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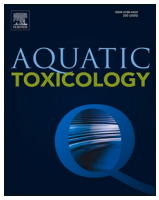

Investigation of potential mechanisms of chronic copper effects on reproduction in zebrafish (*Danio rerio*)

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ABSTRACT

This study investigated copper (Cu) effects on zebrafish (*Danio rerio*) reproduction. Multiple measured endpoints provided insight into potential modes of action. Reproductively active zebrafish were exposed to dissolved Cu concentrations of 0.3 (control), 6.2, 16.8, and 48.8 μg L^{-1} for 21 days, generally following OECD TG 229. Parameters assessed included fecundity, gonado- and liver-somatic index (GSI and LSI), plasma vitellogenin, and two indicators of oxidative stress – catalase (CAT) and glutathione peroxidase (GSH-Px) concentration on days 0, 2, 10, and 21. Additionally, histopathological evaluation of the gill, liver, and gonad was undertaken at test termination. Copper significantly reduced fecundity at 16.8 and 48.8 μg L^{-1} Cu. Correspondingly, CAT and GSH-Px concentrations increased in a concentration- and time-dependent manner in the gonad and liver indicating oxidative stress in these tissues. Plasma vitellogenin concentrations, GSI, and LSI were not affected by Cu exposure. Liver histopathology indicated treatment-related increases in bile duct hyperplasia in male fish. Absence of ovarian post-ovulatory follicles in 16.8 and 48.8 μg L^{-1} group females was consistent with reduced fecundity, but other histopathological findings in gonads were not considered conclusively related to Cu exposure. Overall, the absence of changes in plasma vitellogenin concentrations and endocrine-specific histopathological effects provides further evidence against an endocrine disruption adverse outcome pathway. The lack of overt histopathological damage in the gonads indicates oxidative damage is also likely not driving effects on reproduction. Alternative modalities, such as Cu-induced changes in bioenergetics, could be playing a role in observed reproductive effects and could be investigated.

Introduction

Copper (Cu) is an essential element important to a range of physiological processes in organisms including electron transport, iron transport, hormone synthesis, radical scavenging, and gene transcription (Grosell, 2012; Uauy et al., 1998). In freshwater aquatic systems, Cu is naturally present at concentrations of ~0.2–10 μg L^{-1}, although higher concentrations can be found in a few highly mineralized locations (Luoma and Rainbow, 2008). Copper has a wide variety of uses primarily related to infrastructure, transportation, electricity and water conveyance, and sustainable energy sources such as wind and solar power (Comber et al., 2022; Elshkaki et al., 2016; Hulskotte et al., 2006). Consequently, the extraction and use of Cu to meet various societal needs can lead to release of Cu to aquatic systems and subsequent exposure of aquatic organisms that have the potential to cause toxicity at sufficiently elevated concentrations.

While the mechanisms of acute toxicity of Cu to freshwater aquatic organisms are relatively well understood (Brix et al., 2022; Grosell, 2012), the mechanisms leading to chronic Cu toxicity are not. In fish exposed to Cu, effects on fecundity are often the most sensitive apical endpoint (Driessnack et al., 2017, 2016; Horning and Neiheisel, 1979; Mount, 1968; Pickering et al., 1977). Brix et al. (2022) reviewed

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available information on freshwater vertebrates and developed a preliminary adverse outcome pathway (AOP) network describing multiple potential pathways by which Cu could cause reproductive effects in fish. However, these pathways were largely either incomplete or not fully supported between at least two key events (KEs) due to either limited or conflicting information. Oxidative stress appears to have the most support overall with multiple studies measuring oxidative stress in the gonads and liver, although intermediate KEs linking to reproductive output have not been demonstrated (Craig et al., 2007; Machado et al., 2013; Wu et al., 2019). There is also support for bioenergetic effects related to and/or independent of oxidative stress, but actual measurement of changes in energy allocation to reproduction are lacking (Anni et al., 2019; De Boeck et al., 2006, 1997). Finally, there is also weak support for several other potential pathways including behavioral effects, effects on ion homeostasis, and endocrine disruption. Evidence of endocrine disruption in particular, is confounded by conflicting results and issues with experimental design.

Copper-induced oxidative stress and damage has been reported in various fish species (Craig et al., 2007; Eyckmans et al., 2011), including zebrafish. It presents a potential causal mechanism for reduced fecundity by either direct damage to the ovary and developing oocytes or by damaging ovarian tissue and/or the liver indirectly leading to effects on the endocrine system. Although there is some histopathological evidence to support this (Cao et al., 2019; Garriz et al., 2019; Zhang et al., 2016), there is also uncertainty in the methods and interpretation of available data (Brix et al., 2022). Alternatively, it has been suggested that Cu could directly interact with the endocrine system potentially leading to endocrine disruption (Cao et al., 2019; Garriz et al., 2019; Zhang et al., 2016). Available data from these studies includes changes in expression of genes associated with the HPG axis (gnrh1, gnrh2, fshβ, lhβ) along with reductions in serum FSH and LH. However, some of the data is confounded by testing with fish that do not appear to have been reproductively mature. Although considered an unlikely mode of action (MoA) for Cu effects on fish reproduction based on available data (Brix et al., 2022), endocrine disruption is currently of high environmental regulatory interest because it is critical to communication between cells, tissues, and organs, influencing nearly every physiological function in an organism (ECHA and EFSA, 2018; EU Commission, 2017; USEPA, 2022). Specific to fish reproduction, endocrine disruption has been shown to be an important MoA for numerous organic chemicals (Denslow and Sepulveda, 2007; WHO, 2002).

Considering the above, the aim of this study was to simultaneously test hypotheses that the MoA for Cu effects on fish reproduction involved either oxidative stress or endocrine disruption. Concurrent measurement of these endpoints as a function of both Cu concentration and exposure duration allows for a more robust weight of evidence evaluation of the importance of these two MoAs on reproduction in fish exposed to Cu (Brix et al., 2023). To accomplish this, we used zebrafish (*Danio rerio*) as a model organism and measured multiple endpoints associated with each MoA over time and at multiple Cu concentrations.

Methods

Experimental design and exposure system

The general experimental design for the study included four treatments consisting of a control and nominally 6.7, 20, and 60 μg L^{-1} Cu with six experimental replicates per treatment (Table S1). Test concentrtions were selected based on results from a 14-d preliminary range-finding experiment (0–180 μg L^{-1} Cu). Test concentrations were selected based to ensure a moderate (~50 %) effect on fish reproduction with the objective of trying to isolate the mode of action for this sensitive apical endpoint. The study was generally conducted consistent with OECD TG 229, the Fish Short-Term Reproduction Assay (FSTRA) test method including all endpoints measured in this method (OECD, 2012) (Supplemental Table 1). Additional endpoints related to evaluating oxidative stress and endocrine disruption MoAs were added to the study design as described below.

The exposure system was a flow-through diluter (Benoit Mini-Diluter; ECT, Superior, WI) (Benoit et al., 1982). The system had water-contact components of glass, stainless steel, and Teflon®. Exposure tanks were 10 L glass aquaria (~22.5 × 14.0 × 16.5 cm) equipped with drains and a working tank volume of 4 L (minimum water depth 12 cm). Fresh silicone was used to seal the tanks and prevent cross-contamination from previous experiments. The flow rate to each tank was 1.5 L h^{-1}, providing ~9 vol exchanges d^{-1}. Fluorescent lighting provided a 16 h light and 8 h dark photoperiod with a 2-h light transition period ranging from 540–1000 lx at the water surface. The target water temperature was 25 ± 2 °C, pH between 6.5 and 7.5, and dissolved oxygen (DO) was ≥4.9 mg L^{-1} (≥60 % of the air saturation). Temperature was measured daily, and pH, DO, conductivity, and light intensity were measured twice weekly. Total hardness and alkalinity were measured in the control and one replicate of the highest treatment weekly.

