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Stage-Specific Responses to Maternal Copper Exposure in *Pirata subpiraticus*

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Simple Summary

Spiders are important natural predators that help control pests in agricultural fields. However, soil pollution may threaten their survival and reproduction. Copper is widely used in agriculture, and excessive amounts can accumulate in the soil. In this study, we examined how copper exposure affects female spiders and their offspring. We found that copper exposure negatively affected the health and reproduction of female spiders. Interestingly, even though their offspring were not directly exposed to copper, they also showed changes in their growth and development. Moreover, mothers and offspring responded to copper-related stress in different ways. These findings suggest that copper pollution may affect not only exposed spiders but also future generations. Collectively, our study provides useful information for understanding the environmental risks of copper pollution and protecting beneficial spiders in agricultural ecosystems.

Abstract

Maternal exposure to environmental contaminants can affect both reproductive performance and offspring development, yet the molecular responses of mothers and offspring to metal exposure remain poorly understood in terrestrial arthropods. Here, we investigated the physiological, reproductive, transcriptomic, and metabolic responses to maternal copper (Cu) exposure in the spider *Pirata subpiraticus*. Cu exposure resulted in substantial Cu accumulation and oxidative stress in adult females, accompanied by enhanced antioxidant defenses and reduced body weight. Reproductive performance was also impaired, with delayed egg-sac production and reduced egg number, egg-sac weight, and egg size. Transcriptomic and metabolomic analyses revealed extensive molecular alterations in both adult females and newly hatched spiderlings, but the responses differed between developmental stages. Integrated multi-omics analysis showed that changes in adults were primarily associated with oxidative stress, metal ion homeostasis, immune signaling, and cellular maintenance, whereas spiderlings exhibited broader alterations in carbohydrate and amino acid metabolism, energy production, nucleotide metabolism, and glutathione metabolism. These findings indicate that maternal Cu exposure is associated with biological effects extending from exposed females to their offspring, with molecular responses dependent on developmental stage, highlighting the importance of considering reproductive and early-life responses when evaluating the ecological consequences of metal contamination in terrestrial arthropods.

Keywords: copper; spider; RNA-seq; metabolome; oxidative stress

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1. Introduction

The accumulation of copper (Cu) in agricultural soils has become an environmental concern, particularly in regions affected by mining, smelting, and the long-term application of Cu-containing agrochemicals [1]. Field investigations have reported total Cu concentrations of 82–1295 mg/kg in agricultural soils affected by mining and smelting activities in central Chile [2], while concentrations of up to 690 mg/kg have been documented in European vineyard soils [3]. Such elevated concentrations highlight the potential for substantial Cu contamination in agricultural environments and raise concerns about its effects on soil-dwelling organisms. Arthropods inhabiting contaminated environments are particularly susceptible to metal exposure through direct contact with contaminated substrates and dietary uptake, which can result in metal accumulation and disruption of physiological homeostasis [4,5]. Spiders, as abundant predators in terrestrial and agricultural ecosystems, occupy an important trophic position and can be chronically exposed to metals through both environmental and food-web pathways [6,7]. Previous studies have shown that excessive metal exposure (e.g., cadmium, zinc, and lead) can impair growth, survival, antioxidant defense, and other physiological processes in spiders [8–10]. Importantly, chronic exposure may also compromise reproductive performance, including egg production and offspring development, potentially extending individual-level toxicity to population-level consequences [11,12]. Therefore, understanding how long-term Cu exposure affects spider reproduction is important for evaluating the ecological risks associated with metal contamination.

Reproduction is an energetically demanding process and may be particularly sensitive to metal stress [13]. In female spiders, resources acquired during adulthood are allocated not only to somatic maintenance but also to egg production and offspring development [14]. Exposure to excessive metals can disrupt this allocation by inducing oxidative damage and altering energy and nutrient metabolism, potentially reducing reproductive investment and offspring quality [13]. Indeed, previous studies have reported reduced egg production and hatchling survival in spiders exposed to elevated concentrations of metals, suggesting that the consequences of metal stress may extend beyond directly exposed individuals [9,13]. Maternal exposure is particularly important in this context because contaminants may influence offspring through direct maternal transfer and/or exposure-induced changes in maternal provisioning [15]. Such effects may alter the physiological and metabolic state of offspring during early development, even before they begin independent feeding [15,16]. However, how maternal Cu exposure affects reproductive investment and the early physiological state of spider offspring remains poorly understood.

Previous studies have demonstrated that spiders respond to metal exposure through extensive physiological, transcriptional, and metabolic adjustments [17–19]. For example, Cu and lead exposure have been shown to alter antioxidant enzyme responses, including those involving superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), in ground spiders [8]. Similarly, cadmium exposure significantly increased SOD and malondialdehyde (MDA) levels in the egg sacs of *Pardosa pseudoannulata* [11]. In our previous study, Cu exposure induced oxidative stress and widespread transcriptomic and metabolomic alterations in adult *Pirata subpiraticus*, involving antioxidant defense, cellular homeostasis, and metabolic processes [20]. However, whether these molecular responses are similarly manifested in offspring following maternal Cu exposure remains unknown. Adult females and newly hatched spiderlings differ substantially in their physiological and developmental demands: adults must maintain homeostasis while allocating resources to reproduction, whereas spiderlings undergo rapid development and depend largely on maternally provisioned resources. We therefore hypothesized that maternal Cu exposure would impair reproductive performance and induce distinct molecular responses in

adult females and their offspring. To test this hypothesis, we evaluated Cu accumulation, oxidative stress, and reproductive traits in adult females and compared transcriptomic and metabolomic responses between adult females and newly hatched spiderlings. By integrating physiological, reproductive, transcriptomic, and metabolomic evidence, this study aimed to determine how the consequences of Cu exposure extend from exposed females to their offspring and whether these effects are associated with life-stage-specific molecular responses.

2. Materials and Methods

2.1. Spider Preparation

Adult female *P. subpiraticus* were collected using suction samplers from a controlled rice cultivation field (113.09° E, 28.21° N). Following collection, each female was housed individually in a plastic box with a perforated lid (10 × 12 cm, diameter × height) to prevent interactions among individuals. Mating was conducted under laboratory conditions before the initiation of Cu exposure. Following mating, females were maintained individually until the initiation of egg-sac production. Before the initiation of egg-sac production, all females were maintained under control conditions with uncontaminated artificial soil and distilled water. The spiders were maintained in a controlled environmental chamber at 25 ± 2 °C, with a 16:8 h light:dark photoperiod and 70 ± 10% relative humidity, and fed with fruit flies (*Drosophila* spp.) throughout the experimental period. Females were monitored daily for reproductive activity. The formation of a silken egg-laying pad, indicating the initiation of egg-sac production, was used as the criterion for entry into the exposure experiment.

