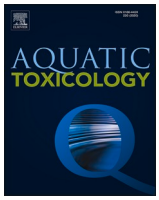
Aquatic Toxicology 272 (2024) 106963

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox



Molecular variations to the proteome of zebrafish larvae induced by environmentally relevant copper concentrations

Sarah L. Green [a,*], Ewen Silvester [a], Sebastian Dworkin [b], Manisha Shakya [c], Annaleise Klein [d], Rohan Lowe [e], Keshava Datta [e], Aleicia Holland [a]

[a] Department of Environment and Genetics, La Trobe University, 133 Mckoy Street, West Wodonga, Albury-Wodonga Campus, Victoria 3690, Australia
[b] Department of Microbiology, Anatomy, Physiology and Pharmacology, La Trobe University, Bundoora Campus, Victoria, Australia
[c] School of Environmental and Rural Science, Faculty of Science, Agriculture, Business and Law, University of New England, Armidale, New South Wales, Australia
[d] Australian Synchrotron, ANSTO, 800 Blackburn Road, Clayton, Victoria 3168, Australia
[e] Proteomics and Metabolomics Platform, La Trobe University, Bundoora Campus, Victoria, Australia

ARTICLE INFO

Keywords:
Proteomics
Metal toxicity
Aquatic toxicology
Amino acid
Loss of equilibrium

ABSTRACT

Contaminants are increasingly accumulating in aquatic environments and biota, with potential adverse effects on individual organisms, communities and ecosystems. However, studies that explore the molecular changes in fish caused by environmentally relevant concentrations of metals, such as copper (Cu), are limited. This study uses embryos of the model organism zebrafish (*Danio rerio*) to investigate effect of Cu on the proteome and amino acid (AA) composition of fish. Wild-type embryos at 24 h post-fertilisation were exposed to Cu (2 μg L^{-1} to 120 μg L^{-1}) for 96 h and the number of healthy larvae were determined based on larvae that had hatched and did not display loss of equilibrium (LOE). The effect concentrations where Cu caused a 10 % (EC_{10}) or 50 % (EC_{50}) decrease in the number of healthy larvae were calculated as 3.7 μg L^{-1} and 10.9 μg L^{-1}, respectively. Proteomics analysis of embryos exposed to the EC_{10} and EC_{50} concentrations of Cu revealed the proteome to differ more strongly after 48 h than 96 h, suggesting the acclimatisation of some larvae. Exposure to excess Cu caused differentially expressed proteins (DEPs) involved in oxidative stress, mitochondrial respiration, and neural transduction as well as the modulation of the AAs (Proline, Glycine and Alanine). This is the first study to suggest that LOE displayed by Cu-stressed fish may involve the disruption to GABAergic proteins and the calcium-dependent inhibitory neurotransmitter GABA. Moreover, this study highlights that proteomics and AA analysis can be used to identify potential biomarkers for environmental monitoring.

1. Introduction

The widespread increase of mining, urbanisation and industrial practices over recent human history has caused metal contamination in freshwater environments (van Beers and Graedel, 2007). This poses a significant risk to aquatic ecosystems and biota as metals have the potential to accumulate in aquatic organisms and disrupt several biological processes, ultimately with harmful consequences to the individual and wider population (Eisler, 1998; Hall et al., 1988). Although many metals, such as copper (Cu) are essential for biological processes, they can become toxic above species-specific limits (Riethmuller, 2000; Shuhaimi-Othman et al., 2015), and their extensive use in multiple industries has caused contamination of associated water bodies at levels that exceed these species-specific limits (Hall et al., 1988; USEPA, 1984). Furthermore, the demand for Cu in the future is expected to increase (Kuipers et al., 2018), enhancing the risk of anthropogenic Cu pollution in the environment.

The contamination of freshwater environments by Cu is a global problem, with Cu concentrations reported to range up to 133 mg L^{-1} in UK waters, with a median of 4.7 μg L^{-1} (Donnachie et al., 2014). In Liaodong Bay, China, Cu concentrations ranged from 6.8 - 11.9 μg L^{-1} (Zhu et al., 2022), while the concentrations in rivers of Osun state, Nigeria, reached 1350 ug L^{-1} with a mean of 407 μg L^{-1} (Titilawo et al., 2018). Copper is an essential micronutrient and occurs at natural background levels; in Australian freshwater environments this is reported to be 0.11 μg L^{-1} (ANZECC, 2000). However, Cu contamination is evident in urban streams and waterways contaminated by mining, with reports of Cu concentrations significantly exceeding this value and

* Corresponding author.
E-mail address: sarah.green@latrobe.edu.au (S.L. Green).

https://doi.org/10.1016/j.aquatox.2024.106963
Received 14 April 2024; Received in revised form 14 May 2024; Accepted 16 May 2024
Available online 17 May 2024

reported to reach up to 40 μg L^{-1} in urban streams (McDonald et al., 2022) and 13,000 μg L^{-1} in an acid mine drainage contaminated river (Shakya et al., 2021b). However, given the likelihood of Cu to complex with dissolved organic matter in water, dissolved Cu concentrations may not reflect Cu bioavailability, and hence, toxicity (Macoustra et al., 2019, 2021). Thus, biomarkers of toxicity may provide a greater and more sensitive understanding of the potential effects of Cu on fish, across various waters that differ in toxicity modifying factors, such as dissolved organic matter. Moreover, as sublethal endpoints require a lower concentration of the metal and occur before changes in growth and reproduction are noticed, they are more sensitive and a better indicator of toxicity (Jezierska et al., 2009). Despite this, an in-depth understanding of the changes at the biomolecular level when organisms are exposed to environmentally relevant concentrations of metals is still limited.

Fish are susceptible to elevated concentrations of metals with earlier life stages such as larvae more sensitive (Jezierska et al., 2009; Wang et al., 2020). Unlike other vertebrates, fish have two methods of nutrient uptake and absorption: the gastrointestinal tract and the gills (Bury et al., 2003). The early developmental stages of fish also have additional exposure routes to waterborne metals, as embryos have a semi-permeable chorion, which hardens as they age, reducing permeability. After hatching the resulting larvae are at the most vulnerable stage with highly permeable skin and without the protection of a hardened chorion (Ganesan et al., 2016; Johnson et al., 2007). At physiological levels, Cu plays a pivotal role in protecting cells from oxidative stress by being an integral component of antioxidants. However, when in excess, the redox nature of Cu causes it to generate harmful reactive oxygen species (ROS) and inhibit antioxidants, placing the cells in a state of oxidative stress (Bopp et al., 2008; Hansen et al., 2006). The imbalance between ROS and antioxidants ultimately leads to cell death via disrupting a plethora of physiological processes (Turrens, 2003). Luzio et al. (2013) found that environmentally realistic concentrations of Cu (12.5 and 100 μg L^{-1}) created increased flux through different apoptotic pathways in zebrafish gill cells at alternate times, suggesting a crosstalk between pathways at different time-points, including through ROS triggering activation of apoptotic-inducer protein p53. Copper has also been shown to cause respiratory distress (De Boeck et al., 1995), changes in muscle metabolism (Beaumont et al., 2000), decreased reproduction (James et al., 2008), changes in gill morphology (Vutukuru et al., 2005) and altered swimming behaviour (Beaumont et al., 1995).

The last decade has revealed the need for chronic toxicity studies and non-traditional assessment methods for risk assessment of contaminants that would more accurately detect exposure pathways in the environment. The push from the scientific community for improved determination of the sublethal effects of metals is not unfounded, as the detrimental effects of sublethal concentrations of metals, such as Cu can cause decreased reproductive output, and changes to growth of individuals that would likely have flow on effects at the population, community and ecosystem scales (James et al., 2008). Elucidating the sublethal and potentially irreversible changes to an organism induced by exposure to metals, such as Cu, is vital and utilising omics tools is one way to reveal changes to biomolecules and pathways (Isani et al., 2011; Jin et al., 2021). The last decade has seen an increase in the use of omics, particularly proteomics, to detect the changes of sublethal concentrations of metals in aquatic biota. In Cu-stressed *Chlorella*, a green alga, proteomics revealed increased energy demands of the algae to cope with Cu stress (Shakya et al., 2022). In the blue mussel (*Mytilus edulis)* exposed to Cu nanoparticles the use of proteomics revealed the impact of Cu on cytoskeletal proteins (Hu et al., 2014). In juvenile carp exposed to Cu nanoparticles, increases in enzymes involved in oxidative stress and the differential expression of liver proteins was observed (Gupta et al., 2016). The use of omics tools can reveal changes to key pathways and is proving an indispensable tool to understand potential mechanisms of toxicity of metals such as Cu (Isani et al., 2011; Jin et al., 2021). Using omics is advantageous in revealing pathways that are affected, particularly those that may be unexpected, and therefore, not targeted in other biomarker assays. Despite the potential for the development of biomarkers for environmental monitoring that may be more accurate at assessing effects of contaminants on organisms in different waters, an in-depth understanding of the changes in proteins etc. using omics in fish exposed to metals is still limited (Kang et al., 2014). Changes to the proteome of model organisms such as zebrafish may be used as a guide to identify potential biomarkers of sublethal effects of contaminants on other fish species and aquatic biota (Dai et al., 2014; Kang et al., 2014). Ultimately, this can guide more accurate risk assessment of potential effects of metals and the development of protection frameworks for fish in the wild.

The sensitivity of zebrafish to Cu is influenced by its life stage, with the earlier life stages reported to have greater sensitivity to waterborne Cu than adult fish (Johnson et al., 2007). Therefore, this study aimed to determine the changes to the proteome and AA composition of fish exposed to environmentally relevant Cu concentrations by exposing zebrafish (*Danio rerio)* embryos to Cu for 96 h. Specifically, this study determined the effect concentrations for Cu based on two endpoints (loss of equilibrium (LOE) and non-hatching) and identified changes to the proteome and amino acid (AA) composition of zebrafish larvae exposed to environmentally realistic Cu concentrations.

2. Methods

2.1. Toxicity bioassays

Laboratory brood stock wildtype (Tuebingen strain) zebrafish housed at the La Trobe University animal research and teaching facility (LARTF), on a standard 14/10 h light/dark cycle, were used in this work; all zebrafish experiments were conducted under an Animal Ethics permit (AEC16–91). Zebrafish were bred by separating males and females with a divider in a sloping breeding (beach) tank overnight, before removing the divider for 15 min. Zebrafish embryos (~ 1 h post ferilisation (hpf)) were kept in petri dishes of synthetic freshwater (Supplementary Table 1) with a hardness of 80–90 mg $CaCO_3$ L^{-1}, pH of 7.2–7.5 in a 28 °C incubator. Preliminary trials for this study showed more than 10 % of control embryos to not develop. Due to this the embryos were first developed in an incubator for 24 h, where only viable embryos (at 24 hpf) were selected for use in the toxicity bioassays. Two 96 h toxicity bioassays were conducted, in accordance with the OECD guidelines (OECD, 2013), at 26 ± 1°C using viable embyros 24 hpf. In the first experiment, 10 embryos in each of the three replicates per treatment were exposed to varying concentrations of Cu (Control, 2 μg L^{-1}, 4 μg L^{-1}, 10 μg L^{-1}, 22 μg L^{-1}, 50 μg L^{-1} and 120 μg L^{-1}; measured concentrations provided in Supplementary Table 2) prepared from a 10 mg-Cu L^{-1} ($CuSO_4{\cdot}5H_2O$; Sigma-Aldrich; Burlington, Massachusetts, United States) stock. Embryos were checked every 24 h, counted and removed if they reached one of two endpoints indicative of forthcoming lethality: (1) coagulation of the embryo or (2) LOE. Embryos that had not hatched but were still translucent (not coagulated) were not removed to give opportunity for delayed hatching. After exposure to Cu for 96 h, larvae (120 hpf) were determined as 'unhealthy' if they had not hatched or were displaying LOE. The use of moribundity endpoints were introduced in acute fish toxicity tests to reduce suffering associated with mortality (Rufli, 2012). Visible moribund abnormalities, including LOE, are indicative of forthcoming mortality (Katsiadaki et al., 2021), and hence, LOE was chosen as a more humane endpoint. Exposure to concentrations > 10 μg L^{-1} resulted in non-hatching, and therefore, these embryos could not count towards LOE and were also counted as an endpoint indicative of imminent death. Effect concentrations were calculated to determine the concentration at which 10 % (EC_{10}) and 50 % of larvae (EC_{50}) were deemed 'unhealthy' based on the endpoints above, for use in the second bioassay.

