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Hypoxia Suppressed Copper Toxicity during Early Development in Zebrafish Embryos in a Process Mediated by the Activation of the HIF Signaling Pathway

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Supporting Information

ABSTRACT: Hypoxia is a global and increasingly important stressor in aquatic ecosystems, with major impacts on biodiversity worldwide. Hypoxic waters are often contaminated with a wide range of chemicals but little is known about the interactions between these stressors. We investigated the effects of hypoxia on the responses of zebrafish (*Danio rerio*) embryos to copper, a widespread aquatic contaminant. We showed that during continuous exposures copper toxicity was reduced by over 2-fold under hypoxia compared to normoxia. When exposures were conducted during 24 h windows, hypoxia reduced copper toxicity during early development and increased its toxicity in hatched larvae. To investigate the role of the hypoxia signaling pathway on the suppression of copper toxicity during early development, we stabilized the hypoxia inducible factor (HIF) pathway under normoxia using a prolyl-4-hydroxylase inhibitor, dimethyloxalylglycine (DMOG) and demonstrated that HIF activation results in a strong reduction in copper toxicity. We also established that the reduction in copper toxicity during early development was independent of copper uptake, while after hatching, copper uptake was increased under hypoxia, corresponding to an increase in copper toxicity. These findings change our understanding of the current and future impacts of worldwide oxygen depletion on fish communities challenged by anthropogenic toxicants.

INTRODUCTION

Hypoxia is one of the most significant stressors affecting aquatic systems worldwide, and its severity and prevalence are projected to rise due to increases in nutrient input and climate change.[1] With rapid industrialization and population growth, agricultural, industrial and domestic effluents containing a wide range of potentially toxic chemicals and nutrients are also increasingly being discharged into aquatic systems, with long-term consequences for aquatic organisms.[2] Therefore, environmental pollutants and hypoxia often co-occur in aquatic systems and, consequently, their potential interacting effects on wildlife must be considered.

To date, few studies have investigated whether chemical toxicity to fish is modified by the availability of oxygen in the water. The chemicals considered in existing studies include polyaromatic hydrocarbons,[3] polychlorinated biphenyls,[3−9] phenols,[10,11] ammonia,[10,12] estrogenic chemicals[13] and toxic metals,[10,14−18] and evidence suggests that alterations in chemical toxicity are highly likely to occur. However, data are often contradictory and hypoxia-induced changes in chemical toxicity appear to vary widely as a function of the chemicals being considered, the model species and its life stage, highlighting this as an essential area for further research.

Among aquatic contaminants, metals are particularly widespread and reach highly toxic concentrations in areas associated with mining and industrial activities.[19] Recent analysis of the relative threat posed by metals to aquatic organisms has identified copper as the most significant metal pollutant in UK waters.[20] Existing studies focusing on the toxicological effects of metals in combination with hypoxia have included copper,[2] cadmium,[15,16] zinc,[10,21] nickel[17] and lead,[10] and have found a suppression of the natural response to hypoxia in the presence of metals, or increased metal toxicity. For copper (Cu), limited data is available but, generally, an increase in toxicity has been suggested. For example, for carp, copper toxicity was shown to increase when exposures occurred under hypoxia,[2,22] and

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similarly, in the mayfly, *Ephoron virgo*, copper-induced mortality increased under hypoxia.[23] However, these studies have not provided any insight on the mechanisms responsible for hypoxia-induced alterations in the observed toxicity.

Here, we present the first study, to our knowledge, investigating the influence of combined exposure to hypoxia and copper on embryonic development, using the zebrafish (*Danio rerio*) as a model fish species. Embryos are particularly vulnerable to chemical exposures due to the sensitive nature of the developmental processes during embryogenesis. In addition, fish embryos are more likely to be exposed to hypoxic conditions than other life stages: eggs of many fish species are deposited in areas of slow water flow and/or high nutrient input, where the co-occurrence of environmental contaminants and hypoxia are likely. Furthermore, embryos lack the ability to avoid unfavorable conditions by moving away from contaminated areas, and rely principally on biochemical response pathways to survive periods of hypoxia. This study aimed to determine the effects of hypoxia on copper toxicity throughout this vulnerable life stage, and the relative susceptibility of developing embryos at various stages of development to these combined stressors. Further, the mechanisms responsible for the effects of hypoxia on copper toxicity were investigated in order to generate a mechanistic understanding of the interactions between copper toxicity and hypoxia, helping to support predictive toxicology in the future.

MATERIAL AND METHODS

Copper Exposures under Normoxia and Hypoxia. Eggs were collected from a breeding population of zebrafish (wild-type WIK strain) according to the procedures described in SI. Fertilized embryos (20 embryos per tank, triplicate tanks per copper concentration) were exposed to concentrations of copper ranging from 0 to 0.1 mg Cu/L from 4 to 100 hpf, to generate cumulative mortality curves under normoxic and hypoxic conditions. For exposures conducted under normoxia (98.4% ± 0.12 air saturation), water was aerated for 1 h before the start of the exposures, and allowed to equilibrate to 28 °C. For exposures conducted under hypoxia (45.3% ± 0.21 air saturation), water was aerated with nitrogen for 1 h, to remove dissolved oxygen, allowed to equilibrate to 28 °C and then mixed with aerated water at the appropriate proportion to obtain the desirable level of air saturation. All tanks were filled with 600 mL of water containing the appropriate air saturation and copper concentration. A large volume of water (30 mL of water per embryo) was used to avoid changes in the water characteristics caused by the metabolic activity of the embryos. For hypoxia treatments, tanks were sealed with a glass plate to prevent gas exchange and reoxygenation of the water. After each 24 h exposure period, the percentage of air saturation was immediately measured in each exposure tank using a calibrated oxygen meter, according to the manufacturer's instruction (Stathkelvin Instruments Oxygen Meter, model 781, UK). Mortalities and hatching (for the 76 and 100 hpf observations) were recorded for each tank. After observations were completed at the end of each 24 h period, water was completely replaced with freshly made exposure water at the appropriate air saturation and copper concentration, as described above.

