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Glyphosate-based herbicide affects reproductive performance and morphological traits across generations in freshwater cladocerans

Josiane Araujo da Silva
araujoda-silva@hotmail.com
Universidade Federal do Rio Grande
Sophia Vallerini
Instituto Federal de Educação
Bernardo dos Santos Vaz
Instituto Federal de Educação
Jenifer Silveira Moraes
Universidade Federal do Rio Grande
Camila de Martinez Gaspar Martins
Universidade Federal do Rio Grande

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Abstract

As global agricultural reliance on glyphosate-based herbicides expands, growing evidence highlights their adverse effects on non-target aquatic organisms. However, most ecotoxicological assessments focus on directly exposed individuals, leaving the generational persistence of these effects largely uncharacterized. Here, we investigated the chronic and generational effects of a commercial glyphosate-based formulation (Roundup Transorb R®) following parental exposure in two freshwater cladocerans, *Daphnia magna* and *Ceriodaphnia dubia*. Organisms were exposed to environmentally realistic concentrations of glyphosate (30, 65 and 100 µg/L) to evaluate reproductive performance, morphological traits, and number of molting. The parental generation (F0) was exposed to the herbicide, whereas subsequent generations (F1 and F2) were reared in contamination-free media to determine whether parental exposure induced persistent effects in unexposed progeny. Herbicide exposure impaired reproduction in both species. In *D. magna*, offspring production decreased significantly in the parental generation (F0), an effect that persisted into F1 but recovered in F2. In contrast, *C. dubia* exhibited reduced fecundity only in the F0 generation. Morphological assessments revealed that body length and width of *C. dubia* decreased relative to controls exclusively in F0. In *D. magna*, however, reductions in body length, body width and tail spine length were detected only in the F1 generation. Number of molting remained unaffected across all generations in both species. Overall, our results demonstrate that the most severe biological impacts occurred in directly exposed organisms and their immediate progeny F1. Across the evaluated endpoints, phenotypic responses returned to baseline levels by the second unexposed generation F2, suggesting potential population recovery once exposure ceases.

Introduction

Pesticides encompass chemical and biological agents applied in agriculture to manage pests and diseases that threaten crop performance and agricultural yield (Sharma et al. 2019; Lopes et al. 2022). Among the most widely applied pesticides are glyphosate-based herbicides, which are very effective in controlling weeds. Glyphosate is currently the most heavily applied active ingredient globally, with an estimated agricultural consumption of approximately 749,000 metric tons annually between 2019 and 2022 (Brookes 2025). Over recent decades, numerous commercial formulations featuring diverse chemical additives have been commercialized. These mixtures incorporate surfactants, adjuvants and solubilizers designed to enhance herbicide efficacy by optimizing physicochemical properties and accelerating cell membrane penetration (Van Bruggen et al. 2018). Consequently, glyphosate is frequently detected in surface waters, groundwater, streams, lakes and drinking water reservoirs worldwide, with environmental monitoring studies reporting concentrations ranging from < 0.3 µg/L to 8.7 mg/L (Battaglin et al. 2014; Ronco et al. 2016; Van Bruggen et al. 2018; Lutri et al. 2020; Lima et al. 2022; Damiani et al. 2023). These findings highlight the widespread environmental contamination and reinforce the need to investigate the effects of glyphosate at environmentally relevant concentrations.

Substantial evidence demonstrates that glyphosate exposure induces toxicological effects across diverse non-target organisms. Mechanisms of glyphosate toxicity in aquatic organisms include inhibition of acetylcholinesterase activity, induction of oxidative stress and alterations in behavioral, morphological and reproductive traits (Bridi et al. 2017; Moraes et al. 2020; Lares et al. 2022; Lopes et al. 2022; Moraes et al. 2023; Barreto et al. 2023). Nevertheless, the vast majority of aquatic toxicity data derives from fish models, whereas toxicological impacts on freshwater invertebrates remain less characterized. In particular, cladocerans represent a critical yet underrepresented group in glyphosate risk assessments, with reported sublethal responses including altered swimming patterns, stunted growth, decreased fecundity and genotoxicity (Hansen and Roslev 2016; Duan et al. 2019; Gustinasari et al. 2021; Lares et al. 2022).

Furthermore, most studies on glyphosate focus on the isolated active ingredient, neglecting the additional components present in commercial formulations. This limitation reduces the environmental relevance of toxicity assessments, since aquatic organisms are exposed to complete formulations, not just pure glyphosate. Several studies have demonstrated that glyphosate-based formulations are more toxic than the active ingredient alone (Tsui and Chu 2003; Demetrio et al. 2014; Bridi et al. 2017; Lares et al. 2022; Spinaci et al. 2022; Abe et al. 2024; Tóth et al. 2024), an effect commonly attributed to the presence of adjuvants (Mottier et al. 2014; Rodrigues et al. 2019; Lopes et al. 2021; Klátyik et al. 2024).

Freshwater cladocerans, such as *D. magna* and *C. dubia*, are key zooplanktonic species widely distributed in lakes and ponds, including waterbodies adjacent to agricultural drainage where pesticide runoff occurs. These microcrustaceans occupy a central position in aquatic food webs as primary grazers of phytoplankton and key prey for macroinvertebrates and fish (Lares et al. 2022). Moreover, *D. magna* and *C. dubia* serve as standard ecotoxicological models (Lares et al. 2022; da Silva et al. 2024; Lopes et al. 2025) owing to their ease of laboratory culture, parthenogenetic reproduction and a rapid life cycle, which allows the assessment of toxicity across several broods within a short period.

Recent research indicates that glyphosate exposure can induce multigenerational toxicity in aquatic organisms, impairing survival and fecundity across generations. Incorporating multi and transgenerational designs into ecotoxicological frameworks provides a more realistic representation of chronic field exposures. This framework yields comprehensive insight into direct toxicity while revealing persistent, cumulative, or delayed effects that manifest in unexposed progeny (Maselli et al. 2017; Suarez et al. 2023). For example, 21-day exposure of the cladoceran *Daphnia exilis* to the commercial glyphosate-based formulation Faena® (2.09, 2.49 and 3.15 mg/L) reduced parental (P1) survival by 50% at 2.49 mg/L. In the F1 generation, continuous exposure under identical conditions significantly reduced survival across all treatments and nearly eliminated offspring production (Rodríguez-Miguel et al. 2021). Similarly, in fish, a 21-day exposure of *Jenynsia lineata* to glyphosate or Roundup Transorb R® (65 µg/L glyphosate equivalent) decreased pregnant female ratios, induced offspring malformations and impaired locomotor activity in the F1 generation (Moraes et al. 2024).

In the present study, we evaluated the chronic and generational toxicity of Roundup Transorb R® in the microcrustaceans *D. magna* and *C. dubia*. We monitored reproductive output, body morphology and number of molting across three successive generations (F0–F2). We hypothesized that the parental generation (F0) would be adversely affected by the herbicide and that these effects would persist across subsequent generations (F1 and F2) in both species.