The second experiment exposed 100 embryos per treatment across 5 petri dishes (20 embryos per petri dish and $n = 5$ replicate dishes per

treatment) to Cu concentrations corresponding to the EC_{10} (3.7 μg L^{-1}, 95 % confidence intervals: 2.4–4.9 μg L^{-1}) and EC_{50} (10.9 μg L^{-1}, 95 % confidence intervals: 9.4–12 μg L^{-1}) derived from the first bioassay (Fig. 1). The Cu concentrations in the second bioassay were measured as 4.9 μg L^{-1} (±1.0; refer to Supplementary Table 3) in the EC_{10} treatment and 10.7 μg L^{-1} (±3.3; refer to Supplementary Table 3) in the EC_{50} treatment. Test solutions were semi-static renewed, with replacement of approximately 80 % of synthetic water in each petri dish every 24 h, and the pH was maintained at 7.7 ± 0.3 for the duration of the test. Embryos that had hatched and did not display LOE in the second bioassay ("healthy") were collected after 48 h, with the majority continuing for 96 h of Cu exposure and collected for downstream analysis; snap frozen and stored at –80°C. For proteomic analysis of larvae at 48 h, 2 larvae were collected from three replicates (petri dishes) chosen at random (of the healthy hatched larvae), to obtain sufficient protein (total 6 larvae, $n = 3$ per treatment). After 96 h, 2 larvae were similarly pooled (of those that did not display LOE) per replicate but from all five replicates per treatment (total 10 larvae, $n = 5$). This was to ensure that even in the EC_{50} treatment there were enough larvae for analysis, regardless if more than 50 % displayed LOE.

2.2. Copper analysis

Test solutions before and after each renewal were filtered through a pre-washed 0.45 μm cellulose acetate membrane filter and preserved with 0.1 % HNO_3. The Cu concentrations in the test solutions were measured before and after introduction into the petri dishes and exposure to embryos. The concentration of Cu was quantified by graphite furnace atomic absorption spectroscopy and was averaged for use in subsequent data analysis. Calibrations were prepared from a 1 g L^{-1} certified standard (Sigma-Aldrich) and were checked alongside blanks and certified standards for quality assurance. Measurement of the bioaccumulation of the Cu in the larvae was attempted, however, due to the size of larvae and analytical detection limits, this was not possible.

2.3. Amino acid analysis

One larvae per replicate, per treatment ($n = 5$), removed after 96 h of Cu exposure was used for AA analysis. For each larvae, 200 μL of 6.6 N HCl with 0.02 % phenol was added to the sample in a 2 mL pyrolysed (550 °C) glass vial, purged with argon and digested for 24 h at 110 °C. Digested samples were placed in a rotary vacuum concentrator (RVC) at 40 °C for 4 h to remove HCl and were then reconstituted in 0.1 % formic acid. The reconstituted samples were then filtered through a 0.2 mm cellulose acetate syringe filter and derivatised with 60 mL borate buffer and 20 mL 6-aminoquinolyl-N-hydrodroxysuccinimidyl (AQC), followed by tyrosine by-product conversion at 55 °C for 10 min, before adding a further 900 μL 0.1 % formic acid. The tagged samples were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS; Shimadzu 8045; Shimazu, Kyoto, Japan) to determine individual AA concentrations, including: Ala, Arg, Asx (Asp + Asn), cystine (Cys-Cys), Glx (Glu + Gln), Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr and Val. Note: Asn and Gln are deaminated during acid hydrolysis to aspartic acid (Asp); these amino acid pairs are reported as Asx (Asp + Asn) and Glx (Glu + Gln). Calibration standards (0.01 – 2 pmol μL^{-1}) were prepared from AA standard H (Waters Corporation; Milford, Massachusetts, United States) and spiked with Gln, Asn and Trp at the same concentration as all other amino acids. Sample queues included: sample blanks (Milli-Q water), quality control (QC; His, Arg, Glu, Lys and Ile) samples and check standards every 10 samples to monitor instrument drift. Bovine Serum Albumin (BSA; Sigma-Aldrich; Burlington, Massachusetts, United States) was used as a hydrolysis recovery QC, with typical recoveries described in Shakya et al. (2021a). The AA values are reported as the relative abundance of each AA (mol%).

2.4. Protein extraction and proteomics

The larvae were homogenised and protein was extracted using a lysis buffer (2 % sodium dodecyl sulfate: 20 mM ammonium bicarbonate) and quantified using a NanoDrop spectrophotometer (Thermo Fisher, Waltham, Massachusetts, United States). Two larvae for each replicate ($n =$

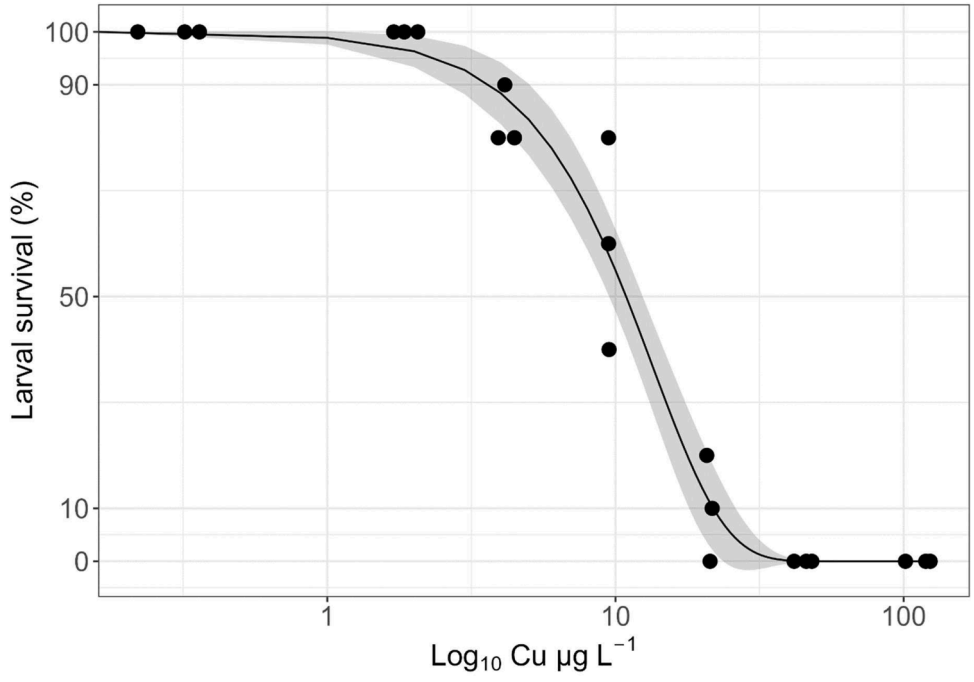


Fig. 1. Dose-response curve of surviving larvae after exposing to a range of copper (Cu) concentrations. Zebrafish larvae at 120-h post fertilisation (hpf) after exposure to copper (Cu) for 96 h that did not display the endpoints of loss of equilibrium (LOE) and non-hatching were represented through survival. Cu concentrations are log-transformed. Shaded areas represent 95 % confidence intervals. Each point represents the percentage of healthy embryos in a replicate petri dish ($n = 3$) at its corresponding measured Cu concentration.

3 at 48 h and $n = 5$ at 96 h per treatment) were combined and homogenised to ensure sufficient concentrations of protein. Samples were then prepared using the single-pot, solid-phase-enhanced sample preparation (SP3) method (Hughes et al., 2019). Samples were analysed through bottom-up proteomics with label-free quantification (LFQ) on a LC-MS/MS (Thermo Ultimate 3000 RSLCnano UHPLC system with a Thermo Q-Exactive HF mass spectrometer, Thermo Fisher, Waltham, Massachusetts, United States). The *D. rerio* reference proteome from uniprot was matched against the results allowing a maximum peptide length of 30 AA, precursor tolerance of 20 ppm, fragment tolerance of 0.05 Da and allowing two missed trypsin cleavages. Total peptide amount was normalised from abundances. Proteins present in at least 2 out of 3 of the 48 h replicate zebrafish or 4 out of 5 of the 96 h replicate zebrafish were used to investigate the impact of Cu on the expression of proteins. Protein expression was compared to their respective controls at 48 h or 96 h.

2.5. Statistical analysis

The EC_{10} and EC_{50} values were calculated (three-parameter log-logistic parameter model) in R studio using the "Dose-Response Curve" (drc) package (Ritz et al., 2016) and visualised in ggplot2 (Villanueva and Chen, 2019). Changes in the composition of AA in the larvae were visualized using a principal component analysis (PCA) plot using the FactoMineR (Lê et al., 2008) and factoextra packages (Kassambara and Mundt, 2020) in R to determine differences between treatments. Permutational multi-variate analysis of variance (PERMANOVA) was used to compare differences in AA composition between treatments and was conducted using the vegan package (Oksanen et al., 2017) and a pairwise test (Martinesz Arbizu, 2017) was used to test for an overall significant variation in AA composition between treatments. One-way analysis of variation (ANOVA) was used to determine statistical differences in abundance of the individual AAs, with the Shapiro test (to determine normality) and the Bartlett test (to test for homogeneity of variance) used to test for assumptions on the residuals from the ANOVA test, of which there were no violations. Tukeys post-hoc test determined statistical differences in abundance between treatments in the individual AAs identified as significant by ANOVA. Significance in all of the AA analyses was determined by $p < 0.05$.

The proteomics results were analysed with the DEP package in R (Zhang et al., 2018). Data was filtered for missing values (at least 4 out of 5 reps required data for 96 h treatment and 2 out of 3 for 48 h treatment), normalised and missing values imputed. Significant differentially expressed proteins (DEPs) were denoted by an adjusted p-value (alpha) of < 0.1 and a log fold-change of at least 1 (a doubling) by comparing the treatments (EC_{10} or EC_{50}) against their respective controls (no added Cu at 48 h or 96 h). The DEPs were visualised using volcano plots generated in ggplot2 (Villanueva and Chen, 2019; Wickham, 2016).

Functional enrichment analysis was performed on significant DEPs in larvae exposed to Cu for 48 h. The Gene Ontology (GO) annotation was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/) to classify the functions of the proteins in response to Cu stress. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis assigned proteins to a functional pathway and were derived through FishEnricher (https://maayanlab.cloud/FishEnrichr/), a gene set enrichment analysis tool specifically developed for the *D. rerio* genome (Chen et al., 2013; Hughes et al., 2019; Kuleshov et al., 2016). Protein-Protein interaction (PPI) networks were constructed using STRING (https://string-db.org/; version 11.5), a database interacting proteins through both physical and functional means (Szklarczyk et al., 2023) and presented using cytoscape (Shannon et al., 2003), with the network uploaded to NDEx, an open-source platform allowing researchers to publish biological network data (Pratt et al., 2015), for visualisation in an interactive format.

3. Results and discussion

3.1. Copper toxicity to zebrafish embryos and larvae

The effect of Cu on the hatching of zebrafish embryos and LOE of larvae (Supplementary Fig. 1) was assessed every 24 h. In zebrafish, hatching from the chorion occurs on average by 72 hpf (Kimmel et al., 1995). In the control and the lowest concentration of Cu treatment (2 μg L^{-1}) all larvae hatched by 72 hpf; this is compared to the highest Cu concentration of 120 μg L^{-1} which resulted in only unhatched coagulated embryos (Supplementary Fig. 2). Within the 4 μg L^{-1} Cu treatment, all larvae had hatched by 96 h, however, 13 % of the larvae exhibited LOE (Supplementary Fig. 2). At 10 μg L^{-1} of Cu exposure, one embryo did not hatch, and of the hatched embryos 37 % displayed LOE, with the remaining 60 % "healthy". The hatched embryos that did not display LOE were deemed "healthy", however, some fish exhibited other abnormal and erratic swimming patterns, such as jerky movements, that were not measured or recorded as an endpoint. Erratic swimming behaviour has also been described previously by Acosta et al. (2016) where zebrafish exposed to 9 μg L^{-1} of Cu for 96 h displayed an observable increase in swimming velocity and distance travelled. In the 22 μg Cu L^{-1} treatment, 70 % of embryos had not hatched by 120 hpf (after 96 h of exposure) and 20 % displayed LOE. In the 50 μg Cu L^{-1} treatment, 93 % of embryos did not hatch with the remaining larvae exhibiting LOE. The highest Cu concentration of 120 μg L^{-1} caused no embryos to hatch and these embryos had coagulated (or begun to coagulate). Copper has previously been reported to inhibit hatching in fish and this is commonly reported in the literature (Jezierska et al., 2009; Johnson et al., 2007).