To investigate the susceptibility of the various stages of embryo development to combinations of copper and hypoxia, exposures were conducted during specific developmental windows at 24 h intervals (4–28, 28–52, 52–76 and 76–100 hpf) to form mortality curves, using a range of copper concentrations (from 0.01 to 0.4 mg Cu/L), under hypoxia and normoxia. These concentrations include environmentally relevant concentrations common in contaminated environments. Embryos (20 per tank) were incubated under control conditions (98.3% ± 0.16 air saturation, 0 mg Cu/L) up to the start of the exposure period, and terminated immediately after the experiments. The percentage of mortalities was recorded after each 24 h exposure experiment, and the percentage of hatched embryos was recorded for the 52–76 hpf exposure window. All experiments were conducted in triplicate, with the exception of the exposures conducted during the developmental period of 76–100 hpf period, which were carried out in quadruplicate.

Effects of the Biochemical Activation of the HIF Pathway on Copper Toxicity during Early Development. We exposed embryos to copper in the presence of a prolyl-4-hydroxylase inhibitor, dimethyloxalylglycine (DMOG), which suppresses oxygen-induced HIF degradation, therefore activating the HIF signaling pathway independently of the presence of oxygen.[24] Embryos were exposed to either 0 or 0.07 mg Cu/L in normoxic water or in water containing 20 μM DMOG (D3695 SIGMA, UK). In parallel, embryos were also exposed to hypoxia alone, and to hypoxia in combination with 0.07 mg Cu/L. Each exposure tank contained 100 mL of exposure water and 10 embryos, and 6 independent tank replicates were included for each treatment group. The concentration of DMOG used was chosen based on a preliminary experiment where a range of concentrations (0.2 to 200 μM) were tested in comparison with a range of hypoxia treatments. The concentration selected was the highest concentration of DMOG where no developmental effects were observed, resembling the level of hypoxia used in this experiment (49.6% ± 0.51 air saturation) that also does not cause any measurable developmental effects in exposed embryos.

Copper Uptake and Quantification of Gene Expression. We hypothesized that hypoxia may cause changes in copper uptake, resulting in differential toxicity. To investigate this, embryos were exposed to 0 or 0.024 mg Cu/L (this concentration caused approximately 10% mortality in the continuous copper exposure) for 24 h under hypoxic or normoxic conditions, for the 4–28, 28–52, 52–76, 76–100 hpf developmental windows, as described above. Copper concentrations in exposed embryos and in the water were measured by ICP-MS. A full description of the experimental setup, sample collection and copper measurements is provided in SI.

Real-time quantitative PCR (RT-QPCR) was used to quantify the transcript profiles of exposed embryos for target genes known to be involved in the responses to copper and/or hypoxia in fish. These included genes involved in pH regulation and gas transport (carbonic anhydrase II (*ca2*), carbonic anhydrase IX (*ca9*)), copper uptake, transport and/or storage (cytochrome c oxidase copper chaperone (*cox17*), ATPase Cu^{2+} transporting, alpha polypeptide (*atp7a*), metallothionein 2 (*mt2*)) and oxidative stress (catalase (*cat*), superoxidase dismutase 1 (*sod1*), glutathione-s-transferase pi 1 (*gstp1*), glutathione S-transferease alpha-like (*gstaI*) and glutathione peroxidase 1 a (*gpx1a*)). Ribosomal protein l8 (*rpl8*) was used as a control gene for normalization purposes. This gene has been shown to remain stable across tissue types and experimental conditions,[25] including under hypoxia in cyprinids[26] and during embryogenesis in zebrafish, in the presence or absence of exposure to silver.[27] Quantitative RT-QPCR assays for each target gene were optimized as previously

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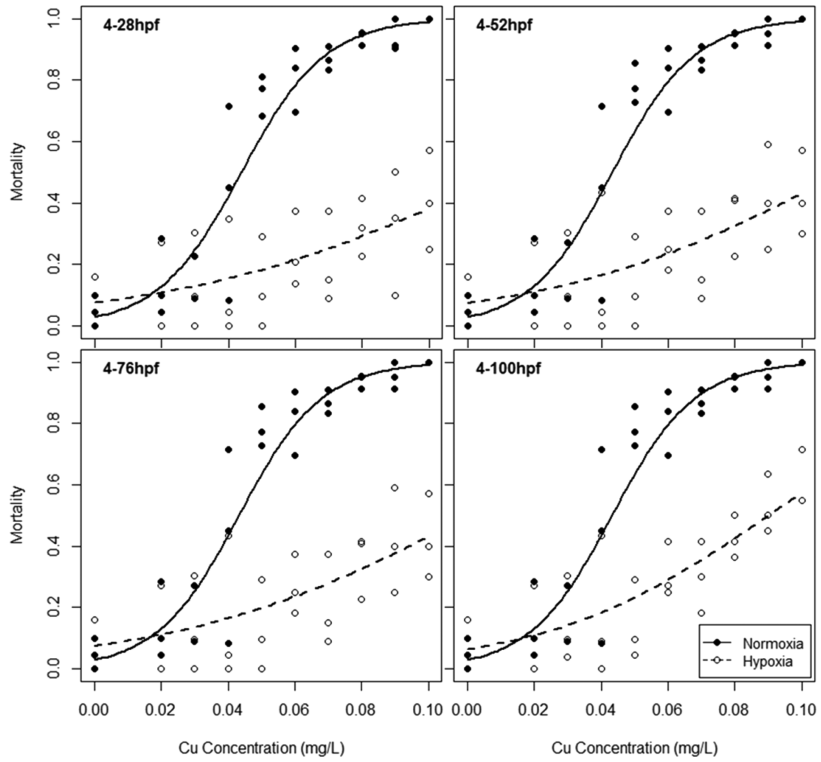

Figure 1. Embryo mortality curves following continuous exposure to copper under normoxia or hypoxia throughout development. Each point on the graph represents the proportion of mortality in an individual replicate tank containing 20 embryos, black and white symbols represent groups exposed to copper under normoxia (98.4% ± 0.12 air saturation) or hypoxia (45.3% ± 0.21 air saturation), respectively, and the lines represent the best fit model for the data, calculated using generalized linear models in R (model output summarized in Table S1a). At the concentrations tested, copper caused an increase in mortality both under hypoxia and normoxia over the whole exposure period ($P < 0.001$). There was a significant difference in copper-induced mortality under normoxia compared to under hypoxia for all time points ($P < 0.001$) and the slope of the dose response curves also differed for all time points ($P < 0.001$).

described[28] and detailed information for each assay is provided in Table S1. A detailed description of these methods is given in SI.

