The Effects of Hypoxia on Chemical Toxicity in Two Model Fish Species

Submitted by

Jennifer Fitzgerald

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature) ……

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 knowledge is limited about the interactions between hypoxia and chemical stressors. Therefore, during this PhD I set out to investigate how the concentration of oxygen in the water influences chemical toxicity in fish. My work focused on two groups of chemicals: toxic metals, of which copper was used as an example, and anti-androgenic chemicals.

Copper is an essential metal, widespread in the aquatic environment, but it can become toxic to aquatic organisms when environmental levels become too high. Copper and hypoxia are likely to co-occur but despite this our understanding of the interactions between these two stressors is limited. To address this, I performed a series of experiments using both the zebrafish (*Danio rerio*) and the three-spined stickleback (*Gasterosteus aculeatus*) as fish models.

I first investigated the effects of hypoxia on copper toxicity to zebrafish embryos during development. Copper toxicity was reduced by over 2-fold under hypoxia compared to normoxia during the first day of development, and I demonstrated that this protective effect was associated with the activation of the hypoxia inducible factor pathway. In contrast, hypoxia increased copper toxicity in hatched larvae, which was deduced to be associated with differential copper uptake.

To test if the interactions between copper and low oxygen observed for the zebrafish also occurred for other fish species, exposures were conducted in a species with a lower tolerance to hypoxia, the three-spined stickleback. The results obtained showed that hypoxia suppressed copper toxicity prior to hatching, but after hatching this effect was reversed, similarly to that observed for the zebrafish. This suggests a potential conserved effect of hypoxia on copper toxicity during embryogenesis across fish species.

To investigate if life stage influences the interactions between low oxygen and copper toxicity, the effects of combined exposures were assessed in the adult male three-spined stickleback. The critical oxygen level (P_{crit}) was determined to

allow appropriate experimental design. The combined exposures to copper and low oxygen resulted in a decreased ability to acclimate to low oxygen. Fish were able to lower their P_{crit} in response to low oxygen conditions when exposed to hypoxia alone but not when exposed to hypoxia in combination with copper. Together, these datasets support the hypothesis that the life stage influences the effects of the combined exposure, as hypoxia protects from copper toxicity during early embryogenesis but increases copper toxicity in hatched embryos and in adults.

I then investigated whether hypoxia can affect the toxicity of another widespread group of pollutants, anti-androgenic chemicals. Male three-spined sticklebacks were exposed to three anti-androgenic chemicals, flutamide, linuron and fenitrothion, under different air saturations. Each chemical had a unique transcriptional response alone and in combination with reduced oxygen saturation. Under both air saturations, spiggin transcription was strongly inhibited by exposure to flutamide. In contrast, exposure to fenitrothion did not result in a significant effect on spiggin transcription. Interestingly, linuron strongly inhibited spiggin under 100% air saturation, but this effect was absent under low air saturation, potentially as a result of interactions between the hypoxia inducible factor pathway and the aryl hydrocarbon receptor pathway. This work illustrates the potential mechanisms responsible for interactions between reduced oxygen and chemical toxicity, especially for aryl hydrocarbon receptor agonists, and highlights how hypoxia can modify the effects of a variety of chemicals with diverse modes of action.

My research highlights the importance of considering the interactions between multiple stressors, and the need to take into account the type of chemical, life stage, and the species tolerance. Understanding these interactions is essential to facilitate the accurate prediction of the consequences of exposure to complex stressors in a rapidly changing environment.

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Research Papers and Author's Declaration

Research Paper 1: Jennifer A. Fitzgerald, Hannah M. Jameson, Victoria H. Dewar Fowler, Georgia L. Bond, Lisa K. Bickley, Tamsyn M. Uren Webster, Nic R. Bury, Robert J. Wilson and Eduarda M. Santos (2016) Hypoxia Suppressed Copper Toxicity during Early Development in Zebrafish Embryos in a Process Mediated by the Activation of the HIF Signaling Pathway. *Environmental Science and Technology*; 50, (8), 4502-451

Research Paper 2: Jennifer A. Fitzgerald, Ioanna Katsiadaki and Eduarda M. Santos. Contrasting effects of hypoxia on copper toxicity during development in the three-spined stickleback (*Gasterosteus aculeatus*). *Manuscript accepted pending corrections in Environmental Pollution.*