The 96 h EC_{10} and EC_{50} for Cu was calculated as 3.7 μg L^{-1} (2.4–4.9: μg L^{-1}: 95 % confidence intervals) and 10.9 μg L^{-1} (9.4–12 μg L^{-1}; 95 % confidence intervals), respectively (Fig. 1). The effect concentrations of Cu on fish vary in the literature, with lethal concentrations of Cu where 50 % of adult zebrafish were affected (LC_{50}) ranging from 12 to 212 μg L^{-1}, depending on the water hardness (Alsop and Wood, 2011; de Oliveira-Filho et al., 2004). A previous study on *Mogurnda adspera* looking at the same sublethal effects of Cu on LOE in sacfry reported an EC_{10} of 12 μg L^{-1} and an EC_{50} of 22 μg L^{-1} (Shakya et al., 2022). However, these fish were exposed after hatching and are significantly larger at maturity, potentially explaining the higher sensitivity of zebrafish to Cu compared to *M. adspersa* (Shakya et al., 2022). Concentrations of Cu as low as 4 μg L^{-1} have previously been reported to cause sublethal effects in fish. A study by Besser et al. (2001) exposed four different newly-hatched fish species to low concentrations of Cu, examining survival and growth. The lowest observable concentration of 4 μg L^{-1} caused a statistically significant reduction of growth by 13 % in fathead minnows. Farkas et al. (2021) subjected Atlantic cod (*Gadus morhua)* embryos at 96 hpf to low concentrations of Cu (0.5 - 6 μg L^{-1}) and suggests that even though these low concentrations of Cu did not increase mortality in the 96 hpf larvae, larvae and embryos exposed immediately after fertilisation would have increased sensitivity to Cu. Given the effect concentrations in the current study are based on sublethal impacts (LOE and non-hatching), the low EC values are unsurprising.

3.2. Amino acid composition of Cu-stressed zebrafish larvae

The AA composition of zebrafish larvae was dominated by: Glx (Gln + Glu), Gly, Asx (Asn + Asp), Lys, Leu, Ala, Ser, Val, Thr and Arg, making up 80 % of the total AAs and contributing between 5 and 13 % each (Fig. 2). The seven remaining measured AAs (Pro, Ile, Phe, Tyr, His, Met and Cys-Cys [dimer]) made up the remaining 20% of total AAs at < 5% each. This result is unsurprising as Li et al. (2009) states that Asp, Asn, Glu and Gln are the major energy substrates in fish. The bulk AA composition of larval fish has been previously shown to alter after exposure to Cu (Shakya et al., 2022). Similarly, in this study, the AA

Fig. 2. Amino acid (AA) composition in zebrafish larvae. The influence of Cu on individual AA proportions (% mol) in zebrafish after 96 h ($n = 5$) exposed to: no Cu (Control: red), 4.9 μg Cu L^{-1} (EC_{10}: green) or 10.7 μg Cu L^{-1} (EC_{50}: blue). Asterisks are used to indicate significant differences between treatments for each AA.

profile of the zebrafish larvae exposed to 4.9 μg Cu L^{-1} (representative of EC_{10}) and 10.7 μg-Cu L^{-1} (representative of EC_{50}) differed to the control (Supplementary Fig. 3), but the differences between treatments for the overall AA profile were not significant (PERMANOVA, $p = 0.069$). The proportion (% mol of AA), however, of some individual AAs (Ala, Pro and Gly) significantly changed in the presence of Cu (Ala ($p = 0.046$), Pro ($p = 0.005$) and Gly ($p = 0.038$)) (Fig. 2 and Supplementary Table 5). However, in the current study, both bulk and free AAs were measured, so changes of the AAs in the current study may also come from digestion of proteins.

Proline (Pro) significantly increased in both the EC_{10} and EC_{50} treatments compared to control, while Gly increased significantly between control and EC_{50} (Supplementary Table 5). Alanine was also found to significantly differ between treatments, with Ala appearing to be higher in the EC_{50} treatment, however, post-hoc Tukey tests did not support a significant difference between Control, EC_{10} and EC_{50}. Overall, the most significant effect of Cu on AAs was for the abundance of Pro in the larvae, with increasing concentrations in both Cu treatments compared to the control. Previous studies have also shown an increase in Pro in a variety of organisms exposed to elevated concentrations of metals, including Cu (Tripathi and Gaur, 2004). There was also a significant decrease in Gly in the EC_{50} treatment, as observed previously in *M. adspersa* exposed to 10 – 60 μg of Cu (Shakya et al., 2022). Although Cu also caused differences in the amounts of Gly and Ala in the larvae, less is known about the effects of metal contamination on these AAs.

3.3. *Differentially expressed proteins identification and functional enrichment analysis*

Label-free quantitative proteomic analysis identified a total of 3437 proteins in the zebrafish samples (Fig. 3a), 3333 of which met the requirements of being identified in at least 2 out of 3 reps at 48 h and 4 out of 5 reps at 96 h for comparison between treatments. This is similar to the number of proteins detected for zebrafish larvae in other studies (Frøyset et al., 2016). Although Cu caused an upregulation and downregulation of proteins after exposure to Cu at both time-points (48 h and 96 h), there were significantly more DEPs at the earlier time point of 48 h (66 DEPs after 48 h at EC_{50}; 20 DEPs after 48 h at EC_{10}; 1 DEP

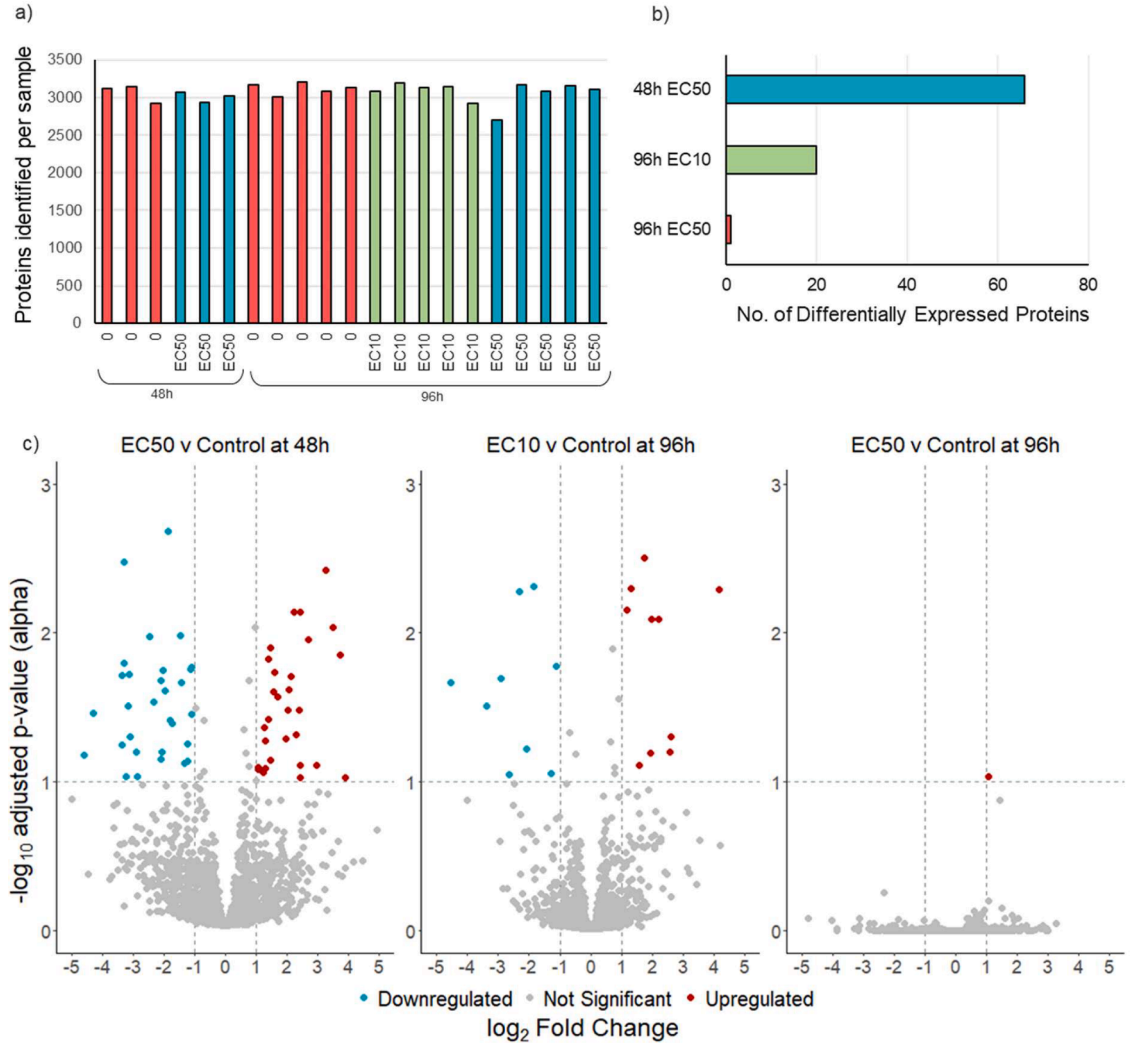


Fig. 3. Protein identification and differentially expressed proteins (DEPs) in copper (Cu) exposed zebrafish larvae after 48 and 96 h. Shown are: (a) The total number of proteins identified in each sample from the zebrafish genome, (b) the number of DEPs between treatments and controls, and (c) volcano plots visualising the upregulated and downregulated DEPs at 48 and 96 h. Differentially expressed proteins are characterised by a $\log_2$ fold change of >1 or <−1 and significance is determined by an adjusted p-value (alpha) of <0.1.

(aminomethyltransferase, Amt) after 96 h at EC_{50} (Fig. 3b). Sonnack et al. (2018) also found Cu influenced the expression of more genes after 48 h of exposure than after 96 h in zebrafish. Volcano plots show that the DEPs are not dominated by either upregulated or downregulated proteins (Fig. 3c).

To gain insight into the functions of the DEPs, gene enrichment analysis was performed by GO (biological process (BP), molecular function (MF), and cellular component (CC)) and KEGG pathway annotation for all treatments (Fig. 4 and Supplementary Table 6), focusing on the significant DEPs in larvae exposed to 10.7 μg L^{-1} of Cu (EC_{50}) for 48 h, as this produced the greatest number of significant DEPs (Fig. 3). The main BP affected by Cu after 48 h of exposure was visual perception, followed by response to stimulus, mitochondrial translation, and neurotransmitter transport, based on categories with the highest counts (Fig. 4a). The proteins most enriched for MF were involved in ATPase activity, followed by ligase activity and structural constituents of the nuclear pore (Fig. 4b). The main cellular localisation of DEPs was in the membrane and in the mitochondria (Fig. 4c). Metabolism, specifically that of AAs (His, Trp, Arg and Pro) dominated the KEGG pathways of the DEPs (Fig. 4d).

