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Influence of Age and Exposure Pathway on Copper and Cadmium Mixture Toxicity: A Study on *Daphnia magna*

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ABSTRACT

Metal contamination in aquatic ecosystems poses substantial risks to freshwater organisms, with mixture effects often deviating from predictions based on single-metal toxicity. Understanding how age-specific sensitivities and multiple exposure pathways influence mixture effects is critical for accurate risk assessment. This study investigated the combined toxicity of copper (Cu) and cadmium (Cd) in *Daphnia magna*. Responses were compared between adults (20–21 days old) and neonates (less than 24 h old) under three exposure scenarios: aqueous-only (0.25 μM Cu, 0.02 μM Cd, and their co-exposure), dietary-only (4.33×10^3 ng Cu/daphnid and 5.72×10^1 ng Cd/daphnid and their co-exposure, delivered via metal-spiked algae *Raphidocelis subcapitata*), and combined aqueous-dietary exposure. Following seven-day exposures, physiological stress indicators (reproduction, growth, and survival), metal accumulation, biochemical and transcriptional changes were evaluated. Co-exposure produced functionally synergistic toxic effects, with neonates showing markedly lower tolerance than adults at equivalent concentrations. Toxicity differed strongly among exposure routes, with combined aqueous–dietary exposure causing the most severe effects, including a 52% reduction in adult reproduction and 30% growth inhibition in neonates. Metal accumulation pattern varied between adults and neonates. Across both life stages, Cu–Cd co-exposures disrupted redox homeostasis, evidenced by elevated reactive oxygen species and significant alterations in antioxidant enzyme activity. These responses were corroborated by pathway-specific transcriptional changes, including upregulation of antioxidant, apoptosis-related, and metal-handling genes, alongside downregulation of development-related genes in neonates. Overall, life stage and exposure pathway emerged as critical determinants of Cu-Cd mixture toxicity. This emphasizes the need of incorporating age-specific sensitivities and realistic multi-metal, multi-pathway exposures into freshwater risk assessment frameworks.

1 | Introduction

Metal contamination is a widespread and persistent threat to aquatic ecosystems globally, driven by continuous anthropogenic inputs from industrial effluents, agricultural runoff, urban discharges, and atmospheric deposition. In natural environments, aquatic organisms are rarely exposed to metals individually; rather they encounter complex mixtures whose combined effects often differ fundamentally from those predicted by single-metal toxicity data [1–3]. Such metal mixtures may exhibit additive, synergistic, or antagonistic interactions [2–8]. These interactions can modify internal metal burdens, shift dominant modes of toxic action, and change both the magnitude and the direction of biological effects, rendering single-metal extrapolations unreliable for ecological risk assessment [2, 3, 9–11]. Despite increasing recognition of

mixture toxicology, major knowledge gaps remain regarding how biological traits and environmental exposure conditions modulate mixture effects under ecologically realistic scenarios.

Two particularly important yet understudied determinants of metal mixture toxicity are life stage and exposure pathway. Age-dependent sensitivity to contaminants has been widely documented, with early life stages often exhibiting increased vulnerability due to underdeveloped detoxification systems, higher metabolic rates, and larger surface area-to-volume ratios [2, 3, 12]. Nevertheless, most metal toxicity studies focus on a single life stage, potentially underestimating risks during sensitive developmental windows. Regulatory frameworks have traditionally emphasized aqueous exposure as the dominant uptake route, despite growing evidence that dietary exposure can contribute substantially to total metal body burden and toxicity [13–15]. Metals associated with suspended particles, biofilms, and prey organisms can be efficiently transferred through food webs, yet dietary pathways remain largely excluded from water quality criteria [16, 17]. The relative contribution of aqueous versus dietary exposure varies among metals, species, and environmental contexts, and their combined influence on mixture toxicity remains poorly characterized [18–20].

Copper (Cu) and cadmium (Cd) represent an ecologically relevant metal pair for investigating mixture toxicity, as they frequently co-occur in freshwater systems, yet differ markedly in their essentiality, toxicokinetics, and mechanisms of action. Copper is an essential, redox-active trace element required for several physiological processes, including enzymatic activity, respiration, neurotransmitter biosynthesis, and antioxidant defense [21–26]. Its ability to cycle between Cu(I) and Cu(II) underlies its role as a cofactor for key enzymes such as superoxide dismutase, cytochrome-c oxidase, and dopamine β-hydroxylase [23]. However, when present in excess, copper shifts from an essential micronutrient to a potent toxicant, inhibiting Na^+/K^+-ATPase and other ion-regulatory enzymes, disrupting mitochondrial respiration, and promoting reactive oxygen species (ROS) generation that leads to lipid peroxidation, DNA damage, and apoptosis [3, 22, 27–29]. Intracellular copper concentrations are therefore tightly regulated by high-affinity transporters and chaperones, including copper transporter 1 (CTR1), the P-type ATPases ATP7A and ATP7B, and antioxidant protein 1 (ATOX1) [23, 30]. Disruption of this homeostatic network results in systemic copper imbalance and severe pathological outcomes, highlighting the narrow margin between Cu essentiality and toxicity [30].

In contrast to the tightly regulated essentiality of Cu, Cd is a non-essential, strongly bioaccumulative, and highly persistent metal, with a distinct mechanism of toxicity [31, 32]. Unlike Cu, Cd does not undergo metabolic detoxification and is poorly excreted, resulting in an extremely long biological half-life and cumulative organ toxicity [33]. Cadmium does not participate in redox cycling but exerts toxicity by binding to sulfhydryl groups of proteins, displacing essential metals such as calcium (Ca), zinc (Zn), and iron (Fe), and impairing metal-dependent enzymes [34–38]. Cadmium induces oxidative stress indirectly by inhibiting antioxidant enzymes, promoting lipid peroxidation, interfering with DNA repair mechanisms, and disrupting mitochondrial function, ultimately leading to carcinogenesis. Accordingly, cadmium and its compounds are classified by the International Agency for Research on Cancer (IARC) as Group 1 carcinogens (carcinogenic to humans) [39]. These fundamental differences between Cu and Cd suggest that their combined effects may be strongly influenced by exposure pathway, internal metal accumulation, and life stage–specific detoxification capacity.

The present study addresses critical knowledge gaps in metal mixture toxicology by investigating the combined effects of Cu and Cd in *Daphnia magna* across different life stages and exposure pathways. *Daphnia magna* is a well-established model organism in aquatic ecotoxicology and an ecologically relevant primary consumer in freshwater food webs [40]. As a filter feeder, *D. magna* is naturally exposed to contaminants through both dissolved uptake across the body surface and dietary ingestion of contaminated algae and particles, making it particularly suitable for multi-pathway exposure studies [41]. Its short life cycle, transparent body, and well-characterized genome further enable age-comparative and mechanistic investigations of contaminant toxicity [42–45].

This study examined: (1) whether Cu-Cd mixture toxicity differs between neonates and adults; (2) how exposure pathway (aqueous-only, dietary-only, or combined aqueous-dietary) influences mixture effects; (3) patterns of metal bioaccumulation under different exposure scenarios; and (4) oxidative stress responses as a mechanistic indicator of mixture toxicity. By integrating age-specific sensitivities with realistic multi-pathway exposure scenarios, this research provides critical insights for improving ecological risk assessment frameworks and enhancing our mechanistic understanding of metal mixture toxicity in freshwater ecosystems.

2 | Material and Methods

2.1 | Experimental Design

In the first exposure setup (aqueous exposure), adults and neonates were exposed to Cu and Cd separately and as a mixture through water (concentration addition exposure). Exposure concentrations were determined by the OECD Test Guidelines No. 202 [46] (see Section 2.3). In the second exposure setup the animals were exposed to the metals through food (dietary exposure) (concentration addition exposure). In this experiment, Cu- and Cd-spiked green algae *Raphidocelis subcapitata* were utilized as food. To determine how much metal was taken up by the algae, experiments on metal uptake were carried out first with *R. subcapitata* (see Section 2.4) followed by exposure of animals with the metal-loaded food. The two exposure pathways were combined in the third experimental setup to assess the influence of the two pathways on toxicity. All the experiments were conducted over a period of 7 days, and toxicity indicators such as growth, reproduction, and survival were monitored. Genes encoding proteins involved in oxidative stress, apoptosis regulation, DNA repair, development, and metal transport were studied and the redox state was evaluated. The overview of the experimental setup is presented in Figure 1.

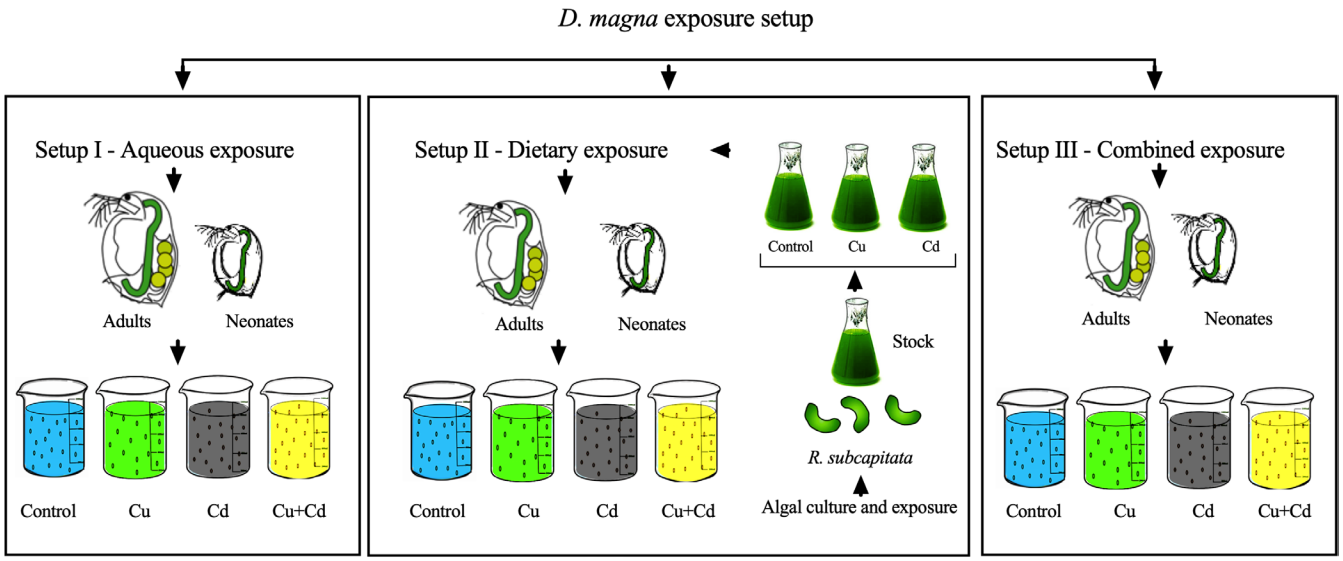

FIGURE 1 | Overview of experimental design. *D. magna* adults (20–21 days old) and neonates (less than 24 h old) were exposed to the single and mixture of Cu and Cd via three exposure set-ups: aqueous (dissolved in water), dietary (spiked with food) and their combination (aqueous + dietary), over 7 days.

