 36 h. When the EC50s and BMCs for 36 and 48 h of exposure were compared, the results clearly indicated that the

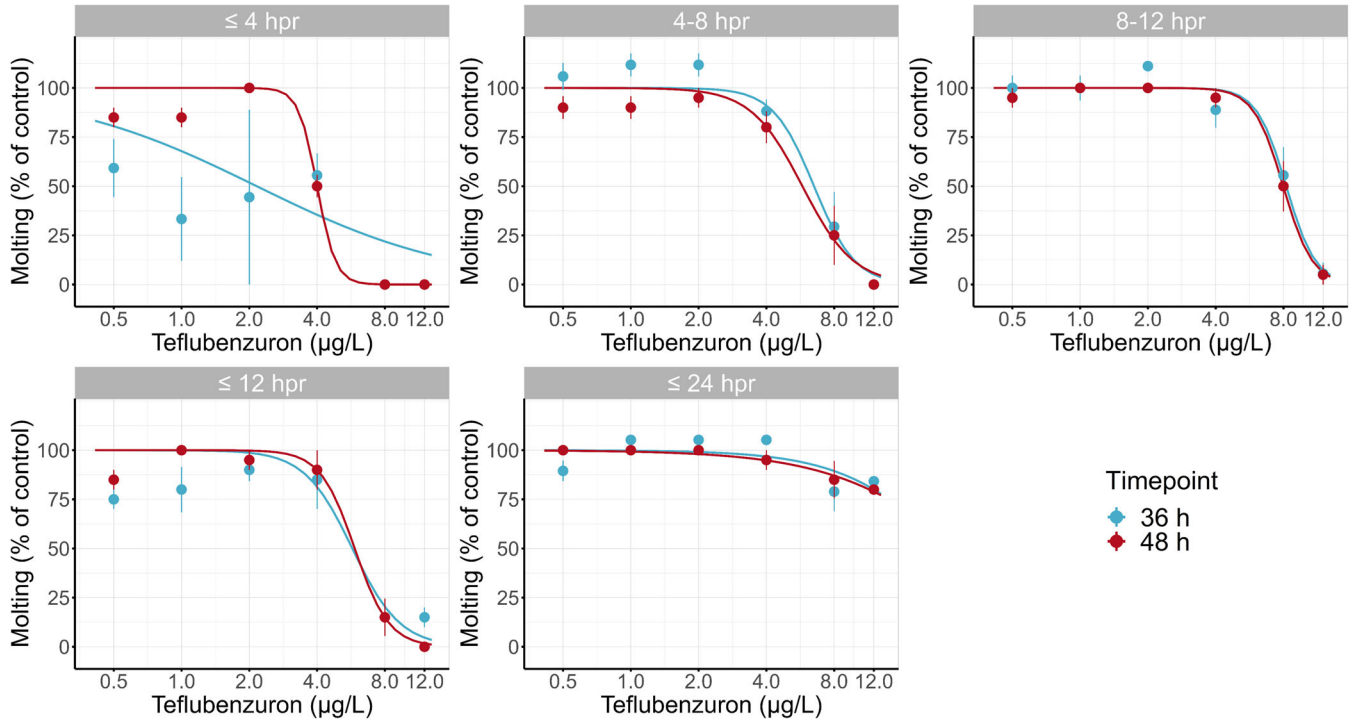

FIGURE 4: Reduced molting of neonatal *Daphnia magna* of different synchronization and age groups (hours post release [hpr]) after 36 and 48 h of exposure to teflubenzuron (TEF); ≤4, 4–8, 8–12, ≤12, and ≤24 hpr. Data are presented as mean ± SEM with fitted concentration–response curves. Observations were made after 36 h (blue line) and 48 h (red line) of exposure to TEF.

≤4-hpr daphnids were the most sensitive among all age synchronization groups (Table 1 and Figure 4, and Supporting Information, Table S2), implying that younger animals and narrow synchronization windows better reflected the molting-related toxicity of TEF on individual animals.

The adverse effect of CSIs (i.e., mortality and molting disruption) becomes visible during the progression of or after a complete molt cycle (de Cock & Degheele, 1998; Harðardóttir et al., 2019; Merzendorfer, 2013), due to the specific mechanism of action of chitin synthase inhibition and its consequence on cuticle formation and molting behavior (Cohen, 2001; Demaeght et al., 2014; Douris et al., 2016; Van Leeuwen et al., 2012; Vincent, 2002). Based on this knowledge, one would expect that the period before the first molt would be the most sensitive time window for the toxic action of CSIs. For example, in the present study, animals synchronized to ≤4 hpr had more exposure time in their sensitive time window compared with animals aged 8–12 hpr and were therefore two times more susceptible to TEF. Moreover, the exposure duration should reflect at least one complete molt cycle to capture the disruption of molting behavior. Thus, a synchronization window of ≤24 hpr might not be representative, because no control exists over age distribution and some animals might therefore already be close to the first molt before the exposure starts. Because chitin synthesis occurs throughout the whole molt cycle (Qu & Yang, 2011; Rocha et al., 2012; Zhu et al., 2016), younger aged populations with narrow age synchronizations (e.g., ≤4 hpr) are expected to be more susceptible to CSIs due to longer total exposure durations in the sensitive time window and increased likelihood of interference with the molting process.