3.4. Amino acid and protein composition is altered in Cu exposed fish from oxidative stress

In this study Pro was shown to significantly increase in zebrafish larvae exposed to both Cu treatments (4.9 μg L^{-1} and 10.7 μg L^{-1}) (Fig. 2). Proteins involved in Pro metabolism were also differentially expressed (Fig. 4, KEGG pathway). The increase in Pro from Cu exposure has been observed in many aquatic organisms, such as some species of algae, where increases in Pro have also been associated with Cu exposure, and with free Pro has been suggested to play a role in reducing the uptake of Cu (Mehta and Gaur, 1999; Wu et al., 1998). The accumulation of Pro does not just aid in reducing metal uptake, but it also acts as an antioxidant by reducing the damage of free radicals, preventing lipid peroxidation (Tripathi and Gaur, 2004). Recent studies by Santos et al. (2020) have reported increased ROS in zebrafish embryos exposed to 15 μg L^{-1} of Cu. While this study did not directly measure ROS production, the increase in Pro could be to alleviate oxidative stress caused by ROS. The role of Pro in the stress response in organisms is widely known with evidence suggesting it enhances antioxidant activity and protects cells against oxidative stress (Liang et al., 2013). However, Pro metabolism has been implicated in not only cellular survival, as once stress has exceeded a certain threshold it has contrasting effects by promoting

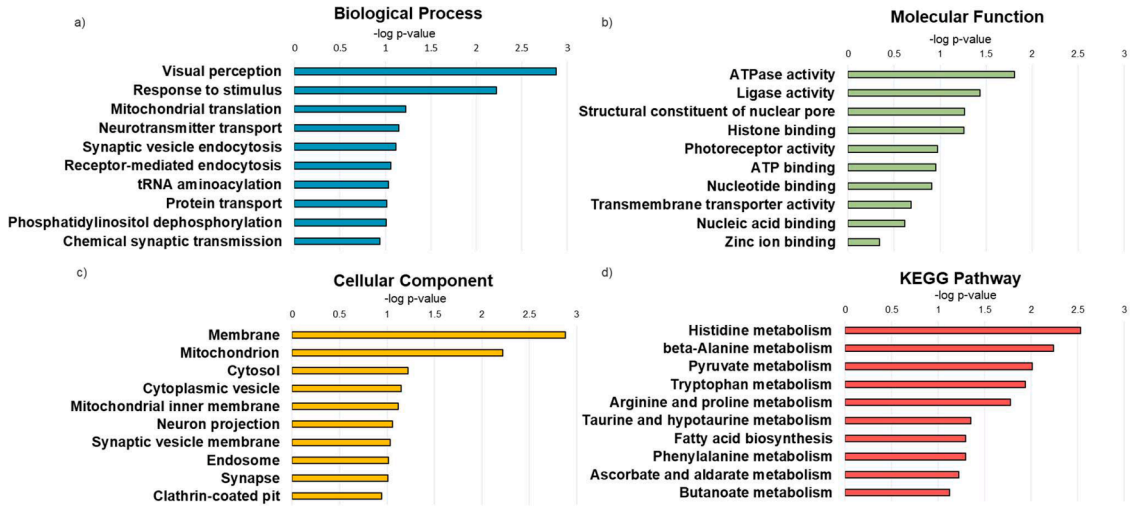


Fig. 4. Functional enrichment analysis of differentially expressed proteins (DEP). Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation of zebrafish larvae exposed to copper (Cu) for 48 h (n = 3). The GO encompassing the: (a) biological processes; (b) molecular function; (c) cellular component categories and (d) KEGG pathways, are shown. The top 10 categories of each, based on the highest number of counts (number of proteins in category) followed by Enrichment Threshold (EASE), set to <0.5, are shown.

cellular apoptosis through increasing ROS production in the mitochondria (Liang et al., 2013). It has also been previously reported in fish that a combination of Ala, Pro and citrulline offers greater protection against oxidative stress and subsequent apoptosis than Pro alone (Li et al., 2013). In the current study, Ala also significantly increased in the presence of Cu (Fig. 2) and DEPs involved in phenylalanine and beta-alanine (derivatives of Ala) metabolism were differentially expressed (Fig. 4, KEGG pathway). While citrulline (an amino acid derived from Arg) was not measured here, Arg abundance did show a trend towards increased abundance in the EC_{10} treatment, although this increase was not significant. Proteins with a KEGG annotation of arginine metabolism were also differentially expressed (Fig. 4, KEGG pathway).

Alongside the changes in AAs, proteins involved in oxidative stress were also differentially expressed in Cu exposed zebrafish in this study. Proteins with a role in oxidative stress were identified in the KEGG/GO analysis, such as mitochondrial function (Fig. 4, biological process) and ATP synthase activity (Fig. 4, molecular function). Moreover, ascorbate depletion occurs under oxidative stress (Domazou et al., 2012) and proteins involved in ascorbate metabolism were differentially expressed in this study (Fig. 4, KEGG pathway). Specifically, peroxiredoxin 6 (Prdx6) was upregulated after 48 h exposure to the EC_{50} Cu treatment. Proteins within the peroxiredoxin family have also been shown to be upregulated in the yeast (*Yarrowia lipolytica*) exposed to Cu (Sánchez-Rojas et al., 2022). This group of proteins are enzymes that act to protect cells against oxidative stress and maintain cell redox homeostasis (a BP which was significant, however, not shown in Fig. 4 due to not being in top 10) by regulating peroxide activity (Perkins et al., 2015).

The ATP-binding cassette (ABC) class of transporter proteins uses diverse substrates and is involved in the efflux of xenobiotics, including metals (Della Torre et al., 2012). In the current study after 48 h of exposure to Cu, zebrafish significantly downregulated ABC-type glutathione S-conjugate transporters (Abcc1), whose molecular function is ATP binding activity (Fig. 4, molecular function). However, many of the studies on fish and ABC transporters show the opposite (Della Torre et al., 2012), including by Long et al. (2011) where exposure of zebrafish embryos to other metals, including cadmium, mercury, lead, and arsenic induced the overexpression of Abcc1 and promoted embryo survival. The impact of Cu on the expression of Abcc1 is less studied. This specific ABC protein is involved in the transport of glutathione (GSH) conjugates (Laberge et al., 2007), and Cu-induced oxidative stress reduces GSH antioxidant activity by Cu directly binding and inhibiting the GSH (Sanchez et al., 2005). Previous studies have demonstrated through HeLa cells, that cells that overexpress Abcc1 potentiate oxidative stress through the efflux of GSH (Laberge et al., 2007), and thus, the zebrafish cells may reduce Abcc1 levels to prevent efflux of the remaining uninhibited GSH, allowing it to play a role in reducing oxidative stress by scavenging ROS. In addition to excess Cu inactivating GSH through the formation of a metal complex (Ngamchuea et al., 2016), production of ROS due to Cu exposure may also decrease intracellular GSH in neuronal cells as ROS mediate the blocking of cysteine via glutamate, required for GSH (Murphy et al., 1989). A number of the DEPs in the larvae exposed to Cu for 48 h were involved in taurine metabolism (Fig. 4, KEGG pathway), a derivative of cysteine produced in oxidative pathways. In the zebrafish exposed to Cu for 96 h, despite the cystine levels between treatments not being significantly different, there was a clear negative correlation between Cys-Cys (cystine, dimer) level and Cu concentration, indicating that Cu-induced ROS may be inhibiting the uptake of cysteine (monomer). Alternatively, GSH is a tripeptide comprised of Gly, Cys and glutamate, and as Cys-Cys was negatively correlated and Gly was significantly decreased, this could suggest the use of these AAs to synthesise GSH to counteract any cellular oxidative damage (Javed and Usmani, 2019). Moreover, although glutamate was not measured, Glx (Glu and Gln) did decrease in the larvae at EC_{10}, although not significant (Fig. 2).

Oxidative stress can lead to cell death via many avenues including through GSH depletion (Laberge et al., 2007) and through ROS-mediated signalling events activating apoptotic pathways (Redza-Dutordoir and Averill-Bates, 2016). A loss of protein kinase C alpha (Prkca), a highly abundant Zn-containing signalling protein, has been attributed to oxidative stress-induced cell death (Reyland, 2009), and thus, it is unsurprising that the zebrafish in the current study upregulated Prkca (Fig. 5, cell signalling), whereby Prkca can modulate members of the mitogen-activated protein kinase (Mapk) kinase family to promote survival (Maher, 2001).

3.5. Protein-protein interaction network

The GO analysis showed vision to be the most impacted biological process (Fig. 4), and proteins associated with visual development and phototransduction were also shown to be affected within the protein-protein interaction (PPI) network (Fig. 5, visual development and phototransduction). The interactions between proteins on the PPI network

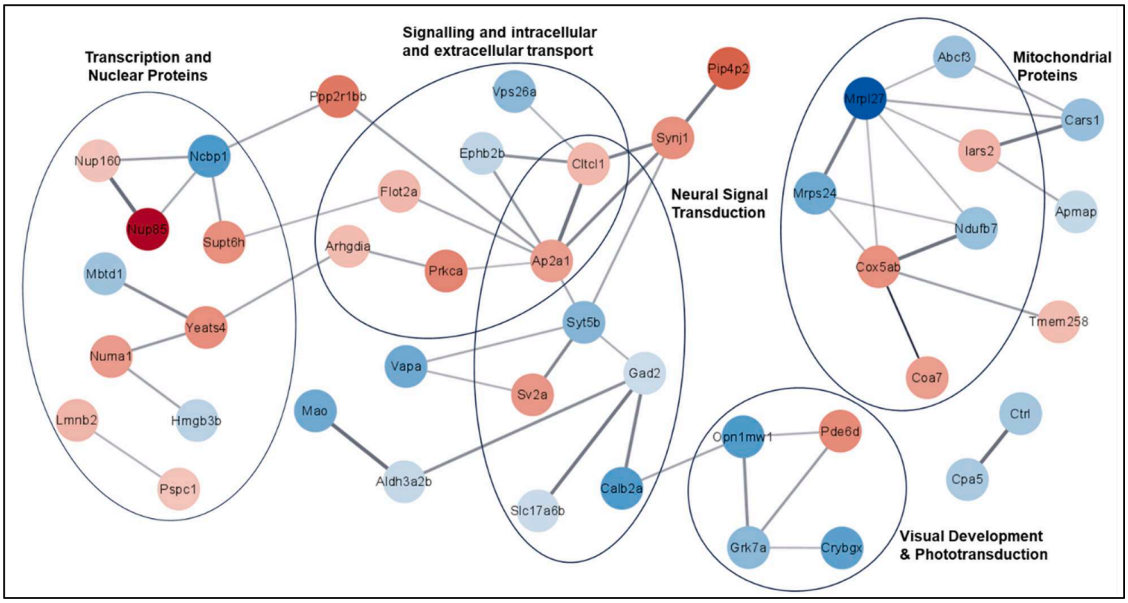


Fig. 5. Function enrichment analysis visualised in a protein-protein interaction (PPI) network of significant differentially expressed proteins (DEPs) in copper (Cu) – stressed zebrafish. Larvae were exposed to 10.7 μg L^{-1} of Cu (EC_{50}) for 48 h and DEPs were sorted by STRING and visualised using Cytoscape (average clustering coefficient 0.261). The network contains 66 nodes and 52 edges, and proteins with no interactions within the network (singletons) were omitted. Nodes are coloured by their log fold change with red filled nodes being upregulated and blue filled nodes being downregulated proteins, with intensity correlating to a greater fold change. Edges indicate protein-protein interactions determined by STRING and intensity is relational to its confidence score.

(Fig. 5) can be either direct (physical) or indirect (functional), where the proteins contribute to the same biological function. Among the PPI subnetworks of DEPs in larval zebrafish affected by exposure to 10.7 μg L^{-1} of Cu for 48 h included proteins involved in neural signal transduction (Fig. 5), mitochondrial proteins and proteins involved in transcription. The network can be further explored in NDEx (https://doi.org/10.18119/N9X02X)https://www.ndexbio.org/#/network/32e571dc-713f-11ee-aa50-005056ae23aa?accesskey=6123684204f30f35966d9201a9fdb76af11a6ea8fd2918fe9a0e854694931925 where selecting a protein will give the GO annotation and selecting the interaction will reveal more data from STRING about its interaction with other proteins.