Statistical Analysis. Statistical analysis to test for differences between the proportion of mortality and hatching following exposure to copper under either hypoxia or normoxia were conducted using generalized linear models in R.[29] A separate model was carried out for each time period after fertilization, using a quasibinomial error structure and logit link to test for effects of copper concentration on the proportion of mortality (as a continuous variable), hypoxia or normoxia (as a categorical variable) and the interaction between the two. Minimum adequate models were derived by model simplification using F tests based on analysis of deviance.[30] A similar approach of model simplification of generalized linear models with quasibinomial error structure was used to test for the effects on the proportion of hatching of copper, hypoxia or normoxia and their interaction. F tests reported refer to the significance of removing terms from the models.

For the data investigating the effects of DMOG, a Kruskal–Wallis test was used to test for overall treatment effects, followed by pairwise Wilcoxon tests correcting for multiple comparisons using the Holm method. Gene expression data was first scrutinized by Chauvenet's criterion to detect outliers for each gene and these were subsequently removed.[31] For both transcript profiles and quantification of copper in exposed embryos, data that did not meet the normality and equal variance criteria was log transformed before a one-way analysis of variance was performed. When a significant effect was identified, pairwise comparisons to determine which groups differed were conducted using the Holm–Sidak post hoc test. All data was considered statistically significant when $p < 0.05$.

RESULTS

Copper Toxicity throughout Development under Hypoxic and Normoxic Conditions. Copper caused mortalities to zebrafish embryos under both hypoxic (45.3% ± 0.21 air saturation) and normoxic (98.4% ± 0.12 air saturation) conditions. However, there were striking differences in the effects of copper when exposures were conducted under hypoxia compared to normoxia, with greater toxicity observed under normoxia throughout development ($P < 0.001$; Figure 1; Table S2). For exposures conducted under normoxic conditions, the vast majority of copper-induced mortalities occurred during the 4–28 hpf exposure period (Figure 1). Between 4–52 hpf and 4–76 hpf, there were no additional mortalities for either normoxic or hypoxic treatments (Figure 1). Under hypoxia, copper-induced mortality increased after 76 hpf, but remained significantly lower than under normoxia throughout the experiment (Figure 1; Table S2).

Copper caused a significant delay in hatching for exposures conducted under normoxia ($P < 0.01$), but not under hypoxia ($P > 0.05$; Figure S1; Table S3a). This delay in hatching was significantly greater for exposures conducted under normoxia compared to hypoxia at 76 hpf ($P < 0.05$; Figure S1a) and at the end of the exposure period (100 hpf; $P < 0.001$; Figure S1b; Table S3a).

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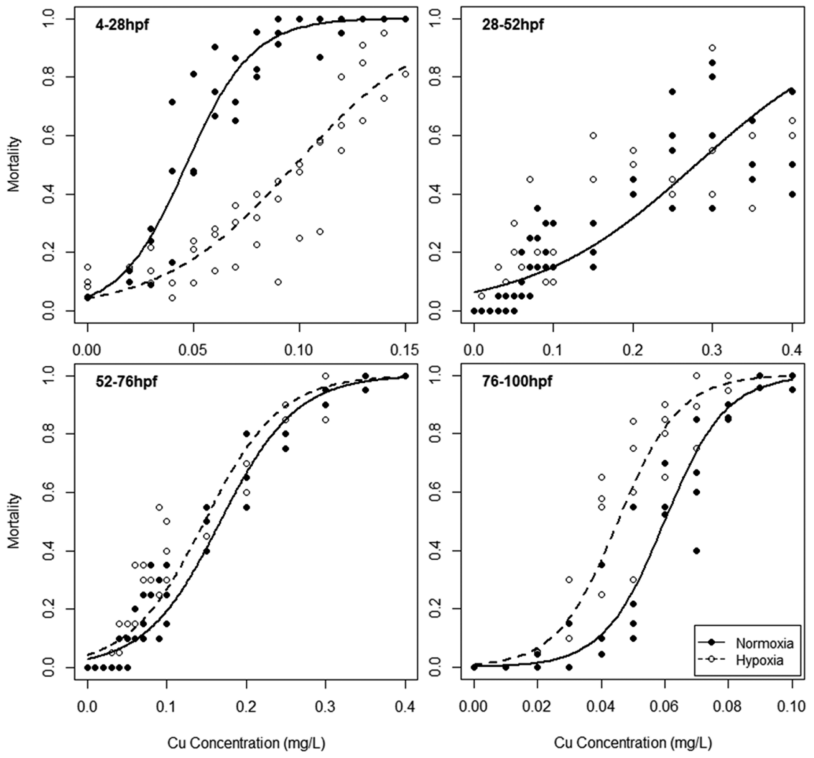

Figure 2. Embryo mortality curves following exposure to copper under normoxia or hypoxia during specific developmental windows. Each point on the graph represents an individual replicate tank containing 20 embryos, black and white symbols represent tanks exposed to copper under normoxia (98.4% ± 0.12 air saturation) or hypoxia (45.3% ± 0.21 air saturation), respectively, and the lines represent the best fit model for the data, calculated using generalized linear models in R (model output summarized in Table S1b). At the concentrations tested, copper caused an increase in mortality both under hypoxia and normoxia in all exposure windows ($P < 0.001$). There was significantly higher mortality following exposure to copper under normoxia compared to hypoxia for embryos exposed during the 4−28 hpf developmental window ($P < 0.001$), and the slope of the dose response curve also differed for hypoxia versus normoxia ($P < 0.001$; Table S1b). In contrast, a significant increase in copper-induced mortality under hypoxia compared to normoxia was observed for embryos exposed during the 52−76 and 76−100 hpf exposure windows ($P < 0.01$, $P < 0.001$ respectively; Table S1b).