2.2 | *Daphnia magna* Culture

The animals used in the study originated from a healthy *D. magna* stock reared in our laboratory (originally supplied by Ghent University, Belgium). The stock culture was maintained under controlled laboratory conditions in medium hard reconstituted water (80–100 mg/L as $CaCO_3$) which was used consistently for stock culture maintenance, acclimation, and as the exposure medium throughout the experiments. Cultures were kept in one liter glass jars at a constant temperature (20°C ± 1°C), pH (8.0 ± 0.2) and a 14 h:10 h, light:dark photoperiod. Daphnids (less than 24 h old) were acclimatized for at least 3 weeks (one generation) under the same physicochemical conditions to minimize handling and environmental stress and to ensure physiological stabilization prior to experimentation. To compare toxicity responses between early developmental and adult life stages, newly hatched neonates (< 24 h old) and fully mature females (20–21 days old) that had completed several molting cycles were used. Neonates produced during the rearing period were discarded daily, except the last brood (6th brood), which was selected for use in the experiments. The culture medium was renewed three times per week by gently transferring daphnids to fresh medium using wide-bore pipettes (internal diameter at least 1.5 times the size of the animals). Care was taken to ensure that the animals were not bumped or crushed during transfer to the new medium. The animals were introduced below the surface of the new medium to avoid trapping air under their carapace. During the rearing period, the daphnids were fed a mixture of the algae *R. subcapitata* and *Chlamydomonas reinhardtii* at a ratio of 3:1 (2×10^7 algal cells/daphnid).

2.3 | Selection of Exposure Concentration

The exposure concentrations were selected based on the OECD acute immobilization test [46]. The acute toxicity of the individual metals and the metal mixtures present in the dissolved phase was determined in a 48-h static, non-renewal lethality test in accordance with the test guidelines (Test No. 202, [46]). One day prior to the start of the toxicity test, a one-molar stock solutions (1 M) of Cu (as $CuSO_4.5H_2O$), and Cd (as $CdCl_2.H_2O$) were prepared using the same reconstituted water that was used for culturing *D. magna* and diluted to concentrations ranging from 0.15–3.0 μM Cu, and 0.02–1.0 μM Cd. Control groups consisted of organisms maintained in reconstituted water without added metals. These ranges were selected based on the published data on Cu and Cd toxicity to *D. magna* [7, 8, 47–50]. The test ranges (Cu = 0.15, 0.25, 0.35, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5 and 3.0 μM; Cd = 0.02, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.5, 0.75, and 1 μM) were applied in a gradient to produce 0% to 100% mortality. A total of 20 organisms (24 h old neonates) were exposed to each metal concentration (singly and in combination) and a control solution. Each metal concentration was tested in 4 replicates in 50 mL polycarbonate cups containing 25 mL of test solution (5 neonates/replicate). After 48 h, the immobilized neonates were counted as an indicator of mortality. Animals were considered immobilized if no movement was observed for 15 s even after gentle probing. The test was repeated twice. Lethal concentrations were determined by plotting the dose—response curve using Prism8 software (GraphPad) and calculated with 95% confidence interval. Exposure concentrations for single and mixture treatments was selected based on 20% of 48 h LC_{50} (Cu = 0.25 μM, Cd = 0.02 μM).

2.4 | Metal Uptake Experiments With *R. subcapitata*

Metal uptake experiments were conducted with green algae *R. subcapitata* to determine the concentration of metals absorbed by algae. The algae were cultured according to the OECD Test Guidelines 201 [51]. The log-phase algae were exposed

to 0.15 μM $CuSO_4.5H_2O$ [96 h IC_{50} = 1.38 μM [52]] and 0.05 μM $CdCl_2$. H_2O [96 h IC_{50} = 0.67 [53]] with two replicates per condition in glass flasks of one liter capacity. Exposures continued for 7 days before they were fed to animals. This is also the time required for completion of 7 day algal growth cycle. The experiments were conducted under continuous illumination and aeration at 20°C ± 1°C. To avoid contamination, the opening of each flask was sealed with aluminum foil and air was filtered through a 0.22 μm nylon filter for aeration. The number of algal cells was monitored daily in each test solution using a Beckman Multisizer Z3 Coulter Counter (Beckman Coulter Inc. Version 3.33). The metal content of each replicate was determined each day according to the procedure followed by Komjarova and Blust [54]. Briefly, 12 mL of the culture was withdrawn from each stock. Ten milliliters of culture was withdrawn to determine the number of algal cells using a Beckman Multisizer Z3 Coulter Counter (Beckman Coulter Inc. Version 3.33). The remaining 2 mL of culture was taken in a separate 50 mL tube, raised to the 40 mL mark with high purity (Milli-Q) water and used for metal analysis of algae. This was followed by shaking and centrifugation. After centrifugation, the supernatant was discarded, and the pellet was oven-dried at 60°C for 24 h. After drying, the samples were digested in 200 μL of ultrapure nitric acid (Trace metal grade—Merck, Darmstadt, Germany) on a hot block at 110°C for 1 h. Digested samples were diluted to 40 mL with ultrapure water (Milli-Q, Bedford, MA, USA). The Cu and Cd concentrations of the samples and blanks were analyzed by inductively coupled plasma mass spectrometry (7700× ICP-MS, Agilent Technologies). The exposure resulted in a Cu load of 2.2×10^{-4} ng/cell dry weight and a Cd load of 2.9×10^{-6} ng/cell dry weight. A reference standard mussel tissue (NIST—2976) was used to assess the validity of the metal analysis protocol.

2.5 | *D. magna* Exposure

For the aqueous exposure, four exposure conditions were chosen: a control (without added metals), 0.25 μM Cu, 0.02 μM Cd singly and a mixture (i.e., concentration addition exposure). Both control and metal-added solutions were prepared from 1 M stock solutions, 1 day before the start of exposure. For each exposure category, 100 adult animals were separately transferred to one liter glass jars filled with 800 mL of exposure medium. To prevent binding of metals to feed particles, food was not added to the aqueous media during the uptake period. The medium was renewed three times per week. For dietary exposure, metal-spiked algae *R. subcapitata* containing 2.2×10^{-4} ng Cu/cell dry weight (equivalent to 4.33×10^{3} ng Cu/daphnid) and 2.9×10^{-6} ng Cd/cell dry weight (equivalent to 5.72×10^{1} ng Cd/daphnid) were fed daily to the respective exposure groups while, control animals were fed uncontaminated diet (without added metals). In the combined scenario the animals were simultaneously exposed to water containing metal concentrations used for aqueous exposure, as well as a diet containing concentrations used in dietary exposure individually (refer to aqueous and dietary exposure details above). In all exposure groups, survival rate and number of neonates produced per female daphnid were monitored daily and collected from the medium. Water quality variables (pH, temperature, dissolved oxygen, and conductivity) and mortality were recorded daily. Animals were considered dead if no movement was observed for 15 s even after gentle shaking. Water samples (10 mL) were taken before and after each feeding to determine the number of algal cells consumed, using a Beckman Multisizer Z3 Coulter Counter (Beckman Coulter Inc. Version 3.33).

2.6 | Metal Accumulation Analysis

All animal samples were analyzed for metal accumulation using inductively coupled plasma mass spectrometry (7700× ICP-MS, Agilent Technologies) following the method previously described in Majid et al. [2]; [3]. At the end of the exposure period, 8 biological replicates were prepared per condition, each consisting of 10 pooled animals. Pooled samples were placed in 1.5 mL Eppendorf tubes and rinsed with deionized water. After the samples were rinsed, the remaining water was removed using a pipette and then snap frozen in liquid nitrogen. The fresh weight of each pooled sample was then measured. Samples were then thawed and oven dried at 60°C for 24 h. Dried tissues were digested in 150 μL ultrapure nitric acid (trace metal grade—Merck, Darmstadt, Germany) by microwave-assisted digestion. The digestion program consisted of sequential power steps of 100 W (3 × 3 min), 180 W (3 × 3 min), and 300 W (1 min), corresponding to a gradual temperature increase to approximately 180°C–200°C, with a total digestion time of approximately 25 min. Digestates were diluted to a final volume of 4 mL with ultrapure water (Milli-Q, Bedford, MA, USA). Copper and Cd concentrations were analyzed in all pooled animal samples and three procedural blanks. Metal concentration was expressed as μg per gram fresh weight (μg gfw^{-1}) (dry weight to fresh weight ratio = 0.01: 0.33). Analytical performance was verified using a certified reference material (SRM 2976, mussel tissue, National Institute of Standards and Technology, USA), which was digested and analyzed in parallel with the samples following the same protocol. Using this quality control procedure, our laboratory obtains recoveries within 5% of the certified values.