Research Paper 3: Jennifer A. Fitzgerald, Mauricio G. Urbina, Nicholas J. Rodgers, Ioanna Katsiadaki, Rod W. Wilson and Eduarda M. Santos. Copper suppresses physiological responses to hypoxia in the three-spined stickleback (*Gasterosteus aculeatus*). *Manuscript in Preparation.*

Research Paper 3: Jennifer A. Fitzgerald, Maciej Tznadel, Ioanna Katsiadaki and Eduarda M. Santos. Effects of combined exposure to anti-androgenic chemicals and low air saturation in the three-spined stickleback (*Gasterosteus aculeatus*). *Manuscript in Preparation.*

Statement of author's contributions:

I, Jennifer Fitzgerald, made the following contributions to the research papers presented in this thesis. Preliminary work was carried out by Victoria Fowler, Georgia Bond and Hannah Jameson, which informed the design of the experiments. I carried out all experiments presented in **paper 1**, conducted the data analysis and wrote the manuscript. Tamsyn Uren Webster, Lisa Bickley, Rob Wilson and myself provided training and supervision for the preliminary work and experiments presented in **paper 1**.

Ioanna Katsiadaki contributed to the design of the experiments and provided training for the techniques used in **paper 2.** I performed all the exposures, conducted the analysis and wrote the manuscript.

For **paper 3**, Ioanna Katsiadaki and Rod Wilson provided guidance for the planning of the experiments. Mauricio Urbina helped design the experiments, specifically for the determination of the critical oxygen level, with additional help from Nicholas Rogers. I planned and conducted all the exposures, conducted all the laboratory analysis, analysed the data and wrote the manuscript. Nic Bury performed the measurement of copper in tissue and water samples for **paper 1 and 3**.

For **paper 4**, I planned the experiments, with help from Ioanna Katsiadaki, and conducted the exposure experiments, performed the RT-QPCR and wrote the manuscript. Maciej Tznadal performed the water chemistry measurements.

For all papers, Eduarda Santos supervised all aspects of the experimental work and the manuscript preparation.

List of General Abbreviations

11KT	11-ketotestosterone
5-HT	serotonin
AhR	aryl hydrocarbon receptor
ANOVA	analysis of variance
AR	androgen receptor
ARNT	aryl hydrogen nuclear translocator
AS	air saturation
ATP	adenosine triphosphate
CAT	catalase
cDNA	complementary DNA
CYP	cytochrome P450
DNA	deoxyribonucleic acid
dpf	days post fertilisation
E_2	oestradiol
EDC	endocrine disrupting chemical
EPO	erythropoietin
ER	estrogen receptor
GSI	gonadosomatic index
HIF	hypoxia inducible factor
hpf	hours post fertilisation
HSI	hepatosomatic index
IDH	isocitrate dehydrogenase
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
mRNA	messenger RNA
MT	metallothionein
NSI	nephrosomatic index
ODD	oxygen-dependent degradation domain
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
P_{crit}	critical oxygen tension
PHD	prolyl hydroxylases
pO_2	partial pressure of oxygen
pVHL	vol Hippel-Lindau protein
RBC	red blood cell
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-QPCR	quantitative real time polymerase chain reaction
SOD	superoxide dismutase
T	testosterone
VTG	vitellogenin

Chapter 1

Introduction

1.1 Stressors in the Environment

Stress can be defined as the response of an individual to unfavourable environmental conditions[1]. Fish inhabiting aquatic environments are continuously exposed to a diverse range of environmental stressors of both natural and anthropogenic origin. These may include changes to the abiotic environment including changes in temperature, noise, pH, salinity, dissolved gases, etc[2]. In addition, the presence of chemical contaminants and/or the alteration of the concentrations of natural substances able to cause adverse physiological effects are another major stressor in aquatic systems. Chemical contaminants in the aquatic environment can be grouped according to their chemical properties and biological effects[3]. They can enter aquatic ecosystems through a number of routes, including via domestic and industrial wastewater effluents, airborne deposition, as well as runoff from urban and agricultural areas[4].