Stage-Dependent Copper Toxicity under Hypoxia and Normoxia. For the experiments conducted during the 24 h time windows, similarly to that reported for the continuous exposures, during the 4−28 hpf exposure period copper was more toxic to embryos under normoxia than under hypoxia ($P < 0.001$; Figure 2; Table S2b). In contrast, for exposures conducted during the 28−52 hpf interval, there was no significant difference in copper toxicity between the two different oxygen concentrations ($P = 0.39$; Figure 2). Furthermore, for both normoxic and hypoxic conditions, copper was less toxic during this developmental stage than during the 4−28 h exposure period (Figure 2). For example, 0.1 mg Cu/L caused 13% mortalities when exposures occurred between 28 and 52 hpf for both hypoxia and normoxia, whereas the same concentration resulted in 97% mortality under normoxia and 49% mortality under hypoxia when exposures to copper occurred between 4 and 28 hpf (Figure 2).

In contrast to the results for the 4−28 hpf, during the 52−76 hpf and 76−100 hpf exposure windows (which correspond to the periods immediately prior and after hatching) copper toxicity was greater when exposures occurred under hypoxia compared to under normoxia ($P < 0.01$; Figure 2, Table S2b). In addition, the sensitivity of zebrafish embryos to copper increased until the 76−100 hpf period (immediately after hatching), both under hypoxia and normoxia.

Similarly to that observed during the continuous exposure, the effects of copper on hatching rate were greater in exposures conducted under normoxia compared to under hypoxia. ($P < 0.001$; Figure S2; Table S3b).

Role of the HIF Signaling Pathway on the Suppression in Copper Toxicity under Hypoxia during Early Development. Under normoxia, exposure to 0.07 mg Cu/L resulted in 75.5% mortalities, whereas under hypoxia, copper-induced mortalities did not occur (1.7%; similar to control levels). In the presence of 20 μM DMOG, exposure to 0.07 mg Cu/L under normoxia resulted in 0% mortalities (Figure 3), supporting the hypothesis that the activation of the HIF pathway is responsible for the decreased copper toxicity observed under hypoxia, during the 4−28 hpf window of development.

Quantification of Copper Uptake. There was a very significant increase in the concentration of copper in whole zebrafish embryos exposed to copper during 4−28 hpf window of development, compared to nonexposed embryos ($P < 0.001$; Figure 4a), independent of the oxygen concentration in the water. Similar results were observed for the 28−52 hpf exposure window ($P < 0.001$; Figure 4c). To determine the relative contribution of the chorion to the copper accumulation seen in exposed embryos, we analyzed the concentration of copper in embryos that were dechorinated after exposure. For both the 4−28 hpf and 28−52 hpf exposure windows, there was no significant change in copper concentrations in dechorinated embryos, irrespective of copper treatment or oxygen concentration ($P = 0.702$ and $P = 0.110$; Figure 4b and Figure 4d,

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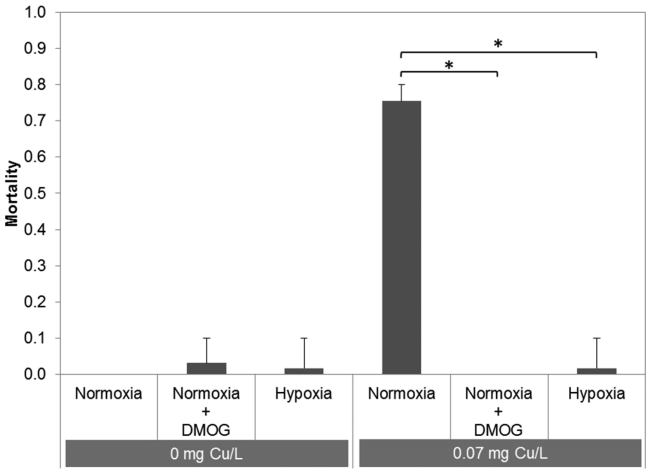

Figure 3. Effects of HIF activation by dimethyloxalylglycine (DMOG) on copper toxicity. Exposures to copper under normoxia, under normoxia in combination with DMOG, and under hypoxia were conducted during the 4−28 hpf developmental period. Six independent tanks containing 10 embryos and 100 mL of exposure water were included for each treatment group. The measured concentrations of oxygen were 98.7% and 49.6% air saturation for normoxia and hypoxia, respectively. Data is presented as mean proportion of embryo mortalities during the exposure + maximum value. For embryos incubated in the absence of copper, there was no significant effect of the treatments on mortality (Kruskal−Wallis test, $\chi^2 = 2.13$, DF = 2, $P = 0.34$), with no significant differences between the three treatments ($P > 0.05$). For embryos incubated in the presence of copper (0.07 mg Cu/L), there was a significant effect of treatment on mortality (Kruskal−Wallis test, $\chi^2 = 14.94$, DF = 2, $P < 0.001$), with significant differences occurring between groups exposed to copper under normoxia compared with (i) those exposed to copper under normoxia in the presence of DMOG ($P < 0.01$), and (ii) those exposed to copper under hypoxia ($P < 0.01$). No differences were detected between groups exposed to copper under normoxia in the presence of DMOG and groups exposed to copper under hypoxia ($P = 0.41$).

respectively). Similarly, for exposures conducted during the 52−76 hpf exposure window, there were no significant differences in copper concentration between any treatment groups ($P = 0.638$; Figure 4e). For hatched embryos exposed between 76 and 100 hpf, a significant increase in copper concentration was observed in embryos exposed to copper under hypoxia compared to the hypoxia control ($P = 0.020$; Figure 4f), but no difference in copper concentration in embryos exposed to copper under normoxia were observed ($P = 0.299$; Figure 4f).