Artificial soil was prepared according to OECD (1984) guidelines and consisted of 70% industrial sand, 20% kaolin clay, and 10% sphagnum peat. Soil pH was adjusted to 6.0 ± 0.5 using $CaCO_3$, and moisture content was maintained at 50% of the maximum water-holding capacity. Cu-contaminated soil was prepared using $CuSO_4{\cdot}5H_2O$ following previously established procedures [8,20]. Briefly, $CuSO_4{\cdot}5H_2O$ was dissolved in distilled water to prepare a stock solution and subsequently diluted and added to the artificial soil to achieve a final nominal Cu concentration of 800 mg/kg based on the dry weight of the artificial soil. The Cu solution was gradually added to the air-dried soil and thoroughly mixed to promote uniform distribution. Control soil received an equivalent volume of distilled water. The exposure concentration was selected based on previous studies examining Cu toxicity in spiders.

Upon formation of the silken egg-laying pad, females were assigned to either the control (CK) or Cu-exposure group, and this time point was designated as day 0 of exposure. Two separate cohorts of adult females were used because physiological and molecular analyses required sampling after 21 d of exposure, whereas reproductive and offspring analyses required continued exposure until egg-sac hatching. One cohort of females was maintained under the respective exposure conditions for 21 d, consistent with previously established Cu-exposure protocols [8,13,20]. At the end of the 21 d exposure period, adult females were collected for determination of Cu accumulation, oxidative stress and antioxidant parameters, and transcriptomic and metabolomic analyses. A separate cohort was continuously maintained under the same CK or Cu conditions through egg-sac development until hatching. Reproductive traits were recorded during this period, and newly hatched spiderlings were collected immediately after emergence for phenotypic measurements and transcriptomic and metabolomic analyses.

2.2. Determination of Cu Content

For each treatment, three biological replicates were prepared, with each replicate consisting of two pooled adult females to obtain sufficient sample mass for Cu determination.

Each pooled sample was placed in a microwave digestion vessel, to which a mixture of 3 mL nitric acid (HNO_3), 2 mL hydrogen peroxide (H_2O_2), and 3 mL distilled water was added. The vessels were then sealed and subjected to microwave digestion at 140 °C for 10 min and at 56 °C for 30 min, which helped heat the samples and facilitate the release of Cu into the solution. After digestion, the Cu concentration in the solution was measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). To determine the actual Cu concentrations in the experimental soil, soil samples were collected from both the control and Cu-treated groups and analyzed using ICP-MS (ICPE-9000, SHIMADZU CORPORATION, JAPAN) following acid digestion. The measured Cu concentrations were 8.34 ± 2.56 mg/kg in the control soil and 685.45 ± 45.23 mg/kg in the Cu-treated soil (mean ± SEM, $n = 3$).

2.3. Determination of Antioxidative Enzyme and MDA Concentration

Spiders (four biological replicates per treatment, with two adult females pooled per replicate) were collected for the analysis of key antioxidant enzymes and malondialdehyde (MDA). Each pooled sample was weighed and then subjected to manual homogenization. This process involved blending each sample with 1 mL of a 10 mM phosphate-buffered solution (i.e., phosphate, sodium chloride, and potassium chloride), which had a pH level of 7.2, in a homogenizer. Following homogenization, the samples were centrifuged at a force of 4000× *g* for 10 min. To measure the activities of SOD, GPX, CAT, and MDA, ELISA kits were employed following the manufacturer's guidelines.

2.4. Reproductive Traits

Reproductive performance was monitored throughout the exposure period. Following the formation of a silken egg-laying pad and assignment to the CK or Cu treatment, females were inspected daily for egg-sac production. A total of 93 adult females were monitored, including 45 in the CK group and 48 in the Cu-treated group. The proportion of females producing egg sacs was calculated for each treatment, and the time to egg-sac production was recorded as the number of days from the initiation of Cu exposure (day 0) to the formation of an egg sac. Following egg-sac formation, the number of eggs per sac, egg-sac weight, and egg size were determined to evaluate reproductive investment. These egg-sac-related traits were analyzed in 25 females from the CK group and 22 females from the Cu-treated group. Females were maintained individually under their respective CK or Cu exposure conditions throughout egg-sac development. Each female and her associated egg sac were considered an independent biological replicate.

2.5. RNA Extraction and Sequencing

Total RNA was extracted from adult females and newly hatched spiderlings (three biological replicates) using a commercial RNA isolation kit according to the manufacturer's instructions. For adult females, each biological replicate consisted of a pooled sample of four individuals. For newly hatched spiderlings, each biological replicate consisted of 20 spiderlings pooled from four different mothers (five spiderlings per mother). The quantity and quality of RNA were assessed using a NanoDrop 2000 spectrophotometer and agarose gel electrophoresis. For each sample, 1.5 μg of total RNA was used for library preparation. Messenger RNA was enriched using poly-T oligo-attached magnetic beads and fragmented under elevated temperature in the presence of divalent cations. First-strand cDNA was synthesized using random hexamer primers and M-MuLV reverse transcriptase, followed by second-strand cDNA synthesis using DNA polymerase I and RNase H. Sequencing libraries were constructed using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® according to the manufacturer's instructions. Library fragments of approximately 250–300 bp were purified using the AMPure XP system, and paired-end sequencing was performed on an

Illumina platform. Raw reads were processed to remove adapter-containing reads, poly-N sequences, and low-quality reads. The quality of the resulting clean reads was evaluated based on Q20, Q30, GC content, and sequence duplication levels. Clean paired-end reads were de novo assembled using Trinity, and transcriptome completeness was assessed using Benchmarking Universal Single-Copy Orthologs (BUSCO). Functional annotation of the assembled unigenes was performed against public databases, including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG), NCBI non-redundant protein (Nr) and nucleotide (Nt) databases, Swiss-Prot, and Pfam. Gene expression levels were quantified using RSEM and expressed as fragments per kilobase of transcript per million mapped reads (FPKM). Differential expression analyses were performed separately for adult females and newly hatched spiderlings by comparing the Cu-exposed group with the corresponding CK group. Genes with a false discovery rate (FDR) < 0.05 and $|\log_2\text{fold change}| > 1$ were considered differentially expressed genes (DEGs). Principal component analysis (PCA) was used to assess overall variation in gene-expression profiles among samples. GO and KEGG enrichment analyses were performed separately for DEGs identified in adult females and spiderlings to characterize their major biological functions and pathways. To further compare transcriptional responses between developmental stages, DEGs shared between adult females and spiderlings were identified. The relationship between Cu-induced expression changes in the two stages was evaluated by Pearson correlation analysis of $\log_2$ fold-change values for the 203 shared DEGs, with each gene represented by paired $\log_2$ fold-change values calculated from the Cu versus CK comparison in adult females and spiderlings, respectively. K-means clustering was performed to group Cu-responsive genes according to their expression patterns across the four experimental groups (Adult_CK, Adult_Cu, Spiderling_CK, and Spiderling_Cu).