Given the increase in human populations and land use, it is expected that the incidence, severity and persistence of stressors in the aquatic environment will continue to increase, with potential adverse effects on the organisms inhabiting affected areas. In this introduction, I first introduce the main stressors investigated in this thesis, including their occurrence in the aquatic environment and how they affect aquatic organisms. I will then discuss the issue of multiple stressors and in particular the current state-of-knowledge about the interactions between chemical contaminants and low environmental oxygen. I also introduce the two model fish species used in this thesis to investigate how hypoxia may affect chemical toxicity, and how their different life strategies were selected to help address the questions posed by this thesis. Finally, I present the aims and hypothesis, and a summary of the approach taken for this thesis.

1.2 Specific Stressors Discussed in this Thesis

1.2.1 Hypoxia

Hypoxia is one of the most significant problems affecting aquatic systems worldwide, and its severity and prevalence is projected to rise due to increases in nutrient inputs and climate change[5]. Hypoxia occurs when water bodies

become depleted in dissolved oxygen to a level that does not support life for most aerobic aquatic organisms[6]. Traditionally, hypoxia was defined as occurring when oxygen concentrations in water bodies fall below 2 mg O_2/L[7], however there has been much dispute over this value, as for many species this threshold is below that which causes hypoxia-driven mortality[8]. Therefore, the exact threshold at which an aquatic system can be considered to be hypoxic is considered to be species and life stage specific. For example, the rainbow trout (*Oncorhynchus mykiss*) is very sensitive to low oxygen levels[9,10], whereas the common carp (*Cyprinus carpio*) can survive for long periods (up to several months) in waters containing very low oxygen levels or even anoxic waters[11,12]. In this thesis, hypoxia refers to the level of air saturation below the threshold to cause stress for an individual organisms.

1.2.1.1 Occurrence of Hypoxia in the Aquatic Environment

The balance between oxygen production and removal in water systems determines the level of dissolved oxygen, therefore imbalances can cause hypoxia. Hypoxia can occur naturally in the aquatic environment, as oxygen solubility in the water is relatively low compared to air[13]. Water column stratification, associated with thermoclines or haloclines that isolate the bottom water from exchange with oxygen-rich surface water, and decomposition of organic matter in the isolated bottom water that consumes oxygen, are factors that lead to the development of hypoxia[14]. Factors affecting vertical mixing, such as wind or seasonal temperature change can also affect dissolved oxygen levels, especially in lakes[15]. If ice and snow cover lakes, it can result in the blocking of photosynthesis and reaeration, resulting in hypoxia[16]. Naturally occurring hypoxic events are most common during the night, known as diel hypoxia, as ongoing respiration, coupled with a cease in photosynthesis, depletes oxygen content in many shallow water systems[17].

There are major concerns over the increases in hypoxic zones in aquatic systems across the globe; reports have shown that since 1960 they have doubled each decade[18]. These increases in hypoxic zones are thought to be caused by a number of anthropogenic factors, including excess nutrient input into aquatic systems and climate change. Eutrophication, where excessive plant or algal growth occurs as a result of large influxes of nutrients entering aquatic

ecosystems, is thought to be a main causative factor contributing to the increase in hypoxic events (Fig.1.). Algae blooms can lead to microbial decomposition and strong depletion of the oxygen content of bottom waters, resulting in hypoxia[19]. In recent decades the prevalence and intensity of these events has increased considerably, driven by the increased use of fertilisers in agriculture and subsequent land run-off, and increased sewage discharges into aquatic environments, containing large amounts of nutrients[6]. Hypoxic zones are generally associated with high population centres[18] and with global population levels set to continue to increase, a corresponding increase in nutrient input into aquatic systems will likely lead to an increase in eutrophication and, in turn, increased incidence, duration and severity of hypoxic events.

Figure.1. Eutrophication of a natural water body (Wiltshire, UK; Fitzgerald *et al* 2016). Eutrophication results in the stimulation of algae and plant growth, resulting in increased photosynthesis, and in turn increased oxygen levels. However, mass die-off of the algal and associated microbial and plant biomass degradation, results in respiration exceeding photosynthesis, causing a fall in oxygen levels.