Total Cu concentrations were measured during the equilibration phase to assess diluter performance. Test solutions from all replicate tanks were measured for total and dissolved Cu on study day (SD) 0. On SD 7, 14, and 21, two replicates of each treatment were randomly sampled for the same parameters. Samples for dissolved Cu were filtered immediately using a 25 mm 0.45 mm PTFE syringe filter and immediately refrigerated.

Test organisms and study initiation

The source of the original in-house culture was USEPA (Cincinnati, OH). The fish used in the study were 5–6 months old, with females weighing at least 0.65 g and males 0.4 g. Selected fish were size normalized such that all fish were ±20 % of the mean sex-based weight. A composite of 4 female and 4 male fish selected for pre-exposure were sent for pathogen screening by PCR at IDEXX Bioanalytical Laboratories (Columbia, MO USA). The fish received limited prophylactic treatment with aquarium salt, until the two-week acclimation period preceding the experiment. The fish were isolated from the main culture and monitored visually several times daily to ensure they remained healthy and free of disease.

Test fish were selected from a laboratory population that was acclimated to test conditions (water quality, illumination) for two weeks prior to experimental initiation at a loading density of <5 g L^{-1}. After the acclimation period in which mortalities were <5 %, fish were impartially assigned to tanks for pre-exposure. After spawning was established in a 14-d pre-exposure phase, five female and five male reproductively active fish were allocated to each replicate using a random block design and the 21-d exposure phase of the study was initiated.

Observations and measurements

Multiple endpoints were assessed during and at the end of the experiment. Fish were examined daily during the experiment, and any external abnormalities (e.g., hemorrhage, discoloration) were noted. Mortalities were recorded and removed from the study. The sex of each fish that died during the exposure was determined by macroscopic evaluation of the gonads. Behavioral changes, such as hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, feeding abstinence, or changes in reproductive behavior such as loss of territorial aggression in males, were noted.

Egg production was also determined daily. As zebrafish spawn in the morning within a few hours of first light, they were not disturbed (except for feeding) until mid-morning, to allow for spawning and water hardening of the eggs. Each test chamber had a grated false bottom with tray underneath to protect eggs from consumption by adults and to facilitate removal. Trays were removed daily, and the total number of eggs and

Table 1
Summary of water quality during experiment.

Parameter	Mean	Standard Deviation	Minimum	Maximum
Temperature (°C)	25.9	0.3	25.5	26.7
pH (SU)	7.6	0.1	7.5	7.9
Dissolved Oxygen (mg L^{-1})	7.5	0.5	6.7	8.8
Hardness (mg L^{-1} as $CaCO_3$)	168	4.3	164	172
Alkalinity (mg L^{-1} as $CaCO_3$)	74	4.8	68	80
Light Intensity (Lux)	835	105	553	999

Table 2
Total and dissolved copper concentrations (mean ± SD).

Treatment	Total Cu (µg L^{-1})	Dissolved Cu (µg L^{-1})
0 (Control)	0.4 ± 0.8	0.3 ± 0.8
6.7	8.0 ± 2.0	6.2 ± 1.3
20	19.9 ± 2.3	16.8 ± 2.7
60	58.6 ± 4.7	48.8 ± 6.4

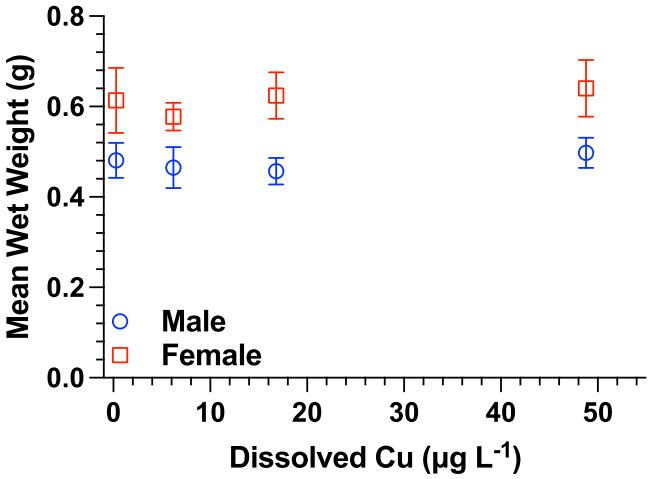

Fig. 1. Adult zebrafish weight at SD 21 (mean ± SEM).

number of fertilized eggs were enumerated.

Prior to processing, fish were euthanized in a 200 mg L^{-1} solution of buffered MS-222. At termination, body weights were determined after which blood samples were collected from the caudal artery/vein using a heparinized microhematocrit tube. Depending on the size of the fish, blood volumes typically ranged from 20 to 40 µL. Plasma was separated from the blood by centrifugation (15,000 g for 3 min) and stored with protease inhibitors at −20 °C until analyzed for vitellogenin. Liver and gonad tissues were then removed and rinsed in ice-cold PBS (pH 7.4) to remove excess blood. Tissues were weighed, minced and then homogenized on ice in PBS (1:9 tissue mass:PBS volume) with a glass homogenizer. Homogenates were then centrifuged (12,000 rpm for 15 min at 4 °C), after which the supernatant was collected and frozen at −20 °C until analyzed.

Immediately after blood collection, fish from two replicates per test concentration were fixed in modified Davidson's solution (14 % v/v of 95 % ethanol, 37 % of 37 % formaldehyde, and 6.25 % glacial acetic acid) for 48 h to prevent autolysis and cellular deterioration. The abdomen of each fish was partially opened prior to fixation to allow for rapid perfusion. After fixation in Davidson's solution, fish were transferred to 10 % neutral buffered formalin until they were processed for histopathology of the gonad, liver, and gill.

Histopathology evaluations were conducted at Environmental Pathology Laboratories (Sterling, VA, USA). Upon receipt, the heads and tails of the fixed fish were excised, and the remaining trunk segments were decalcified using a commercial decalcifying solution (Formical 2000; StatLab). The carcasses were embedded left side down for