2.6. Metabolomic Sequencing and Data Analysis

Adult females and newly hatched spiderlings (six biological replicates) from the CK and Cu groups were subjected to untargeted metabolomic analysis. For adult females, each biological replicate consisted of four individuals. For newly hatched spiderlings, each biological replicate consisted of 20 spiderlings pooled from four different mothers. Frozen samples were homogenized and metabolites were extracted using the same procedure as previously described. Following centrifugation, the supernatants were collected for liquid chromatography–mass spectrometry (LC-MS) analysis. Quality-control (QC) samples were prepared by pooling equal aliquots of individual samples and were analyzed throughout the analytical sequence to evaluate the stability and reproducibility of the LC-MS system. Raw LC-MS data were processed for peak detection, alignment, retention-time correction, and normalization. Metabolites were identified by matching accurate mass, retention time, and MS/MS fragmentation information against available metabolite databases. Multivariate analyses, including principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA), were performed to evaluate overall differences in metabolic profiles among treatment groups. PLS-DA models were evaluated using R^2Y and Q^2 values. R^2Y was used to assess model fit, whereas Q^2 was used to evaluate predictive performance through cross-validation. Permutation testing was performed to assess model robustness and the potential risk of overfitting. Differential metabolite analyses were performed separately for adult females and newly hatched spiderlings by comparing the Cu-exposed group with the corresponding CK group. Differentially expressed metabolites (DEMs) were identified based on variable importance in projection (VIP), statistical significance, and fold-change criteria [VIP > 1, $p < 0.05$, and $|\log_2\text{fold change}| \geq 1$]. Identified DEMs were subsequently mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and pathway

enrichment analysis was performed to characterize metabolic pathways affected by Cu exposure in adult females and spiderlings.

2.7. RT-qPCR Validation

To validate the RNA-seq results, six representative genes (*SOD*, *CAT*, *GPX*, *CACNA1D*, *ATG7*, and *PDHA1*) were selected for quantitative real-time PCR (qRT-PCR) analysis in adult females and newly hatched spiderlings. For reverse transcription, 1 µg of total RNA from each sample was used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. qRT-PCR was performed using the synthesized cDNA, gene-specific forward and reverse primers (Table 1), and iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The thermal cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 45 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 60 s. A melting-curve analysis was subsequently performed from 65 to 95 °C, with temperature increments of 0.5 °C, to confirm amplification specificity. Relative expression levels of the target genes were normalized by glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [21] and calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primers used in the study.

Gene	Gene Name	Primer	Primer Sequence (5′–3′)
SOD	Superoxide dismutase	SOD-F SOD-R	AGTGCGTCTCCTCTGTCAAG AATTCGGCAACAGCAGATCC
CAT	Catalase	CAT-F CAT-R	AGATCCCCGTGGATTTGCTG TTGGTGGGTGGTTTCAGGTC
GPX	Glutathione peroxidase	GPX-F GPX-R	CGGCTACGAGGGATGTCTTC ATTTGCGTTGATTGGCGAGG
CACNA1D1	Muscle calcium channel subunit alpha-1	CACNA1D1-F CACNA1D1-R	GCTTCGATTGCTTCGTCGTC AGTTAAAACGGCCGCCAAAC
ATG7	Autophagy-related protein 7	ATG7-F ATG7-R	GGGTGGCCACTACGAAACAT AGAGTGCCAGCTCCAAACAA
PDHA1	Pyruvate dehydrogenase E1 subunit alpha 1	PDHA1-F PDHA1-R	GCCGCGCATATCATCAGTTTT ACTGCAATTCCTGCAAAGCC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH-F GAPDH-R	AAAGATGTGGCGAGATGGCA AGCTTCCTTACCAAGACGGC

2.8. Data Analysis

Statistical analyses were performed using GraphPad Prism (v9, GraphPad Software, Boston, MA, USA) and R software (v4.6.2, R Foundation for Statistical Computing, Vienna, Austria). The normality of the data was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated using the Brown–Forsythe test. Comparisons of Cu accumulation, oxidative stress and antioxidant parameters, and reproductive traits between the CK and Cu groups were performed using independent-samples two-tailed *t*-tests when the assumptions of normality and homogeneity of variance were satisfied. Differences in the proportion of females producing egg sacs between treatments were analyzed using Fisher's exact test. Statistical significance was set at $p < 0.05$. For integrated transcriptomic and metabolomic analysis, DEGs and DEMs from the corresponding Cu-versus-CK comparisons were mapped to KEGG pathways to identify biological processes represented at both molecular levels. Data visualization was performed using the "pheatmap" and "ggplot2" packages in R, and figures were assembled using Adobe Illustrator.

3. Results

3.1. Cu Accumulation and Oxidative Stress Responses in Adult Females

Cu content in adult female *P. subpiraticus* was markedly higher in the Cu-exposed group than in the CK group ($p < 0.001$; Figure 1A), whereas body weight was significantly reduced following Cu exposure ($p < 0.05$; Figure 1B). MDA levels were significantly elevated in Cu-exposed females ($p < 0.01$; Figure 1C). The activities of the antioxidant enzymes SOD and GPX were also significantly higher in the Cu group than in the CK group ($p < 0.05$; Figure 1D,E), and CAT activity showed a similar increase ($p < 0.01$; Figure 1F). Thus, Cu exposure was associated with increased lipid peroxidation together with elevated antioxidant enzyme activities in adult females.