2.7 | Growth, Reproduction, and Survival Analysis

Growth and reproduction were assessed in accordance with OECD Test Guideline No. 211 [40], with minor adaptations. Growth was determined at the end of the exposure period by measuring the length of the carapax from the top of the head to the base of the spine in individual daphnids (10 individuals per exposure condition). The measurements were done by capturing digital images using a CCD camera (DFK 41AF02 FC, Imaging source) mounted on a trinocular stereomicroscope (Nikon, SMZ 800). The pictures were processed to measure length using ImageJ software (version 1.52u, National Institutes of Health, Bethesda, MD, USA). For the assessment of reproduction, adult daphnids were maintained individually under each exposure condition (10 females per condition). The number of live offspring produced per surviving female daphnid was counted daily and then removed after counting. Survival was monitored throughout the exposure period by recording the number of dead individuals, following the criteria described in the OECD daphnia immobilization test guideline (OECD Test No. 202). Animals were considered dead when no movement was observed even after gentle shaking.

2.8 | Molecular and Biochemical Analysis

2.8.1 | Gene Expression

Gene expression was evaluated in adults and neonates in all the exposure setups. For each exposure condition, 8 biological replicates were prepared per condition, each consisting of 4–5 pooled animals to obtain sufficient RNA for downstream analysis. The nucleotide sequences of some of the selected genes were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/) (Table S1). Other primer sequences used were obtained from previously published data [49, 55–59]. To optimize the design of the primers across exon boundaries, primers were first manually designed for each gene and then subsequently analyzed using the primer analysis software LightCycler probe design software (version 3.5, Roche Molecular Biochemicals, Germany). Primer efficiencies between 85% and 100% were determined by using the Ct slope method. All primer sequences are listed in Table 1.

RNA was extracted using a phenol-chloroform extraction procedure [60] and precipitated using Na-acetate and 70% ethanol. RNA concentration and quality were determined spectrophotometrically using Nanodrop ND-1000 (NanoDrop ND-1000, ISOGEN Life Science). All RNA samples were adjusted to a concentration of 200 ng. Genomic DNA was removed using Turbo DNA free kit (Ambion Thermo Fisher Scientific). After RNA extraction, cDNA synthesis was performed using the Superscript TM III first-strand synthesis supermix (Thermo-fisher Scientific) according to the manufacturer's instructions. cDNA

TABLE 1 | Nucleotide sequence of specific primer pair of *D. magna* used in the study.

Gene	Forward primer	Reverse primer
Act[a]	CCACACTGTCCCCATTTATGA A	CGC GAC CAGCCAAAT CC
cyp[a]	GACTTTCCACCAGTGCCATT	AAC TTTCCATCGCATCATCC
18s[a]	CGCTCTGAATCAAGGGTGTT	TGTCCGACCGTGAAGAGAGT
28s[a]	GAGGCGCAATGAAAGTGAAG	TGTTCGAGACGGGATCA
β-act[a]	CCACACTGTCCCCATTTATGA A	CGCGACCAGCCAAATCC
sdh[a]	TGCCAT TTAGTCGCACTC AG	GTGAGCTTGTCCTCCTTTGC
cat	CCGTTACAACACTGCCGATGA	AAGGCTGTGCGTTCTTTAGATG
CuZn-sod	GAGACCTGGGCAACATTGTG	GACTCTGGCCCGCTAAGTG
Mn-sod	GATGTTTGGGAGCATGCCTAC	GGACCGACACATCTTTCCAG
gst	GGGAGTCTTTTACCACCGTTTC	TCGCCAGCAGCATACTTGTT
grx	TGAAGCAGCTGACCCATAAG	GCAACTTGGTTTCGTTGAGA
gpx	CGTGGCTACTTACTGAGGGTTT	CGGACGAACGTAACGGATT
hsp70	ACTGATGCCGTGATTACTGTTC	CCTTGTGATGCTGGTGTAGAA
hsp90	CCGAGGAAGAGAAACCAAAG	CGTCGACCGAATACTTCTCC
p73-like	GCCTGGGCATTTGAACTTTA	ATGGAAGTGATCAGCCTTGG
riv1	ATGCGTTAGGCGTCAATACC	TACAAGGTTTGCCCTTGCTT
rad17like	GCGAATCATTCAAACCACTAC	CCATTTCTCTGAGTTCGATCT
aif1	CCTTACATGAGGCCACCACT	ACTGAAACGCCACCATTTTC
ai5	TCTGAATGACGCGAAAGATG	ACAGGTCAAACATGGCATCA
vtg	CTG TTC CTC GCT CTG TCT TG	CCA GAG AAG GAA GCG TTG TAG
opsin	TCCTCGTGCTTGAAGAC	GCGCTTGTTTCGGATAC
vri	CATCCACATCACCAGCATCAC	CGCGACAACGACCAATCT
cp	TCTGCTGGGAAAGTTGAAGATG	CAAATGGACGAGAAGACGATG
notch2	GCCACATCGAACACTGAAT	CATGTTGAGCACTTCACCT
mt-a	TTGCCAAAACAATT GCTCAT	CACCTCCAGTGGC ACAAAT
ctr1	GGGAGTACCTGAGTCGGA	ATGTAATGGGACGCACTG
zip9	CGTTCGTATTGTTCCTCTACAG	CCCAAATTACTCTTGCCAC
Serca	TCGCAAAGAAGTCT TCGACTC	AAACACCAATAC GACGGCAG

[a]Housekeeping genes (HKG).

samples were stored at −20°C until their use for amplification by the real-time PCR.

Real-time qPCR was performed in a 96-well plate (Applied Biosystems, Thermo Fisher Scientific) using the Fast Syber Green master mix (Applied Biosystems, Thermo Fisher Scientific) amplified and detected by using the 7500 Fast Real-time PCR system (Applied Biosystems, Life Technologies). Primer efficiencies calculated as $E = 10^{-1/slope}$, were evaluated using four-point standard curves, prepared by a 1:3 serial dilution of cDNA. Efficiencies ranging from 0.85 to 1.15 were accepted. Potential reference genes were selected according to the method given by Rongying et al. [61] (see Table 1). GeNorm was used for the analysis of the most stable reference genes. Gene expression analysis was performed following MIQE guidelines [62] (Table S2). Gene expression (transcriptional) profiles of the genes related to the cellular redox state, apoptosis, DNA repair mechanisms, reproduction, development and metal transport were studied in all exposure setups. The relative quantification of each gene expression level was normalized according to the expression of at least three most stable reference genes.

2.8.2 | Hydrogen Peroxide Quantification

The concentration of hydrogen peroxide (H_2O_2) was determined spectrophotometrically using an OxiSelect hydrogen peroxide assay kit (Cell Biolabs Inc., San Diego, US) following the instructions provided by the manufacturer. For each exposure condition, 8 biological replicates were prepared per condition, each consisting of 3–4 pooled animals. Samples were snap-frozen in liquid nitrogen and homogenized (using glass beads) in a 1× buffer. The homogenates were centrifuged at 13000 rpm (rpm) for 1–2 min at 4°C and the supernatant obtained was used for the quantification of H_2O_2. A small volume of the supernatant (10 μL) was diluted in a ratio of 1:10 and used for protein estimation. Next, ADHP (10-Acetyl-3, 7-dihydroxyphenoxazine) and HRP (horseradish peroxidase) working solutions were prepared. The ADHP/HRP working solution was added (50 μL) to each sample and incubated at room temperature for 30 min in dark. The absorbance was measured at 544 nm in a FLUOstar Omega multi–mode microplate reader (BMG Labtech, Ortenberg, Germany). The H_2O_2 content was determined using a standard curve. The total protein content was quantified separately using Bradford 1× dye reagent and with Bovine Serum Albumin (BSA) serving as the standard.

2.8.3 | Glutathione Assay

Glutathione content has been utilized as an indicator of metal toxicity, particularly in the context of Cu stress. Glutathione, as a key antioxidant molecule, was selected to assess the antioxidant defense capacity of the daphnids under all metal exposure conditions. To measure glutathione content, a spectrophotometric method was used, based on the method originally described by Tietze [63]. For each exposure condition, eight independent biological replicates were analyzed. Each daphnid was photographed to determine the mean total body size (mm^2) for normalization. Next, the daphnids (3–4 animals/sample) were snap-frozen in liquid nitrogen and stored at −70°C. Frozen animals were dissolved in 320 μL of 0.2 M HCl, equipped with glass beads measuring 2 μm in diameter and then disrupted using a Retsch Mixer Mill MM 200 (Retsch) for 1 min at a frequency of 30 Hz. The samples were kept on ice throughout the procedure. After this step, 280 μL of the homogenate was transferred to a new tube, neutralized with 30 μL of 0.2 M NaH_2PO4 followed by adjusting the pH to 5.6 with approximately 230 μL of 0.2 M NaOH. Next, 340 μL of each sample was transferred to a new tube for (oxidized) glutathione, glutathione disulfide (GSSG) analysis and the remaining 180 μL was used for glutathione reductase (GSH) assay. To quantify total GSH, four separate aliquots of 40 μL of the neutralized extract were added in a 96-well plate (Greiner Bio-One, Kremsmünster, Austria). The reaction mixture, with a total volume of 200 μL, comprised 0.1 M NaH_2PO_4 (pH 7.5), 5 mM EDTA, 0.5 mM NADPH, and 0.6 mM DTNB. Once again, the reaction was initiated by the adding of 0.2 U of GR to all but one sample (no-GR control). To quantify GSSG, samples were first treated with 1 μL of 2-vinyl pyridine (2-VP) for 30 min at RT to complex GSH. In order to remove surplus 2-VP, the solution that had undergone derivatization was subjected to two rounds of centrifugation. Four aliquots of 80 μL neutralized extract were added to 96-plate wells. The total reaction volume of 200 μL also contained 0.1 M NaH_2PO_4 (pH 7.5), 5 mM EDTA, 0.5 mM NADPH, and 0.6 mM DTNB. Again, the reaction was initiated by the addition of 0.2 U of GR to all samples but one (no-GR control). After mixing the reaction mixture on a shaker, absorbance (GR-dependent reduction of DTNB) was monitored for 15 min with the FLUOstar Omega multi-mode microplate reader (BMG Labtech, Ortenberg, Germany). The standards were run simultaneously alongside the same plates as duplicate assays. A negative control with milli-Q water was used as a blank measurement.