3.6. Cu alters expression of mitochondrial proteins

In the current study, the presence of excess Cu caused a mixed expression of mitochondrial proteins in zebrafish larvae (Fig. 5, mitochondrial proteins), including a few involved in oxidative phosphorylation. Oxidative phosphorylation utilises an ETC on the mitochondrial inner membrane comprised of complexes I to IV and electron transporters to efficiently produce large amounts of ATP. The ATP is produced by ATP synthase which is powered by a mitochondrial membrane potential created by the translocation of protons from complexes I, III and IV (Zorova et al., 2018). Elevated Cu has previously been shown to affect energy production in fish (Abou Anni et al., 2019). Long-term exposure to Cu has also been shown to decrease enzymatic activity in the gills and muscle of killfish (*Piecilia vivipara*) but increase ATP production in the liver (Abou Anni et al., 2019). The zebrafish larvae exposed to Cu upregulated cytochrome C oxidase subunit 5Ab (Cox5ab), a Cu-containing metalloenzyme, part of complex IV of the ETC. Moreover, cytochrome C oxidase assembly factor 7 (Coa7) involved in the assembly of cytochrome C was also enriched in zebrafish exposed to Cu. However, in contrast, NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 7 (Ndufb7) of complex I in the ETC was downregulated. A proposed mechanism of reducing the production of mitochondrial ROS in cells is through lowering NADH production and inhibiting complex I (Liemburg-Apers et al., 2015). This complex contains subunits sensitive to oxidative damage and the reversible glutathionylation of it has been shown to protect it from otherwise irreversible deactivation from oxidative damage (Hurd et al., 2008). Increased mitochondrial membrane potential associated with complexes I and III have also been implicated in ROS production (Brand, 2010; Miwa et al., 2003). Fish, therefore, may inhibit complex I to reduce potential ROS production, and hence oxidative stress, and this may be why Ndufb7is downregulated in this study. However, cells still require extra energy to cope with stressors such as Cu, and thus, complete deactivation of the ETC would be detrimental. Previously, Shakya et al. (2022) reported the upregulation of proteins and metabolites responsible for the creation of succinate (the sixth molecule in the TCA cycle) in algae exposed to Cu. Succinate can be produced through Val oxidation through metabolism into methylmalonyl-coA (Hutson et al., 2005) and Val was found to be increased in Cu exposed fish in this study, although not significant at $p = 0.06$. The conversion of succinate to fumarate in the TCA cycle catalyses the conversion of FAD to $FADH_2$ for use by complex II of the ETC. Fish in our study that were exposed to Cu potentially bypass the need for NADH and complex I, increasing the flux from complex IV by utilising $FADH_2$ as the main electron carrier to support the increased energy demands. Moreover, complex IV is said to be a pace setter for ATP synthesis due to it being a highly regulated enzyme (Srinivasan and Avadhani, 2012), and thus, the upregulation of a cytochrome C oxidase subunit and an assembly protein could suggest an increase in energy production to meet demands of the cell. Alternative functions of cytochrome C could also offer an explanation, one being that release of cytochrome C from the mitochondria induces apoptotic pathways, expected in severely stressed fish (Jiang and Wang, 2004). More likely, as the proteins were upregulated in larvae trying to cope with the metal stress, is that cytochrome C acts as an antioxidant, where it can mediate the removal of superoxides (Pereverzev et al., 2003) and in doing so, reduce oxidative damage and improve the survival outcome of the larvae.

3.7. Upregulation of nuclear proteins

Changes in the transcription and expression of certain proteins from Cu exposure is consistently demonstrated (Abou Anni et al., 2019; Shakya et al., 2022). This is not surprising as the increased demand for proteins to combat Cu stress and maintain homeostasis would result in increased transcription. The current study resulted in predominantly

DEPs involved in the transcription of nuclear proteins, mRNA export and translation, identified in the network analysis (Fig. 5) and in the GO/KEGG annotation (Fig. 4, biological process (nucleotide binding, nuclear pore structural constituent and histone binding)). Upregulation of RNA binding and regulation of DNA-templated transcription proteins, like paraspeckle component 1 (Pspc1; Fig. 5, transcription and nuclear proteins) were shown in this study. Paraspeckles have been suggested to alter gene regulation in times of stress to assist in pro-survival pathways (McCluggage and Fox, 2021). Lamin B2 (Lmnb2) is a protein involved in protein localisation to the nuclear envelope, heterochromatin formation and nucleus organisation and was upregulated under the elevated Cu conditions. Likewise, lamins such as Lmnb2have a role in regulating cell longevity during oxidative stress and increased ROS (Shimi and Goldman, 2014). The Lmnb2 isoform specifically is involved in supressing p53 and promoting proliferation and longevity, explaining why despite literature consistently reporting an upregulation of p53 from oxidative stress including from Cu, upregulated p53 was not observed in this study (Luzio et al., 2013; Shimi and Goldman, 2014). As only the proteomes of healthy larvae were determined in this study, it may be that a key contributor to survival and acclimatisation from toxic concentrations of Cu is by inhibition by Lmnb2 of the p53-mediated apoptotic pathway induced by ROS. The YEATS domain containing 4 (Yeats4) protein which was also upregulated, plays a role in nuclear organisation, and is involved in the remodelling of chromatin, histone acetylation and the regulation of transcription by RNA polymerase II. Previously, environmental stressors have been found to modify the structure of chromatin and histones (Pawlak and Deckert, 2007). In particular, Cu has been found to inhibit histone acetyltransferase *in vivo* human liver cells (Kang et al., 2004) and potentially the upregulation of an alternative histone acetylator, mediated, may counteract the impact Cu is having in the nucleus. Moreover, nucleoporin 85 (Nup85) and nucleoporin 160 (Nup160), components of a nuclear pore complex also involved in RNA transport/export were shown to be upregulated in response to exposure to Cu (Fig. 5, transcription and nuclear proteins). These two proteins are often co-expressed and were strongly upregulated, making their biological process annotation, structural constituent of nuclear pore (Fig. 4) among the most enriched process impacted by Cu, suggesting the significance of RNA export and translation in combatting Cu-induced stress. The changes to the proteome of the larvae reflects Cu-stressed larvae that are adapting via increased transcription and alternative protein pathways to counteract the effects of Cu on targeted proteins.

3.8. Cu disrupts proteins involved in neural signalling and vision

One of the most significant interaction of proteins that were differentially expressed because of Cu exposure were linked to neural transmission, with proteins related to visual development and phototransduction also affected (Fig. 5). This is consistent with previous studies reporting affected neurotransmission and cognitive dysfunction from exposure to sublethal concentrations of other metals (Karri et al., 2016). Santos et al. (2022) observed a modulation of the antioxidant system and induction of apoptosis in the brain of zebrafish accompanied by affected locomotor control and changes in social behaviour when exposed to Cu. Changed behaviour in fish has been attributed to disrupted neurotransmission, and in the current study, zebrafish larvae displayed neural distress through their inability to maintain equilibrium and their erratic swimming behaviour. Equilibrium in fish is maintained through coordination between the visual and vestibular systems with the retina involved in the dorsal light reflex (Kasumyan, 2004), and disruption to proteins related to visual development and phototransduction along with disruption to neural signalling likely led to the increased LOE displayed in zebrafish larvae in this study.

Cu affected many proteins involved in the glutamate to γ-aminobutyric acid (GABA) pathway, a main inhibitory neurotransmitter regulated by calcium signalling with a suggested role in locomotion (Barnaby et al., 2022). Glutamate decarboxylase 2 (Gad2) is an enzyme involved in the synthesis of GABA and was downregulated in Cu exposed fish in this study. Moreover, synaptic transmembrane vesicle glycoprotein (Sv2a) was upregulated and is involved in regulating the release of GABA through expression of synaptogamins (Stout et al., 2019; Tokudome et al., 2016), which were shown to be downregulated (Synaptotagmin 5b; Syt5b) in this study. Syt5b is involved in Ca^{2+}-dependent exocytosis, required for synaptic transmission in the retina and brain (Henry et al., 2022), alluding to its function in neural signalling and vision. Clathrin (Cltc1), the main component of clathrin coated vesicles (CCV) and Ap2a1, an adaptor protein complex involved in the endocytosis of CCV were also differentially expressed (upregulated) in Cu exposed larvae. Endocytosis of the neuronal receptors that bind GABA ($GABA_A$ receptors) occurs via the use of CCVs and is facilitated by Ap2a1 (Jurd and Moss, 2010). Previous research has shown that glutamate decarboxylases such as Gad2 and $GABA_A$ receptors control swimming behaviour in larval zebrafish, with reduced expression of glutamate decarboxylases and GABA receptors associated with hyperactivity in larval zebrafish (Barnaby et al., 2022; Lüffe et al., 2021). The inhibition of GABA and downregulation of Gad2 has been shown previously in larval zebrafish displaying impaired locomotor control upon exposure to other metals and toxins (Stehr et al., 2006; Zhao et al., 2019). Larvae unable to acclimatise to Cu displayed LOE, however, the proteome of stressed, but 'healthy' larvae at 48 h revealed the inhibition of the GABA pathway. The disruption to this pathway, causing a decrease in the inhibitory neurotransmitter GABA, could be a potential cause or contributor to the LOE in larvae unable to acclimatise to Cu after 96 h, where the behaviour of the larvae only changes once the inhibitory GABA signal goes under a certain threshold.

Expression of other proteins involved in neural signalling via calcium, and that play a role in locomotion and phototransduction, were also altered. Exposure to Cu caused the downregulation of calretinin (Calb21), a calcium-binding protein involved in neural function, synaptic plasticity, optic nerve regeneration and is associated with motoneurons responsible for high-speed swimming and the escape response in zebrafish (Berg et al., 2018; García-Crespo and Vecino, 2004). Additionally, a glutamic-acid rich protein (Garp), Garp2, was the only shared DEP between the 48 h EC_{50}, 96 h EC_{10} and 96 h EC_{50} Cu treatments and was upregulated in the zebrafish larvae (although significant ($p = 0.028$) at EC_{50} for 96 h, with a fold change of 0.8 it did not quite meet the fold change threshold of a doubling to be considered a significantly DEP) (Table S5). Garp are known to play a role in the function of rod photoreception (Goldberg et al., 2016) and the overexpression of Garp2 has been shown to regulate phototransduction gain and recovery (Sarfare et al., 2014). Phototransduction also plays a pivotal role in the movement of fish as it allows fish to adapt to light and navigate using visual cues, including that of predators and prey (Eilertsen et al., 2022; Rinner et al., 2005; Zang and Neuhauss, 2021). This may have also affected the swimming ability of fish in this study, and with predator avoidance, social behaviours and feeding requiring effective swimming, the implications of this altered behaviour on fish in the wild exposed to low concentrations of Cu is vast. In *M. adspersa* sacfry Cu localised to the eye and brain tissue (Shakya et al., 2022), this might also have occurred in the zebrafish larvae in this study, therefore, future studies should determine whether Cu also localises to these areas in zebrafish larvae and other fish species.

Copper was shown to directly alter the expression of proteins involved in the synthesis, release and uptake of GABA, a primary inhibitory neurotransmitter related to locomotion and behaviour in fish, along with calretinin involved in neural function associated with swimming and escape behaviours, and Garp2 involved in phototransduction. Thus, changes in swimming behaviour and LOE displayed by fish exposed to environmentally relevant Cu concentrations in this study may be due to disruption to calcium dependent, GARP and GABA dependent neural and visual pathways.

3.9. Acclimatisation of larvae to Cu stress

Zebrafish larvae at 48 h exhibited the largest change in their proteome from Cu, with 66 DEPs proteins compared to the 20 DEPs and 1 DEP in larvae after exposure to 4.9 μg L^{-1} (EC_{10}) and 10.7 μg L^{-1} (EC_{50}) of Cu for 96 h, respectively. A study by Sonnack et al. (2018) on the transcriptomic response of zebrafish embryos to Cu (11 ug L^{-1}) also found that embryos at 48 h had far more differentially expressed genes than at 96 h. The larvae in Sonnack et al. (2018) had significant changes to the transcripts of proteins involved in oxidative phosphorylation, transcription and histone modification, also reported in the current study. Similarly, the larvae had changes to pathways targeting the nervous system, brain development, camera-type eye development and eye morphogenesis (Sonnack et al., 2018). By contrast, the larvae in this study were found to have significant differences more in neural signalling and eye functioning than in eye development. The differential expression of more proteins in the current study, and transcripts in the study by Sonnack et al. (2018), at 48 h rather than 96 h suggests that some embryos were able to acclimatise to Cu at this environmentally realistic Cu concentration over the 96 h period and reach a state of homeostasis. Acclimatisation to Cu has been demonstrated in fish previously, with Kamunde and Wood (2004) proposing two net processes required for acclimatisation to be reached: (1) recovery from the disruption caused by the Cu and (2) regulation of the Cu uptake. A study by McGeer et al. (2000) on rainbow trout followed the recovery of fish after exposure to sublethal concentrations of Cu through monitoring of sodium influx. In this study by Kamunde and Wood (2004), Na^+ homeostasis was disrupted within 2 days but had recovered by a week. This suggests that the use of biomarkers to assess risks may need to be undertaken at earlier timeframes than that of traditional chronic and acute toxicity bioassays. Moreover, in future studies, the proteome and AA composition of larvae unable to acclimatise should be compared to larvae that successfully acclimatise. This comparison would enhance biomonitoring efforts that use biomarkers by understanding what is occurring in aquatic biota and ensure that stressed, but acclimatised fish are not ignored.