Global climate change is another major contributor to hypoxia. A rise in water temperature in streams and lakes can reduce the oxygen solubility and increase respiration rates, which may, in turn, lower the dissolved oxygen concentrations. The risk of hypoxia is greater in the summer, when low flow rates in rivers and stratification in lakes can interact with temperature to exacerbate the occurrence of hypoxia[20]. In addition, increases in global water temperatures will likely strengthen stratification, trapping hypoxic zones at the bottom of water bodies, and altered wind patterns have the potential to expand dead zones[6]. Increased frequency of extreme weather events, such as storms and El Niño events, may also increase hypoxia through increased run-off and surface temperatures, respectively[21]. A combination of these factors has culminated in an increase in the incidence, severity and duration of hypoxic events over the last 50 years, and this has been predicted to continue to increase, in line with current global warming models[22].

1.2.1.2 Effects of Hypoxia

The effects of hypoxia on fish and their adaptations to low oxygen environments have been longstanding areas of interest in both environmental research and management. Fish respond to hypoxia through a wide range of molecular, physiological, anatomical, and behavioural adaptations that vary among species, life stages, and habitats.

Metabolic and molecular responses to hypoxia are important to enhance the ability of fish to survive low oxygen levels, in particular when oxygen concentrations drop below a species' critical oxygen level (P_{crit}). P_{crit} is defined as the environmental oxygen tension at which an organism's oxygen consumption rate transitions from being independent of environmental oxygen (oxyregulator) to being dependent on environmental oxygen levels (oxyconformer)[23] (Fig. 2.). As a result, the P_{crit} for a species is considered by many as an indicator of hypoxia tolerance[24]. Many physiological adjustments can affect the P_{crit} and how fish adapt to low oxygen, including those involved in maintaining oxygen transport to target tissues. Hyperventilation is one of the first responses to low oxygen conditions in most fish species. It causes an increase of the flow of water over the gills, raising the gradient of blood to water PO_2 (partial pressures of oxygen) and resulting in an elevation of arterial PO_2. In

addition, hyperventilation also produces a respiratory alkalosis due to the reduction in arterial PCO_2 (partial pressure of carbon dioxide). This elevation of the red blood cell pH in turn increases the haemoglobin's oxygen affinity[25]. Increased red blood cell production and haemoglobin affinity also can occur, promoting oxygen transport to target tissues[26]. These physiological adjustments result in decreases in P_{crit} and thus an enhancement of hypoxia tolerance.

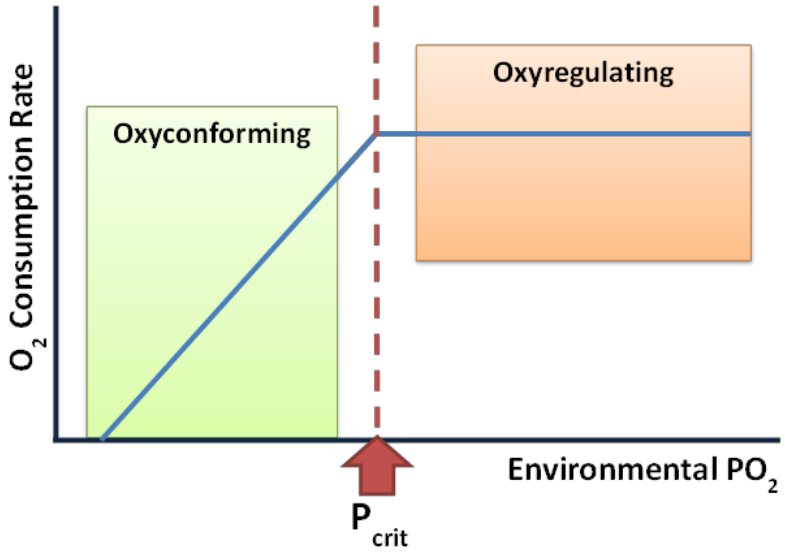

Figure.2. Schematic representation of the concept of critical oxygen tension (P_{crit}). P_{crit} is the point at which organisms' O_2 consumption rate transitions from oxyregulator (O_2 consumption is independent of the environmental PO_2) to oxyconformer (O_2 consumption is dependent on environmental PO_2)