2.9 | Statistical Analysis

Statistical analysis was performed using Prism8 statistical software (GraphPad). In all experiments, each parameter was evaluated separately with an appropriate control. Data were evaluated for normality using the Shapiro–Wilk test. As the assumptions for parametric tests were not met, non-parametric statistical analyses were employed. For each endpoint, differences among exposure conditions were assessed using the Kruskal–Wallis test, followed by Dunn's multiple comparison. A p-value less than 0.05 was considered statistically significant.

3 | Results

3.1 | Metal Accumulation

In both adults and neonates, Cu accumulation was comparable across all exposure pathways following both single-metal and co-exposures ($p < 0.05$; Figure 2). In contrast, Cd accumulation in adult daphnids was generally higher when exposed to Cd alone and reduced in the presence of Cu aqueous: 25% decrease [$p < 0.05$], dietary: 42% decrease [$p < 0.05$], combined: 37% decrease [$p < 0.05$]. In neonates, Cd concentrations increased significantly following both single and mixture exposures ($p < 0.05$). Similar to adults, neonates generally accumulated

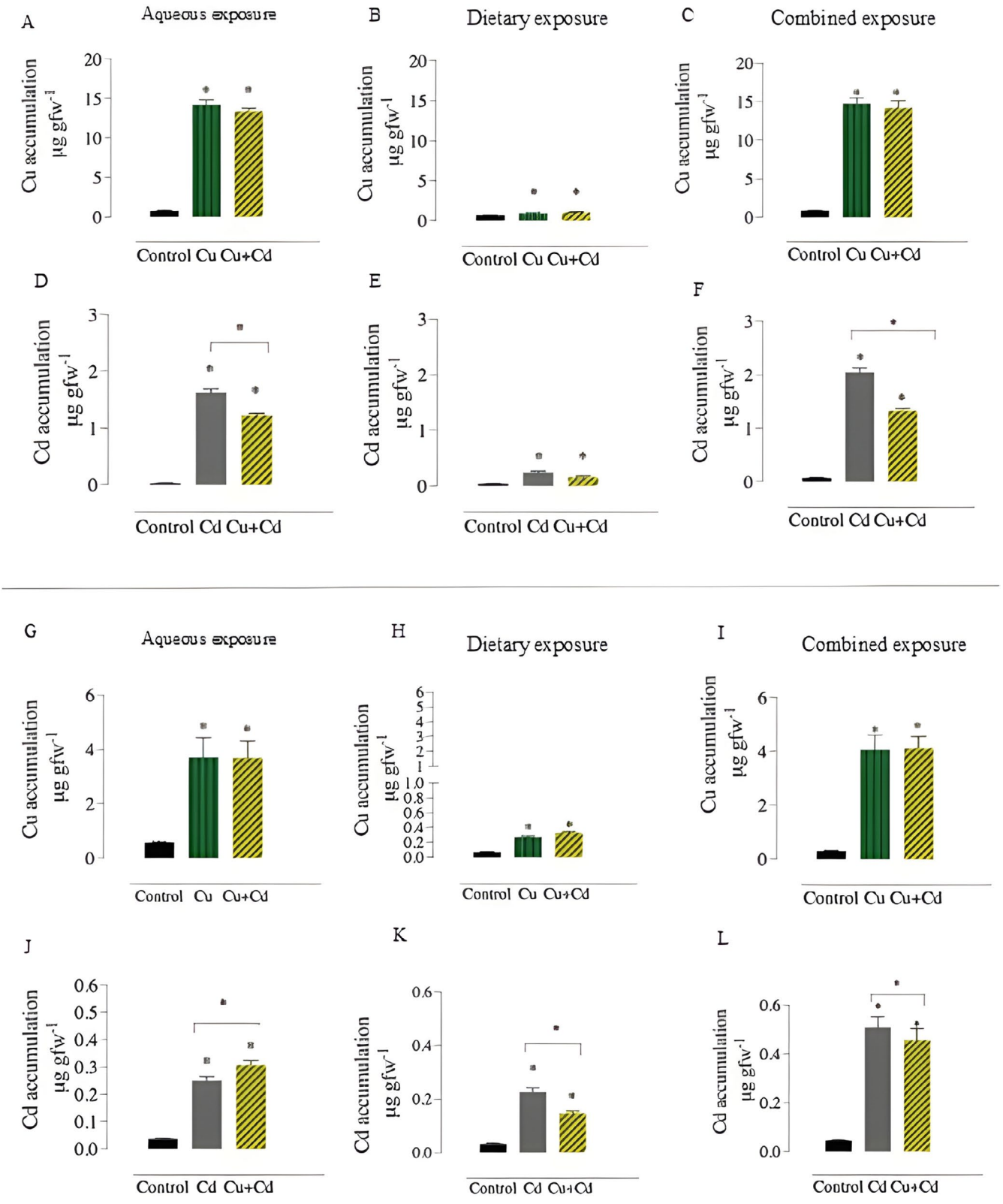

FIGURE 2 | Metal accumulation in *D. magna* adults and neonates. Graphs representing metal concentrations in adults (20–21 days old) (A–F) and neonates (less than 24 h old) (G–L) exposed to aqueous metals (0.25 µM Cu, 0.02 µM Cd and in combination); dietary metals (4.33×10^3 ng Cu/daphnid, 5.72×10^1 ng Cd/daphnid and in combination) and combined exposure (aqueous + dietary Cu and Cd provided either alone or together). The values presented in the figure represent pooled data, showing the average ± standard error of the mean (SEM) from a minimum of 16 biological replicates. Two independent experiments were performed, and the results of two experiments confirm each other. Significant differences relative to controls or between treatments are indicated by asterisks $*p < 0.05$. fw, fresh weight.

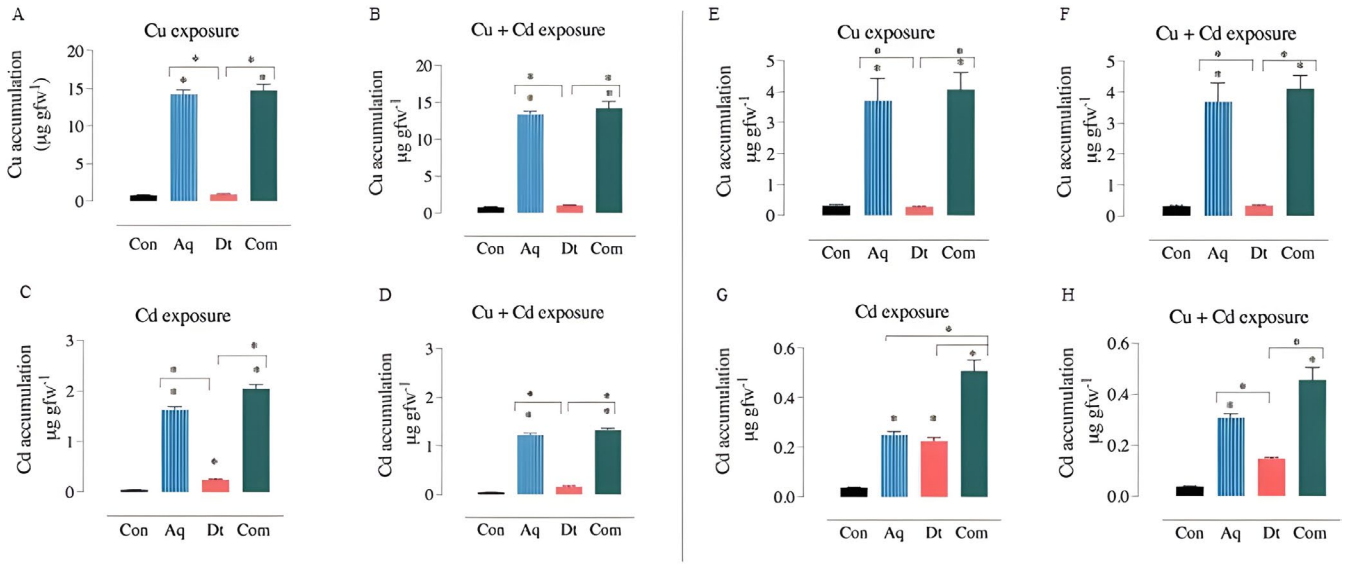

FIGURE 3 | Comparative Cu and Cd accumulation in *D. magna* among exposure pathways. Panels (A–D) show metal accumulation in adults: (A) Cu accumulation during Cu-only exposure; (B) Cu accumulation during Cu + Cd co-exposure; (C) Cd accumulation during Cd-only exposure; (D) Cd accumulation during Cu + Cd co-exposure. Panels (E–H) show metal accumulation in neonates: (E) Cu accumulation during Cu-only exposure; (F) Cu accumulation during Cu + Cd co-exposure; (G) Cd accumulation during Cd-only exposure; (H) Cd accumulation during Cu + Cd co-exposure. For each panel, daphnia were exposed via: Con, control (no added metals); Aq, aqueous exposure (0.25 µM Cu, 0.02 µM Cd, and in combination); Dt, dietary exposure (4.33×10^3 ng Cu daphnid^{-1}, 5.72×10^1 ng Cd daphnid^{-1}; and in combination) and the combined pathway (aqueous + dietary). The values presented in the figure represent pooled data, showing the average ± standard error of the mean (SEM) from a minimum of 16 biological replicates. Two independent experiments were performed, and the results of two experiments confirm each other. Significant differences relative to controls or between treatments are indicated by asterisks *$p < 0.05$.

less Cd when exposed to the mixture, with reductions of 40% under dietary exposure and 11% under combined exposure ($p < 0.05$). However, aqueous exposure resulted in a significant increase in Cd accumulation in neonates (26%, $p < 0.05$). Comparison among exposure pathways showed that Cu and Cd accumulation was higher following aqueous and combined exposures than dietary exposures alone ($p < 0.05$) (Figure 3). Overall, metal accumulation was generally lower in neonates than in adults across all exposure conditions ($p < 0.05$).