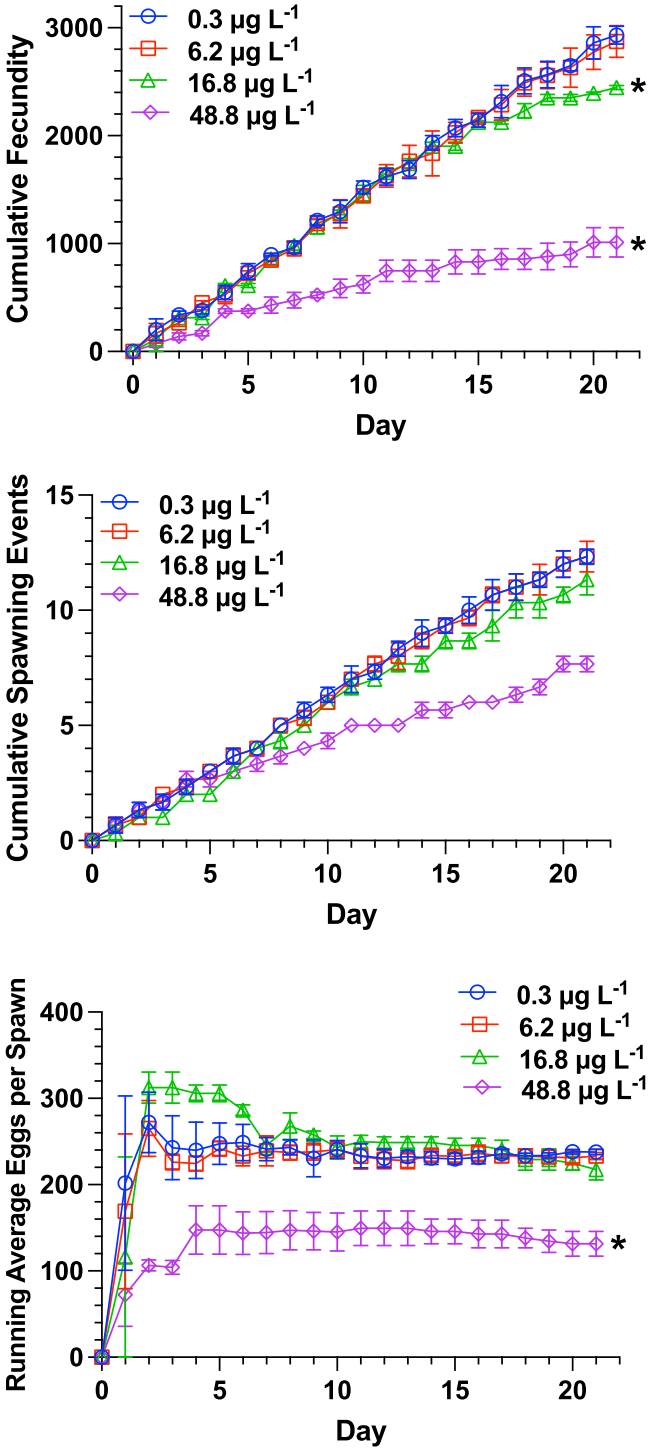

Fig. 2. Effects of copper on fecundity and spawning (mean ± SEM). *=significantly different from the control ($p < 0.05$).

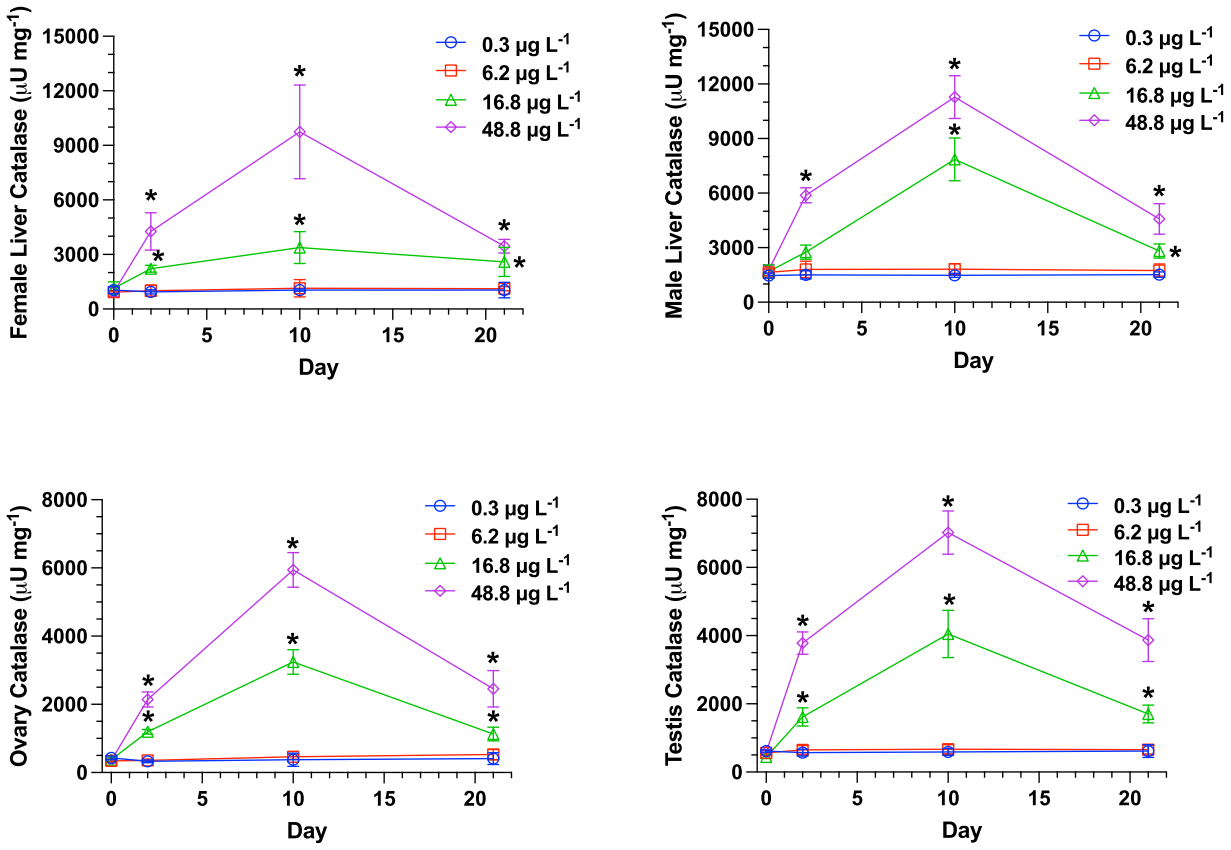

Fig. 3. Liver and gonad catalase concentrations (mean ± SEM). *=significantly ($p < 0.05$) different from the control when analyzed within a given study day.

sectioning in the sagittal plane, prior to routine processing for paraffin embedding using an automated tissue processor (Tissue-Tek VIP; Sakura Finetek). Using a manual microtome (MICROM International), six sections (~4–5 μm thickness) were acquired from each fish, and these were placed on three glass slides. All sections were stained with hematoxylin and eosin (HE; Hematoxylin 2 and Eosin-Y; Richard-Allan Scientific) and covered with glass coverslips using a commercial mounting medium (Permount Solution; Fisher Scientific).

Gonad, liver, and gill tissue in all sections were evaluated by brightfield microscopy. During the evaluation, the pathologist was aware of the treatment-group status of individual fish, as recommended by the FSTRA guidance (OECD, 2012). Histopathologic findings were scored for severity according to the following grading system: Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate, and Grade 4 = severe (see Supplemental Information for details).

Biochemical analysis and analytical chemistry

Catalase and GSH-Px concentrations were measured in the gonad and liver by competitive binding ELISA using commercially available assays specific for fish (AFG Bioscience, Northbrook, IL). The assays were stored at 4 °C prior to use and used following the manufacturer's instructions. In these assays, antigens in the samples compete with biotinylated antigens for binding with an enzyme-specific capture antibody. Unbound antigen was removed by washing and a horseradish peroxidase-conjugated antibody was added and incubated. After incubation, any unbound antibody was removed by washing and a tetramethylbenzidine substrate was added to elicit a color change. This reaction was halted by addition of an acidic stop solution, after which optical density was measured at 450 nm using a BioTek 800 TS microplate reader (BioTek, Winooski, VT) and interpolated from a standard curve.