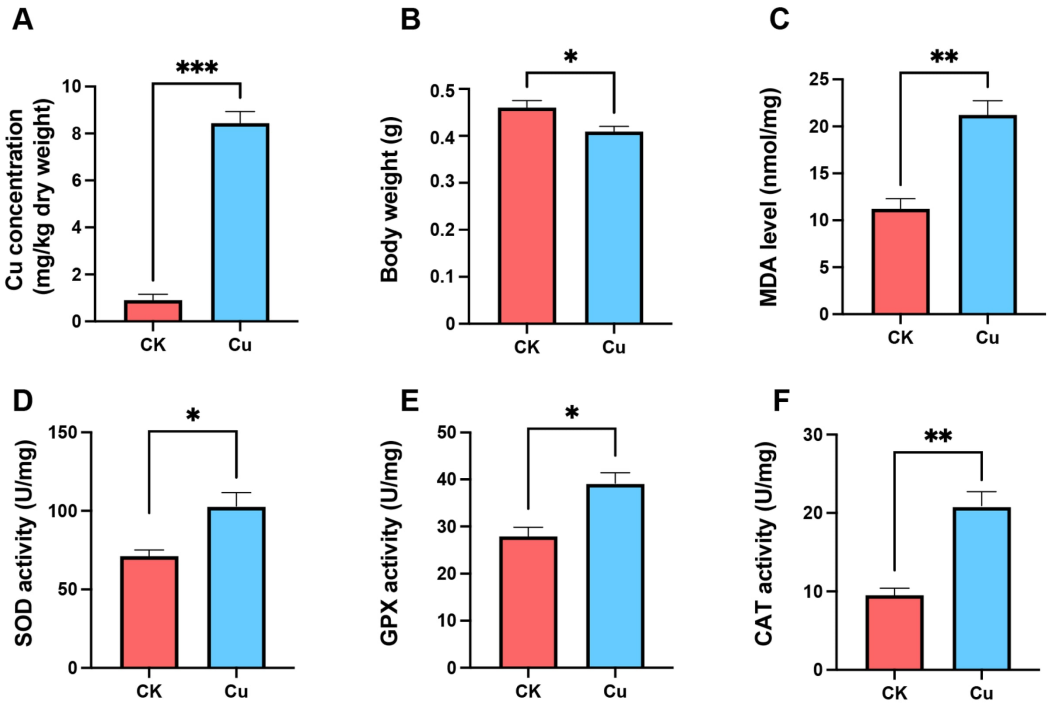


Figure 1. Cu accumulation, body weight, and oxidative stress responses in adult female *Pirata subpiraticus* following Cu exposure. (**A**) Cu content in adult females. (**B**) Body weight. (**C**) Malondialdehyde (MDA) content. Activities of (**D**) superoxide dismutase (SOD), (**E**) glutathione peroxidase (GPX), and (**F**) catalase (CAT). Data are presented as mean ± SEM. Sample sizes were $n = 3$ for Cu accumulation (**A**), $n = 20$ for body weight (**B**), and $n = 4$ for oxidative stress parameters (**C**–**F**), per group. Differences between the CK and Cu groups were analyzed using independent-samples two-tailed *t*-tests. Asterisks indicate statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2. Effects of Cu Exposure on Reproductive Traits

Cu exposure significantly altered multiple reproductive traits in female *P. subpiraticus* (Figure 2). The proportion of females producing egg sacs was lower in the Cu group than in the CK group (Figure 2A). The time to egg-sac production was significantly longer in Cu-exposed females, increasing from approximately 22 days in the CK group to 35 days in the Cu group ($p < 0.001$; Figure 2B). In addition to the prolonged time to egg-sac production, the number of eggs per sac differed significantly between treatments. Females in the CK group produced more eggs compared with those in the Cu group ($p < 0.001$; Figure 2C). Egg-sac weight was also significantly lower in Cu-exposed females than in CK females ($p < 0.05$; Figure 2D). Similarly, egg size was significantly reduced in the Cu group ($p < 0.001$; Figure 2E). Overall, Cu-exposed females showed a longer time to egg-sac

production and lower values for the number of eggs per sac, egg-sac weight, and egg size compared with CK females.

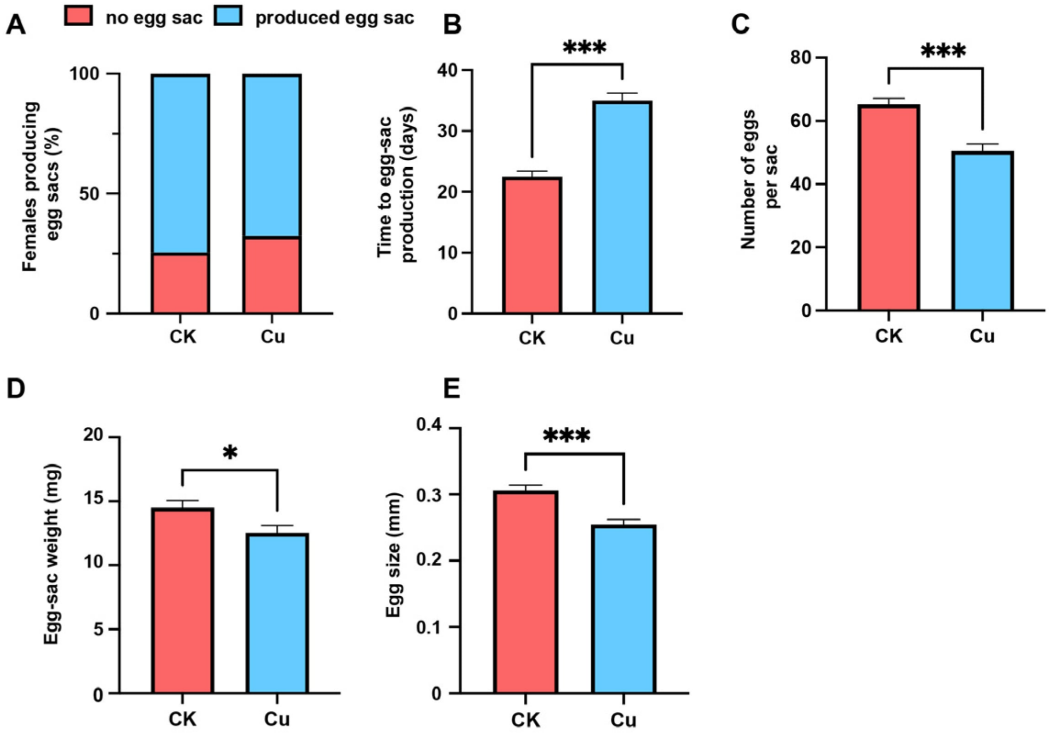


Figure 2. Effects of Cu exposure on reproductive traits of female *Pirata subpiraticus*. (**A**) Proportion of females producing egg sacs. (**B**) Time to egg-sac production. (**C**) Number of eggs per sac. (**D**) Egg-sac weight. (**E**) Egg size. Data in graphs (**B**–**E**) are presented as mean ± SEM. Sample sizes were $n = 45$ and 48 females in the CK and Cu groups, respectively, for panel (**A**). For panels (**B**–**E**), sample sizes were $n = 25$ and 22 independent females or egg sacs in the CK and Cu groups, respectively. Differences between the CK and Cu groups were analyzed using independent-samples two-tailed *t*-tests. Asterisks indicate statistical significance (* $p < 0.05$, *** $p < 0.001$).

3.3. Stage-Specific Transcriptomic Responses to Cu Exposure

Transcriptomic profiling revealed distinct gene expression responses to Cu exposure in adult females and newly hatched spiderlings (Figure 3). Principal component analysis (PCA) showed separation among the four experimental groups, with samples from the same treatment and developmental stage generally clustering together (Figure 3A). Differential expression analysis identified 463 DEGs in adult females, including 132 upregulated and 331 downregulated genes in the Cu group relative to the corresponding CK group (Figure 3B). In spiderlings, 419 DEGs were identified, of which 276 were upregulated and 143 were downregulated following Cu exposure (Figure 3C). Comparison of the transcriptional responses between adult females and spiderlings identified 203 DEGs shared between the two developmental stages. The $\log_2$ fold-change values of these shared DEGs showed a strong negative correlation between adults and spiderlings (Pearson $r = -0.986$, $p < 0.001$; Figure 3D), with genes showing increased expression in one stage generally exhibiting decreased expression in the other.