3.2 | Reproduction, Growth and Survival

Reproductive capacity, growth, and mortality were assessed as general toxicity endpoints. Reproduction, was quantified as the number of neonates produced per adult female daphnid. Under combined aqueous–dietary exposure, reproductive output decreased significantly in all metal treatments (Cu: $p < 0.05$, Cd: $p < 0.05$, Cu + Cd: $p < 0.001$) (Figure 4, Table 2). The reduction was greatest in animals exposed to the Cu and Cd mixture with 52% reduction relative to control($p < 0.001$), compared with significantly smaller reductions observed under single-metal exposures (Cu: 21%, $p < 0.05$; Cd: 17%, $p < 0.05$). In addition, exposure to the Cu and Cd mixture significantly reduced reproductive capacity under both aqueous and dietary exposure pathways ($p < 0.05$). On pathway wise comparison, a decrease in reproductive capacity was found in combined exposure irrespective of single or co-exposure.

In neonates, co-exposure to the Cu–Cd significantly impaired growth across all exposure pathways, resulting in a marked reduction in body size compared with controls ($p < 0.05$). Growth inhibition was most pronounced under the combined aqueous–dietary exposure, with a 30% decrease in body size ($p < 0.05$), compared with smaller but still significant reductions under aqueous (14%, $p < 0.05$) and dietary (9%, $p < 0.05$) exposure alone (Figure 5; Table 2). In addition, neonates exposed to Cu alone exhibited significant growth impairment under both aqueous and combined exposure conditions ($p < 0.05$).

No mortality was observed in adult *Daphnia magna* across any exposure group during the experimental period. In contrast, neonates exhibited significant mortality under both aqueous-only and combined aqueous–dietary exposure pathways for all metal treatments. The strongest reduction in survival occurred in neonates exposed to the Cu–Cd mixture via the combined exposure pathway, resulting in a 30% decrease in survival compared with controls ($p < 0.05$).

3.3 | Gene Expression Changes in Adults

In adults, gene expression responses varied markedly among exposure pathways (Figure 6). Aqueous exposure primarily induced genes involved in metal detoxification, with metallothionein (*mta*) strongly upregulated in Cd- and mixture-exposed animals ($p < 0.01$ and $p < 0.05$, respectively). Under dietary exposure, a more limited response was observed, with the Cu and Cd mixture inducing the detoxification gene *gst* ($p < 0.05$). In contrast, the combined exposure pathway elicited broad transcriptional responses affecting multiple biological processes. Genes associated with oxidative stress (*Mn-sod*), detoxification (*gst*),

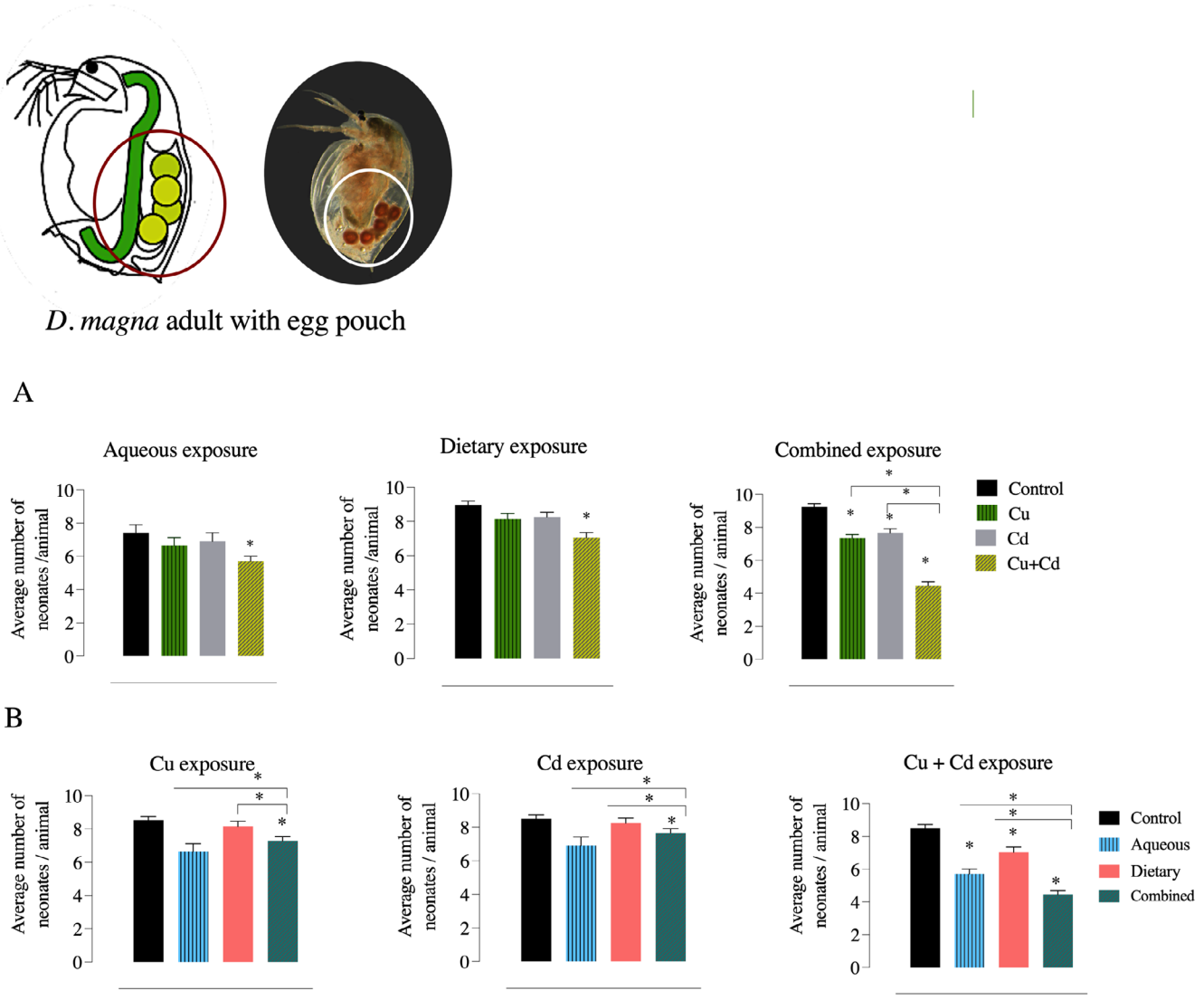

FIGURE 4 | Reproduction in *D. magna* adults. Top panel: Schematic (left) and photograph (right) of a *Daphnia magna* adult with visible egg pouch, indicating the anatomical location of developing embryos used for neonate production assessment. The graphs represent (A) Average reproductive output (number of neonates/animal) in adults (20–21 days old) exposed to the respective Cu and Cd concentrations alone and in a mixture for 7 days via aqueous, dietary, and combined pathways. (B) Comparison of reproductive output across exposure pathways (aqueous, dietary, combined) for each metal treatment (Cu alone, Cd alone, and Cu + Cd mixture). The values indicated in figure are the average ± standard error of mean (SEM) of minimum 20 biological replicates. Two independent experiments were performed, and the results of two experiments confirm each other. Bars represent mean ± SEM. Significant differences relative to controls or between treatments are indicated by asterisks *$p < 0.05$. Scale bar = 500 μm.

heat shock response (*hsp90*), apoptosis (*aif1*, *api5*), reproduction (*vtg*), and Cu transport (*ctr1*) were all significantly upregulated in mixture-exposed animals ($p < 0.05$–0.001). Conversely, *mta* was significantly downregulated under combined mixture exposure ($p < 0.05$), indicating a pathway-specific alteration in metal-handling mechanisms.

3.4 | Gene Expression Changes in Neonates

In neonates, expression patterns were more complex (Figure 7). In addition to the genes analyzed in adults, development-related genes (*opsin*, *vri*, *cp*, *notch2*) were also examined in neonates. Following aqueous exposure, mixture-exposed neonates showed upregulation of antioxidant and stress-response genes, including *Mn-sod*, *gst*, *hsp90*, as well as apoptosis- and transport-related genes (*aif1*, *api5*, *ctr1*; $p < 0.05$). This exposure pathway also resulted in downregulation of *mta*. Dietary exposure induced a more selective response. Cu-alone and mixture exposure upregulated the calcium transport gene *serca* ($p < 0.05$), whereas mixture exposure led to downregulation of antioxidant (*CuZn-sod*) and development-related genes (*riv1*, *vri*; $p < 0.05$–0.01). The most pronounced transcriptional response occurred under the combined exposure pathway. Mixture-exposed neonates exhibited widespread upregulation of genes involved in oxidative stress (*cat*, *Mn-sod*, *gst*, *gpx*), cellular stress (*hsp90*), apoptosis (*p73-like*, *aif1*), reproduction (*vtg*), metal handling (*mta*, *serca*), and development (*opsin*; $p < 0.05$–0.001). In contrast, genes related to circadian regulation (*vri*), development (*cp*), and Cu transport (*ctr1*) were downregulated ($p < 0.05$–0.01). Exposure to Cu alone also resulted in downregulation of *cp* and *ctr1* ($p < 0.05$). Across all

exposure pathways, a consistent response to mixture exposure in neonates was the upregulation of the reproduction-related gene *vtg* ($p < 0.05$).

TABLE 2 | Toxicity responses in *D. magna* adults (20-21 days old) and neonates (less than 24 h old) after single and co-exposure to Cu and Cd across three exposure pathways (aqueous, dietary, and combined).

***D. magna* adults**				
Reproduction				
Pathway	**Cu**	**Cd**	**Cu + Cd**	**Effect of co-exposure**
Aqueous	−10%	−7%	−23%	More than additive
Dietary	−9%	−8%	−21%	More than additive
Combined	−21%	−17%	−52%***	More than additive
***D. magna* neonates**				
Growth				
Pathway	**Cu**	**Cd**	**Cu + Cd**	**Effect of co-exposure**
Aqueous	−6%	−3%	−14%*	More than additive
Dietary	−2%	−2%	−9%*	More than additive
Combined	−12%	−6%	−30%*	More than additive

Note: The effect was classified as more than additive when combined effect of the individual exposures was significantly greater (denoted by asterisk sign) than the sum of the effects of individual exposures alone.
*$p < 0.05$.
***$p < 0.001$.