Materials and methods

Culturing of D. magna and C. dubia

D. magna cultures were maintained in accordance with ABNT NBR 12713 guidelines (ABNT 2022). Organisms were held in environmental chambers (B.O.D. incubators) at a density of 30 individuals per 1.5 L of natural water under optimal physicochemical conditions: pH 7.6–8.0 and total hardness 125–225 mg $CaCO_3$/L. Culture and test were maintained at 20 ± 2°C under a 16:8 h light/dark photoperiod. Organisms were fed the microalga *Raphidocelis subcapitata* (1×10^6 cells per adult) supplemented with a complex feed mixture comprising fermented fish diet (Tretamin®), multivitamin solution Centrum®, and dried baker's yeast (1 mL complex feed per 1.5 L medium).

C. dubia cultures were maintained according to ABNT NBR 13373 guidelines (ABNT 2022). Organisms were held in environmental chambers (B.O.D. incubators) at a density of 30 individuals per 0.5 L of MS reconstituted medium under the following parameters: pH 7.0–7.6 and total hardness 40–48 mg $CaCO_3$/L. Culture and test temperature was set to 25 ± 2°C with a 16:8 h light/dark photoperiod. The diet consisted of *R. subcapitata* (1×10^5 cells per adult) supplemented with 500 µL of the complex feed mixture per 0.5 L culture medium.

Acute tests with the reference substance sodium chloride (NaCl) were performed periodically to confirm culture health and batch sensitivity. The mean concentration values producing an effect in 50% of the organisms (EC_{50}) for NaCl were 5.82 ± 0.24 g/L (48-h exposure for *D. magna*) and 1.57 ± 0.06 g/L (7-day exposure for *C. dubia*). In compliance with ABNT NBR 12713/2022 and 13373/2022 standards, control chart coefficients of variation remained well below the 30% threshold (4.1% for *D. magna* and 3.8% for *C. dubia*).

Generational experiments

D. magna and *C. dubia* were exposed to a control treatment (uncontaminated culture medium: natural water for *D. magna* and MS medium for *C. dubia*) and three nominal concentrations of Roundup Transorb R® (RTR): 30, 65 and 100 µg/L glyphosate acid equivalents. The formulated product contains 588 g/L of glyphosate potassium salt (equivalent to 480 g/L of glyphosate acid) and 739 g/L of other ingredients not specified by the manufacturer (Monsanto do Brasil Ltda.). Test concentrations were chosen based on Moraes et al. (2023). The 65 µg/L concentration represents the maximum allowable threshold for Brazilian legislation freshwater bodies, while 30 µg/L and 100 µg/L represent environmentally realistic

sub and supra-threshold concentrations, respectively. An RTR stock solution (0.1 g/L glyphosate acid equivalent) was prepared in ultrapure water.

For each exposure group, neonates (n = 15 per treatment) were individually isolated in glass beakers containing 30 mL of test medium (Fig. 1). Assays were conducted under semi-static conditions, with complete medium renewal every 72 h. Organisms were fed *R. subcapitata* at species-specific cell densities alongside 50 µL of complex feed at test initiation and at each renewal point. Temperature, pH and dissolved oxygen were recorded prior to and following every medium change (Tables S2 and S3).

For *D. magna*, neonates (2–26 h old) were exposed for 21 days (OECD Guideline 211). For *C. dubia*, neonates (6–24 h old) were exposed for 8 days (ABNT NBR 13373). Only the parental generation (F0) was exposed to the herbicide. Subsequent generations (F1 and F2) were reared in herbicide-free medium. To establish the F1 generation, neonates from the third brood of F0 mothers were harvested after approximately 15 days of exposure for *D. magna* and 6 days for *C. dubia*. Neonates were pooled and randomly assigned to start the reproduction assay according to OECD 211. The F2 generation was established using identical procedures from F1 mothers. Across all subsequent generations, 15 individual replicates per treatment were monitored under the same feeding and environmental conditions as the F0 generation, including temperature, photoperiod, and feeding regime, but in contaminant-free media, with semi-static renewals twice weekly.

Assessment of reproductive and morphological parameters

For each generation (F0–F2), the cumulative number of molts and total offspring produced per female during the experimental period were recorded for each species. At test termination, surviving adults were photographed under a stereomicroscope (OPZTR; Opticam Microscopy Technology®) equipped with a digital camera (OPTHD 14 MP; Opticam Microscopy Technology®). Morphometric measurements were quantified using ImageJ software. Evaluated morphological traits for *D. magna* included body length, body width and tail spine length (Figure S1). For *C. dubia*, body length and body width were evaluated (Figure S2).

Glyphosate analytical quantification

For glyphosate determination, water samples (15 mL) were collected in triplicate from experimental units of *D. magna* at the beginning (0 h) and end (72 h) of each exposure period, immediately before medium renewal, and stored in polypropylene tubes at 4°C in the dark until analysis (Kylin, 2013). For *C. dubia*, glyphosate quantification in the exposure medium was not feasible due to matrix interference from the MS culture medium, which contains salts and nutrients that compromise derivatization and chromatographic detection. Therefore, quantification was performed only for the stock solution (0.1 g/L).

Prior to analysis, samples (except the stock solution) were frozen at − 70°C, lyophilized until complete water removal, and reconstituted in ultrapure water to 10% of the original volume (5 mL), yielding a 10-fold concentration factor. Glyphosate determination was carried out by high-performance liquid chromatography with diode array detection (HPLC-DAD) at 210 nm, following pre-column derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) according to the protocol described by Moraes et al. (2024). All analyses were performed in triplicate.

The analytical calibration curve was prepared at five concentration levels: 10, 50, 100, 500, and 1000 µg/L, using a 1 g/L glyphosate stock solution (PESTANAL® analytical standard, Sigma-Aldrich) subjected to the same sample preparation procedure. The curve yielded a coefficient of determination $r^2 = 0.998$. The limits of detection (LOD) and quantification (LOQ) were 5 µg/L and 15 µg/L, respectively, calculated from the standard deviation of the intercept (σ) and the slope (S) of the regression line ($LOD = 3.3 \times \sigma/S$; $LOQ = 10 \times \sigma/S$) and confirmed by signal-to-noise ratios (S/N ≥ 3:1 and ≥ 10:1) at the lowest calibration level. For the stock solution (0.1 g/L), samples were diluted 10-fold and a separate calibration curve (5–20 mg/L) was used.

To ensure data quality and reliability, the following quality control measures were adopted: (i) reagent blanks (ultrapure water) and matrix blanks (culture medium without glyphosate) were analyzed in each analytical sequence and showed no co-eluting peaks at the glyphosate retention time; (ii) accuracy was assessed by spike recovery tests (triplicate) at 30, 65, and 100 µg/L (corresponding to the nominal exposure concentrations), submitted to the same lyophilization and derivatization protocol; mean recoveries ranged from 85% to 112%, with relative standard deviation (RSD) < 10% for all levels; (iii) method precision was verified by repeatability (intra-assay) and intermediate precision (inter-assay), with coefficients of variation below 5% and 12%, respectively; and (iv) the metabolite AMPA (aminomethylphosphonic acid) was qualitatively monitored in the samples at the same wavelength (260 nm), but quantitative validation was not possible due to lack of linearity of the calibration curve ($r^2 < 0.99$) under the tested conditions; therefore, AMPA results are reported only as detected or not detected.