In order to be able to tolerate hypoxia, organisms decrease their basal metabolic rate by limiting the use of ATP, and increase anaerobic respiration. At the molecular level, transcriptomic studies in the zebrafish (*Danio rerio*) and mudsucker (*Gillichthys mirabilis*) have shown decreased transcription of genes involved in aerobic metabolism and increased transcription of those involved in anaerobic metabolism following exposure to hypoxia[27]. In addition, decreases in the expression of genes that code for proteins involved in fat metabolism, cellular uptake and transport, including acyl-CoA dehydrogenase, intestinal fatty acid binding protein and other metabolite binding proteins were observed in the zebrafish following exposure to severe hypoxic conditions for 3 weeks[28]. At the protein level, hypoxia was shown to decrease the activity of Na^+/K^+-ATPase in

the Amazonian cichlid (*Astronotus ocellatus*), which resulted in a decrease in ATP demands[29].

Reactive oxygen species (ROS) are highly reactive molecules that can cause damage to cell membranes, proteins, enzymes and DNA[30]. Steady state ROS level is provided by the balance between ROS generation and elimination. The decrease in environmental oxygen concentrations can disrupt ROS balance inducing oxidative damage[31]. Under hypoxia, oxidative stress occurs as a result of accumulated electrons available for ROS formation using whatever oxygen remains, leading to a rise in ROS production and in turn an increase in antioxidant responses[32]. The exact mechanism responsible for the increase in electrons is not yet known but is has been suggested it may result from greater reduction of carriers in the electron-transport chain, with the additional electron reacting with oxygen and forming ROS[31]. Oxidative stress as a result of hypoxia has been shown in the goldfish (*Carassius auratus*)[33] and in the goby (*Perccottus glenii*)[34]. For the common carp[35], exposure to anoxia increased superoxide dismutase (SOD) and catalase (CAT) activities in the liver. In addition, in the medaka (*Oryzias latipes*), exposure to hypoxia resulted in increased levels of glutathione-S-transferase [36].

Hypoxia can induce strong behavioural responses in fish, which are dependent on the species and its life stage. These responses include avoidance of hypoxic zones, changes in the levels of activity and use of aquatic surface respiration [27]. Importantly, these behavioural responses to hypoxia can result in alterations in a number of important processes for population sustainability, including predator-prey interactions, schooling behaviour, formation and maintenance of dominance hierarchies, and parental care[37].

Long term exposures to hypoxia can result in a reduction of parameters with population level relevance, including growth and reproduction[38]. A number of studies have provided evidence for the adverse effects of hypoxia on reproduction in fish, both in the laboratory and in the field. For example, changes in endocrine function, egg quality, sperm motility, fertilisation and larval survival were observed in the common carp exposed to very low levels of dissolved oxygen[39]. A field study in the Atlantic croaker (*Micropogonias undulatus*) reported widespread reproductive impairment and disruption of sex

steroid signalling in both males and females after chronic exposure to hypoxia in its natural environment[40].

Together, these studies demonstrate that hypoxia can have a wide range of effects on fish, and highlight why hypoxia is an important stressor to consider when managing the aquatic environment.

1.2.1.3 Hypoxia Inducible Factor Pathway

At the molecular level, cellular oxygen sensing and the activation of downstream responses are mediated via the hypoxia inducible factor (HIF) pathway (Fig. 3.). HIF is a heterodimeric transcription factor comprised of alpha and beta subunits. HIF-1α is an oxygen dependent protein, that is continuously expressed in the cell[41]. HIF-1α stability is partially regulated by a group of oxygen sensitive enzymes, prolyl hydroxylases (PHDs)[42]. In the presence of oxygen, the HIF-1α subunit is degraded by the family of HIF-PHDS. This allows for the recognition of HIF-1α by the protein-ubiquitin ligase complex containing the von Hippel-Lindau protein (pVHL), leading to HIF-1α ubiquitination and degradation by the proteasome pathway[43].