3.5 | Redox State in Adults and Neonates

The effects of metal exposure on redox homeostasis were evaluated by quantifying hydrogen peroxide (H_2O_2) levels and the glutathione redox balance (GSH:GSSG ratio) in adult daphnids and neonates (Figure 8). In adults, exposure to the Cu and Cd mixture via the combined pathway resulted in a significant increase in H_2O_2 levels ($p < 0.05$), whereas no significant changes were observed following aqueous or dietary exposures. Consistent with this oxidative shift, the GSH:GSSG ratio was significantly reduced in mixture-exposed adults under the combined exposure pathway ($p < 0.05$), indicating increased oxidative stress.

Neonates exhibited a more pronounced and pathway-dependent redox response. Similar to adults, mixture exposure via the combined pathway resulted in a significant decrease in the GSH:GSSG ratio ($p < 0.05$). However, in contrast to adults, neonates showed significantly elevated H_2O_2 levels following mixture exposure via both aqueous and dietary pathways ($p < 0.05$). In addition, exposure to Cu or Cd alone under the combined pathway led to a significant decrease in H_2O_2 levels in neonates ($p < 0.05$).

4 | Discussion

4.1 | Age and Pathway-Dependent Metal Accumulation Under Cu–Cd Co-Exposure

Our study shows that Cu–Cd co-exposure produces complex, age- and pathway-dependent metal accumulation patterns (Figures 2 and 3). In adults, Cu consistently suppressed Cd accumulation across exposure pathways, suggesting antagonistic interactions at the level of uptake. In contrast, neonates exhibited divergent accumulation patterns, reflecting age-dependent

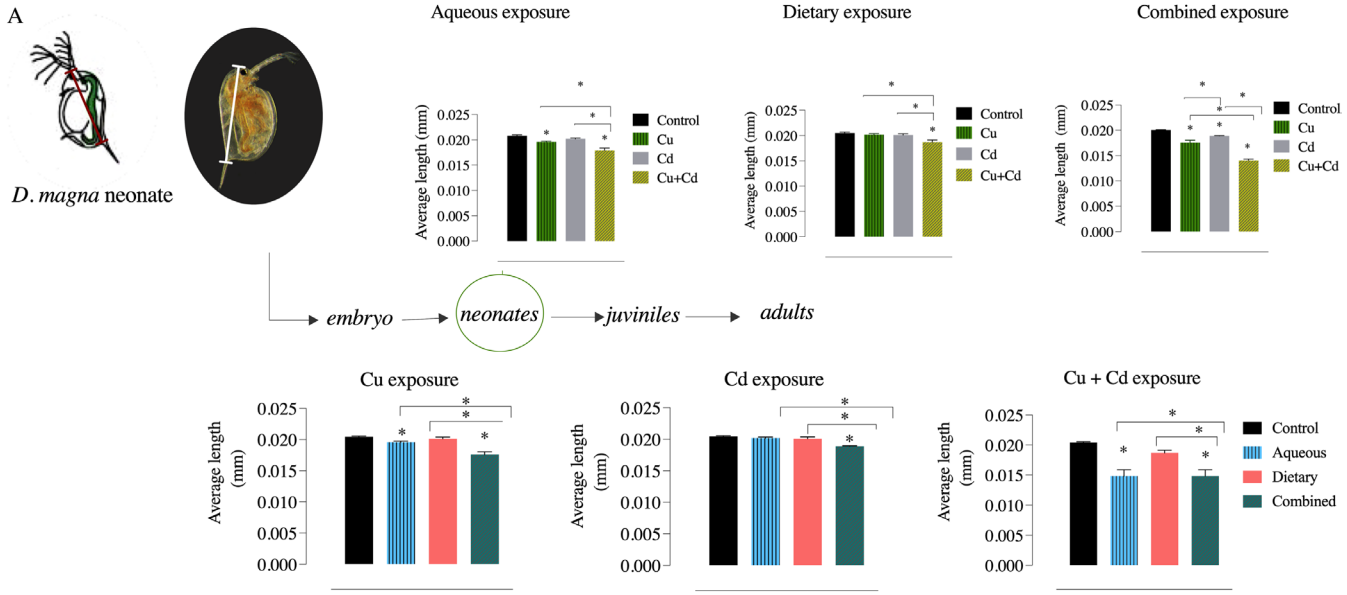

FIGURE 5 | Growth responses of *Daphnia magna* neonates. The graphs in the upper panel represent average body length (mm) of neonates after 7 days aqueous, dietary, and combined aqueous–dietary exposures to single-metal or mixture treatments compared with controls. The lower panel summarizes overall growth responses across exposure types, showing pathway-specific effects for Cu-only, Cd-only, and Cu + Cd mixture treatments. Growth inhibition was most pronounced under the combined pathway, consistent with increased toxic pressure under multi-route exposure. Bars represent ± standard error of mean (SEM). Significant differences relative to controls or between treatments are indicated by asterisks (*$p < 0.05$).

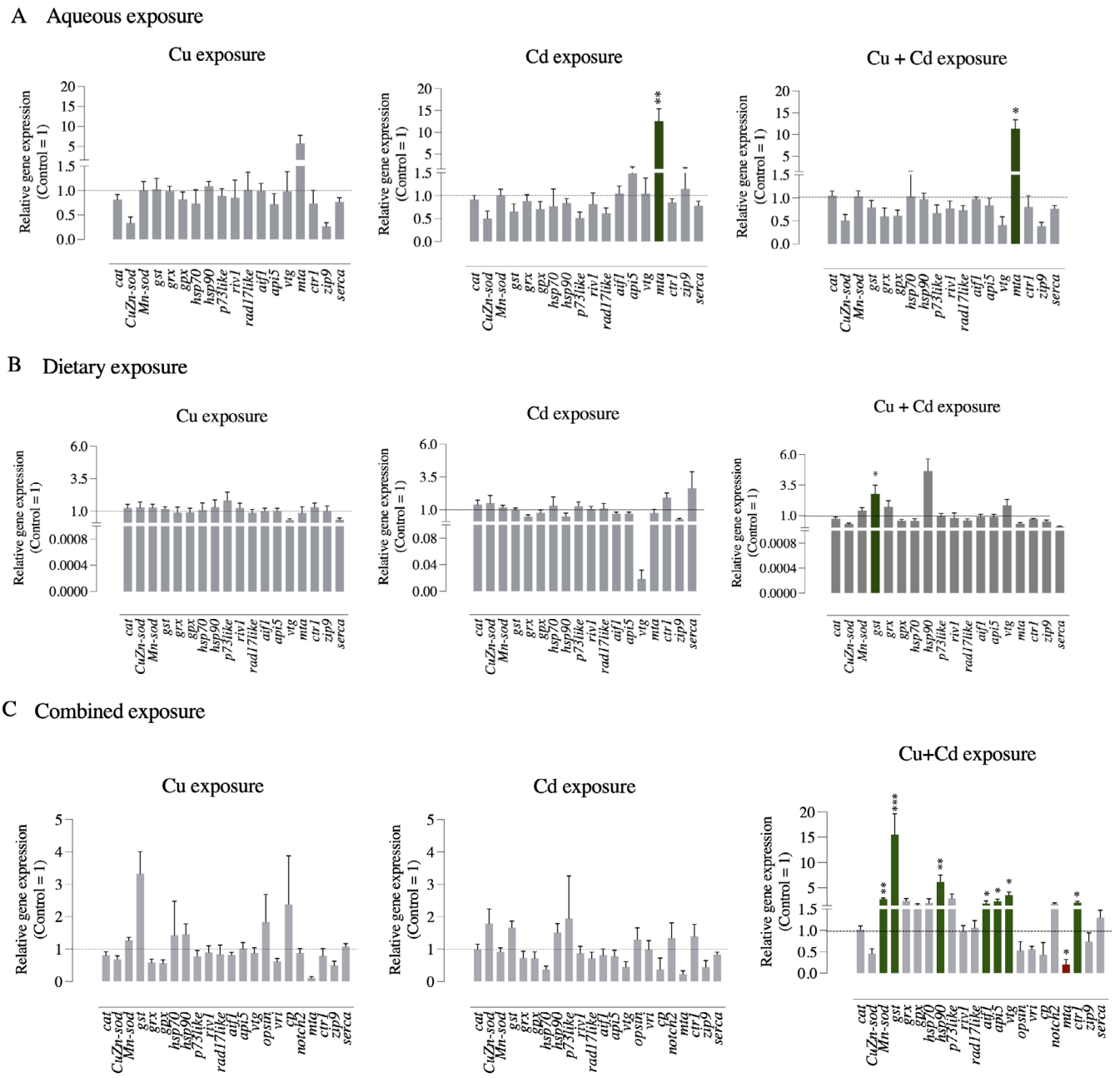

FIGURE 6 | Molecular responses in *D. magna* adults. Gene expression in adults (20–21 days old) exposed for 7 days to (A) 0.25 μM Cu, 0.02 μM Cd alone and in combination via aqueous medium; (B) 4.33×10^3 ng Cu/daphnid and 5.72×10^1 ng Cd/daphnid alone and in combination via dietary pathway; (C) combined exposure to the respective Cu and Cd concentration alone and a combination of Cu and Cd. The green color represents the upregulation of genes relative to control. The values indicated in figure are the average ± standard error of mean (SEM) of minimum 8 biological replicates. Significant differences relative to controls or between treatments are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

differences in metal handling. This aligns with our prior findings in planarians, where developing and adult animals showed contrasting Cd accumulation trends [2], as well as with our previous work in zebrafish embryos and adults, where Cu–Cd co-exposure differentially affected metal accumulation and physiological responses across life stages, with embryos showing higher vulnerability and distinct internal metal dynamics compared to adults [3]. Similar developmental shifts in metal handling have also been reported in larval white sturgeon and rainbow trout, where the relative contribution of glutathione-based defenses and metallothionein-mediated sequestration changed across developmental stages [64, 65]. Despite their substantially lower body mass, neonates exhibited increased Cd accumulation under aqueous co-exposure, indicating that physiological regulation, rather than size-normalized exposure, governs age-specific accumulation [66, 67]. Such regulation likely reflects developmental differences in transporter expression, membrane permeability, gut physiology, and detoxification capacity across life stages [2, 3, 12]. Together, these developmental transitions provide a mechanistic explanation for why neonates accumulated Cd differently than adults under otherwise identical exposure conditions.