Statistical analysis

Statistical evaluations were performed using SigmaPlot 14.5 software. Data normality and homogeneity of variances were assessed using Shapiro-Wilk and Brown-Forsythe tests, respectively. Parametric data were analyzed via one-way Analysis of Variance (ANOVA) followed by Dunnett's *post-hoc* test to identify significant differences relative to the control group. Non-parametric datasets were analyzed using Kruskal-Wallis tests followed by Dunn's *post-hoc*. Statistical significance was defined $p < 0.05$. All data are presented as mean ± standard error (*SE*).

Results

Analytical quantification of glyphosate

For *D. magna* exposure media, mean measured glyphosate concentrations at 0 h were 46.6 ± 14.4 µg/L, 87.7 ± 30.0 µg/L and 168.5 ± 26.8 µg/L for nominal targets of 30, 65 and 100 µg/L, respectively. At the end of the 72 h renewal period, measured concentrations dropped to 20.1 ± 2.7 µg/L, 50.2 ± 12.5 µg/L and 93.0 ± 24.8 µg/L, corresponding to concentration declines of 43.1%, 57.2% and 55.2%, respectively. Glyphosate was not detected in control media. The stock solution mean concentration was 0.165 ± 0.007 g/L. AMPA was qualitatively detected in all samples after 72 hours of exposure, except in the control group.

Molting and reproduction in D. magna and C. dubia

Molting number was not significantly altered by RTR exposure in either species across any evaluated generation (Fig. 2). In terms of reproductive output, directly exposed (F0) *D. magna* exhibited significant reductions in offspring production across all herbicide treatments, with fecundity decreases of 31.1% ($p = 0.002$), 30.4% ($p < 0.001$) and 15.2% ($p < 0.001$) at 30, 65 and 100 µg/L, respectively, relative to control (Fig. 3A, Table S1). In the F1 generation, a statistically significant reduction in offspring production persisted exclusively in the 65 µg/L treatment (14.9% decrease; $p = 0.001$), whereas no reproductive impairments were detected in the F2 generation at any concentration (Fig. 3B, C; Table S1). Furthermore, premature brood loss (abortions) occurred in exposed F0 *D. magna*, with 1, 5 and 2 aborted embryos observed at 30, 65 and 100 µg/L, respectively (Figure S3), compared to zero instances in the control group.

For *C. dubia*, reproductive output in the F0 generation was significantly reduced only at the highest exposure concentration (100 µg/L), decreasing by 32.1% relative to control ($p = 0.015$; Fig. 3D, Table S1). Subsequent unexposed generations (F1 and F2) displayed no significant differences in total offspring production (Fig. 3E, F). Mortality remained below the 20% threshold established by the test guidelines across all generations and treatments in *D. magna*, as well as in the F0 and F1 generations of *C. dubia*. In *C. dubia*, elevated mortality (26.6%) was observed only in the F2 generation at 65 and 100 µg/L.

Morphological responses in D. magna and C. dubia

Morphological evaluations revealed contrasting species-specific sensitivity patterns across generations. In *D. magna*, parentally exposed (F0) adults showed no significant alterations in body dimensions (Fig. 4A, 5A, 6A). However, morphological changes emerged in the F1 progeny (Fig. 4B, 5B, 6B). Specifically, adult body width was significantly reduced across all F1 treatment groups, decreasing by 2.9% ($p = 0.011$), 5.1% ($p < 0.001$) and 2.97% ($p = 0.025$) at 30, 65 and 100 µg/L, respectively. Tail spine length was also significantly shortened in F1 by 30.0% ($p < 0.001$), 27.5% ($p < 0.001$) and 17.5% ($p = 0.045$) across the same respective treatment groups. Body length in F1 *D. magna* decreased significantly at 30 µg/L (2.6% reduction; $p = 0.020$) and 65 µg/L (3.2% reduction; $p = 0.006$). By the F2 generation, all morphometric endpoints in *D. magna* had recovered to control levels (Figs. 4C, 5C, 6C). Conversely, *C. dubia* exhibited direct morphological sensitivity in the parentally exposed (F0) generation (Figs. 4D, 5D). Body length decreased significantly across all treatments (30 µg/L: 15.2%; 65 µg/L: 14.9%; 100 µg/L: 15.4%; $p < 0.001$ for all), as did body width (30 µg/L: 13.5%, $p = 0.003$; 65 µg/L: 15.5%, $p < 0.001$; 100 µg/L:

16.0%, $p < 0.001$). In subsequent unexposed generations (F1 and F2), no significant morphological differences were detected relative to control organisms (Figs. 4E, 5E, 4F, 5F).

Discussion

Our study demonstrates that chronic exposure to the commercial herbicide Roundup Transorb R® adversely impacts reproduction and body size in the freshwater cladocerans *D. magna* and *C. dubia*. Notably, these toxicological effects occurred at environmentally relevant glyphosate concentrations and considered compliant with current regulatory standards. Directly exposed (F0) *D. magna* exhibited consistent reproductive suppression across all tested herbicide levels alongside an increased incidence of embryonic abortion. In contrast, *C. dubia* showed direct reproductive impairment only at the highest exposure level (100 µg/L). The greater sensitivity observed in *D. magna* may be partially explained by differences in standard assay durations (21 days for *D. magna* vs. 8 days for *C. dubia*). Extended exposure time can allow toxic effects on reproductive output to accumulate. Manar et al. (2012) reported a similar phenomenon when evaluating the pesticide chlordane; extending the test duration for *C. dubia* from 7 to 14 days yielded reproductive inhibition comparable to that observed in *D. magna* after 21 days. In standard testing frameworks, the shorter assay duration for *C. dubia* accounts for its faster developmental rate and earlier onset of reproduction, with first broods released around day 3, whereas *D. magna* requires approximately 12 days to reach reproductive maturity (Manar et al. 2012).

In unexposed progeny, reproductive toxicity in *D. magna* persisted into the F1 generation at 65 µg/L, whereas *C. dubia* exhibited complete reproductive recovery in both F1 and F2. The absence of persistent reproductive impairment in the F2 generation across both species suggests that generational toxicity was not maintained under the conditions tested. From an ecological standpoint, these findings suggest that cladoceran populations retain the capacity for recovery once chemical stress ceases, such as following transient agricultural runoff events. However, under continuous or recurring application regimes, repeated reproductive inhibition could disrupt zooplankton population dynamics and alter trophic structure in shallow freshwater systems (Elser and Goldman 1991; Carpenter et al. 2001). The reproductive impairment observed in unexposed F1 *D. magna* at 65 µg/L indicates that parental exposure to regulatory-compliant pesticide concentrations can induce carryover effects in immediate progeny. This generational response may stem from several non-exclusive pathways: (1) altered maternal nutrient provision or physiological stress transferred to developing eggs; (2) indirect herbicide exposure of developing embryos within the maternal brood chamber; or (3) short-term direct exposure of neonates prior to isolation (up to 24 h post-birth in contaminated media) (Sumiya et al. 2014; Miyakawa et al. 2018). Although reproductive deficits attenuated by F2 despite the presence of germline cells during F0 exposure (Jeremias et al. 2022), transient population dips in primary consumers can nevertheless trigger ecological shifts.