3.10. Potential biomarkers of Cu contamination in aquatic environments

The concentrations of Cu used in the current study (4.9 μg L^{-1} and 10.7 μg L^{-1}) are commonly found in the environment, for example, in urban streams in Melbourne, Australia, which contain Cu concentrations of up to 40 μg L^{-1} (McDonald et al., 2022). As the vast majority of Cu in natural waters is likely complexed with dissolved organic matter (Macoustra et al., 2019, 2021), measuring the concentration of dissolved Cu is not always a reliable indicator of potential effects. Consequently, total concentrations of Cu do not necessarily equate to its bioavailability, and thus, biomolecular indicators of stress could give a more accurate detection of the potential ecotoxicological effects. Potential biomarkers identified in this study include the 'Garp' protein which was the only DEP common among the Cu treatments. Another potential biomarker is Gad2, or other proteins involved in GABAergic signalling, as this neural pathway was the most significantly affected pathway by Cu in the current study (Fig. 5, neural signal transduction). One of the major impacts Cu has on many aquatic species is through oxidative stress and proteins or AAs that respond to this stress could also be used as biomarkers, such as Lmnb2 or ABC transporter proteins. Additionally, this study adds to a large body of literature regarding AAs that has consistently reported an increase in Pro from metal stress and so further research should aim to define normal and abnormal concentrations of Pro in a range of organisms for potential use as a biomarker. The current study identified biomarkers of Cu toxicity in zebrafish larvae, however excess Cu has different exposure routes in embryos and larvae than adult fish (Ganesan et al., 2016; Johnson et al., 2007). Therefore, future studies should determine if the DEPs (and Pro) identified in the current study as potential biomarkers also differ in adult fish in response to excess Cu, and hence, make suitable biomarkers of Cu contamination.

4. Conclusions

Zebrafish embryos are highly sensitive to Cu with EC_{10} and EC_{50} values of 3.7 and 10.9 μg L^{-1}, respectively. This study demonstrated that exposure to Cu at these environmentally realistic concentrations over short time periods (48 h and 96 h) causes measurable differences in the proteome and amino acid composition of zebrafish larvae. The interest in understanding the effects of metals on organisms at a molecular level is justified as disruption to multiple processes occurred in the zebrafish larvae. The greatest changes were observed at the earlier time point of 48 h, with the zebrafish seemingly able to either acclimatise to the Cu by 96 h or were displaying endpoints that could be lethal (LOE or non-hatching). At 48 h, disruption to neural signal transduction was shown to be the main processes affected by Cu and may be why some fish by 96 h displayed LOE and erratic swimming behaviour when exposed to Cu. This has repercussions for fish in the wild as disrupted swimming behaviourand altered neural functioning may affect and limit survival outcomes. Additionally, proteomic analysis of the zebrafish larvae revealed the differential expression of proteins that cause and protect against oxidative stress and apoptosis. Biomolecules including proteins and AAs are more sensitive than traditional endpoints to elevated concentrations of metals and, therefore, the monitoring and risk assessment of contaminated waterways should include detection at this sensitive level. For this, further research should explore whether the Cu affected proteins and AA pathways identified in the current study, are similar in other teleosts, to aid in the development of biomarkers of Cu toxicity to fish.

CRediT authorship contribution statement

Sarah L. Green: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Ewen Silvester:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision. **Sebastian Dworkin:** Methodology, Writing – review & editing. **Manisha Shakya:** Writing – review & editing. **Annaleise Klein:** Writing – review & editing. **Rohan Lowe:** Data curation, Formal analysis. **Keshava Datta:** Data curation. **Aleicia Holland:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2024.106963.

References

Abou Anni, I.S., Zebral, Y.D., Afonso, S.B., Moreno Abril, S.I., Lauer, M.M., Bianchini, A., 2019. Life-time exposure to waterborne copper III: effects on the energy metabolism of the killifish *Poecilia vivipara*. Chemosphere 227, 580–588. https://doi.org/10.1016/j.chemosphere.2019.04.080.

Acosta, D.D.S., Danielle, N.M., Altenhofen, S., Luzardo, M.D., Costa, P.G., Bianchini, A., Bonan, C.D., da Silva, R.S., Dafre, A.L., 2016. Copper at low levels impairs memory of adult zebrafish (*Danio rerio*) and affects swimming performance of larvae. Comp.

Biochem. Physiol. Part C Toxicol. Pharmacol. 185-186, 122–130. https://doi.org/10.1016/j.cbpc.2016.03.008.
Alsop, D., Wood, C.M., 2011. Metal uptake and acute toxicity in zebrafish. Common mechanisms across multiple metals. Aquat. Toxicol. 105 (3), 358–393. https://doi.org/10.1016/j.aquatox.2011.07.010.
ANZECC (Australian and New Zealand Environment and Conservation Council). (2000). *National Water Quality Management Strategy – Australian and New Zealand Guidelines For Fresh and Marine Water Quality*. (4).
Barnaby, W., Dorman Barclay, H.E., Nagarkar, A., Perkins, M., Teicher, G., Trapani, J.G., Downes, G.B., 2022. GABAA α subunit control of hyperactive behavior in developing zebrafish. Genetics 220 (4). https://doi.org/10.1093/genetics/iyac011.
Beaumont, M.W., Butler, P.J., Taylor, E.W., 1995. Exposure of brown trout, Salmo trutta, to sub-lethal copper concentrations in soft acidic water and its effect upon sustained swimming performance. Aquat. Toxicol. 33 (1), 45–63. https://doi.org/10.1016/0166-445X(95)00007-Q.
Beaumont, M.W., Butler, P.J., Taylor, E.W., 2000. Exposure of brown trout, *Salmo trutta*, to a sub-lethal concentration of copper in soft acidic water: effects upon muscle metabolism and membrane potential. Aquat. Toxicol. 51 (2), 259–272.
Berg, E.M., Bertuzzi, M., Ampatzis, K., 2018. Complementary expression of calcium binding proteins delineates the functional organization of the locomotor network. Brain Struct. Funct. 223 (5), 2181–2196. https://doi.org/10.1007/s00429-018-1622-4.
Besser, J., Dwyer, F., Ingersoll, C., Wang, N., 2001. Early Life-Stage Toxicity of Copper to Endangered and Surrogate Fish Species. U.S. Environmental Protection Agency, Ofiice of Research and Development, Gulf Breeze, Florida.
Bopp, S.K., Abicht, H.K., Knauer, K., 2008. Copper-induced oxidative stress in rainbow trout gill cells. Aquat. Toxicol. 86 (2), 197–204. https://doi.org/10.1016/j.aquatox.2007.10.014.
Brand, M.D., 2010. The sites and topology of mitochondrial superoxide production. Exp. Gerontol. 45 (7), 466–472. https://doi.org/10.1016/j.exger.2010.01.003.
Bury, N.R., Walker, P.A., Glover, C.N., 2003. Nutritive metal uptake in teleost fish. J. Exp. Biol. 206 (Pt 1), 11–23. https://doi.org/10.1242/jeb.00068.
Chen, E.Y., Tan, C.M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G.V., Clark, N.R., Ma'ayan, A., 2013. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinform. 14, 128. https://doi.org/10.1186/1471-2105-14-128.
Dai, Y.J., Jia, Y.F., Chen, N., Bian, W.P., Li, Q.K., Ma, Y.B., Chen, Y.L., Pei, D.S., 2014. Zebrafish as a model system to study toxicology. Environ. Toxicol. Chem. 33 (1), 11–17.
Della Torre, C., Zaja, R., Loncar, J., Smital, T., Focardi, S., Corsi, I., 2012. Interaction of ABC transport proteins with toxic metals at the level of gene and transport activity in the PLHC-1 fish cell line. Chem. Biol. Interact. 198 (1), 9–17. https://doi.org/10.1016/j.cbi.2012.04.008.
De Boeck, G., De Smet, H., Blust, R., 1995. The effect of sublethal levels of copper on oxygen consumption and ammonia excretion in the common carp, *Cyprinus carpio*. Aquat. Toxicol. 32 (2), 127–141. https://doi.org/10.1016/0166-445X(94)00086-6.
de Oliveria-Filho, E.C., Lopes, R.M., Paumgartten, F.J.R., 2004. Comparative study on the susceptability of freshwater species to copper-based pesticides. Chemosphere 56 (4), 369–374. https://doi.org/10.1016/j.envpol.2021.117536.
Domazou, A.S., Zelenay, V., Koppenol, W.H., Gebicki, J.M., 2012. Efficient depletion of ascorbate by amino acid and protein radicals under oxidative stress. Free Radic. Biol. Med. 53 (8), 1565–1573. https://doi.org/10.1016/j.freeradbiomed.2012.08.005.
Donnachie, R.L., Johnson, A.C., Moeckel, C., Pereira, M.G., Sumpter, J.P., 2014. Using risk-ranking of metals to identify which poses the greatest threat to freshwater organisms in the UK. Environ. Pollut. 194, 17–23. https://doi.org/10.1016/j.envpol.2014.07.008.
Eilertsen, M., Dolan, D.W.P., Bolton, C.M., Karlsen, R., Davies, W.I.L., Edvardsen, R.B., Furmanek, T., Sveier, H., Migaud, H., Helvik, J.V., 2022. Photoreception and transcriptomic response to light during early development of a teleost with a life cycle tightly controlled by seasonal changes in photoperiod. PLoS Genet. 18 (12), e1010529 https://doi.org/10.1371/journal.pgen.1010529.
Eisler, R., 1998. Copper Hazards to Fish, Wildlife, and Invertebrates: A Synoptic Review. US Department of the Interior, US Geological Survey.
Farkas, J., Svendheim, L.H., Jager, T., Ciesielski, T.M., Nordtug, T., Kvæstad, B., Hansen, B.H., Kristensen, T., Altin, D., Olsvik, P.A., 2021. Exposure to low environmental copper concentrations does not affect survival and development in Atlantic cod (Gadus morhua) early life stages. Toxicol. Rep. 8, 1909–1916. https://doi.org/10.1016/j.toxrep.2021.11.012.
Frøyset, A.K., Khan, E.A., Fladmark, K.E., 2016. Quantitative proteomics analysis of zebrafish exposed to sub-lethal dosages of β-methyl-amino-L-alanine (BMAA). Sci. Rep. 6 (1), 29631. https://doi.org/10.1038/srep29631.
García-Crespo, D., Vecino, E., 2004. Differential expression of calretinin in the developing and regenerating zebrafish visual system. Histol. Histopathol. 19 (4), 1193–1199. https://doi.org/10.14670/hh-19.1193.
Ganesan, S., Anaimalai Thirumurthi, N., Raghunath, A., Vijayakumar, S., Perumal, E., 2016. Acute and sub-lethal exposure to copper oxide nanoparticles causes oxidative stress and teratogenicity in zebrafish embryos. J. Appl. Toxicol. 36 (4), 554–567. https://doi.org/10.1002/jat.3224.
Goldberg, A.F.X., Moritz, O.L., Williams, D.S., 2016. Molecular basis for photoreceptor outer segment architecture. Prog. Retin. Eye Res. 55, 52–81. https://doi.org/10.1016/j.preteyeres.2016.05.003.
Gupta, Y.R., Sellegounder, D., Kannan, M., Deepa, S., Senthilkumaran, B., Basavaraju, Y., 2016. Effect of copper nanoparticles exposure in the physiology of the common carp (*Cyprinus carpio*): biochemical, histological and proteomic approaches. Aquac. Fish. 1, 15–23. https://doi.org/10.1016/j.aaf.2016.09.003.
Hall, W.S., Bushong, S.J., Hall, L.W., Lenkevich, M.J., Pinkney, A.E., 1988. Monitoring dissolved copper concentrations in Chesapeake Bay, U.S.A. Environ. Monit. Assess. 11 (1), 33–42. https://doi.org/10.1007/BF00394510.
Hansen, B.H., Rømma, S., Søfteland, L.I., Olsvik, P.A., Andersen, R.A., 2006. Induction and activity of oxidative stress-related proteins during waterborne Cu-exposure in brown trout (*Salmo trutta*). Chemosphere 65 (10), 1707–1714. https://doi.org/10.1016/j.chemosphere.2006.04.088.
Henry, D., Joselevitch, C., Matthews, G.G., Wollmuth, L.P., 2022. Expression and distribution of synaptotagmin family members in the zebrafish retina. Jo. Comp. Neurol. 530 (4), 705–728. https://doi.org/10.1002/cne.25238.
Hu, W., Culloty, S., Darmody, G., Lynch, S., Davenport, J., Ramirez-Garcia, S., Dawson, K.A., Lynch, I., Blasco, J., Sheehan, D., 2014. Toxicity of copper oxide nanoparticles in the blue mussel, *Mytilus edulis*: a redox proteomic investigation. Chemosphere 108, 289–299. https://doi.org/10.1016/j.chemosphere.2014.01.054.
Hughes, C.S., Moggridge, S., Müller, T., Sorensen, P.H., Morin, G.B., Krijgsveld, J., 2019. Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. Nat. Protoc. 14 (1), 68–85. https://doi.org/10.1038/s41596-018-0082-x.
Hurd, T.R., Requejo, R., Filipovska, A., Brown, S., Prime, T.A., Robinson, A.J., Fearnley, I.M., Murphy, M.P., 2008. Complex I within oxidatively stressed bovine heart mitochondria is glutathionylated on cys-531 and cys-704 of the 75-kda subunit: potential role of cys residues in decreasing oxidative damage. J. Biol. Chem. 283 (36), 24801–24815. https://doi.org/10.1074/jbc.M803432200.
Hutson, S.M., Sweatt, A.J., LaNoue, K.F., 2005. Branched-chain amino acid metabolism: implications for establishing safe intakes. J. Nutr. 135 (6), 1557S–1564S. https://doi.org/10.1093/jn/135.6.1557S.
Isani, G., Andreani, G., Carpenè, E., Di Molfetta, S., Eletto, D., Spisni, E., 2011. Effects of waterborne Cu exposure in gilthead sea bream (*Sparus aurata*): a proteomic approach. Fish Shellfish Immunol. 31 (6), 1051–1058. https://doi.org/10.1016/j.fsi.2011.09.005.
James, R., Sampath, K., Jothilakshmi, S., Vasudhevan, I., Thangarathinam, R., 2008. Effects of copper toxicity on growth, reproduction and metal accumulation in chosen ornamental fishes. Ecohydrol. Hydrobiol. 8 (1), 89–97. https://doi.org/10.2478/v10104-009-0007-y.
Javed, M., Usmani, N., 2019. An overview of the adverse effects of heavy metal contamination on fish health. Proc. Natl. Acad. Sci. India Sect. B Biol. Sci. 89 (2), 389–403. https://doi.org/10.1007/s40011-017-0875-7.
Jezierska, B., Ługowska, K., Witeska, M., 2009. The effects of heavy metals on embryonic development of fish (a review). Fish Physiol. Biochem. 35 (4), 625–640. https://doi.org/10.1007/s10695-008-9284-4.
Jiang, X., Wang, X., 2004. cytochrome c-mediated apoptosis. Annu. Rev. Biochem. 73 (1), 87–106. https://doi.org/10.1146/annurev.biochem.73.011303.073706.
Jin, W., Li, Z., Ran, F., Huang, S., Huo, K., Li, J., Han, Q., Wang, G., Wang, Z., Jian, S., Li, K., Li, C., 2021. Transcriptome analysis provides insights into copper toxicology in piebald naked carp (*Gymnocypris eckloni*). BMC Genom. 22 (1), 416. https://doi.org/10.1186/s12864-021-07673-4.
Johnson, A., Carew, E., Sloman, K.A., 2007. The effects of copper on the morphological and functional development of zebrafish embryos. Aquat. Toxicol. 84 (4), 431–438. https://doi.org/10.1016/j.aquatox.2007.07.003.
Jurd, R., Moss, S.J., 2010. Impaired GABA(A) receptor endocytosis and its correlation to spatial memory deficits. Commun. Integr. Biol. 3 (2), 176–178. https://doi.org/10.4161/cib.3.2.10742.
Kamunde, C.N., Wood, C., 2004. Environmental chemistry, physiological homeostasis, toxicology, and environmental regulation of copper, an essential element in freshwater fish. Australas. J. Ecotoxicol. 10, 1–20.
Kang, J., Lin, C., Chen, J., Liu, Q., 2004. Copper induces histone hypoacetylation through directly inhibiting histone acetyltransferase activity. Chem. Biol. Interact. 148 (3), 115–123. https://doi.org/10.1016/j.cbi.2004.05.003.
Kang, N., Kang, H.I., An, K.G., 2014. Analysis of fish DNA biomarkers as a molecular-level approach for ecological health assessments in an urban stream. Bull. Environ. Contam. Toxicol. 93 (5), 555–560.
Karri, V., Schuhmacher, M., Kumar, V., 2016. Heavy metals (Pb, Cd, As and MeHg) as risk factors for cognitive dysfunction: a general review of metal mixture mechanism in brain. Environ. Toxicol. Pharmacol. 48, 203–213. https://doi.org/10.1016/j.etap.2016.09.016.
Kassambara, A., & Mundt, F. (2020). Extract and visualise the results of multivariate data analyses. R package version 1.0.5.
Kasumyan, A., 2004. Vestibular system and sense of equilibrium in fish. J. Ichthyol. 44, S224–S268.
Katsiadaki, I., Ellis, T., Andersen, L., Antczak, P., Blaker, E., Burden, N., Fisher, T., Green, C., Labram, B., Pearson, A., Petersen, K., Pickford, D., Ramsden, C., Rønneseth, A., Ryder, K., Sacker, D., Stevens, C., Watanabe, H., Yamamoto, H., Sewell, F., Hawkins, P., Rufli, H., Handy, R.D., Maynard, S.K., Jacobs, M.N., 2021. Dying for change: a roadmap to refine the fish acute toxicity test after 40 years of applying a lethal endpoint. Ecotoxicol. Environ. Saf. 223, 112585 https://doi.org/10.1016/j.ecoenv.2021.112585.
Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev.Dyn. 203 (3), 253–310. https://doi.org/10.1002/aja.1002030302.
Kuipers, K.J.J., van Oers, L.F.C.M., Verboon, M., van der Voet, E, 2018. Assessing environmental implications associated with global copper demand and supply scenarios from 2010 to 2050. Glob. Environ. Chang. 49, 106–115. https://doi.org/10.1016/j.gloenvcha.2018.02.008.
Kuleshov, M.V., Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M., Lachmann, A., McDermott, M.G., Monteiro, C.D., Gundersen, G.W., Ma'ayan, A., 2016. Enrichr: a comprehensive gene