Transcript Profiling. The majority of the alterations in transcript profiles observed occurred in embryos exposed to copper during the 28−52 hpf exposure period and predominantly under normoxia. Transcripts involved in the response to oxidative stress were the most significantly affected by the exposures. Significant down-regulations in transcript profiles were measured for *gstp1*, *gsta1* *gpx1a*, following exposure to copper during the 28−52 hpf exposure period, under normoxia, but not under hypoxia (Figure 5; Figure S4). In contrast, for *cat*, a significant down-regulation was observed following exposure to copper under hypoxia, but not under normoxia (Figure 5; Figure S4). In addition, *gstaI* and *gstp1* were significantly down-regulated following exposure to copper under normoxia, but not under hypoxia for the 52−76 hpf and 76−100 hpf exposure periods, respectively (Figure 5; Figures S5−6).

For transcripts involved in copper transport and binding, a complex pattern of response was observed. The metal binding protein, *mt2*, was significantly up-regulated in embryos maintained under hypoxia alone compared to those kept under normoxia, at the end of the 52−76 hpf exposure period, but this gene was unaffected by the copper exposure throughout this study (Figure 5; Figure S5). *cox17*, a gene involved in metal coupling, was significantly down-regulated following exposure to copper under normoxia but not under hypoxia, during the 28−52 hpf period (Figure 4). In addition, the copper transporter, *atp7a*, was not affected by the exposure conditions at any of the developmental stages analyzed (Figure 5).

Genes involved in carbon dioxide (CO_2) dynamics and pH regulation were also investigated. *ca9* was significantly down-regulated as a result of exposure to copper under normoxia but not under hypoxia during the first 24h developmental window (Figure 5; Figure S3). In addition, *ca2* was significantly down-regulated following exposure to copper under hypoxia, but not under normoxia during the 28−52 hpf period (Figure 5; Figure S4).

DISCUSSION

The objective of this study was to determine the influence of oxygen availability on copper toxicity in developing fish. Our data demonstrate a strong influence of the concentration of oxygen on the toxicity of copper to zebrafish embryos, dependent on the embryonic stage of development, for both the amplitude and direction of hypoxia-induced changes in copper toxicity. During early development, hypoxia strongly suppressed copper toxicity in a process mediated by the activation of the HIF signaling pathway; whereas after hatching this effect was reversed and copper toxicity increased in a process likely related to increased copper uptake under hypoxia. This is the first time that these contrasting effects of hypoxia on copper toxicity are documented during embryogenesis in a model fish species.

Effects of Hypoxia on Copper Toxicity during Embryogenesis. Copper toxicity was significantly greater under normoxia compared to hypoxia when exposures occurred continuously throughout embryogenesis. In contrast, for exposures conducted during specific developmental windows, hypoxia suppressed copper toxicity during early development but increased its toxicity after hatching, demonstrating that the role of hypoxia on copper toxicity is fundamentally dependent on the stage of development. Continuous exposures to hypoxia through embryogenesis are likely in some natural environments, where hypoxic events can persist for long periods of time. In seasonal environments, during the warm season, hypoxia is associated with the formation of thermoclines and increased primary production in surface waters, resulting in excess oxygen consumption as organic materials decompose in lower water layers.[32] Hypoxia can also occur due to nocturnal decreases in photosynthesis and continued respiration, resulting in significant daily oxygen fluctuations in water bodies.[33] Despite the widespread occurrence of hypoxia in water systems, assessment of chemical toxicity for regulatory purposes does not consider the influence of oxygen on the effects of chemicals on aquatic organisms and guidelines for embryo testing request oxygen to be constant and above 80% saturation.[34] The very pronounced shifts in toxicological responses to copper shown

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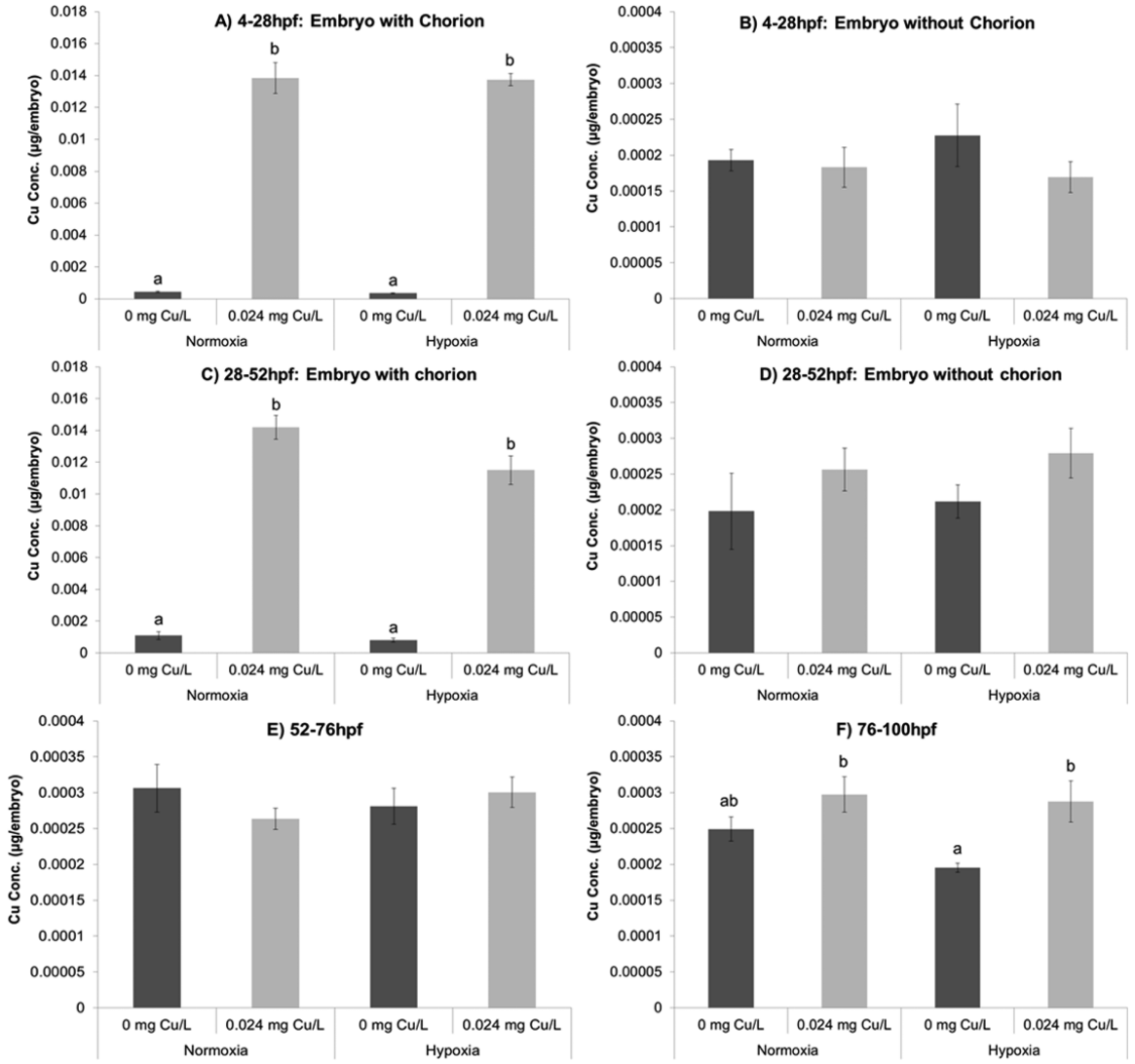