Generational outcomes following pesticide exposure vary across aquatic organisms, exhibiting both recovery and exacerbation patterns across generations. In the estuarine rotifer *Proales similis*, a 48 h parental exposure to glyphosate pure active ingredient (1–1000 µg/L) reduced intrinsic population

growth rates (r) through the F2 generation, with population recovery occurring only from F3 onward (Arreguin-Rebolledo et al. 2023). Conversely, continuous multigenerational exposure of *Daphnia exilis* to the formulation Faena® (2.09–3.15 mg/L) caused reductions in survival and reproduction of the parental generation (F0) and resulted cumulative toxicity, with reproduction in F1 being almost completely suppressed. This evidence a generational effect in which accumulated toxic responses may contribute to the population decline (Rodríguez-Miguel et al. 2021).

In addition to reproductive effects, morphological alterations were also observed in *D. magna* and *C. dubia*, indicating generation-dependent responses and suggesting distinct coping strategies under exposure to the stressor. In *C. dubia*, parental exposure to RTR significantly reduced adult body length and width in F0, but this growth inhibition was completely reversed in F1 and F2. In contrast, *D. magna* maintained normal body dimensions during direct F0 exposure, but its F1 progeny exhibited significant reductions in body width, body length and tail spine length before recovering in F2. Changes in body size in these organisms may reflect energetic reallocation, whereby resources that would normally be allocated to growth are redirected toward cellular defense mechanisms, such as antioxidant responses and detoxification processes, or toward reproduction as an adaptive strategy to ensure species survival under adverse conditions (Jacobasch et al. 2014; Poulsen et al. 2021; Sahilli and Korkmaz 2025).

The morphological changes observed in the F1 generation of *D. magna*, as well as the reproductive effects, may be related to maternal transfer, indirect exposure of F1 offspring during development, or direct exposure of F1 offspring, even for a short period. In cladocerans, body morphology—particularly body size and tail spine length—is closely associated with defense against predation. Under natural environmental conditions, such changes may be detrimental to the species, as smaller individuals tend to exhibit lower fecundity and greater vulnerability to predation (Lynch 1980), potentially compromising population dynamics. Unlike *C. dubia*, *D. magna* may have allocated more energy to maintaining essential physiological functions during the exposure period rather than to reproduction, resulting in smaller offspring that subsequently recovered their morphological characteristics in the F2 generation (Jacobasch et al. 2014).

Regarding molting, RTR did not affect its dynamics of the organisms. Molts is an essential process that occurs frequently in cladocerans such as *D. magna* and *C. dubia* as organisms reach reproductive maturity, since it is necessary for them to reach adult size. In addition, the reproductive cycle occurs synchronously with the molting cycle. After molting, females ovulate new eggs from the ovary into the brood chamber located in the dorsal region. At the end of each reproductive cycle, the brood is released from the brood chamber into the surrounding water. After this release, molts occurs again and the cycle repeats (Sumiya et al. 2014; Miyakawa et al. 2018). Thus, alterations in molts frequency may directly affect growth, development time, and reproduction. The absence of effects on the number of molts suggests that the herbicide, at the concentrations evaluated, perhaps did not significantly affect the endocrine and enzymatic mechanisms regulating ecdysis. However, it would be necessary to evaluate cellular and biochemical parameters to confirm the absence of an effect.

Another noteworthy point is the absence of dose-response effects for *D. magna*. We highlight that significant effects were observed at both concentrations; however, reproductive and morphological effects were more pronounced at the 65 µg/L concentration than at 100 µg/L. A possible explanation is differential activation of protective cellular mechanisms across concentration thresholds. For instance, Moraes et al. (2023) observed increased lipid peroxidation in *Jenynsia multidentata* exposed to 65 µg/L glyphosate (96 h), whereas at 100 µg/L lipid damage subsided alongside a marked induction of glutathione S-transferase (GST) activity. A similar compensatory mechanism may operate in *D. magna*, where higher herbicide stress (100 µg/L) may trigger stronger induction of detoxification enzymes and antioxidant defenses, thereby partially attenuated the reproductive and morphological effects, rendering them less pronounced than those recorded at the 65 µg/L concentration.

The occurrence of generation-dependent effects reinforces the importance of considering not only the immediate impacts on organismal health and reproduction, but also potential generational consequences in the ecological risk assessment of glyphosate-based formulations. Non-genetic inheritance mechanisms, including epigenetic modifications (e.g., DNA methylation, histone modifications and non-coding RNA expression), may contribute to mediating transgenerational phenotypic plasticity under chemical stress (Kovalchuk 2012; Jeremias et al. 2022). For example, parental exposure of *D. magna* to copper (0.021 mg/L) altered global DNA methylation in F0, and these epigenetic alterations persisted epigenetic persisted through three unexposed generations (F1–F3) (Jeremias et al. 2022). Investigating epigenetic marks following exposure to glyphosate-based formulations represents an important direction for future research.

Glyphosate is the most widely used herbicide worldwide, and most studies on glyphosate evaluate only the isolated active ingredient, disregarding the other components present in commercial formulations. The use of a commercial formulation in the present study reinforces its ecological relevance, as it allows the effects of one of the most widely used glyphosate-based formulations to be investigated under conditions that more realistically reflect environmental exposure. Several studies have demonstrated that glyphosate-based formulations exhibit greater toxicity to organisms than the isolated active ingredient (Bridi et al. 2017; Lares et al. 2022; Spinaci et al. 2022; Abe et al. 2024; Tóth et al. 2024). This greater toxicity has been attributed to the presence of other ingredients in these formulations, such as the surfactant polyoxyethylene amine (POEA), which has been reported to be toxic to several species (Mottier et al. 2014; Rodrigues et al. 2019; Lopes et al. 2021; Klátyik et al. 2024).

The measured glyphosate concentrations decreased after 72 h. This reduction may be associated with several processes occurring in the experimental system, such as microbial activity, given that the culture media were not autoclaved. In addition, the possibility of glyphosate bioaccumulation by microcrustaceans cannot be excluded. Although glyphosate is expected to exhibit low bioaccumulation potential in biota due to its low octanol–water partition coefficient (log Kow: −3.4), evidence indicates that the herbicide can accumulate in the tissues of various organisms following exposure through water or diet (Tongo et al. 2022; Yan et al. 2023; Drechsel et al. 2024; Li et al. 2026). However, even with the decrease in glyphosate concentrations over the exposure period, toxic effects were still observed in the

tested organisms. This may be related to the degradation by-products of the herbicide. Degradation may occur through cleavage of C–N bonds, forming AMPA, a potentially more toxic metabolite, or through cleavage of C–P bonds, generating sarcosine and subsequently glycine, which are less toxic products (Sun et al. 2019).