In the absence of oxygen, prolyl hydroxylation does not occur, leading to stabilisation and accumulation of HIF-1α[44]. HIF-1α then translocates to the nucleus and dimerises with aryl hydrogen nuclear translocator (ARNT; also known as HIF-1β). This complex then binds to hypoxia response elements (HREs) in the promotor or enhancer regions of hypoxia-inducible genes, along with transcriptional co-activators (e.g. CBP/p300), resulting in the regulation of the transcription of a wide range of hypoxia-responsive genes, including genes involved in glycolysis and erythropoiesis[45]. These molecular responses then cascade into a series of biochemical and physiological adjustments, enabling organisms to better survive under hypoxic conditions[46].

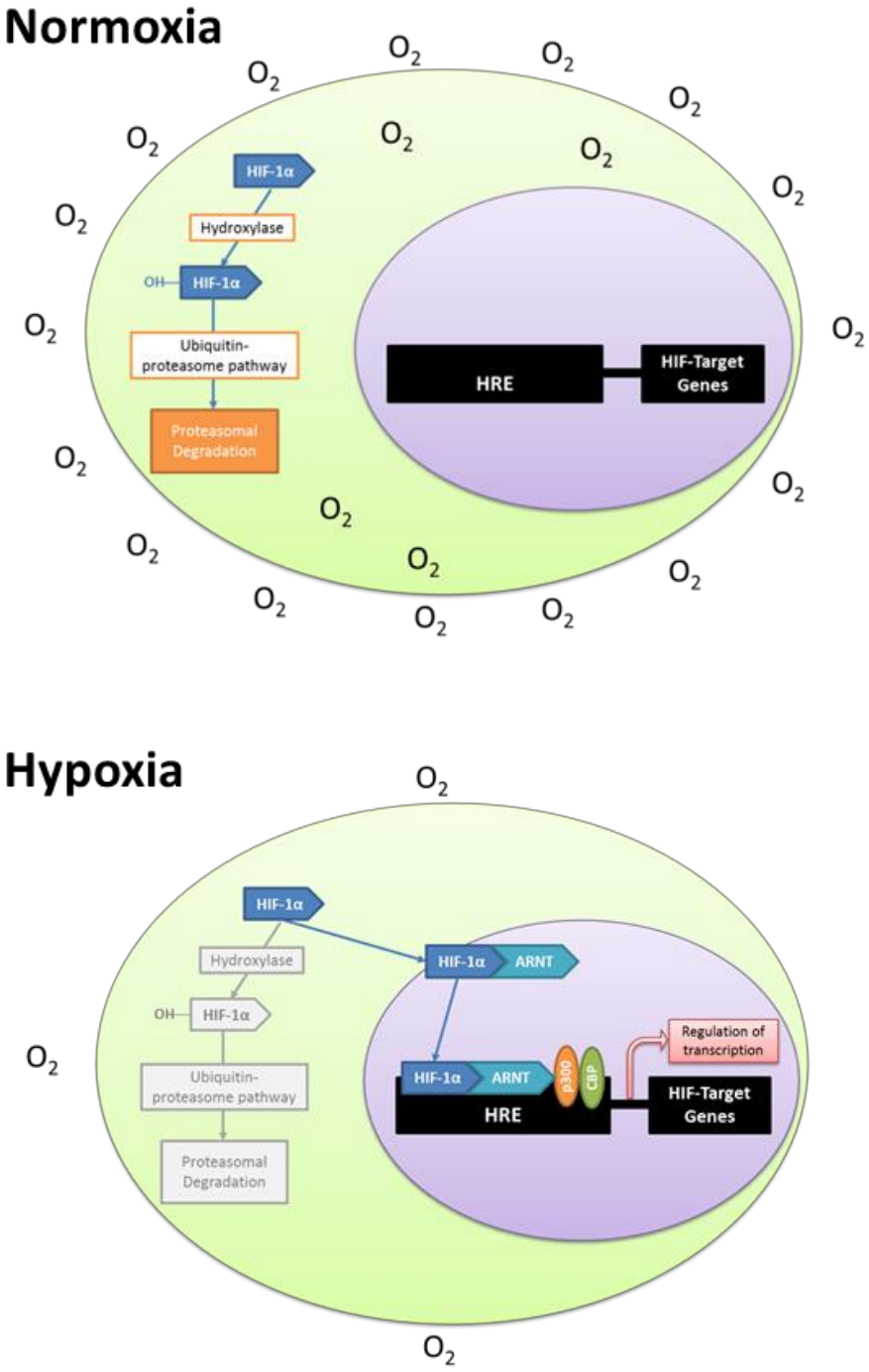

Figure.3. The regulation of the HIF pathway under high oxygen levels (normoxia) and low oxygen levels (hypoxia). Under normoxia, HIF-1α is targeted for degradation, through hydroxylation by proline hydroxylase (PHD) enzymes. This results in the recognition of HIF-1α by the von-Hippel-Lindau (VHL) protein, resulting in the degradation of HIF-1α via the ubiquitin-proteasome pathway. Under hypoxia, the activity of PHDs is decreased resulting in the stabilisation of HIF-1α. HIF-1α is then translocated into the nucleus where it forms a dimer with the aryl hydrogen receptor nuclear translocator (ARNT). The dimer recruits further

transcriptional regulators (e.g. CBP/p300) and binds to the hypoxia response elements in the promotor or enhancer regions of genes involved in the hypoxia response.

1.2.2 Metals as Environmental Stressors

Metals are important contaminants of aquatic environments worldwide and they are considered hazardous to aquatic life because of their persistence, high toxicity, and bioavailability in aquatic systems[47]. Metal pollution is increasing as a result of increased industrial activity, mining, intensive agriculture, household waste and motor traffic. They can accumulate in aquatic organisms and persist in water and sediments, and when they reach certain concentration thresholds, they can cause adverse effects to exposed organisms[47]. Metal toxicity in aquatic systems is strongly influenced by a number of factors including their speciation, solubility and complexation, which in turn are dependent on the physical/chemical properties of the water, including its pH, salinity, and organic matter[48]. The toxic modes of action and the target biological systems affected by metals are variable but involve the impairment of functions such as locomotion, osmoregulation, respiration and reproduction. In this thesis I have chosen copper as a metal pollutant of interest and will discuss below its known environmental impacts and effects.