Clustering analysis further separated the Cu-responsive genes into four major expression patterns, comprising 97, 205, 263, and 114 genes in clusters 1–4, respectively (Figure 3E). Cluster 1 was characterized predominantly by increased expression in Cu-exposed adults and decreased expression in Cu-exposed spiderlings, whereas cluster 3 displayed the opposite pattern, with decreased expression in Cu-exposed adults and increased expression in Cu-exposed spiderlings. Cluster 2 showed a pronounced reduction in expression in

Cu-exposed adults, while cluster 4 exhibited increased expression in Cu-exposed adults and relatively elevated expression in CK spiderlings. GO enrichment analysis showed that genes associated with these expression patterns were enriched in distinct functional categories (Figure 3F,G). Enriched terms included extracellular matrix structural constituent, 3-hydroxyanthranilate 3,4-dioxygenase activity, dihydrofolate reductase activity, collagen trimer, and processes associated with folic acid-containing compounds and tetrahydrofolate metabolism and biosynthesis. Additional enriched terms included cellular response to DNA damage stimulus, G-protein-coupled receptor activity, DNA repair, response to stress, DNA replication, double-stranded DNA binding, mismatch repair, and mismatched DNA binding.

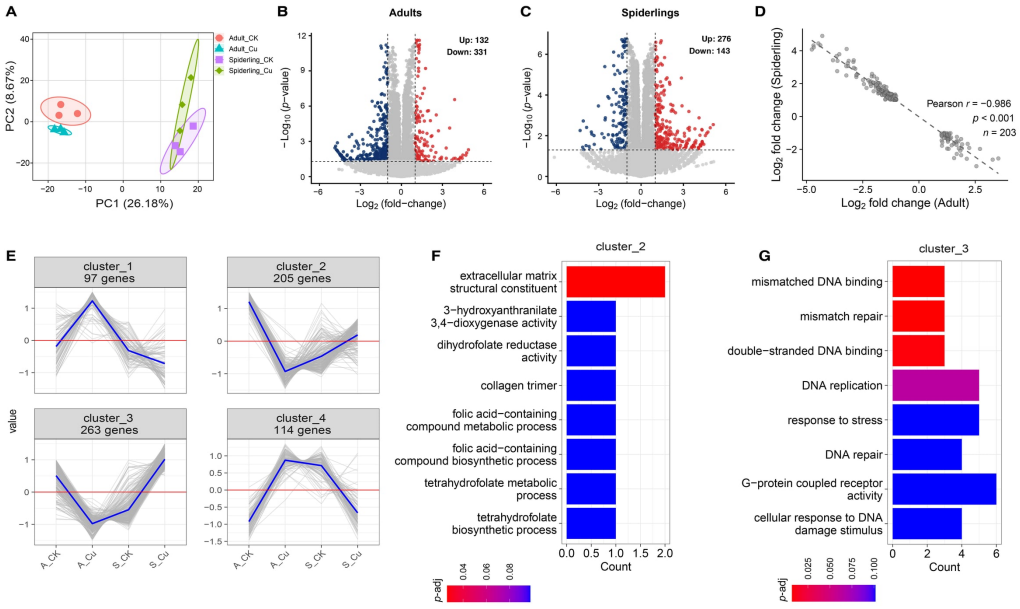


Figure 3. Transcriptomic responses to Cu exposure in adult female and newly hatched *Pirata subpiraticus* spiderlings. (**A**) Principal component analysis (PCA) of transcriptomic profiles from Adult_CK, Adult_Cu, Spiderling_CK, and Spiderling_Cu groups. Shaded ellipses represent the 95% confidence regions for each group. (**B**,**C**) Volcano plots showing differentially expressed genes (DEGs) between the Cu and CK groups in adult females (**B**) and spiderlings (**C**), respectively. Red and blue points represent upregulated and downregulated genes, respectively, whereas gray points represent genes that were not significantly differentially expressed. (**D**) Correlation of log2 fold changes for the 203 DEGs shared between adults and spiderlings. The Pearson correlation coefficient and corresponding *p* value are shown. (**E**) K-means clustering analysis of Cu-responsive genes across the four experimental groups, identifying four distinct expression patterns. Gray lines represent individual genes, and blue lines represent the mean expression pattern of genes within each cluster. (**F**,**G**) Gene Ontology (GO) enrichment analysis of genes in clusters 2 (**F**) and 3 (**G**), respectively.

3.4. Metabolome Sequencing and DEMs Analysis

Untargeted metabolomic analysis revealed distinct metabolic profiles between the CK and Cu groups in both adult females and newly hatched spiderlings (Figure 4). Multivariate analysis showed separation between the treatment groups (Figure 4A,B). Differential metabolite analysis further showed distinct patterns of metabolic changes between the two developmental stages. In adult females, 51 differentially expressed metabolites (DEMs) were identified between the Cu and CK groups, including 34 upregulated and 17 downregulated metabolites (Figure 4C). In spiderlings, 59 DEMs were detected, including 13 upregulated and 46 downregulated metabolites (Figure 4D).