Conclusion

The results of this study indicate that the most pronounced effects occurred in directly exposed organisms and in the first generations following exposure. Based on the endpoints evaluated, responses tended to return to baseline levels from the second generation onward. This finding may be considered encouraging, as it suggests that glyphosate does not induce persistent effects across successive generations of cladocerans. However, it is important to note that significant reproductive and morphological alterations were observed at relatively low, environmentally relevant concentrations. Under more complex environmental scenarios, including climate-related stressors and co-exposure to other contaminants, these effects could be amplified and lead to more severe ecological consequences than those observed in the present study.

Declarations

Authors and Affiliations

Programa de Pós-graduação em Ciências Fisiológicas, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande. Av, Itália km 8, 96203-900, Rio Grande, RS, Brazil.

Josiane Araujo da Silva, Jenifer Silveira Moraes & Camila de Martinez Gaspar Martins

Instituto Federal de Educação, Ciência e Tecnologia Sul-rio-grandense, Campus Pelotas. Praça 20 de Setembro, 455, 96015-360, Pelotas, RS, Brazil.

Sophia Vallerini & Bernardo dos Santos Vaz

Conflict of interests

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.

Author Contribution

J.A.S: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Visualization, Writing - Original Draft, Writing - Review & Editing. S.V: Methodology and Validation. B.S.V: Methodology, Validation, Formal analysis, Resources, Writing - Review & Editing. J.S.M:

Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Review & Editing. C.M.G.M: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Review & Editing, Resources, Supervision, Funding acquisition. All authors have read and approved the final manuscript.

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Data Availability

Data are available from the corresponding author upon reasonable request.

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Figures

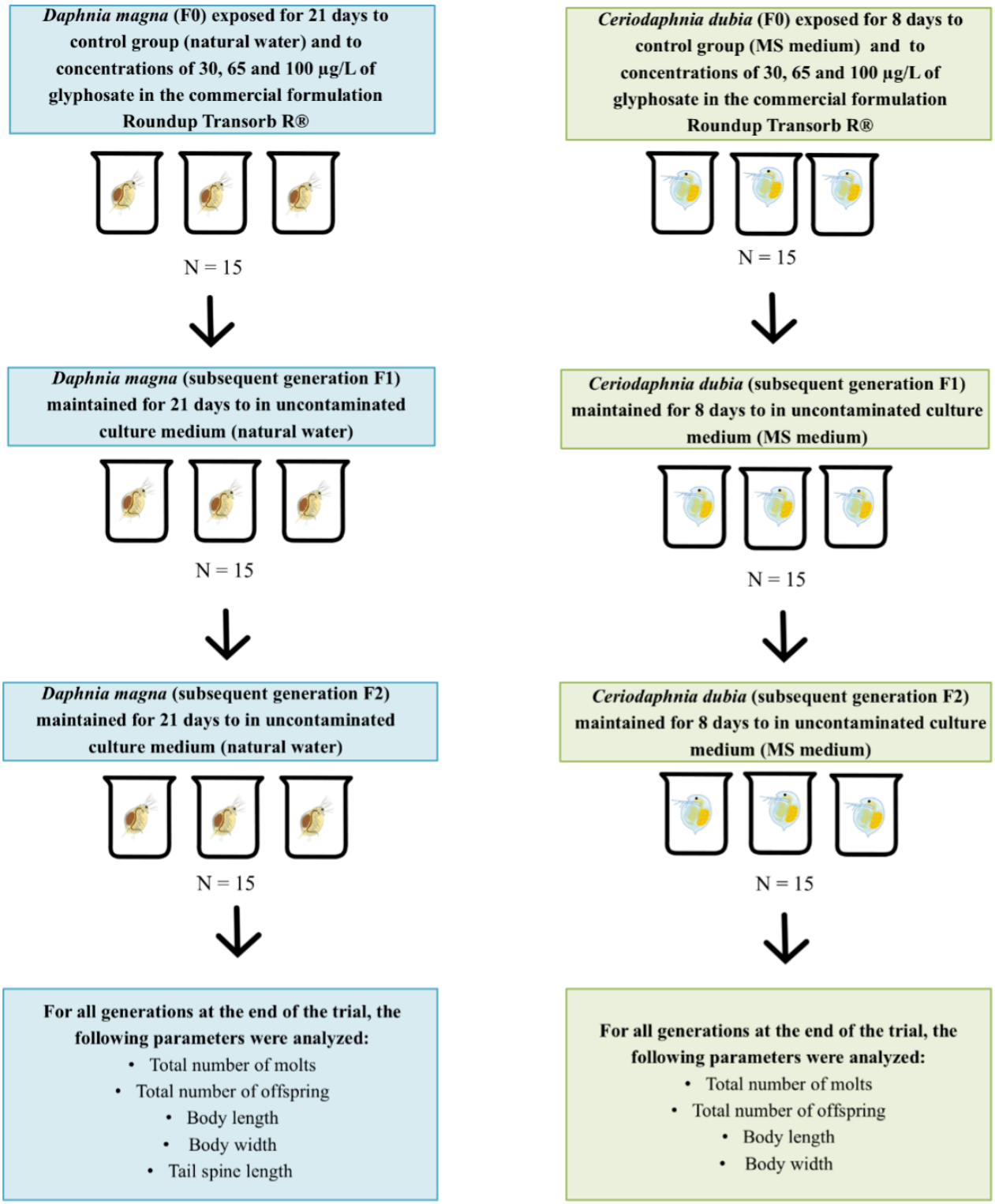


Figure 1

Experimental design of the chronic toxicity assays with the cladocerans *Daphnia magna* (21-day exposure) and *Ceriodaphnia dubia* (8-day exposure). The parental generation (F0) was exposed to four treatments: control and three glyphosate concentrations (30, 65 and 100 µg/L) as the commercial formulation Roundup Transorb R®. Subsequent generations (F1 and F2) were maintained under identical

experimental conditions in herbicide-free culture media. n = 15 individually housed organisms per treatment.