Plasma vitellogenin concentration was measured by sandwich ELISA using a commercial assay following the manufacturer's instructions (Biosense Laboratories, Bergen, Norway). The assays were stored at 4 °C prior to use. In this assay, samples were added to microplate wells precoated with a capture antibody that binds vitellogenin. An HRP-labelled detection antibody was then added to bind the captured vitellogenin. The presence of vitellogenin was visualized by the addition of a peroxidase enzymatic substrate to produce a color change, and optical density was measured at 492 nm using a BioTek 800 TS microplate reader (BioTek, Winooski, VT). The sample concentration of vitellogenin was quantified via interpolation from a standard curve.

Copper analyses of test solutions were conducted by Accurate Environmental Laboratories (Stillwater, OK). Samples were analyzed by ICP-MS in accordance with USEPA method 200.8 (USEPA, 1994) with a practical quantitation limit of 1 μg L^{-1} and included analysis of method blanks and matrix spikes. Dissolved organic carbon was analyzed using a total organic carbon analyzer (Shimadzu) with a practical quantitation limit of 0.5 mg L^{-1}. Hardness and alkalinity were analyzed by colorometric titration.

Data analysis

The objective of this study was to evaluate whether Cu-induced effects on zebrafish reproduction were linked to an endocrine-mediated mechanism of action or oxidative stress in the gonads and/or liver. It was not designed to generate effect endpoints such as NOEC/LOEC and ECx due to the limited number of treatments that were spaced relatively far apart. Consequently, these types of endpoints were not estimated. Statistical analysis was performed in general accordance with OECD guideline 229 (OECD, 2012). Endpoint responses were compared between treatments using analysis of variance, treating males and females separately. When normality and equal variance assumptions were not

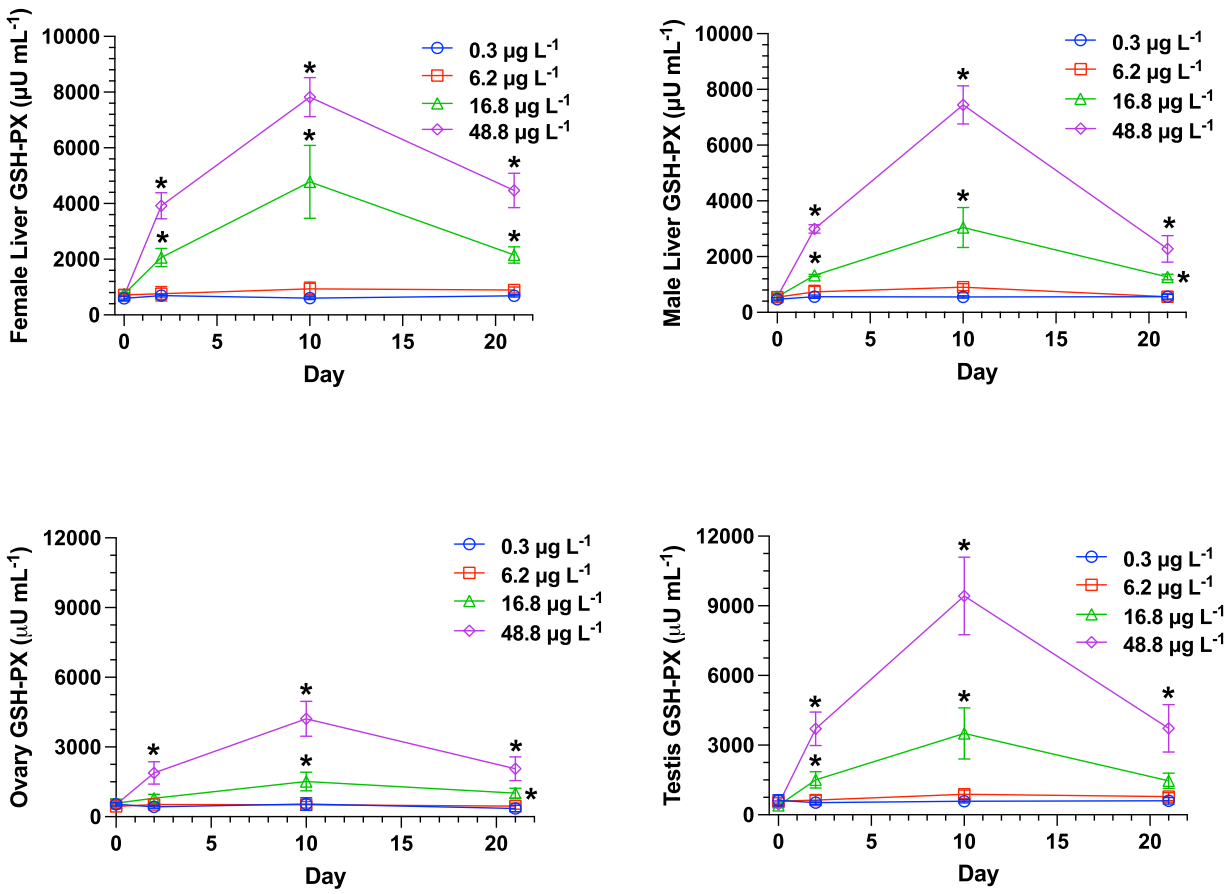

Fig. 4. Liver and gonad glutathione peroxidase (GSH-Px) concentrations (mean ± SEM). *=significantly different from the control ($p < 0.05$) when analyzed within a given study day.

met, data were transformed and re-evaluated. If assumptions were still not met, non-parametric methods were applied. The specific test used for each endpoint is provided in the results. All analyses were conducted in GraphPad Prism (v10.4).

Results

Water quality

All water quality parameters were maintained within acceptable ranges throughout the experiment for temperature (25.9 ± 0.3), pH (7.6 ± 0.1), and dissolved oxygen (7.5 ± 0.5) (mean ± SD, Tables 1 and S1). Dissolved oxygen remained above 60 % saturation throughout the study, and aeration was not required. Water hardness (168 ± 4.3 mg L^{-1} as $CaCO_3$) and alkalinity (74 ± 4.8 mg L^{-1} as $CaCO_3$) were stable throughout the exposure. Samples for DOC analysis of the laboratory dilution water were inadvertently not collected during the experiment. Total organic carbon concentration in dilution water samples collected 3–4 months prior were <2 mg L^{-1} ($n = 2$), while DOC in dilution water collected 4 months after the experiment was <0.5 mg L^{-1} ($n = 1$).

Measured copper concentrations

Based on results from an earlier range-finding study (not described in the current paper), target total Cu concentrations selected for the definitive study were 0, 6.7, 20, and 60 µg L^{-1}. Since the time interval between sample collection was consistent throughout the study (weekly), the mean measured concentration represented a time-weighted average of treatment concentrations (Tables 2, S2, S3). Measured total Cu concentrations closely approximated nominal concentrations, with dissolved Cu concentrations 17–27 % lower than total concentrations. The stability of test concentrations, as characterized by the coefficient of variation (CV), was concentration-dependent, with the lowest CVs (8–13 %) associated with the highest treatment increasing to 21–25 % in the 6.7 µg L^{-1} nominal treatment. The CVs in the control group were high on a relative basis (202–295 %), but variation was low on an absolute basis (SD <1 µg L^{-1}). Throughout the remainder of this paper, biological responses are described as a function of the mean measured dissolved Cu concentrations (0.3 (control), 6.2, 16.8, and 48.8 µg L^{-1}), which best represent the bioavailable Cu concentration (Di Toro et al., 2001).