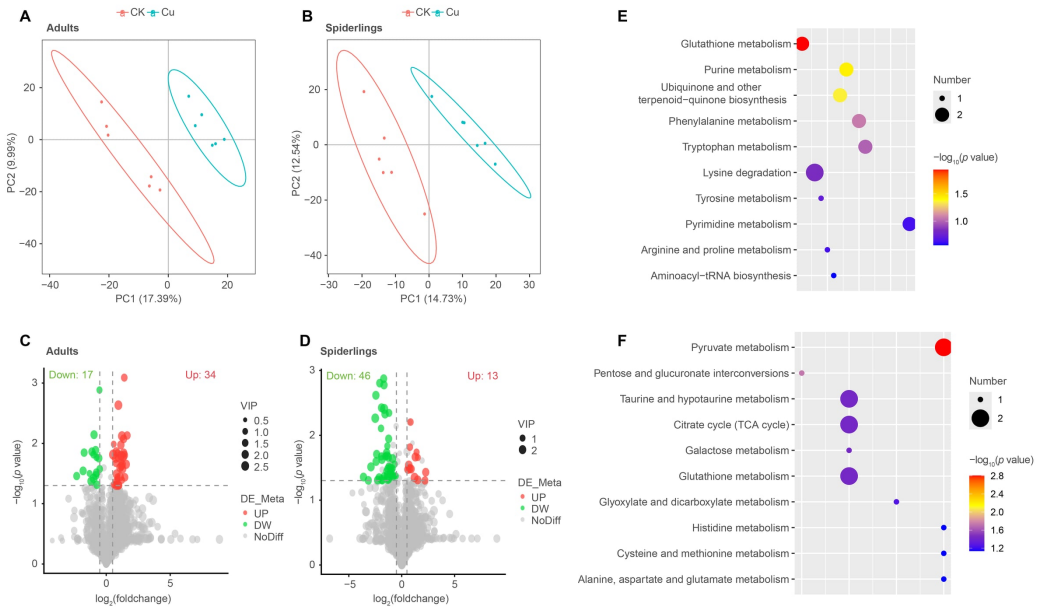


Figure 4. Metabolic responses to Cu exposure in adult female and newly hatched *Pirata subpiraticus* spiderlings. (**A**,**B**) Principal component analysis (PCA) of metabolomic profiles between the CK and Cu groups in adult females (**A**) and spiderlings (**B**), respectively. Ellipses represent the 95% confidence regions for each treatment group. (**C**,**D**) Volcano plots showing differentially expressed metabolites (DEMs) between the Cu and CK groups in adult females (**C**) and spiderlings (**D**), respectively. Red and green points represent upregulated and downregulated metabolites, respectively, whereas gray points represent metabolites without significant differences. Point size represents the variable importance in projection (VIP) value. (**E**,**F**) KEGG pathway enrichment analysis of DEMs identified in adult females (**E**) and spiderlings (**F**), respectively. Bubble size represents the number of DEMs associated with each pathway, and bubble color represents the −log10(*p* value).

KEGG enrichment analysis showed that DEMs in adult females were associated with several amino acid, nucleotide, and redox-related metabolic pathways. The enriched pathways included aminoacyl-tRNA biosynthesis, arginine and proline metabolism, pyrimidine metabolism, tyrosine metabolism, lysine degradation, tryptophan metabolism, phenylalanine metabolism, ubiquinone and other terpenoid-quinone biosynthesis, purine metabolism, and glutathione metabolism (Figure 4E). In spiderlings, DEMs were enriched in a different set of metabolic pathways, including alanine, aspartate and glutamate metabolism, cysteine and methionine metabolism, histidine metabolism, glyoxylate and dicarboxylate metabolism, glutathione metabolism, galactose metabolism, the citrate cycle (TCA cycle), taurine and hypotaurine metabolism, pentose and glucuronate interconversions, and pyruvate metabolism (Figure 4F). Glutathione metabolism was represented among the enriched pathways in both adult females and spiderlings.

3.5. Conjoint Transcriptome and Metabolome Analysis

Integrated transcriptomic and metabolomic analyses revealed distinct molecular response profiles to Cu exposure in adult females and spiderlings (Figure 5). In adult females, coordinated transcriptomic and metabolic changes were mainly associated with oxidative stress response, metal ion response, Toll and IMD signaling, arachidonic acid metabolism, and lysosomal processes (Figure 5A). Genes associated with the oxidative stress response included *SOD1*, *CAT*, *GPX*, *PRDX*, and *HMOX*, while genes related to metal ion response included *MT*, *ATP7A*, *ATP7B*, *CTR1*, *SLC31A1*, and *SLC30A1*. Cu exposure altered the expression of multiple genes within these two functional groups, accompanied by changes in the corresponding metabolites. Changes were also observed in immune- and cellular-

process-related pathways in adult females. Genes associated with Toll and IMD signaling, including Relish, Dif, Dorsal, Toll, IMD, and PGRP, exhibited differential expression between the CK and Cu groups. Arachidonic acid metabolism contained changes in *CYP2J*, *CBR1*, *PTGES2*, and *HPGDS*, together with associated metabolites. In addition, lysosomal processes involved differential expression of *ATG5*, *ATG7*, *LC3*, *LAMP1*, and *CTSL* and corresponding metabolite changes (Figure 5A).

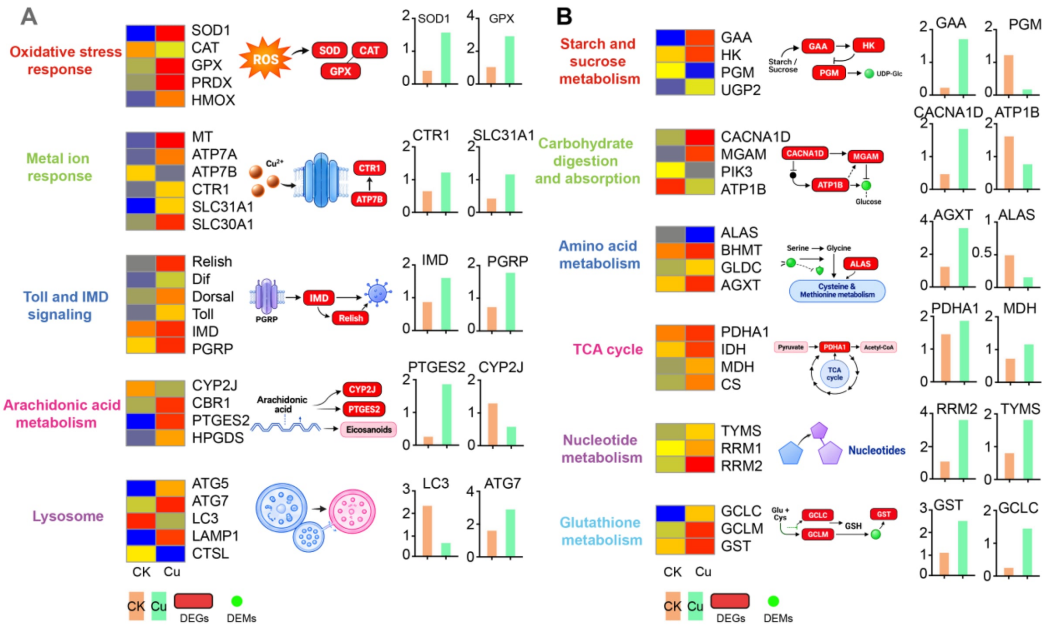


Figure 5. Integrated transcriptomic and metabolomic responses to Cu exposure in adult female and newly hatched *Pirata subpiraticus* spiderlings. (**A**) Integrated analysis of major Cu-responsive biological processes in adult females, including oxidative stress response, metal ion response, Toll and IMD signaling, arachidonic acid metabolism, and lysosomal function. (**B**) Integrated analysis of major Cu-responsive metabolic processes in spiderlings, including starch and sucrose metabolism, carbohydrate digestion and absorption, amino acid metabolism, the TCA cycle, nucleotide metabolism, and glutathione metabolism. Heatmaps show the relative abundance patterns of representative differentially expressed genes (DEGs) between the CK and Cu groups. Red indicates higher expression levels, whereas blue indicates lower expression levels. Pathway diagrams illustrate representative genes and metabolites associated with each biological process. Bar plots show the relative levels of selected DEGs in the CK and Cu groups. Red rectangles and green circles in the pathway diagrams represent DEGs and DMs, respectively.