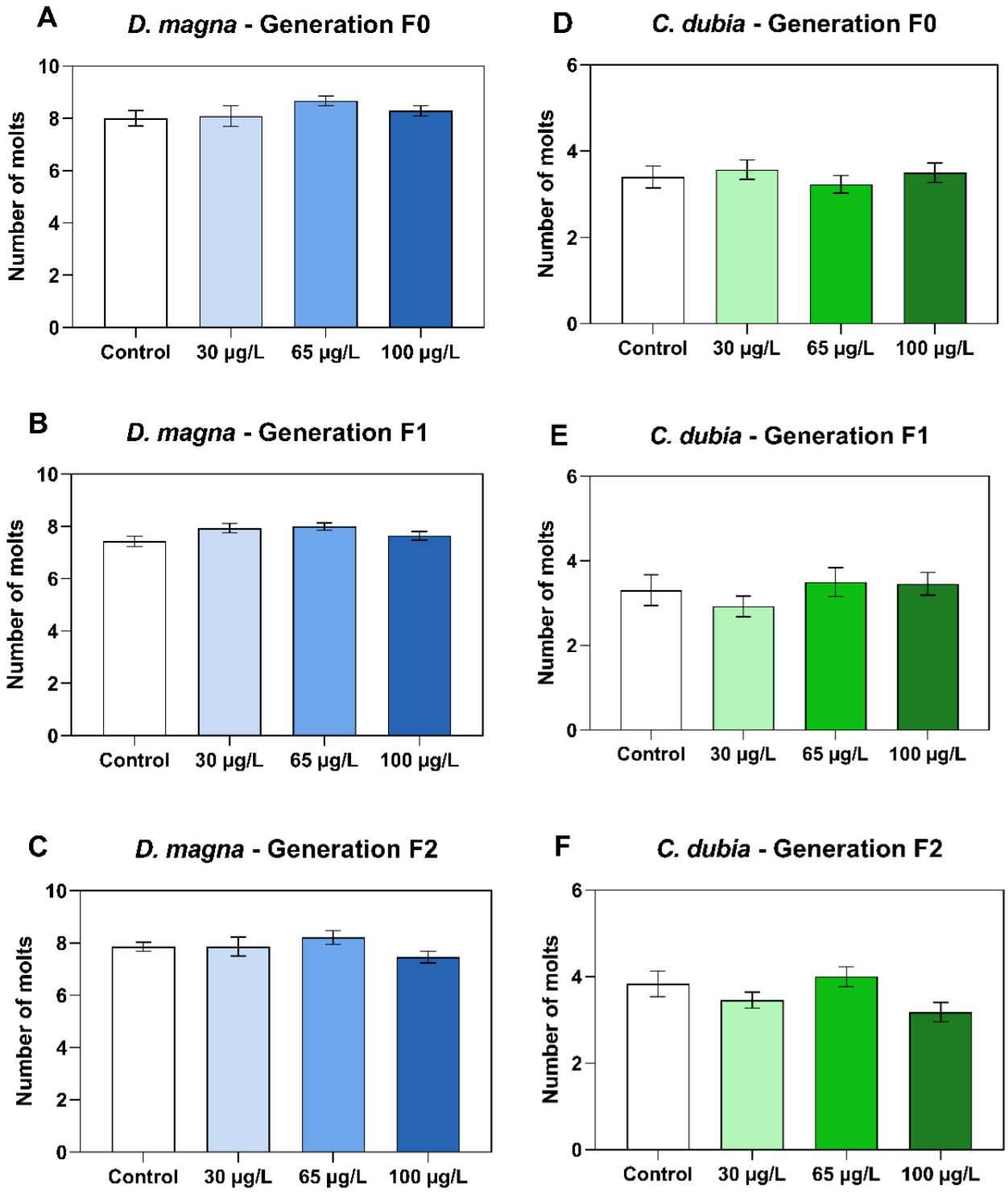


Figure 2

Number of molts in *Daphnia magna* (A–C; 21-day assay) and *Ceriodaphnia dubia* (D–F; 8-day assay) across three successive generations (F0, F1 and F2). Data are presented as mean ± standard error.

Asterisks indicate statistically significant differences relative to the control group ($p < 0.05$). Sample size (n) for each treatment and generation are provided in Table S4. For *D. magna*, data for the F1 generation were analyzed via one-way ANOVA followed by Dunnett's *post-hoc*test ($p < 0.05$), whereas non-parametric Kruskal–Wallis tests followed by Dunn's *post-hoc* tests ($p < 0.05$) were used for F0 and F2. For *C. dubia*, data for the F2 generation were analyzed via one-way ANOVA with Dunnett's *post-hoc* test ($p < 0.05$), while F0 and F1 were evaluated using Kruskal–Wallis tests with Dunn's *post-hoc* tests ($p< 0.05$).