Adult weight, survival, and behavior

Adult zebrafish survival was 100 % in all treatments through the 21-d study (Table S4). Similarly, no significant (ANOVA; $p = 0.88$) differences were observed in male or female fish weight compared to the control group (Fig. 1 and Table S4), and no changes in zebrafish behavior were noted in any treatment at any point in the study (Table S5).

Reproduction

Zebrafish reproduction was evaluated using several metrics to understand how overall fecundity was being affected by Cu exposure. Because there was 100 % survival of adult fish in all treatments, it was not necessary to normalize reproductive data to the number of females. The analysis focused on the three replicates that were maintained for the entire 21-d exposure. At the end of the 21-d exposure, total fecundity was significantly affected at both 16.8 µg L^{-1} (Dunnett's, $p = 0.03$) and

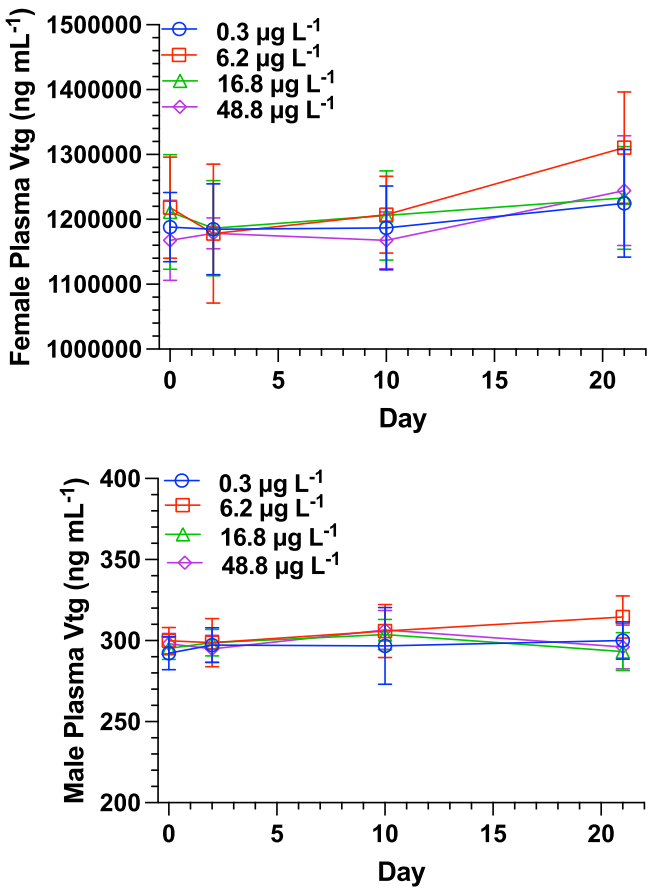

Fig. 5. Plasma vitellogenin concentrations in female and male fish (mean ± SEM). Note female vitellogenin concentrations are ~400-fold higher than concentrations in males.

48.8 µg L^{-1} (Dunnett's, $p < 0.0001$) dissolved Cu (Fig. 2 and Table S4). The cumulative number of spawning events was also reduced at 48.8 µg L^{-1} dissolved Cu, although it was marginally non-significant (Kruskal Wallis, $p = 0.06$) (Fig. 2). Reproductive data were also evaluated by calculating a running average of the number of eggs per spawning event (Fig. 2). Using this metric, a significant reduction (Dunnett's, $p < 0.0001$) in eggs per spawning event was observed in the 48.8 µg L^{-1} treatment.

Catalase and glutathione peroxidase concentrations in tissues

Catalase and GSH-Px concentrations in the liver and gonads exhibited similar patterns, with increasing concentrations in the 16.8 and 48.8 µg L^{-1} treatment groups on SD 2 and 10. This was followed by decreasing concentrations on SD 21, although these were still significantly elevated above control concentrations. Catalase and GSH-Px concentrations were generally significantly ($p < 0.05$) elevated compared to the control in these two treatments on SD 2, 10, and 21 (Figs. 3 and 4, Tables S6-S9).

Plasma vitellogenin concentrations

Circulating plasma vitellogenin concentrations in female and male fish were relatively consistent over the course of the exposure period, with no significant ($p > 0.05$) difference observed between treatments at any sampled time point (Fig. 5, Tables S6-S9). Mean plasma vitellogenin concentrations in males were ~0.3 µg mL^{-1} for all treatment groups, while in the females, vitellogenin concentrations ranged from ~1180–1300 µg mL^{-1}.

Gonado- and liver-somatic indices

Liver somatic and gonado-somatic indices varied somewhat over the course of the study, with several significant ($p < 0.05$) differences detected within a given study day. However, these differences were not persistent across multiple sampling time points and did not appear to be a function of either Cu concentration or time (Fig. 6, Tables S6-S9).

Histopathology

No exposure related changes in gill morphology were observed in male or female zebrafish (Table S10). In male zebrafish, there was an increased prevalence of bile duct hyperplasia (overall severity range = minimal to moderate) in the liver that appeared to follow a concentration-response pattern (Table 3, Fig. 7). There were also slight increases in minimal to mild peribiliary fibrosis in male fish in the 48.8 µg L^{-1} treatment and in female fish exposed to 16.8 µg L^{-1} Cu. However, given the small percentage of fish affected (i.e., 2/10) in those groups, and absence of a dose-response pattern in females, it is uncertain whether this was result of Cu exposure.

There was no change in testis stage scores and only a slight increase in minimal to mild germ cell degeneration in male testes at 16.8 and 48.8 µg L^{-1} Cu. Given that germ cell degeneration can be observed spontaneously in untreated male cyprinids (Wolf et al., 2024), it is unclear whether the low incidence of this finding was exposure-related in the current study (Table 4). In females, post-ovulatory follicles were absent from the ovaries of females in the 16.8 and 48.8 µg L^{-1} Cu treatments but were present at low frequencies in the control and 6.2 µg L^{-1} Cu-treated females (Table 4, Fig. 7). Post-ovulatory follicles are remnants of the granulosa/theca cell sheaths that persist temporarily post-spawning; thus their presence is an indication of recent spawning activity. The lack of post-ovulatory follicles in the 16.8 and 48.8 µg L^{-1} group females was consistent with the reduced fecundity observed in those two groups. There was a pronounced increase in ovarian oocyte atresia in the 16.8 µg L^{-1} but not 48.8 µg L^{-1} Cu treatment (Table 4, Fig. 7). Because variable background levels of physiologic oocyte atresia occur naturally in females (Wolf et al., 2024), the absence of a dose-response effect in this case suggests that the increased atresia in the 16.8 µg L^{-1} group was a spurious result unrelated to treatment. Most ovaries received Stage 3 scores except for the 6.2 µg L^{-1} copper treatment group, where most were recorded as Stage 2 (Table 4). As for oocyte atresia, a convincing case for treatment-effect could not be made due to the lack of a concentration-response pattern. Other findings tended to be present in low numbers of fish per group, were consistent with background changes, and were therefore not considered to be exposure related.