In spiderlings, the integrated molecular profile was characterized by a different set of pathways (Figure 5B). Transcriptomic and metabolic changes were observed in starch and sucrose metabolism and carbohydrate digestion and absorption, involving genes such as *GAA*, *HK*, *PGM*, *UGP2*, *CACNA1D*, *PIK3*, and *ATP1B*. Amino acid metabolism was represented by changes in *ALAS*, *BHMT*, *GLDC*, and *AGXT*. Changes associated with central energy metabolism were also observed in the TCA cycle, including differential expression of *PDHA1*, *IDH*, *MDH*, and *CS*. Additional changes in spiderlings were associated with nucleotide and glutathione metabolism. The nucleotide-related genes *TYMS*, *RRM1*, and *RRM2* showed altered expression following Cu exposure, while glutathione metabolism included changes in *GCLC*, *GCLM*, and *GST*. These transcriptional changes occurred together with differences in metabolites associated with the corresponding metabolic processes (Figure 5B).

3.6. qRT-PCR Validation of Representative Genes

qRT-PCR analysis was performed for six representative genes (*SOD*, *CAT*, *GPX*, *CACNA1D*, *ATG7*, and *PDHA1*) in adult females and spiderlings (Figure 6). In adult females, the expression levels of *SOD*, *CAT*, and *GPX* were significantly higher in the Cu group than in the CK group ($p < 0.05$). *ATG7* and *PDHA1* were also significantly upregulated following Cu exposure ($p < 0.05$), whereas no significant difference was detected for *CACNA1D*. In spiderlings, *SOD*, *CAT*, *GPX*, and *CACNA1D* were significantly upregulated in the Cu group ($p < 0.05$), whereas *ATG7* was significantly downregulated ($p < 0.05$). *PDHA1* showed a modest increase, but the difference was not statistically significant. Overall, the qRT-PCR results were consistent with the expression directions observed in the transcriptomic analysis.

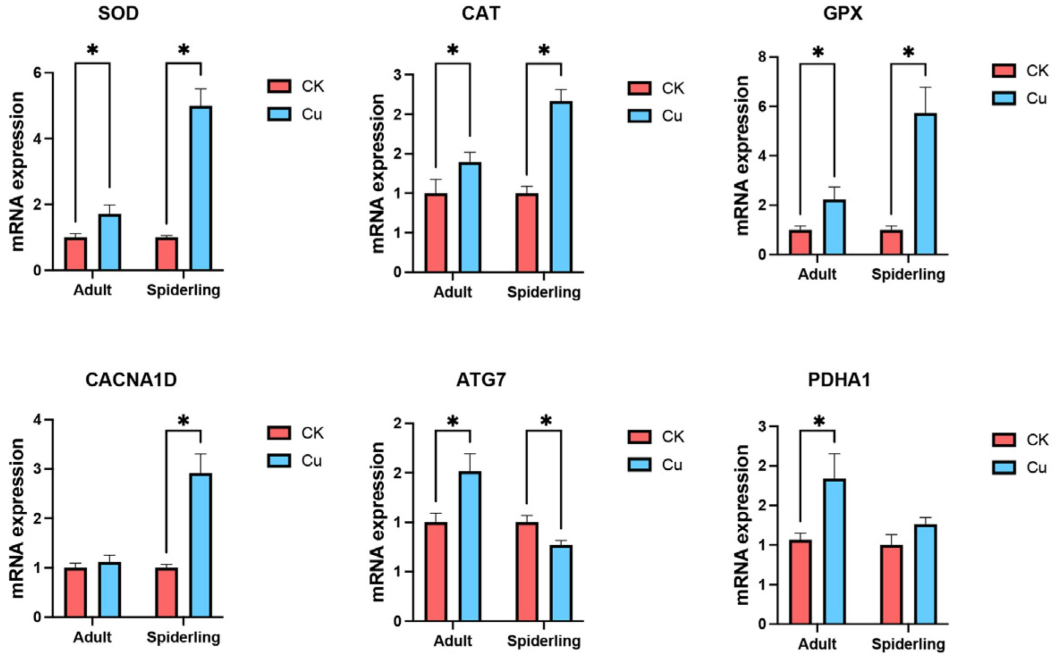


Figure 6. qRT-PCR analysis of representative Cu-responsive genes in adult female and newly hatched *Pirata subpiraticus* spiderlings. Relative mRNA expression levels of *SOD*, *CAT*, *GPX*, *CACNA1D*, *ATG7*, and *PDHA1* were determined in adult females and spiderlings from the CK and Cu groups. Data are presented as mean ± SEM. Each group included $n = 3$ independent biological replicates. Differences between the CK and Cu groups within each developmental stage were analyzed using independent-samples two-tailed *t*-tests. Asterisks indicate significant differences (* $p < 0.05$).

4. Discussion

The present study demonstrates that maternal Cu exposure affects multiple levels of biological organization in *P. subpiraticus*, ranging from maternal physiological condition and reproductive performance to molecular responses in newly hatched offspring. These findings suggest that the consequences of Cu exposure may extend beyond directly exposed females, potentially influencing offspring physiology through maternally mediated effects. Notably, the distinct molecular responses observed in adults and spiderlings highlight the importance of developmental stage in shaping responses to maternal Cu exposure. Together, these findings emphasize the need to consider both maternal and offspring responses when assessing the ecological risks of Cu contamination in terrestrial arthropods.

The substantial accumulation of Cu in adult females was accompanied by increased lipid peroxidation and activation of antioxidant defenses. Elevated MDA levels are consistent with oxidative damage caused by excessive metal exposure, whereas the concurrent

increases in SOD, GPX, and CAT activities likely represent an enzymatic response to increased oxidative challenge [22–24]. Similar responses have been observed in metal-exposed arthropods, in which oxidative stress is accompanied by enhanced antioxidant defenses that contribute to the maintenance of cellular redox homeostasis [24,25]. Importantly, the biochemical responses observed here were supported at the transcriptional level: *SOD*, *CAT*, and *GPX* were all significantly upregulated in Cu-exposed adult females, consistent with the increased activities of their corresponding enzymes. Such concordance across transcriptional and biochemical levels provides complementary evidence for activation of the antioxidant defense system during Cu exposure. Nevertheless, elevated MDA levels persisted despite the enhanced antioxidant response, suggesting that increased antioxidant activity did not fully counteract Cu-associated oxidative damage [26]. The concurrent reduction in body weight further suggests a broader physiological cost of Cu exposure. This physiological disturbance may be particularly consequential during reproduction, when females must simultaneously maintain somatic homeostasis and allocate resources to egg production and development [27,28], providing an important physiological context for the reproductive alterations observed in the present study.