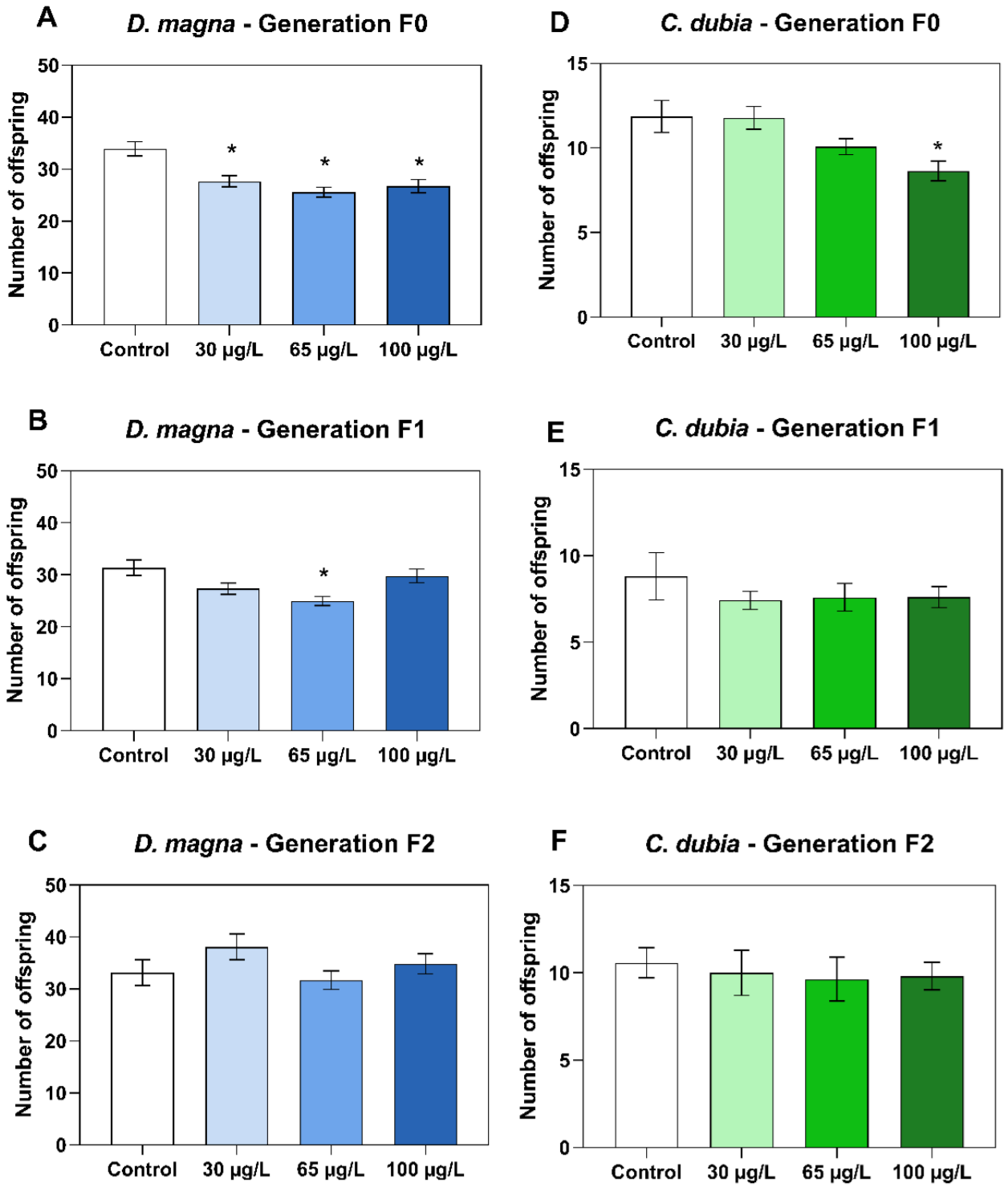


Figure 3

Number of offspring in *Daphnia magna* (A–C; 21-day assay) and *Ceriodaphnia dubia* (D–F; 8-day assay) across three successive generations (F0, F1 and F2). Data are presented as mean ± standard error. Asterisks indicate statistically significant differences relative to the control group ($p < 0.05$). Sample size (n) for each treatment and generation are provided in Table S4. For *D. magna*, data across all generations were analyzed via one-way ANOVA followed by Dunnett's *post-hoc*test ($p < 0.05$). For *C. dubia*, non-parametric Kruskal–Wallis tests followed by Dunn's *post-hoc* tests ($p < 0.05$) were applied across all generations.

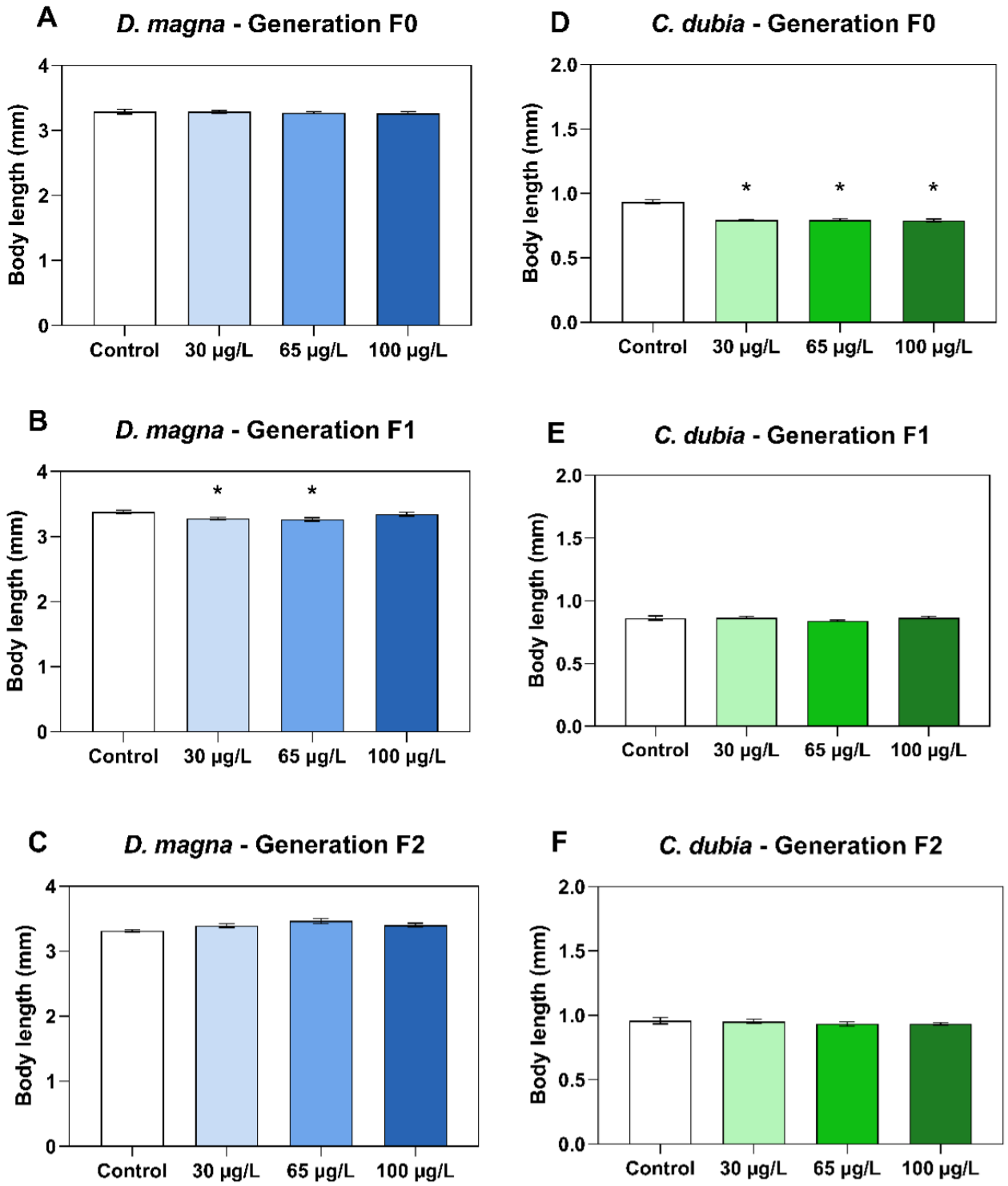


Figure 4

Body length (mm) in *Daphnia magna* (A–C; 21-day assay) and *Ceriodaphnia dubia* (D–F; 8-day assay) across three successive generations (F0, F1 and F2). Data are presented as mean ± standard error. Asterisks indicate statistically significant differences relative to the control group ($p < 0.05$). Sample size (n) for each treatment and generation are provided in Table S4. For both species, data for the F1 generation were analyzed via one-way ANOVA followed by Dunnett's *post-hoc* test ($p< 0.05$), whereas

non-parametric Kruskal–Wallis tests followed by Dunn's *post-hoc*tests ($p < 0.05$) were applied for the F0 and F2 generations.