set enrichment analysis web server 2016 update. Nucleic Acids Res. 44 (W1), W90–W97. https://doi.org/10.1093/nar/gkw377.
Laberge, R.M., Karwatsky, J., Lincoln, M.C., Leimanis, M.L., Georges, E., 2007. Modulation of GSH levels in ABCC1 expressing tumor cells triggers apoptosis through oxidative stress. Biochem. Pharmacol. 73 (11), 1727–1737. https://doi.org/10.1016/j.bcp.2007.02.005.
Lê, S., Josse, J., Husson, F., 2008. FactoMineR: an R package for multivariate analysis. J. Stat. Softw. 25, 1–18.
Li, H.T., Feng, L., Jiang, W.D., Liu, Y., Jiang, J., Li, S.H., Zhou, X.Q., 2013. Oxidative stress parameters and anti-apoptotic response to hydroxyl radicals in fish erythrocytes: protective effects of glutamine, alanine, citrulline and proline. Aquat. Toxicol. 126, 169–179. https://doi.org/10.1016/j.aquatox.2012.11.005.
Li, P., Mai, K., Trushenski, J., Wu, G., 2009. New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. Amino Acids 37 (1), 43–53. https://doi.org/10.1007/s00726-008-0171-1.
Liang, X., Zhang, L., Natarajan, S.K., Becker, D.F., 2013. Proline mechanisms of stress survival. Antioxid. Redox Signal. 19 (9), 998–1011. https://doi.org/10.1089/ars.2012.5074.
Liemburg-Apers, D.C., Willems, P.H.G.M., Koopman, W.J.H., Grefte, S., 2015. Interactions between mitochondrial reactive oxygen species and cellular glucose metabolism. Arch. Toxicol. 89 (8), 1209–1226. https://doi.org/10.1007/s00204-015-1520-y.
Long, Y., Li, Q., Cui, Z., 2011. Molecular analysis and heavy metal detoxification of ABCC1/MRP1 in zebrafish. Mol. Biol. Rep. 38 (3), 1703–1711. https://doi.org/10.1007/s11033-010-0283-z.
Lüffe, T.M., D'Orazio, A., Bauer, M., Gioga, Z., Schoeffler, V., Lesch, K.P., Romanos, M., Drepper, C., Lillesaar, C., 2021. Increased locomotor activity via regulation of GABAergic signalling in foxp2 mutant zebrafish-implications for neurodevelopmental disorders. Transl. Psychiatry 11 (1), 529. https://doi.org/10.1038/s41398-021-01651-w.
Luzio, A., Monteiro, S.M., Fontaínhas-Fernandes, A.A., Pinto-Carnide, O., Matos, M., Coimbra, A.M., 2013. Copper induced upregulation of apoptosis related genes in zebrafish (*Danio rerio*) gill. Aquat. Toxicol. 128-129, 183–189. https://doi.org/10.1016/j.aquatox.2012.12.018.
Macoustra, G., Holland, A., Stauber, J., Jolley, D.F., 2019. effect of various natural dissolved organic carbon on copper lability and toxicity to the tropical freshwater microalga *Chlorella sp*. Environ. Sci. Technol. 53 (5), 2768–2777. https://doi.org/10.1021/acs.est.8b04737.
Macoustra, G.K., Koppel, D.J., Jolley, D.F., Stauber, J.L., Holland, A., 2021. Effect of dissolved organic matter concentration and source on the chronic toxicity of copper and nickel mixtures to *Chlorella sp*. Environ. Toxicol. Chem. 40 (7), 1908–1918. https://doi.org/10.1002/etc.5038.
Maher, P., 2001. How protein kinase C activation protects nerve cells from oxidative stress-induced cell death. J. Neurol. 21 (9), 2929–2938. https://doi.org/10.1523/jneurosci.21-09-02929.2001.
Martinesz Arbizu, P. (2017). pairwiseAdonis: pairwise miultilevel comparison using adonis. *R package, Version 0.4.1*.
McCluggage, F., Fox, A.H., 2021. Paraspeckle nuclear condensates: global sensors of cell stress? Bioessays 43 (5), 2000245. https://doi.org/10.1002/bies.202000245.
McDonald, S., Holland, A., Simpson, S.L., Gadd, J.B., Bennett, W.W., Walker, G.W., Keough, M.J., Cresswel, T., Hassell, K.L., 2022. Metal forms and dynamics in urban stormwater runoff: new insights from diffusive gradients in thin-films (DGT) measurements. Water Res. 209, 117967 https://doi.org/10.1016/j.watres.2021.117967.
McGeer, J.C., Szebedinszky, C., McDonald, D.G., Wood, C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: iono-regulatory disturbance and metabolic costs. Aquat. Toxicol. 50 (3), 231–243. https://doi.org/10.1016/S0166-445X(99)00105-8.
Mehta, S.K., Gaur, J.P., 1999. Heavy-metal-induced proline accumulation and its role in ameliorating metal toxicity in *Chlorella vulgaris*. New Phytol. 143 (2), 253–259. https://doi.org/10.1046/j.1469-8137.1999.00447.x.
Miwa, S., St-Pierre, J., Partridge, L., Brand, M.D., 2003. Superoxide and hydrogen peroxide production by *Drosophila* mitochondria. Free Radic. Biol. Med. 35 (8), 938–948. https://doi.org/10.1016/S0891-5849(03)00464-7.
Murphy, T.H., Miyamoto, M., Sastre, A., Schnaar, R.L., Coyle, J.T., 1989. Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. Neuron 2 (6), 1547–1558. https://doi.org/10.1016/0896-6273(89)90043-3.
Ngamchuea, K., Batchelor-McAuley, C., Compton, R.G., 2016. the copper(II)-catalyzed oxidation of glutathione. Chem. Eur. J. 22 (44), 15937–15944. https://doi.org/10.1002/chem.201603366.
OECD, 2013. Test No.236: Fish Embryo Acute Toxicity (FET) Test. OECD. https://doi.org/10.1787/9789264203709-en.
Oksanen, F.J., Simpson, G.L., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Solymos, P., Stevens, M.H.M., Szoecs, E., & Weedon, J. (2017). Vegan: community ecology package. *R package, Version 2*. 4–3.
Pawlak, S., Deckert, J., 2007. Histone modifications under environmental stress. Biol. Lett. 44 (2), 65–73.
Pereverzev, M.O., Vygodina, T.V., Konstantinov, A.A., Skulachev, V.P., 2003. Cytochrome c, an ideal antioxidant. Biochem. Soc. Trans. 31 (Pt 6), 1312–1315. https://doi.org/10.1042/bst0311312.
Perkins, A., Nelson, K.J., Parsonage, D., Poole, L.B., Karplus, P.A., 2015. Peroxiredoxins: guardians against oxidative stress and modulators of peroxide signaling. Trends Biochem. Sci. 40 (8), 435–445. https://doi.org/10.1016/j.tibs.2015.05.001.
Pratt, D., Chen, J., Welker, D., Rivas, R., Pillich, R., Rynkov, V., Ono, K., Miello, C., Hicks, L., Szalma, S., Stojmirovic, A., Dobrin, R., Braxenthaler, M., Kuentzer, J., Demchak, B., Ideker, T., 2015. NDEx, the network data exchange. Cell Syst. 1 (4), 302–305. https://doi.org/10.1016/j.cels.2015.10.001.
Redza-Dutordoir, M., Averill-Bates, D.A., 2016. Activation of apoptosis signalling pathways by reactive oxygen species. Biochim. Biophys. Acta 1863 (12), 2977–2992. https://doi.org/10.1016/j.bbamcr.2016.09.012.
Reyland, M.E., 2009. Protein kinase C isoforms: multi-functional regulators of cell life and death. Front. Biosci. 14 (6), 2386–2399. https://doi.org/10.2741/3385 (Landmark Ed).
Riethmuller, N., 2000. *The Effect of True Water Hardness and Alkalinity on the Toxicity of Cu and U to Two Tropical Australian Freshwater Organisms* (Publication Number 28907729. ProQuest One Academic, Ann Arbor [Master's, Charles Darwin University (Australia)].
Rinner, O., Makhankov, Y.V., Biehlmaier, O., Neuhauss, S.C., 2005. Knockdown of cone-specific kinase GRK7 in larval zebrafish leads to impaired cone response recovery and delayed dark adaptation. Neuron 47 (2), 231–242. https://doi.org/10.1016/j.neuron.2005.06.010.
Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2016. dose-response analysis using R. PLoS ONE 10 (12), e0146021. https://doi.org/10.1371/journal.pone.0146021.
Rufli, H., 2012. Introduction of moribund category to OECD fish acute test and its effect on suffering and LC50 values. Environ. Toxicol. Chem. 31 (5), 1107–1112. https://doi.org/10.1002/etc.1779.
Sánchez-Rojas, T., Espinoza-Culupú, A., Ramírez, P., Iwai, L.K., Montoni, F., Macedo-Prada, D., Sulca-López, M., Durán, Y., Farfán-López, M., Herencia, J., 2022. Proteomic study of response to copper, cadmium, and chrome ion stress in *Yarrowia lipolytica* strains isolated from andean mine tailings in peru. Microorganisms 10 (10). https://doi.org/10.3390/microorganisms10102002.
Sanchez, W., Palluel, O., Meunier, L., Coquery, M., Porcher, J.M., Aït-Aïssa, S., 2005. Copper-induced oxidative stress in three-spined stickleback: relationship with hepatic metal levels. Environ. Toxicol. Pharmacol. 19 (1), 177–183. https://doi.org/10.1016/j.etap.2004.07.003.
Santos, D., Félix, L., Luzio, A., Parra, S., Cabecinha, E., Bellas, J., Monteiro, S.M., 2020. Toxicological effects induced on early life stages of zebrafish (*Danio rerio*) after an acute exposure to microplastics alone or co-exposed with copper. Chemosphere 261, 127748. https://doi.org/10.1016/j.chemosphere.2020.127748.
Santos, D., Luzio, A., Félix, L., Cabecinha, E., Bellas, J., Monteiro, S.M., 2022. Microplastics and copper induce apoptosis, alter neurocircuits, and cause behavioral changes in zebrafish (*Danio rerio*) brain. Ecotoxicol. Environ. Saf. 242, 113926 https://doi.org/10.1016/j.ecoenv.2022.113926.
Sarfare, S., McKeown, A.S., Messinger, J., Rubin, G., Wei, H., Kraft, T.W., Pittler, S.J., 2014. Overexpression of rod photoreceptor glutamic acid rich protein 2 (GARP2) increases gain and slows recovery in mouse retina. Cell Commun. Signal. 12, 67. https://doi.org/10.1186/s12964-014-0067-5.
Shakya, M., Holland, A., Klein, A.R., Rees, G.N., Laird, J., McCallum, J.C., Ryan, C.G., Silvester, E., 2022. Biomolecular modifications in the sacfry of *Mogurnda adspersa* in response to copper stress. Aquat. Toxicol. 248, 106179 https://doi.org/10.1016/j.aquatox.2022.106179.
Shakya, M., Silvester, E., Holland, A., Rees, G., 2021a. Taxonomic, seasonal and spatial variation in the amino acid profile of freshwater macroinvertebrates. Aquat. Sci. 83 (2) https://doi.org/10.1007/s00027-021-00789-5.
Shakya, M., Silvester, E., Rees, G., Stitz, L., Holland, A., 2021b. Spatial variation in the amino acid profile of four macroinvertebrate taxa along a highly polluted river. Environ. Pollut. 284, 117536 https://doi.org/10.1016/j.envpol.2021.117536.
Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13 (11), 2498–2504.
Shimi, T., Goldman, R.D., 2014. Nuclear lamins and oxidative stress in cell proliferation and longevity. Adv. Exp. Med. Biol. 773, 415–430. https://doi.org/10.1007/978-1-4899-8032-8_19.
Shuhaimi-Othman, M., Yakub, N., Ramle, N.A., Abas, A., 2015. Comparative toxicity of eight metals on freshwater fish. Toxicol. Ind. Health 31 (9), 773–782. https://doi.org/10.1177/0748233712472519.
Sonnack, L., Klawonn, T., Kriehuber, R., Hollert, H., Schäfers, C., Fenske, M., 2018. Comparative analysis of the transcriptome responses of zebrafish embryos after exposure to low concentrations of cadmium, cobalt and copper. Comp. Biochem. Physiol. Part D Genom. Proteom. 25, 99–108. https://doi.org/10.1016/j.cbd.2017.12.001.
Srinivasan, S., Avadhani, N.G., 2012. Cytochrome c oxidase dysfunction in oxidative stress. Free Radic. Biol. Med. 53 (6), 1252–1263. https://doi.org/10.1016/j.freeradbiomed.2012.07.021.
Stehr, C.M., Linbo, T.L., Incardona, J.P., Scholz, N.L., 2006. The developmental neurotoxicity of fipronil: notochord degeneration and locomotor defects in zebrafish embryos and larvae. Toxicol. Sci. 92 (1), 270–278. https://doi.org/10.1093/toxsci/kfj185.
Stout, K.A., Dunn, A.R., Hoffman, C., Miller, G.W., 2019. The synaptic vesicle glycoprotein 2: structure, function, and disease relevance. ACS Chem. Neurosci. 10 (9), 3927–3938. https://doi.org/10.1021/acschemneuro.9b00351.
Szklarczyk, D., Kirsch, R., Koutrouli, M., Nastou, K., Mehryary, F., Hachilif, R., Gable, A. L., Fang, T., Doncheva, N.T., Pyysalo, S., Bork, P., Jensen, L.J., von Mering, C., 2023. The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest. Nucleic Acids Res. 51 (D1) https://doi.org/10.1093/nar/gkac1000. D638-d646.
Titilawo, Y., Adeniji, A., Adeniyi, M., Okoh, A., 2018. Determination of levels of some metal contaminants in the freshwater environments of Osun State, Southwest Nigeria: a risk assessment approach to predict health threat. Chemosphere 211, 834–843. https://doi.org/10.1016/j.chemosphere.2018.07.203.