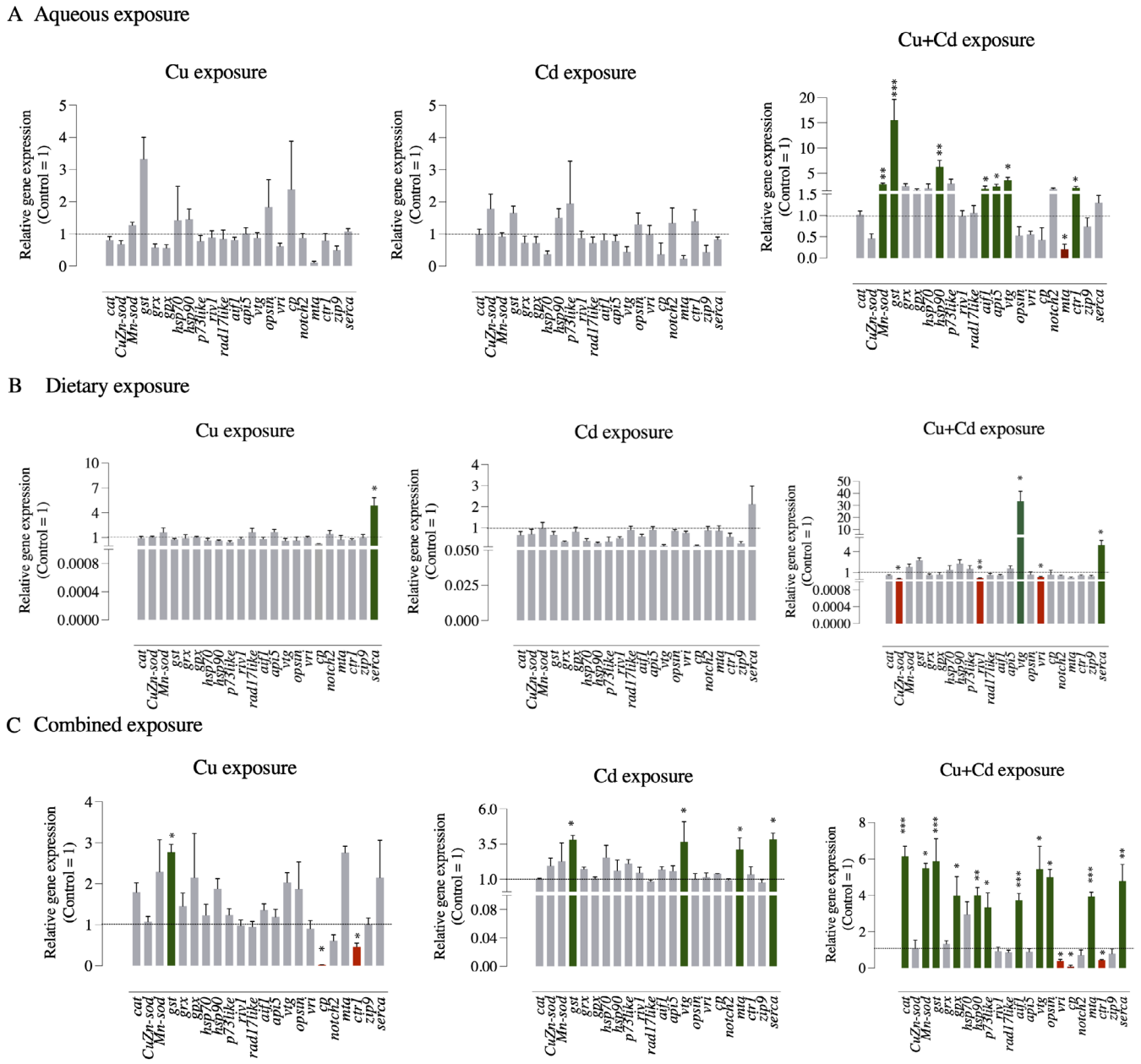

FIGURE 7 | Molecular responses in *D. magna* neonates. Gene expression in neonates (less than 24 h old) exposed for 7 days to (A) 0.25 μM Cu, 0.02 μM Cd alone and in combination via aqueous medium; (B) 4.33×10^3 ng Cu/daphnid and 5.72×10^1 ng Cd/daphnid alone and in combination via dietary pathway; (C) combined exposure to the respective Cu and Cd concentration alone and a combination of Cu and Cd. The green color represents the upregulation of genes relative to control. The values indicated in figure are the average ± standard error of mean (SEM) of minimum 8 biological replicates. Significant differences relative to controls or between treatments are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Age-dependent toxicokinetic differences and mixture-specific uptake interactions remain poorly integrated into current risk-assessment frameworks, which typically rely on simplified exposure–effect relationships and rarely account for developmental variation in metal handling under co-exposure scenarios. This omission is particularly consequential given that interactions between Cd and Cu during uptake are highly complex and species specific. For instance, Cd inhibited Cu uptake in *Daphnia magna* [68], whereas the opposite effect was observed in *Danio rerio* [50, 69]. Such interactions can occur even at low metal concentrations [68, 70] and are frequently mediated by ionic or molecular mimicry, whereby metals compete with essential elements for transporter binding, alter transport dynamics, or form toxic metal complexes [71, 72].

Across exposure pathways, waterborne uptake dominated over dietary uptake. This dominance of waterborne uptake has also been reported in other aquatic taxa supporting the generality of our observations. For example, in two species of calanoid copepods (*Pseudodiaptomus annandalei* and *Eurytemora affinis*), uptake from the aqueous phase was substantially higher than from dietary exposure, even when dietary metal concentrations were elevated [73]. This pattern is ecologically relevant because dissolved metals persist in aquatic

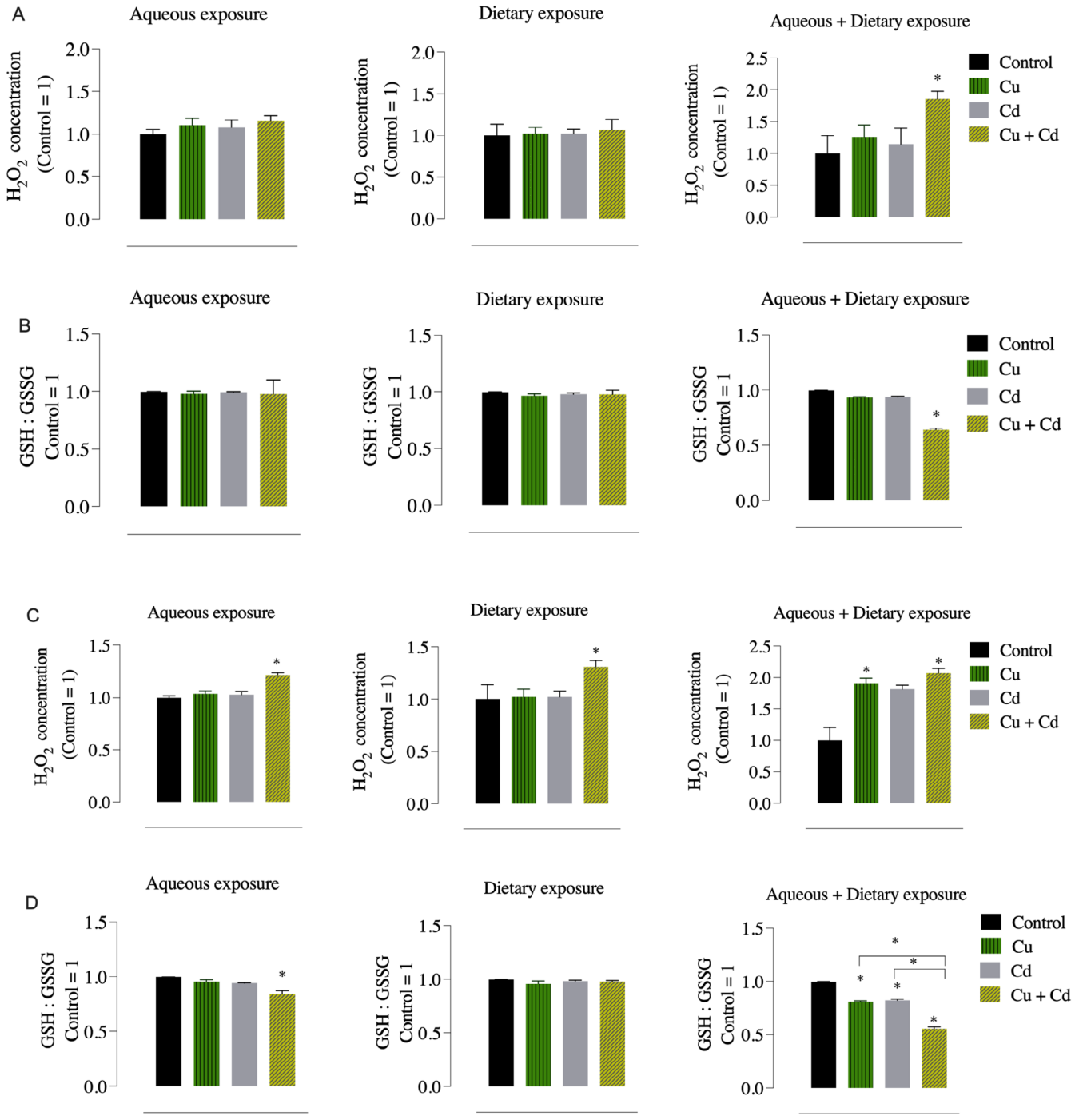

FIGURE 8 | Redox-state in *D. magna* adults and neonates. Graphs depicting hydrogen peroxide (H_2O_2) levels in (A) adults and (B) neonates and GSH:GSSG in (C) adults and (D) neonates exposed via aqueous, dietary and combined pathway. The values indicated are the average ± standard error of mean (SEM) of minimum 8 biological replicates. Two independent experiments were performed, and the results of two experiments confirm each other. Significant differences relative to controls or between treatments are indicated by asterisks (*$p < 0.05$).

environments and remain continuously bioavailable through direct contact with permeable surfaces, such as gills and the body wall, whereas dietary uptake is constrained by feeding behavior, assimilation efficiency, and gut regulatory processes [74–76]. Mechanistically, the dominance of waterborne metal uptake can be explained by chemical speciation and metal interactions at biological surfaces, as described by the biotic ligand model, whereby toxicity occurs when metal binding at epithelial sites leads to an internal dose exceeding a critical threshold at the site of action [77–80]. Overall, these results show that metal accumulation under Cu–Cd co-exposure is governed by a dynamic interplay between exposure pathway and developmental stage, mediated by age-specific uptake mechanisms and internal regulatory capacity. The suppressive effect of Cu on Cd accumulation in adults, contrasted with divergent responses in neonates suggest that predicting metal bioaccumulation and toxicity requires explicit consideration of life stage, exposure route, and co-occurring metals.