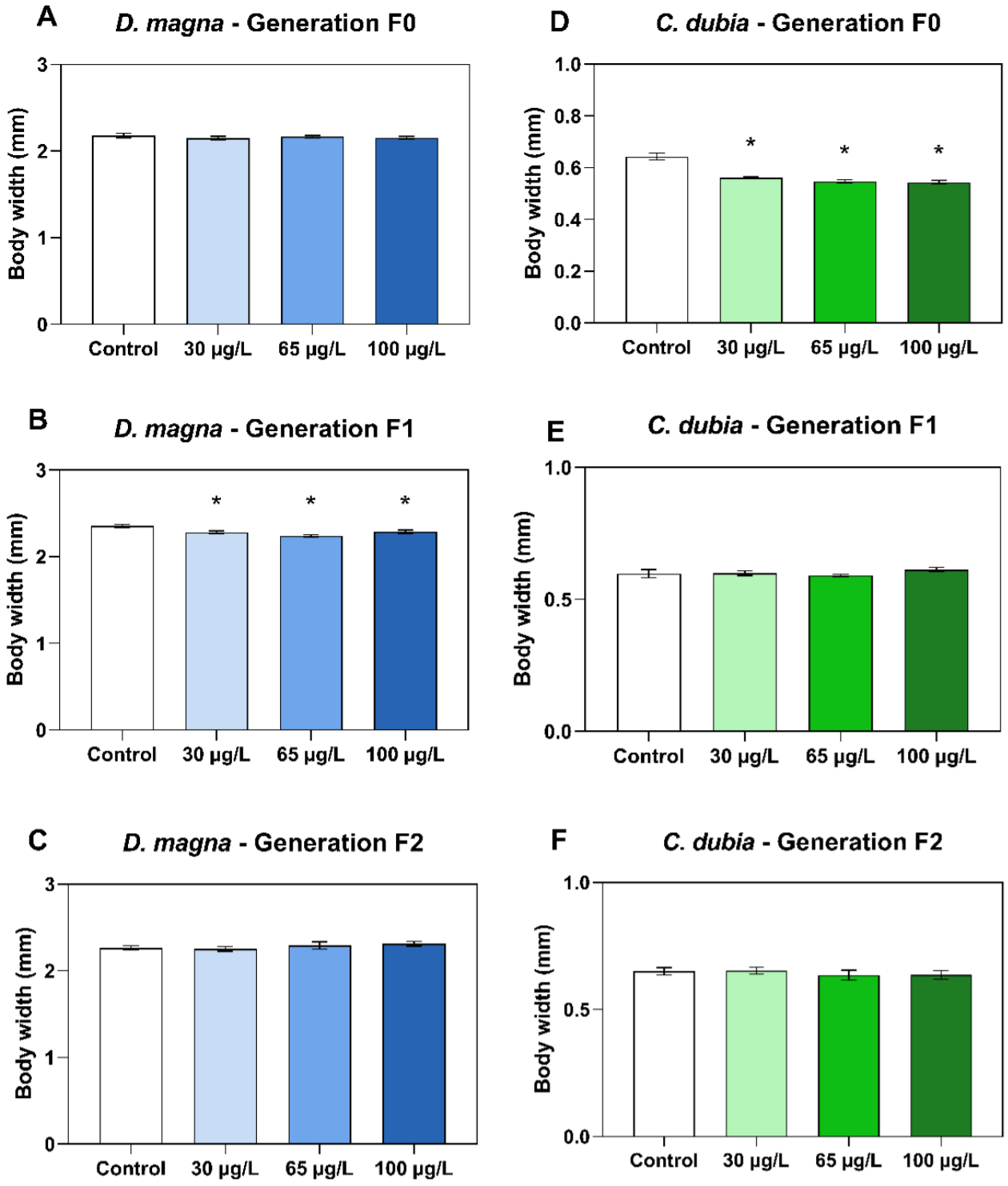
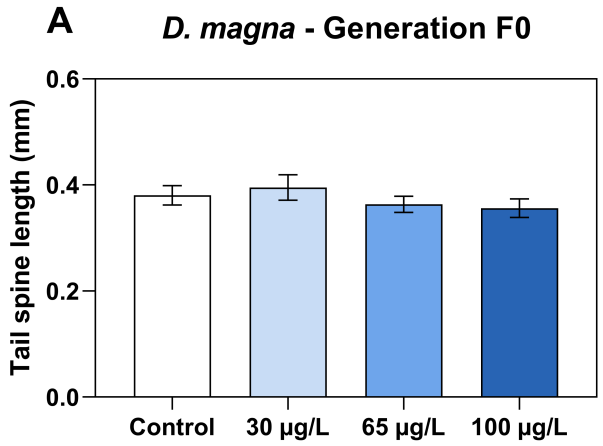
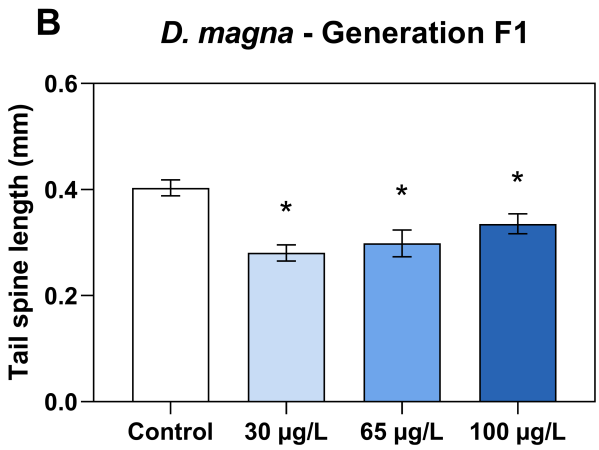


Figure 5

Body width (mm) in *Daphnia magna* (A–C; 21-day assay) and *Ceriodaphnia dubia* (D–F; 8-day assay) across three successive generations (F0, F1 and F2). Data are presented as mean ± standard error.

Asterisks indicate statistically significant differences relative to the control group ($p < 0.05$). Sample size (n) for each treatment and generation are provided in Table S4. For *D. magna*, data for the F0 and F1 generations were analyzed via one-way ANOVA followed by Dunnett's *post-hoc*test ($p < 0.05$), whereas the F2 generation was evaluated using non-parametric Kruskal–Wallis tests followed by Dunn's *post-hoc* tests ($p< 0.05$). For *C. dubia*, data for the F2 generation were analyzed via one-way ANOVA with Dunnett's *post-hoc* test ($p < 0.05$), while the F0 and F1 generations were evaluated using Kruskal–Wallis tests with Dunn's *post-hoc* tests ($p < 0.05$).





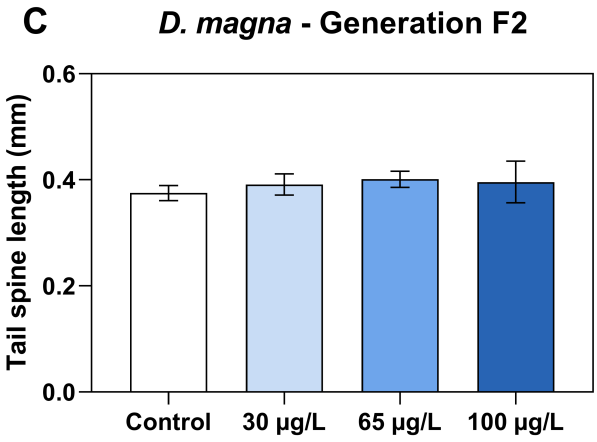


Figure 6

Tail spine length (mm) in *Daphnia magna* (A–C; 21-day assay) across three successive generations (F0, F1 and F2). Data are presented as mean ± standard error. Asterisks indicate statistically significant differences relative to the control group ($p < 0.05$). Sample size (n) for each treatment and generation are provided in Table S4. For the F0 and F1 generations, data were analyzed via one-way ANOVA followed by Dunnett's *post-hoc*test ($p < 0.05$), whereas the F2 generation was evaluated using non-parametric Kruskal–Wallis tests followed by Dunn's *post-hoc* tests ($p< 0.05$).

Supplementary Files

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