Figure 4. Measured copper concentrations in zebrafish embryos exposed to copper under normoxia or hypoxia. Zebrafish embryos were exposed to 0 or 0.024 mg Cu/L for 24 h during 4 developmental windows (A, B: 4−28. C, D: 28−52. E: 52−76. F: 76−100 hpf), under hypoxia (43.2% ± 0.55 air saturation) or normoxia (98.9% ± 0.22 air saturation). For the first two time windows, embryos were sampled either as whole embryos (embryo with chorion; A, C) or dechorinated (embryo without the chorion; B, D). Each treatment consisted of 4 replicate tanks containing 25 embryos in 600 mL of exposure water, and two pools of 5 embryos were collected from each replicate tank for determination of total copper content, by ICP-MS (n = 8 pools of embryos for each treatment group). Data is presented as mean μg Cu/embryo ± standard error mean. Letters indicate significant differences between treatment groups, with groups identified with different letters as significantly different (one-way ANOVA followed by pairwise comparisons using the Holm−Sidak post hoc test; $P < 0.05$).

here, demonstrate the strong influence of variable oxygen concentrations on copper toxicity and highlight the importance of considering more realistic environmental conditions, in which variable concentrations of oxygen often occur, when determining safety thresholds for chemical toxicity. This information is fundamental for determining the most sensitive set of environmental conditions and life stages for a given chemical that poses a risk to aquatic organisms.

Interactions between Copper and Hypoxia during Early Development. The most significant hypoxia-induced differences in copper toxicity were observed during the first 24 h of exposure, when a significant suppression in embryo mortality was observed. Furthermore, analysis of the effects of hypoxia on copper toxicity during specific developmental windows demonstrated that the decrease in copper toxicity observed under hypoxia compared to normoxia was unique to this stage of development. This suggests that during this early developmental window (4−28 hpf) the physiological responses to hypoxia protect embryos from the toxicological effects of copper. We hypothesized that the activation of the HIF signaling pathway in embryos exposed to copper under hypoxia was responsible for the reduction in toxicity observed during this early developmental period. HIF-1α acts as an oxygen sensing molecule in the cytoplasm and is constitutively expressed in vertebrates[35] and strongly expressed in zebrafish embryos during development.[36] HIF-1α stability is partially regulated by a group of oxygen-sensitive enzymes, prolyl hydroxylases (PHDs).[37] In the presence of oxygen (normoxia), the family of HIF-PHDs modify the HIF-1α subunit, allowing for HIF-1α recognition by a protein-ubiquitin ligase complex containing the von Hippel−Lindau tumor suppressor protein (pVHL), and leading to HIF-1α degradation by the proteasome.[38] However, when intracellular oxygen concentrations are low, PHD activity is inhibited, which, in turn,

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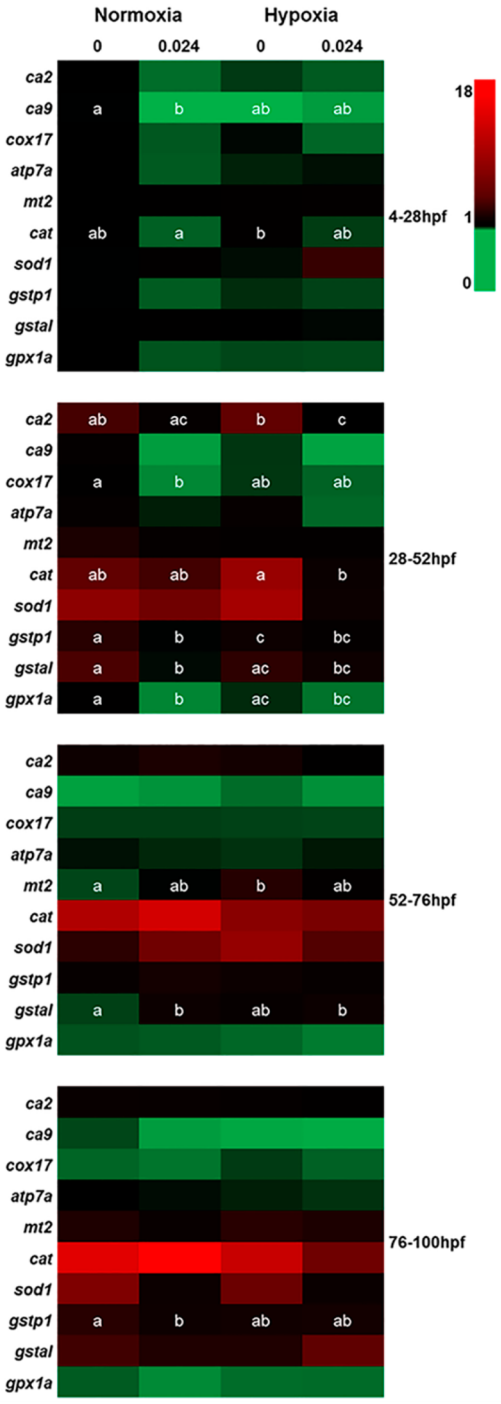