Discussion and conclusions

Two possible modes of action for copper-induced reproductive toxicity in fish are endocrine disruption and oxidative stress. The endocrine disruption hypothesis is supported in some studies by gene expression data that show both upregulation and downregulation of multiple genes in the reproductive steroidogenic pathway, as well as downregulation of the estrogen receptor in the liver in several fish species (Cao et al., 2019; Driessnack et al., 2017, 2016; Zhang et al., 2016). Alternatively, direct measurement of anti-oxidant concentration or activity, along with ovarian histopathology, suggests that direct oxidative damage to the ovary may result in increased apoptotic signaling and oocyte atresia (Cao et al., 2019; Garriz et al., 2019; Wu et al., 2019). For both hypotheses, concerns have been raised about experimental findings for some of the supporting studies (see Brix et al. (2022) for discussion), introducing uncertainty. Overall, prior to this study, the existing weight of evidence supported the oxidative damage hypothesis as the most likely mode of action for effects on fish reproduction. However, the lack of concurrent testing of both hypotheses in

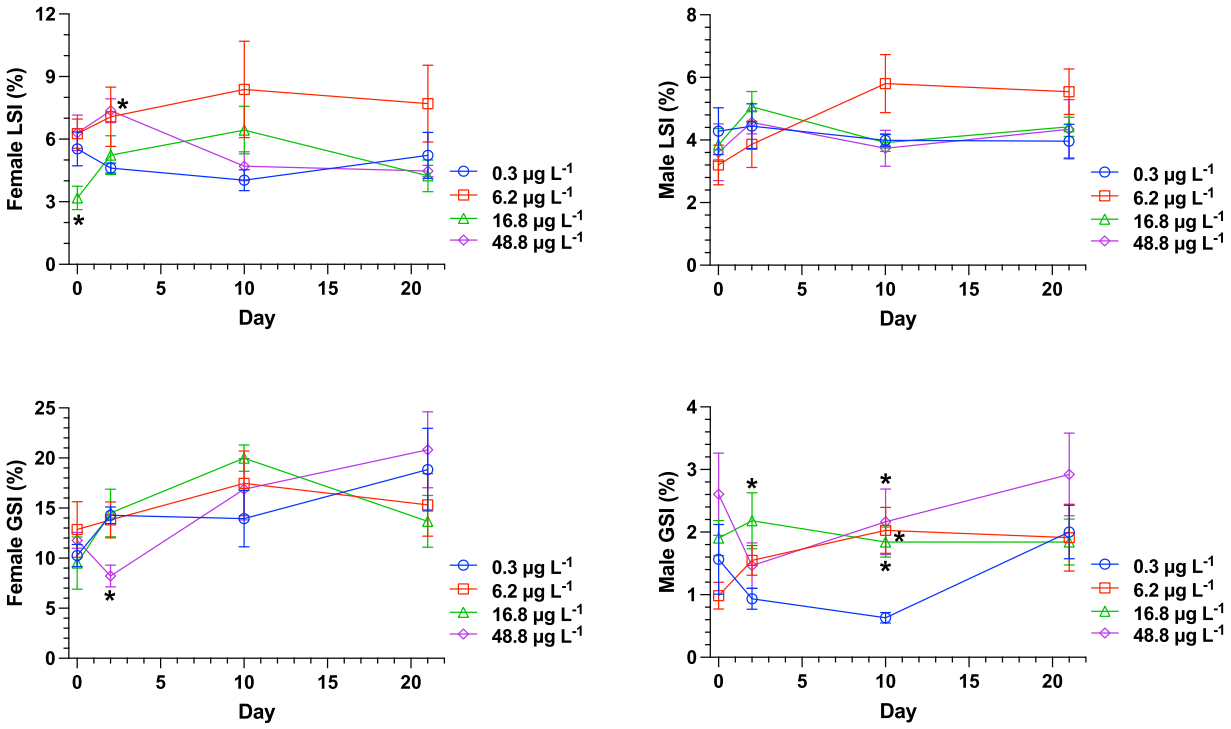

Fig. 6. Female and male liver somatic index (LSI) and gonado-somatic index (GSI) (mean ± SEM). *=significantly different from the control ($p < 0.05$) when analyzed within a given study day.

Table 3
Summary of liver histopathology ($n = 10$).

	Males				Females			
Copper Concentration (µg L^{-1})	0.3	6.2	16.8	48.8	0.3	6.2	16.8	48.8
Hyperplasia, bile duct	2	4	4	7	1	2	0	1
Minimal	1	3	2	6	1	1	0	1
Mild	1	0	2	1	0	1	0	0
Moderate	0	1	0	0	0	0	0	0
Cholangiofibrosis	0	0	0	1	1	0	0	0
Minimal	0	0	0	0	1	0	0	0
Mild	0	0	0	1	0	0	0	0
Fibrosis, peribiliary	0	0	0	2	0	2	1	1
Minimal	0	0	0	2	0	1	1	1
Mild	0	0	0	0	0	1	0	0
Necrosis, bile duct epithelium	1	0	0	2	2	1	1	1
Minimal	1	0	0	2	1	0	1	1
Mild	0	0	0	0	1	1	0	0
Vacuolation	10	10	10	10	10	10	10	10
Minimal	1	0	0	0	6	5	4	3
Mild	5	7	8	7	4	5	6	7
Moderate	4	3	2	3	0	0	0	0

the same test species under the same conditions has hindered a conclusive determination of the mode of action.

The objective of this study was to simultaneously test both hypotheses by exposing reproductively active zebrafish to Cu (0.3, 6.2, 16.8, and 48.8 µg L^{-1}) and measuring fish weight and reproductive output, anti-oxidant concentrations (catalase and glutathione peroxidase), plasma vitellogenin, LSI and GSI, and conducting a histopathological evaluation of fish gills, liver, and gonads at the end of the exposure.

Significant effects on zebrafish reproduction were observed in the 16.8 and 48.8 µg L^{-1} Cu treatments. Cumulative fecundity was reduced by 66 % at the highest Cu concentration, with a corresponding 39 % reduction in the cumulative number of spawning events. At this highest concentration, there was also 45 % reduction in the mean number of eggs per spawning event (Fig. 2), which was apparent compared to the controls from the onset of exposure at SD 1. Cumulative fecundity showed a similar early deviation from control values, while the number of spawning events did not deviate until ~SD 7. Effects on both spawning frequency and number of eggs per spawn have also been observed in fathead minnows (*Pimephales promelas*) exposed to Cu, which suggests that a similar etiology drives reduced fecundity in that species (Driessnack et al., 2017, 2016; Pickering et al., 1977).

Plasma vitellogenin is considered a mechanistic biomarker for endocrine effects and is used in regulatory screening studies in fish to investigate the potential for endocrine disruption. In this study, plasma vitellogenin concentrations in male zebrafish in all groups were low (as would be expected) and unaffected by Cu exposure (Fig. 5), providing evidence that Cu does not induce an estrogenic or anti-androgenic effect in male fish. Plasma vitellogenin concentrations in female zebrafish were at levels typically observed for reproductively active fish and were also not affected by Cu exposure (Fig. 5). These results suggest that observations by Cao et al. (2019), in which Cu elicits multiple changes in gene expression of hormones, hormone receptors and enzymes along the HPG axis, do not translate to effects on vitellogenin, a protein at the terminal end of this axis. This is consistent with studies on yellow catfish, where similar gene expression effects along the HPG axis did not affect plasma vitellogenin concentrations (Zhang et al., 2016). Overall, the vitellogenin results from this study continue to strengthen the weight of evidence that Cu is not affecting fish reproduction via disruption of the endocrine system.