4.2 | Contrasting Accumulation and Toxicity Patterns Under Cu–Cd Co-Exposure

Although metal accumulation provides important insight into exposure, the observed toxicity patterns did not directly scale with internal metal accumulation. In general, individual metal body burdens reduced in co-exposure scenarios, yet strong effects were observed (reduced growth and reproduction) (Figures 4 and 5). This indicates that the bioavailable fraction of metals and their interactions at the cellular and molecular level, rather than total internal burden alone, govern biological effects under mixture scenarios [2, 3]. This decoupling indicates that mixture toxicity arises not solely from total internal metal burden but from functional interactions at cellular and molecular targets. It is important to note that the bioavailable fraction of metals was not directly measured in the current study, and our conclusions regarding its role are inferred from toxicity patterns, molecular stress responses, and previous studies on metal cellular interactions [2, 3].

A plausible mechanism underlying these effects is that Cu and Cd interact at key physiological and molecular targets rather than solely at the level of uptake. Copper is an essential metal with tightly regulated homeostasis [21–26], whereas Cd is non-essential and disrupts cellular processes by binding to sulfhydryl groups and displacing essential metals from enzymes and structural proteins [31, 32, 36–38]. During co-exposure, Cu-induced oxidative stress may compromise cellular defense systems, such as antioxidant enzymes and metallothioneins, thereby increasing organismal sensitivity to Cd even when Cd accumulation is reduced [3]. Conversely, Cd may interfere with Cu-dependent metabolic pathways, which can trigger downstream signaling responses [81]. These interactions often activate stress-response genes, proteins, and metabolic pathways aimed at mitigating or tolerating damage, creating a decoupling between bioaccumulation and toxicity [3, 82]. Our transcriptional data provide clear evidence of these stress and compensatory responses, though gene expression changes do not always mirror protein levels or enzymatic activity [83, 84]. Integrating transcriptomics with proteomics or metabolomics could further reveal the molecular consequences of metal exposure.

Elevated expression of stress-related genes and biomarkers supports the occurrence of synergistic toxicity under Cu–Cd co-exposure (Figures 6 and 7, Table 2). The induction of heat shock proteins, antioxidant enzymes, and metal-binding proteins indicates that co-exposed organisms experienced increased and multifaceted cellular stress. Importantly, these responses likely reflect not only metal-specific molecular damage but also a more generalized physiological burden arising from concurrent activation of stress response and detoxification pathways. Such competing demands on cellular defense systems may amplify adverse effects even when internal metal burdens are reduced. This shows that enhanced effects can arise from overlapping modes of action and shared detoxification or stress-response pathways, rather than from simple additivity of internal doses alone [85, 86]. Taken together, these results show that under Cu–Cd co-exposure, internal metal concentrations alone are insufficient to predict toxicity, as effects are driven more by bioavailable metals, cellular interactions, and overlapping stress responses than by total accumulation alone.

4.3 | Linking Oxidative Stress to Age-Dependent Impairment of Growth and Reproduction

Oxidative stress emerged as a mechanistic link between Cu–Cd co-exposure and impaired growth and reproductive performance. Across life stages, the combined aqueous–dietary exposure elicited coordinated molecular and biochemical responses indicative of redox imbalance, including the upregulation of antioxidant and stress-response genes involved in reactive oxygen species (ROS) detoxification and protein stabilization, together with clear shifts in redox biomarkers (Figures 4–8). Although ROS are unavoidable by-products of aerobic metabolism, their accumulation becomes detrimental when antioxidant defenses are overwhelmed [87], leading to oxidative damage and disruption of energy- and growth-dependent physiological processes. In the present study, oxidative stress was assessed using complementary indicators that capture both ROS burden and redox buffering capacity. Hydrogen peroxide (H_2O_2) and the glutathione redox ratio (GSH:GSSG) provided converging evidence of oxidative disturbance. Under Cu–Cd co-exposure, particularly via the combined pathway, H_2O_2 levels increased significantly, indicating enhanced ROS production and/or impaired detoxification. Concurrently, the GSH:GSSG ratio decreased, reflecting depletion of reduced glutathione and a shift toward a more oxidized intracellular environment. Together, these changes demonstrate that antioxidant buffering capacity was insufficient to counteract oxidative challenge under mixture exposure.

In adults, redox imbalance coincided with altered regulation of reproduction-related processes. Vitellogenin (vtg), which is essential for yolk formation and oocyte development, was significantly upregulated under Cu–Cd co-exposure. Adults co-exposed to Cu and Cd exhibited significant upregulation of vtg, potentially as a compensatory response to maintain reproductive output under oxidative pressure [88–90]. Concurrently, antioxidant defense genes (*Mn-sod*, *gst*) were elevated, consistent with redox stress, and biochemical markers (elevated H_2O_2, reduced GSH:GSSG ratio) supported that antioxidant defenses were challenged [91]. This oxidative stress also underpins the increased vulnerability observed in neonates. Neonates consistently exhibited higher sensitivity than adults, as evidenced by significant mortality, severe growth inhibition, and more pronounced molecular stress responses (Figures 4 and 7). In contrast, adults showed no mortality under any exposure condition, indicating the greater susceptibility of early life stages. This sensitivity likely reflects the morphological, physiological, and biochemical immaturity of neonates, including limited detoxification capacity and underdeveloped homeostatic regulation [2, 3, 12]. To differentiate toxic effects from natural physiological responses, such as molting, we evaluated the expression of cuticular protein (cp) genes, which are essential for exoskeletal development and the molting process in crustaceans 125. Under normal developmental conditions, cp genes are differentially upregulated across molt cycle, with many showing elevated expression during inermoult and/or cuticle-forming stages, reflecting active exoskeleton synthesis and maintenance [65]. We observed a significant downregulation

of cp in neonates exposed to Cu and the Cu + Cd mixture via the combined route, suggesting that Cu toxicity disrupts normal molting, potentially impairing growth and structural integrity. Supporting this interpretation, the copper transporter gene ctr1 was downregulated under combined exposure, likely reflecting a regulatory response to limit Cu uptake. Concurrently, metallothionein a (mta) was upregulated, consistent with a detoxification response to excess intracellular Cu. These molecular responses indicate a coordinated effort to modulate metal homeostasis and mitigate Cu-induced toxicity in neonates.

At the molecular level, neonates also exhibited oxidative stress signatures similar to adults but with more severe consequences. Elevated *vtg* expression in mixture-exposed neonates likely represents a stress-related compensatory antioxidant and immune response rather than reproductive investment [92, 93]. Additional transcriptional changes in developmental genes, including altered vrille, serca, and opsin expression, suggest disruption of multiple physiological pathways. Exclusive upregulation of hsp90 under combined Cu–Cd exposure reflects a robust cellular response to oxidative stress [94]. Neonates showed elevated H_2O_2 levels and reduced GSH:GSSG ratios under both Cu-alone and combined exposures, as well as under individual aqueous or dietary exposures, underscoring their limited capacity to buffer oxidative stress (Figures 7 and 8). Overall, while adults appear capable of partially compensating for redox imbalance to preserve reproductive function, neonates lack sufficient antioxidant and homeostatic capacity, resulting in disrupted development, reduced growth, and increased mortality.

5 | Conclusion

This study shows that Cu and Cd co-exposure produces pathway-dependent, age-specific toxicity in *Daphnia magna* that cannot be predicted from single-metal effects. The key findings are: (1) Cu–Cd interactions during uptake are context-dependent, with Cu generally suppressing Cd accumulation in adults but producing divergent patterns in neonates depending on exposure route; (2) mixture toxicity is functionally synergistic at the organismal level despite antagonistic accumulation, indicating that internal metal concentrations alone are insufficient predictors of toxicity; (3) combined aqueous + dietary exposure produces the most severe effects, underscoring the importance of multi-route exposure in ecologically realistic scenarios; (4) neonates are significantly more vulnerable than adults, exhibiting higher mortality, greater growth inhibition, and more pronounced molecular stress responses; (5) oxidative stress is a unifying mechanism of mixture toxicity, with consistent upregulation of antioxidant defenses and biochemical evidence of redox imbalance; and (6) pathway-specific molecular signatures reveal that aqueous, dietary, and combined exposures engage distinct (though overlapping) stress response pathways. These results have direct relevance for the environmental management of metal-contaminated aquatic ecosystems. Current risk assessment frameworks, which typically evaluate single metals and emphasize aqueous exposure, may substantially underestimate the toxicity of metal mixtures encountered by aquatic organisms. Vulnerability of early life stages is also highlighted, which are often underrepresented in standard toxicity testing protocols. Future research should focus on: (1) integrating exposure route, life stage, toxicokinetics, and molecular mechanisms in risk assessment of metal mixtures; (2) investigating transporter-level interactions between metals to elucidate uptake mechanisms in a mixture scenario; (3) assessing the generality of metal interaction patterns across genotypes, species, and environmental conditions. Addressing these knowledge gaps can allow more predictive, mechanistically grounded approaches to assessing the ecological risks of metal mixtures in a changing environment.

Author Contributions

Sanah Majid: conceptualisation, methodology, investigation, data processing, original draft preparation, editing, funding acquisition. **Karen Smeets:** conceptualisation, supervision, review, funding acquisition. **Mubiana K. Valentine:** methodology. **Ronny Blust:** conceptualisation, supervision, review, funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Accession number and nucleotide sequences of genes obtained from NCBI database. **Table S2:** MIQE guidelines concerning qPCR experiment.