1.2.2.1 Copper

Copper is an essential trace metal for most living organisms, where it acts as a co-factor for a wide range of enzymatic reactions including those involved in iron acquisition, oxygen transport and cellular metabolism[49]. However, when concentrations in the environment are elevated it can become highly toxic to living organisms. This is especially true for freshwater fish species, as decreased water hardness has been shown to strongly increase copper toxicity[50].

1.2.2.1.1 Occurrence of Copper in the Aquatic Environment

Copper is present naturally in the Earth's crust and, as such, is generally found in aquatic environments, with naturally occurring copper concentrations reported to reach up to 30 µg/L in freshwater systems[51]. However, these

concentrations are increasing, as a result of anthropogenic activities. In areas impacted by historical and contemporary mining, concentrations can reach extremely high levels. For example, for the River Lee and the River Hayle, in the UK, measured concentrations of 193 µg/L[52] and ~417 µg/L[53], respectively, have been reported and attributed to historic mining activities. Other sources of environmental copper include fabricated metal products, electrical equipment and the leather industry[54]. Agricultural run-off also adds to the copper burden, as many new fertilisers use copper as their main constituent. Copper is also being used as an alternative addition to antifouling paints after the ban of Tributyltin[55], which contributes directly to its increase in aquatic systems. Copper was identified as the metal causing the greatest threat to the aquatic environment in the UK, based on its current measured concentrations and toxic effects[56], demonstrating its strong environmental relevance.

1.2.2.1.2 Effects of Copper Exposure in Fish

Copper can have a large range of effects on fish and has been shown to alter locomotion activity, disturb metabolic processes by causing inhibition of respiratory enzymes, or gas exchange by inducing gill damage, and disrupt osmoregulation homeostasis through inhibition of gill Na^+-K^+-activated ATPase[48]. Copper can also impact the mechanoreceptors of lateral lines, impair the function of olfactory organs, and is associated with changes in blood chemistry, enzyme activity and corticosteroid metabolism[48]. Studies in the brook trout (*Salvelinus fontinalis*)[57] and bluntnose minnow (*Pimephales notatus*)[58] have shown that concentrations of copper well below lethal levels reduced reproductive output, potentially as a result of reducing energy directed to reproduction to compensate for the cost of copper-induced physiological changes.