Reproduction appeared to be particularly sensitive to maternal Cu exposure, with effects evident in both the timing of egg-sac production and reproductive investment. The pronounced delay in egg-sac production suggests that Cu exposure affected the progression of reproduction rather than solely reducing its final output [29,30]. At the same time, reductions in egg number, egg-sac weight, and egg size indicate decreased investment in individual reproductive events. These reproductive changes may be linked to metabolic disturbances associated with Cu exposure. In adult females, alterations in amino acid and nucleotide metabolism suggest changes in metabolic processes that support protein synthesis, cellular maintenance, and reproductive development. Meanwhile, increased antioxidant activity under Cu-induced oxidative stress may require additional metabolic resources, potentially limiting their availability for egg production. Reproductive impairment following metal exposure has also been reported in other arthropods, where reduced fecundity, delayed reproduction, and altered egg production have been associated with the energetic and physiological costs of coping with contaminant stress [31,32]. Reproduction is especially resource demanding because females must balance the allocation of energy and nutrients between somatic maintenance and the production and development of offspring [33,34]. Under Cu exposure, the simultaneous occurrence of oxidative damage, enhanced antioxidant defenses, and reduced body weight suggests that maternal physiological demands were elevated during this reproductive period [35–37]. Such demands could potentially constrain the resources available for reproduction, although resource allocation was not directly measured in the present study. Importantly, the reduction in egg size may have consequences beyond maternal reproductive output, because egg provisioning establishes the initial resources available to developing offspring before independent feeding [38]. Thus, the reproductive effects observed here provide an important link between maternal Cu exposure and the subsequent molecular alterations detected in newly hatched spiderlings. In addition to altered maternal reproductive investment, the potential transfer of accumulated Cu from females to developing embryos may represent another pathway through which maternal exposure influences offspring. If such transfer occurs, embryonic Cu exposure could contribute to the molecular alterations observed in newly hatched spiderlings, although this possibility remains unverified in the present study.

The molecular analyses further showed that maternal Cu exposure was associated with distinct responses in adult females and newly hatched spiderlings. Although substantial transcriptomic and metabolic alterations were detected at both stages, the biological processes represented by these changes differed considerably. In adult females, integrated

transcriptomic and metabolomic analyses highlighted processes associated with oxidative stress, metal ion homeostasis, Toll and IMD signaling, arachidonic acid metabolism, and lysosomal function. These responses are broadly consistent with the physiological demands of directly exposed females [39], in which Cu accumulation requires coordinated regulation of redox balance, metal handling, and cellular homeostasis [40,41]. The upregulation of antioxidant-related genes, including SOD, CAT, and GPX, together with increased antioxidant enzyme activities, suggests a coordinated transcriptional and physiological response to Cu-induced oxidative stress. These responses, alongside the observed metabolic alterations, may increase the physiological costs of maintaining cellular homeostasis and potentially affect resource availability for reproduction. This provides a possible link between molecular responses and the observed reproductive impairment, although the causal involvement of specific genes and metabolic pathways remains to be established. In contrast, the molecular changes observed in spiderlings were concentrated more strongly in carbohydrate and amino acid metabolism, the TCA cycle, nucleotide metabolism, and glutathione metabolism [42]. Alterations in these processes may be particularly relevant during early development, when energy production, substrate utilization, and biosynthetic activity are required to support rapid growth and cellular development [43,44]. The transcriptomic comparison further showed that shared Cu-responsive genes were regulated differently between adults and spiderlings, reinforcing the broader stage-specific patterns observed in the integrated analyses. A notable example of this stage-specific response was *ATG7*, a key gene involved in autophagy, which was upregulated in adult females but downregulated in spiderlings following maternal Cu exposure. The increased *ATG7* expression in adults may reflect an adaptive response associated with cellular maintenance under direct Cu stress, whereas its reduced expression in spiderlings may indicate altered regulation of autophagy-related processes during early development. These contrasting responses could reflect differences in developmental requirements and exposure conditions between the two stages. Additionally, glutathione-related processes were represented in the metabolic responses of both stages, suggesting that redox regulation may constitute a common component of the response to maternal Cu exposure [45], despite substantial differences in the broader molecular profiles. Overall, these multi-omics results suggest that the consequences of Cu exposure are strongly dependent on developmental context: adult females predominantly exhibited molecular changes related to stress management and cellular homeostasis, whereas newly hatched spiderlings showed broader alterations in metabolic and biosynthetic processes.

Collectively, our findings show that maternal Cu exposure affects not only the physiological condition and reproductive performance of adult *P. subpiraticus*, but is also associated with substantial molecular alterations in newly hatched offspring. The concurrent changes in maternal oxidative status, reproductive traits, and offspring molecular profiles suggest that the consequences of Cu exposure can extend beyond directly exposed females and influence the next generation during early development. Importantly, adults and spiderlings exhibited distinct molecular responses, highlighting developmental stage as an important factor shaping the biological consequences of metal exposure. Future studies combining measurements of maternal-to-offspring Cu transfer with offspring growth, survival, and reproductive performance would help determine the persistence and ecological consequences of these effects. Given the important role of spiders as predators in agricultural ecosystems, reproductive and offspring-level effects of Cu exposure may have implications extending beyond individual toxicity. Specifically, delayed egg-sac production and reduced egg numbers could decrease offspring recruitment, potentially affecting population maintenance under prolonged Cu contamination. If such reproductive effects persist across generations, they may ultimately influence spider abundance and the biological control services these preda-

tors provide in agricultural ecosystems. However, these population-level consequences remain hypothetical and require validation under field conditions. Additionally, the use of a single Cu concentration limits our ability to assess concentration-dependent responses, including potential effects at lower exposure levels. Future studies incorporating a broader range of Cu concentrations would help clarify the ecological relevance of these findings under different environmental conditions. Furthermore, the relatively small sample size used for antioxidant analyses may limit statistical power, and future studies with larger sample sizes would help further validate these findings.

5. Conclusions

This study provides evidence that the biological consequences of maternal Cu exposure in *P. subpiraticus* extend beyond the directly exposed female and are associated with changes in offspring at the earliest stage of independent life. Importantly, the distinct molecular profiles of adult females and newly hatched spiderlings emphasize that the effects of maternal metal exposure are strongly shaped by developmental context rather than being uniformly expressed across life stages. By linking maternal physiological stress and reproductive impairment with offspring molecular responses, our findings broaden the understanding of Cu toxicity from individual-level effects to maternal-offspring consequences. These results highlight the importance of incorporating reproductive and early-life responses into ecological assessments of metal contamination, particularly for predatory arthropods occupying important trophic positions in agricultural ecosystems.

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