Elevated Cu concentrations are known to generate reactive oxygen species (ROS) in tissues (Regoli, 2012). The significant increases in liver and gonad CAT and GSH-Px in the 16.8 and 48.8 µg L^{-1} treatments, where effects on reproduction occurred, indicate sustained high levels of ROS in those tissues (Figs. 3 and 4). In both tissues, these antioxidants increased significantly with the first measurement on SD 2. They continued to increase through SD 10 before decreasing on SD 21 to concentrations comparable to those observed on SD 2 (i.e., still significantly elevated). This type of pattern is not unusual, and may result from

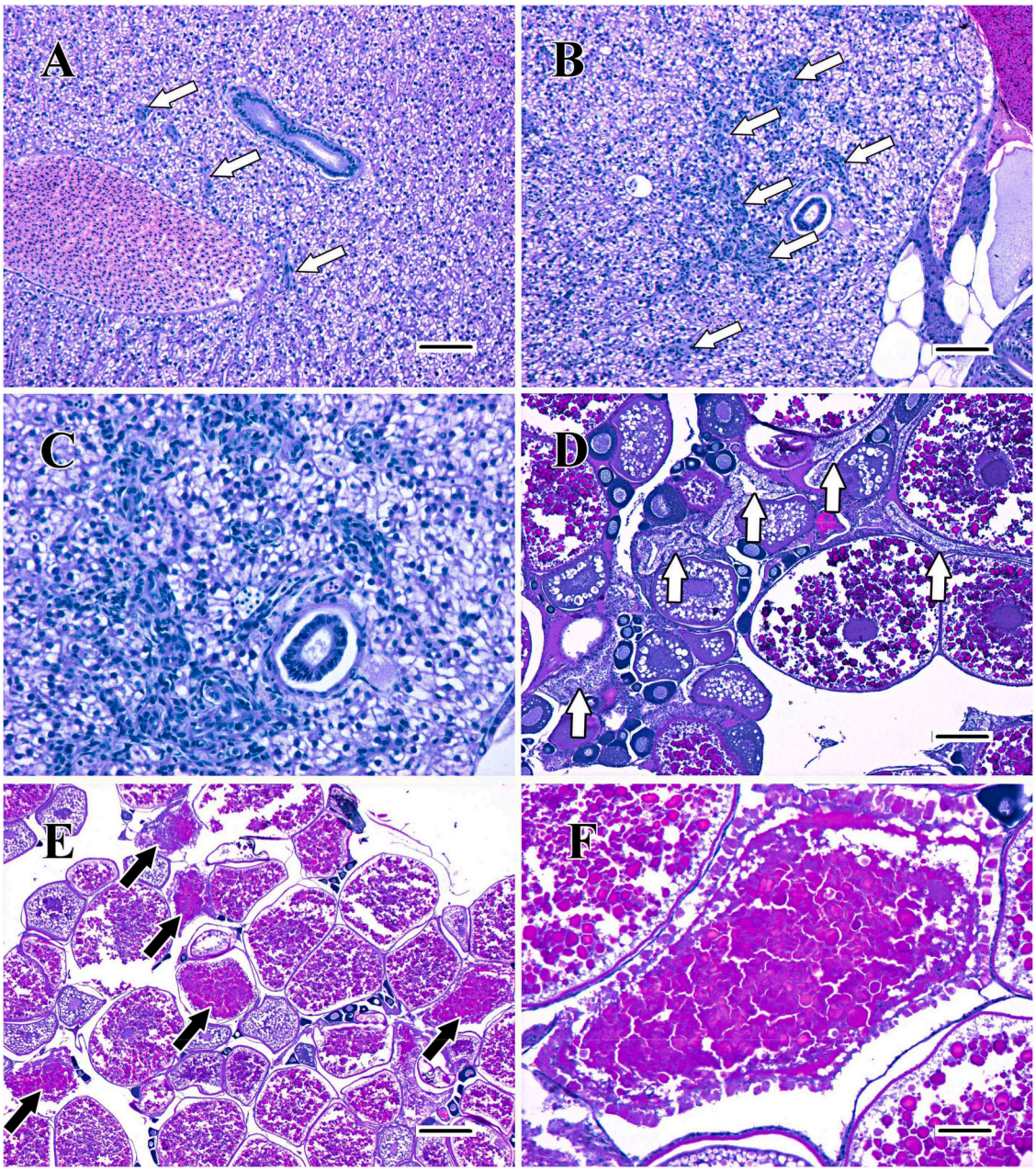

Fig. 7. Histopathology images. **A.** Liver from a control male. A few small bile ductules are evident (white arrows). **B.** Liver from a male exposed to 48.8 µg/L copper. Mild bile duct hyperplasia (white arrows). **C.** Higher magnification of liver in B to better demonstrate proliferating small bile ducts. **D.** Ovary from a female exposed to 6.2 µg/L copper. White arrows indicate post-ovulatory follicles. **E.** Ovary from a female exposed to 16.8 µg/L copper. Post-ovulatory follicles are absent, and instead there are numerous atretic follicles (black arrows). **F.** Higher magnification of ovary in E to better demonstrate an atretic follicle (center). Magnification: A, B and F, bar = 100 µm; C, bar = 50 µm; D and E, bar = 250 µm.

sustained elevated ROS disrupting Nrf2 and/or NFκB signaling, which are important transcription factors for regulating antioxidant genes (Lee and Johnson, 2004; Wu et al., 2019). Alternatively, upregulation of detoxification mechanisms such as metallothioneins, may have led to reduced ROS and antioxidant activity in the latter half of the exposure (Craig et al., 2009).

Although we only measured indicators of oxidative stress, previous studies on zebrafish in the same Cu concentration range had measured indicators of oxidative damage (increased malondialdehyde (MDA) production, a marker of lipid peroxidation) in zebrafish liver suggesting this was also likely occurring in our study (Craig et al., 2007). The observations on gonadal oxidative stress in this study are also consistent with a previous study on rare minnows (*Gobiocypris rarus*) exposed to Cu that demonstrated upregulation of both activity and gene expression of antioxidants in ovaries along with a significant (~3-fold) increase in MDA production (Wu et al., 2019).

In the liver, the primary histopathological observation was a concentration-dependent increase in bile duct hyperplasia in male fish (Table 3), which was interpreted as the exacerbation of a background finding that occurred at a substantially lower frequency in control males. This particular effect is not surprising, as Cu is preferentially accumulated in the liver as the primary storage organ and then excreted via the bile as part of normal Cu homeostasis. However, during elevated Cu exposures, biliary Cu excretion increases, resulting in high bile Cu concentrations and likely oxidative damage to the bile duct tissues (Grosell et al., 1998, 2001). While this effect is consistent with the

Table 4
Summary of gonad histopathology ($n = 10$).

	Males				Females			
Copper Concentration (μg L^{-1})	0.3	6.2	16.8	48.8	0.3	6.2	16.8	48.8
Germ cell degeneration	0	0	2	2				
Minimal	0	0	2	1				
Mild	0	0	0	1				
Testis/Ovary stage								
Stage 1	1	2	0	1	0	0	0	0
Stage 2	9	7	9	9	1	8	1	0
Stage 3	0	1	1	0	9	1	9	10
Oocyte atresia, increased					2	2	7	2
Minimal					1	0	2	2
Mild					1	1	4	0
Moderate					0	1	1	0
Post-ovulatory follicles					2	3	0	0
Minimal					1	0	0	0
Mild					1	1	0	0
Moderate					0	2	0	0

excretion pathway of Cu and localized hepatic effects of high Cu accumulation, the absence of a similar outcome in females is unexplained. Overall, the liver effects were largely minimal to mild in severity, and did not produce consistent concentration-dependent effects on LSI.