Figure 5. Transcript profiles for selected target genes following exposure to copper under hypoxia and normoxia during specific developmental windows. Embryos were exposed to 0 or 0.024 mg Cu/L under hypoxia (43.2% ± 0.55 air saturation) or normoxia (98.9% ± 0.22 air saturation) during specific 24 h developmental windows (4–28, 28–52, 52–76 and 76–100 hpf). Immediately after the exposure period embryos were sampled and grouped in pools of 5 embryos per treatment group and transcript profiles were determined using RT-QPCR. Ten target genes were analyzed including: carbonic anhydrase II (*ca2*), carbonic anhydrase IX (*ca9*), cytochrome c oxidase copper chaperone (*cox17*), ATPase Cu++ transporting, alpha polypeptide (*atp7a*), metallothionein 2 (*mt2*), catalase (*cat*), superoxidase dismutase 1 (*sod1*), glutathione-s-transferase pi 1 (*gstp1*), glutathione S-transferase alpha-like (*gstaI*) and glutathione peroxidase 1 a (*gpx1a*). Six pools of embryos were analyzed for each treatment group. Data are presented as average relative expression (normalized against the expression of the control gene *rpl8*). Individual data points classified as outliers, identified by Chauvenet's criterion, and points for which the expression was below the detection limit of the assay were excluded from the analysis, resulting in a replication of n = 4–6 pools per treatment group. Letters within each box indicate significant differences between treatment groups, with groups identified with different letters being significantly different from each other (one-way ANOVA followed by pairwise comparisons using the Holm–Sidak post hoc test; $P < 0.05$).

results in the stabilization of HIF-1α. Accumulated HIF-1α then dimerizes with the aryl hydrocarbon nuclear translocator (ARNT, also known as HIF1-β[39]), and the HIF-1α-ARNT dimer acts as a transcription factor, binding to hypoxia response elements (HRE) and resulting in the regulation of transcription of a wide range of hypoxia-responsive genes,[40] which regulate the physiological responses to hypoxia in vertebrate organisms. We investigated the role of HIF-1α on the suppression of copper toxicity under hypoxia during early embryogenesis by using the prolyl-4-hydroxylase inhibitor, DMOG, to stabilize HIF-1α and activate hypoxia signaling pathways under normoxia.[24] Our results showed that when exposures were conducted in the presence of DMOG, copper toxicity was greatly reduced, similarly to that observed when exposures were conducted under hypoxia. The results demonstrate that the biochemical and physiological responses resulting from the activation of the HIF pathway confer protection from copper toxicity during the 4–28 h developmental window.

Molecular responses to hypoxia include regulation of their intracellular pH to compensate for the increased acidosis caused by anaerobic metabolism, via up-regulation of *ca9*.[41] This enzyme catalyzes the conversion of extracellular CO_2 to carbonic acid[42] and is known to be induced by mild hypoxia in tumor cells.[43] The pH of the internal media is an important factor contributing to copper speciation and toxicity. Cu toxicity is known to be altered with changes in pH, for example as a result of copper complexes forming at higher pH, reducing the bioavailability of toxic copper ions. Plasma pH is influenced by the proportion of circulating bicarbonate ions resulting from CO_2 conversion to bicarbonate, catalyzed by carbonic anhydrase.[44] Exposure to hypoxia did not induce alterations in *ca9* expression, but a significant down regulation of *ca9* following exposure to copper under normoxia was observed, suggesting that copper may have disrupted CO_2 transport and pH regulation under normoxia, but not under hypoxia. These findings are supported by previous studies demonstrating a significant inhibition of carbonic anhydrase activity following copper exposure *in vitro*[45] and *in vivo*,[46,47] contributing to the toxicological effects of copper via disruption of acid–base balance.[45] Interestingly, under hypoxia, copper did not affect the transcription of carbonic anhydrase during early development, suggesting that its adverse effect on pH regulation was absent under hypoxia during this developmental window. These findings have implications for the regulation of copper speciation and bioavailability, which is known to be influenced by the pH of circulating fluids.[48]

Hypoxia Induced Alterations in Copper Toxicity Are Dependent on the Stage of Embryonic Development. Analysis of the effects of hypoxia on copper toxicity during four developmental windows comprising early embryogenesis to 24

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Environ. Sci. Technol. 2016, 50, 4502–4512

h posthatching revealed that hypoxia influences copper toxicity in contrasting ways at different developmental stages. During early development, hypoxia significantly decreased copper toxicity, followed by a period where hypoxia did not alter copper toxicity (28−52 hpf) and finally hypoxia increased copper toxicity in hatched embryos. In addition, we identified time periods particularly sensitive to copper, namely during early development (4−28 hpf) and immediately after hatching (76−100 hpf).

At the transcriptional level, the most significant differences in transcript profiles were observed in embryos exposed to copper during the second developmental window tested (28−52 hpf), which coincided with the time period where copper was least toxic and where hypoxia did not influence its toxicity. Furthermore, the majority of copper-induced transcriptional changes observed occurred in embryos exposed to copper under normoxia. These findings suggest that the ability of embryos to deploy compensatory mechanisms in response to copper may be responsible for the reduced toxicity occurring during this time window. This is likely to be particularly important for embryos exposed to copper under normoxia, where the protective effects resulting from the activation of the HIF-1α pathway are absent, and for which changes in gene transcription were strongly evident.

Genes regulated by copper exposure during the 28−52 hpf developmental windows include a down-regulation of transcripts encoding for oxidative stress responsive genes (*gst* isoforms and *gpx*) and the copper chaperone *cox17* under normoxia but not under hypoxia. These findings contrast with some of the literature, where copper-induced increases in the activity of glutathione S-transferase[49] and glutathione peroxidise enzymes[50] have been reported. It is important to note that the concentration of copper chosen for these exposures (0.024 mg Cu/L) was relatively low and well below those causing mortalities during this developmental window. Many of the copper responsive transcripts have non-monotonic dose response curves with opposite effects at low and high concentrations.[51−53] This may explain the unusual changes in transcript profiles measured here. Nevertheless, the fact that a wide range of transcriptional changes occurred following exposure to copper under normoxia but were limited to two genes under hypoxia (decrease in the transcript encoding *cat* and *ca2*), supports the hypothesis that, during this time window, the ability of embryos to activate transcriptional responses to copper increased their tolerance to this toxic metal.