In line with our findings, the naupliar stage (0–3 h old) of the marine copepod *Tisbe battagliai* was also reported to be 25 times more susceptible to TEF than that observed for the later copepodid stage (Macken et al., 2015). In addition, the second instar larvae of the terrestrial arthropod Colorado potato beetle (*Leptinotarsa decemlineata)* were found to be two times more susceptible to TEF than the third instar larvae (Malinowski & Pawinsk, 1992).

It should be noted that in the present study, the estimated LC50 for the effect of TEF on *D. magna* survival in the ≤24-hpr group was 16.8 µg/L, which was 60 times higher than previously reported (Medeiros et al., 2013). This large discrepancy might arise from the use of a commercial formulation of TEF by Medeiros and coworkers. It was previously suggested that certain formulations of TEF could be more toxic to *D. magna*, and also that these studies were conducted under different experimental conditions than our setup (Scheepmaker, 2008). Furthermore, strain differences might also explain the discrepancies in sensitivity across studies, as reported for studies with juvenile hormone analogs and 3,4-dichloroaniline toxicity in *D. magna* (Oda et al., 2006, 2007). Recently, higher capability of chitin metabolism was suggested to be a potential reason for sensitivity differences observed across *D. magna* strains after exposure to the CSI diflubenzuron (Kato et al., 2022). In addition, differences in media compositions could also lead to differences in LC50s, although this has mainly been shown for metals (Loureiro et al., 2011).

When comparing the EC50 values for molting and survival, it seems that the latter was in most cases lower. For instance, complete or partial inhibition of molting only occurred at concentrations higher than 4 µg/L, whereas 2 µg/L TEF was sufficient to produce up to 25% mortality (in the ≤4-hpr group). This suggests that molting disturbances were likely not the only cause for mortality in our studies. Although it is expected that lack of successful molting causes mortality (Song, Evenseth, et al., 2017; Song, Villeneuve, et al., 2017), it is still unclear whether lethality is a direct effect of failed molting or other associated effects (e.g., starvation due to reduced swimming ability or suffocation). Because chitin plays a vital role in the sclerotization (hardening) of the arthropod cuticle (Andersen, 2010), impaired chitin synthesis may also contribute to disruption of other cuticle functions including subsequent reduction in protection against predators (Beckerman et al., 2013).

Collectively, results from the present study clearly suggested that both age and age synchronization window may be key factors in the outcome of a standard acute toxicity test for TEF and potentially also other CSIs. The main purpose of our study was to better assess the toxicity of TEF to individual organisms of defined development rather than to consider a natural population containing diverse life stages. The differences in sensitivity due to age synchronization that we observed are thus expected to be of largest practical importance to standard laboratory tests with short and consistent test conditions. However, when considering ecologically relevant effects of CSIs such as TEF, our results might indicate demographic changes in exposed crustacean populations. The importance of age- and age synchronization-related differences in sensitivity is seldomly addressed, and studies to assess the role of these factors in naturally occurring populations are thus warranted.

More extensive studies that would characterize the molecular and biochemical changes underlying phenotypic effects such as mortality and molting defects are needed, to explain the significant differences we found. Such studies should also undertake a broader assessment of other molting-disrupting mechanisms for a better understanding of the differences in life stage-dependent sensitivity and molting-associated mortality of other CSIs and possibly also endocrine-disrupting chemicals.

In the regulatory context, molting has previously been proposed as an additional endocrine disruption-related endpoint to be included in the *Daphnia* sp. Reproduction assay (OECD, 2003). As demonstrated by our results, a narrower synchronization window closely following brood release of neonates in standard toxicity tests such as OECD (2004) test guideline 202 would yield a more conservative estimate of acute toxicity for these and potentially other molting-disruptive chemicals. Moreover, it is strongly recommended to report both age and age synchronization in standardized toxicity tests, to enhance the reliability and reproducibility of such tests in general.

Future efforts should also address the translatability of the present results to other CSIs (e.g., diflubenzuron, lufenuron, and buprofezin) and compounds impacting molting through different

modes of action (e.g., EcR agonists such as halofenozide or tebufenozide). Such assumptions would be supported by the basic principles of causality between initial molecular events along well-defined toxicity pathways converging on the same apical targets, and specifically exemplified by the AOPs that have been developed for these groups of chemicals (Schmid et al., 2021; Song, Villeneuve, et al., 2017). In addition to these AOPs, more detailed testing and ring tests with a larger assembly of test chemicals would be required to demonstrate the reproducibility of the present results.

CONCLUSIONS

We have demonstrated the key roles of age and age synchronization window in the sensitivity of *D. magna* to TEF. A higher susceptibility to TEF was observed for *D. magna* at a younger age and within a narrow age synchronization window. Our results further indicated that the synchronization window stipulated by acute standard toxicity test guidelines for *Daphnia* sp. might underestimate the adverse effects of molting-disruptive compounds such as TEF and be less protective for population effects of these compounds. In addition, we have demonstrated the importance of age and age synchronization for detecting the nonregulatory but highly relevant endpoints such as molting in acute toxicity tests that have been the focus of several conceptual and formal invertebrate-specific AOPs. Taken together, our results suggest that both age and age synchronization window should be reported in standardized toxicity tests and modifications to test designs and test guidelines for the results to be sufficiently conservative and protective need to be adequately discussed.

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Data Availability Statement—Data, associated metadata, and calculation tools are available from the github repository https://github.com/ssschmid/Age_Synchronization_TEF_D.magna.

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