In the gonads, there was no effect on GSI (Fig. 6) in either sex, and no concentration-dependent effects on ovarian developmental stage (Table 4). The lack of an effect on GSI is consistent with previous studies (Driessnack et al., 2017, 2016), and is perhaps not surprising, given that it is a relatively coarse measurement of gonadal development and cyclicity. While the absence of ovarian post-ovulatory follicles was consistent with the reduced fecundity in the 16.8 and 48.8 μg L^{-1} groups, relationships between other histopathologic findings in the gonads (e.g., germ cell degeneration in males, and oocyte atresia or ovarian stage score differences in females) and Cu exposure were considered inconclusive. In some reproductive studies that use fractionally spawning species such as zebrafish, apparent discordance between effects on reproduction and morphological changes in the gonads may occur because the histopathological appearance of the gonads primarily reflects activity in those organs during the 2–3 d day period prior to sacrifice, as opposed to the entire exposure duration (Wolf et al., 2024). Alternatively, or in addition, the relatively small sample size ($n = 10$), that was a consequence of trying to test two hypotheses simultaneously, might have contributed to some ambiguity, although the sample size employed in this study was consistent with OECD recommendations (OECD, 2009). We also note that the effects on reproduction were relatively consistent across and within treatments in the last 10 days of the exposure, suggesting the sample size should have been sufficient.

Previous studies have also resulted in somewhat inconclusive histopathology results. Cao et al. (2019) suggested a modest effect on zebrafish ovarian stage by Cu, but the study used fish just entering reproductive maturity, and it is unclear whether this was a reproductive effect or reflective of variability in fish reaching sexual maturation during the study. In another study involving rare minnows, a ~4-fold increase (~20 % frequency) in oocyte atresia was observed when fish were exposed to a single Cu concentration with corresponding indicators of oxidative stress and damage (Wu et al., 2019). However, examination of photomicrographs from that publication reveals that oocytes indicated as atretic are actually tangential sections through normal perinucleolar phase oocytes. This and other questionable or clearly erroneous microscopic diagnoses render the histopathology results from that particular study unreliable.

Consistent with previous recommendations, we have attempted to place these new findings within an AOP network describing potential pathways by which Cu might affect fish reproduction (Brix et al., 2022, 2023). In addition to the oxidative stress molecular initiating events (MIEs), Brix et al. (2022) previously summarized at a high level, support for a potential bioenergetics modality for Cu effects on fish reproduction. After further review of this summary, we provide a more detailed AOP network for Cu effects on fish reproduction including pathways evaluated in this study and detailing a potential bioenergetics AOP network.

Using the available data three MIEs were identified leading to five potential AOPs that could be contributing to bioenergetic effects on fish reproduction (Fig. 8). One set of pathways is centered on a Cu-induced stress response leading to increased cortisol concentrations and associated downstream effects. The pathways involve increased alterations in liver metabolism and an increase in hepatic energy demand which includes an interaction with the cortisol pathways. Key events (KE) and key event relationships (KERs) are relatively well supported for each of the AOPs, which all converge on a single KE characterizing reduced energy availability and/or allocation for reproduction. Importantly, the

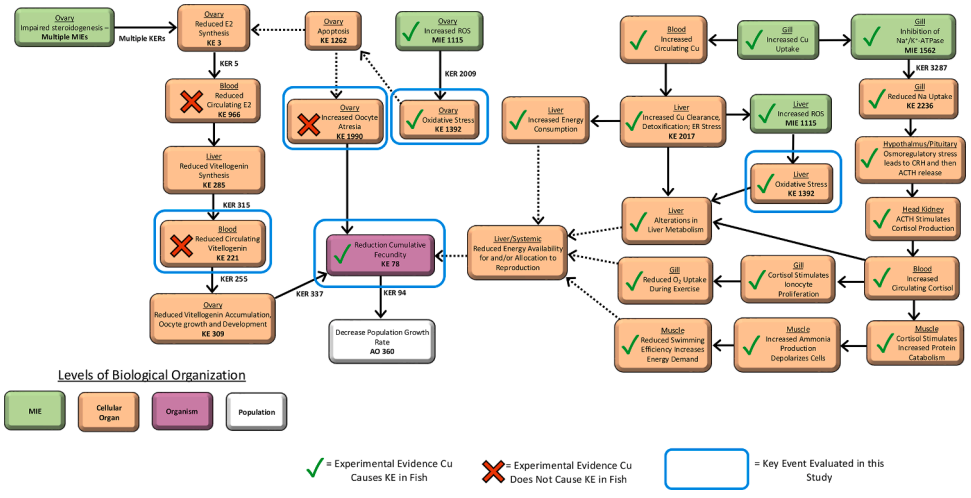

Fig. 8. AOP network describing several pathways that can potentially affect fish reproduction (chemically agnostic). Key events (KEs) and key event relationships (KERs) with numbers are described on the AOPwiki (https://aopwiki.org). Green checks indicate KEs confirmed to occur during Cu exposure (see Brix et al. 2022 and this a paper for details). Key events with a red X are part of known or hypothesized pathways that have been confirmed not to occur during Cu exposure. Solid black arrows indicate KERs well supported on the AOPwiki or in the scientific literature. Dashed arrows are KERs either hypothesized or not well supported.

KERs linking each AOP to this converging KE are not well supported in the literature. While we judge the linkages to be plausible, we did not identify any studies to robustly support or refute these KERs (i.e., absence of investigation) and so the potential for Cu to affect fish reproduction through a bioenergetic modality remains somewhat speculative. Finally, we note that while we have focused on bioenergetics as, in our judgement, it is a plausible modality for Cu-induced reproductive effects, we do not rule out the possibility that other MoAs may be driving or contributing to reproductive effects.

In conclusion, we demonstrated that Cu decreased reproduction in zebrafish exposed to 16.8 and 48.8 μg L^{-1} Cu. At these concentrations, zebrafish experienced significant and persistent oxidative stress, accompanied by elevated levels of antioxidants in both the liver and gonads. However, we did not identify a mechanistic link between this oxidative stress and observed reductions in fecundity through histopathology investigations. While oxidative stress is known to cause localized tissue damage (as we observed in the liver), we did not observe clear effects of oxidative damage in the gonads based on histopathology and GSI, which suggests that oxidative damage is not a significant contributor to reproductive effects via direct damage to the gonads. Our results also did not provide evidence that endocrine disruption via the HPG axis contributed to the observed reproductive effects. Specifically, the absence of changes in plasma vitellogenin concentrations, combined with the lack of endocrine-specific histopathological effects, or effects on GSI or LSI, do not support an AOP in which Cu disrupts the HPG axis leading to reduced vitellogenesis and corresponding reductions in fecundity. Given these results, available weight of evidence indicates alternative modalities may cause Cu-induced effects on reproduction and we suggest effects on organism bioenergetics as a plausible mode action.

CRediT authorship contribution statement

Kevin V. Brix: Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Jeffrey C. Wolf:** Writing – review & editing, Visualization, Investigation, Conceptualization. **Stijn Baken:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Tara Miller:** Writing – review & editing. **Yamini Gopalapillai:** Writing – review & editing, Project administration. **Douglas J. Fort:** Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kevin Brix reports financial support and article publishing charges were provided by International Copper Association Ltd. Jeff Wolf reports financial support was provided by International Copper Association Ltd. Doug Fort reports financial support was provided by International Copper Association Ltd. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2025.107698.

Data availability

All data is provided in Supplemental Information

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