Tokudome, K., Okumura, T., Shimizu, S., Mashimo, T., Takizawa, A., Serikawa, T., Terada, R., Ishihara, S., Kunisawa, N., Sasa, M., Ohno, Y., 2016. Synaptic vesicle glycoprotein 2A (SV2A) regulates kindling epileptogenesis via GABAergic neurotransmission. Sci. Rep. 6 (1), 27420. https://doi.org/10.1038/srep27420.

Tripathi, B.N., Gaur, J.P., 2004. Relationship between copper- and zinc-induced oxidative stress and proline accumulation in *Scenedesmus sp.* Planta 219 (3), 397–404. https://doi.org/10.1007/s00425-004-1237-2.

Turrens, J.F., 2003. Mitochondrial formation of reactive oxygen species. J. Physiol. 552 (Pt 2), 335–344. https://doi.org/10.1113/jphysiol.2003.049478.

USEPA, 1984. Ambient Water Quality Criteria for Copper. United States Environmental Protection Agency, Washington DC.

van Beers, D., Graedel, T.E., 2007. Spatial characterisation of multi-level in-use copper and zinc stocks in Australia. J. Clean. Prod. 15 (8), 849–861. https://doi.org/10.1016/j.jclepro.2006.06.022.

Villanueva, R.A.M., Chen, Z.J., 2019. ggplot2: elegant graphics for data analysis (2nd ed.). Meas. Interdiscip. Res. Perspect. 17 (3), 160–167. https://doi.org/10.1080/15366367.2019.1565254.

Vutukuru, S.S., Suma, C., Madhavi, K.R., Juveria, J., Pauleena, J.S., Rao, J.V., Anjaneyulu, Y., 2005. Studies on the development of potential biomarkers for rapid assessment of copper toxicity to freshwater fish using *Esomus danricus* as model. Int. J. Environ. Res. Public Health 2 (1), 63–73. https://www.mdpi.com/1660-4601/2/1/63.

Wang, R.F., Zhu, L.M., Zhang, J., An, X.P., Yang, Y.P., Song, M., Zhang, L., 2020. Developmental toxicity of copper in marine medaka (*Oryzias melastigma*) embryos and larvae. Chemosphere 247, 125923. https://doi.org/10.1016/j.chemosphere.2020.125923.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. R package.

Wu, J.T., Hsieh, M.T., Kow, L.C., 1998. Role of proline accumulation in response to toxic copper in *Chlorella sp.* (chlorophyceae) cells. J. Phycol. 34 (1), 113–117. https://doi.org/10.1046/j.1529-8817.1998.340113.x.

Zang, J., Neuhauss, S.C.F., 2021. Biochemistry and physiology of zebrafish photoreceptors. Pflüg. Arch. Eur. J. Physiol. 473 (9), 1569–1585. https://doi.org/10.1007/s00424-021-02528-z.

Zhang, X., Smits, A., van Tilburg, G., Ovaa, H., Huber, W., Vermeulen, M., 2018. Proteome-wide identification of ubiquitin interactions using UbIA-MS. Nat. Protoc. 13, 530–550.

Zhao, G., Wang, Z., Xu, L., Xia, C.X., Liu, J.X., 2019. Silver nanoparticles induce abnormal touch responses by damaging neural circuits in zebrafish embryos. Chemosphere 229, 169–180. https://doi.org/10.1016/j.chemosphere.2019.04.223.

Zhu, Y.J., Zhu, X.Y., Xu, Q.J., Qian, Y.H., 2022. Water quality criteria and ecological risk assessment for copper in Liaodong Bay, China. Mar. Pollut. Bull. 185, 114164.

Zorova, L.D., Popkov, V.A., Plotnikov, E.Y., Silachev, D.N., Pevzner, I.B., Jankauskas, S.S., Babenko, V.A., Zorov, S.D., Balakireva, A.V., Juhaszova, M., Sollott, S.J., Zorov, D.B., 2018. Mitochondrial membrane potential. Anal. Biochem. 552, 50–59. https://doi.org/10.1016/j.ab.2017.07.009.