From 52 to 100 hpf, we observed a switch in the effects of hypoxia from a protective role during early development to increasing copper toxicity during late development. This switch coincided with the initiation of hatching, where the metabolic activity of vertebrate embryos is known to increase[54] and the protection of the chorion is removed. We hypothesize that the switch in the effect of hypoxia on the toxicity of copper is likely to be associated with the progressive change in hypoxia tolerance threshold that occurs in zebrafish embryos as development progresses. Zebrafish embryos have been shown to progressively lose the ability to survive anoxia after 30 hpf,[55] and the expression of HIF isoforms inducible by hypoxia also changes progressively throughout development,[36] with consequent changes in the activation of hypoxia-responsive downstream genes.

It is important to note that the most significant effects of hypoxia on the toxicity of copper to zebrafish embryos occurred during the stages of development where copper is most toxic (during early development (4−28 hpf) and after hatching (76−100 hpf)), and contrasting effects of hypoxia on copper toxicity were observed during these windows of development. To investigate if alterations in copper accumulation in exposed embryos were associated with the differences in toxicity observed, we measured the concentrations of copper in embryos exposed to 0.024 mg Cu/L under hypoxia or normoxia during each developmental window. The data revealed that for 4−28 hpf and 28−52 hpf, there was a very significant increase in copper concentration in embryos exposed to copper, independent of the concentration of oxygen in the water. This increase in copper content was linked with the presence of the chorion, and in dechorinated embryos there were no changes in copper concentration in exposed embryos compared to controls. The chorion is known to bind copper, providing a barrier preventing copper from reaching the embryonic cells.[56−58] In addition, hypoxia did not affect copper concentrations in embryos with or without the chorion demonstrating that this is unlikely to be the mechanism responsible for the hypoxia-induced reduction in copper toxicity during early development. This is supported by the measured transcript profiles for *atp7a*, which indicated that there were no alterations in the transcription of this key copper transporter.[59] For the final window of exposure, 76−100 hpf, there was an increase in copper concentration in embryos exposed to copper under hypoxia compared to embryos exposed to hypoxia alone, but an increase in copper concentration was not observed in embryos exposed to copper under normoxia. This effect of hypoxia on copper uptake could explain, at least in part, the increase in copper toxicity observed under hypoxia in hatched embryos.

Overall, our study demonstrated that hypoxia caused very significant changes in copper toxicity during the embryonic development of a model fish species, in a stage-specific manner. The changes observed included a strong decrease in copper toxicity during early development followed by an increase in toxicity during late development. We demonstrated that the suppression in copper toxicity during early development was associated with the activation of the HIF signaling pathway and the increase in copper toxicity observed in hatched embryos may be as a result of differential copper uptake.

The progressive increase in the incidence, severity and prevalence of hypoxic events in both marine and freshwater systems worldwide is likely to continue due to factors associated with climate change, human population growth and migration toward coastal zones. In parallel, chemical contamination of aquatic systems continues to increase, and consequently the likelihood of aquatic organisms being exposed simultaneously to hypoxia and chemical pollutants during development will continue to increase. The very strong alterations in copper toxicity caused by hypoxia exemplify the importance of considering the concentrations of oxygen in the environment when defining the impact of chemical exposures on aquatic organisms. In addition, it is important to consider the tolerance to hypoxia of fish species, and within each species, the relative tolerance of each life stage. As demonstrated here, the effects of combined exposures during life stages with different tolerances to hypoxia resulted in dramatically different outcomes, with hypoxia strongly suppressing copper toxicity during early development when embryos are able to tolerate extended periods of anoxia, but increasing copper toxicity in the relatively hypoxia sensitive hatched embryos. To protect better

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Environ. Sci. Technol. 2016, 50, 4502−4512

the sustainability of aquatic ecosystems, it is fundamental to generate a mechanistic understanding of the interactions between the most environmentally relevant groups of chemicals and hypoxia, for a range of teleost species with varying hypoxia tolerance. This information will, in turn, facilitate accurate predictions of the consequences of worldwide expansion in oxygen depletion to fish communities challenged by anthropogenic toxicants.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b01472.

Material and Methods; Figure S1, proportion of hatched embryos at 4−76 hpf and 4−100 hpf; S2, proportion of hatched embryos at 52−76 hpf; S3, transcript profiles for target genes in embryos exposed from 4 to 28 hpf; S4, transcript profiles for target genes in embryos exposed from 28 to 52 hpf; S5, transcript profiles for target genes in embryos exposed from 52 to 76 hpf; S6, transcript profiles for target genes in embryos exposed from 76 to 100 hpf; Table S1, details for the qPCR assays employed in this study; S2, generalized linear models for the combined effects of hypoxia and copper on zebrafish mortality; S3, generalized linear models for the combined effects of hypoxia and copper on hatching rates; References (PDF).

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Author Contributions

J.A.F. and E.M.S. conceived and designed the experiments. J.A.F., E.M.S., V.H.D.F., N.R.B., G.L.B. and H.M.J. performed the experiment. J.A.F., E.M.S., R.J.W. analyzed the data. E.M.S., J.A.F., T.M.U.W., L.K.B. and R.J.W. provided training and supervision throughout the project. J.A.F. wrote the first version of the paper. The paper was written through contributions of all authors. All authors have given approval to the final version of the paper.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

atp7a	ATPase, Cu++transporting, alpha-polypetide
ca2	carbonic anhydrase II
ca9	carbonic anhydrase XI
cox17	cytochrome c oxidase copper chaperone
cat	catalase
DMOG	dimethyloxalylglycine
gpx1a	glutathione peroxidase 1 a
gstaI	glutathione-s-transferase alpha-like
gstp1	glutathione S-transferase pi 1
hpf	hours postfertilization
HIF-1α	hypoxia inducing factor-1-alpha
mt2	metallothionein 2
rpl8	ribosomal protein L8
sod1	superoxidase dismutase 1

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