Copper is frequently used as a fungicide in aquaculture, but it has also been reported to increase the susceptibility to viral and bacterial diseases in fish[59, 60]. Copper also has the potential to affect behaviour in fish, likely mediated through interactions with the olfactory epithelium. Impacts of copper on olfaction by copper have been associated with disruption of fright responses[61], feeding[62] and steroid pheromones[61]. In addition, copper was shown to disrupt fish

migration as a result of fish avoiding copper contaminated spawning grounds[63,64].

Exposure to copper has been shown to cause oxidative stress in fish as a result of increased levels of reactive oxygen species which can lead to lipid peroxidation, protein and DNA damage[65-68]. Copper can cause oxidative stress by inhibition of the activity of antioxidant enzymes, alterations in the mitochondrial electron-transfer chain and the formation of ROS[48,69]. For antioxidant enzymes, diverse responses have been reported. Catalase gene expression and enzymatic activity has been reported to increase[67, 70] and decrease[71] in the gill, liver and kidney of fish exposed to copper. Similarly, for glutathione peroxidase, copper was reported to result in increases in mRNA expression in the brown trout (*Salmo trutta L.*)[72] but no changes were observed in the goldfish[70]. This diverse range of responses is considered to be as a result of a complex interaction between a need to defend against accumulation of ROS and the direct inhibitory action of copper on the antioxidant enzymes themselves[48].

An important molecular response to copper toxicity is the induction of metallothionein (MT). Metallothionein is a metal binding protein that detoxifies and stores metal ions through the binding and removal of their redox potential[73]. A single *mt* isoform is predominately induced by metals in fish, which bind to metal response elements in its promotor region, stimulating transcription. Metallothionein has been used as an indicator and an early warning system for heavy metal pollution as a result of the wide acknowledgment of its accounting for a major proportion of the cellular storage of copper in fish[48]. Increases in metallothionein synthesis have been shown to occur as a response to copper, in both short and long term exposures, in both lab and field studies[52,70,74,75].

The embryo-larval and the early juvenile stages are considered to be the most sensitive life stages to copper toxicity[50]. The sub-lethal effects of copper on fish during these stages of development include reductions in yolk sac utilisation and decreased body length, potentially suggesting retarded development, as well as reduction in neuromasts in embryos, which has the potential to cause effects on later lateral line function and on behaviour[76-78].

1.2.3 Endocrine Disrupting Chemicals

The endocrine system regulates many essential functions for life, including development, growth, and reproduction. It consists of a network of glands and/or cells which secrete hormones that act on target cells, regulating their function. Hormones can be grouped structurally into four categories including peptides, steroids, amines and fatty acid derivatives, and they activate molecular responses in target cells via activation of specific hormone receptors[79]. Hormone regulated processes can be disrupted by chemical pollutants which can interact with hormone receptors by mimicking or antagonising endogenous hormones, or they can disrupt hormone synthesis, transport, storage, metabolism or excretion[80]. A wide range of evidence has emerged over recent years demonstrating that many natural and man-made chemicals have the potential to have adverse health effects on organisms through disruption of the endocrine system[81]. These chemicals are generally termed as endocrine disrupting chemicals (EDCs).

Chemical disruption of the sex steroid hormone signalling is the most well studied within the field of endocrine disruption. Sex steroids are mainly produced in the gonads and regulate not only gonadal development and reproduction, but also a wide variety of other processes in the body, including growth, development, metabolism and immune function. They act in target cells through binding and activating nuclear receptors, which act as transcription factors and elicit the regulation of extensive molecular pathways. In addition, some sex steroids also act via membrane bound receptors, generating rapid responses following hormone stimulation[82]. There are three primary classes of steroid hormones involved in the control of reproduction; androgens, oestrogens and progestogens[82,83]. These sex steroids have a number of roles in the regulation of reproduction. The predominant role of progesterone in fish is to promote the final maturation of gametes in both the male and the female. In males, androgens are responsible for controlling all stages of spermatogenesis and sexual development, and in fish the main biologically active androgen is 11-ketotesterone (11-KT)[84]. In females, oestrogens are involved in similar roles of sexual development and oogenesis, with the principal biologically active oestrogen in fish being 17β-oestradiol; E_2[82]. Environmental chemicals that mimic oestrogens and androgens are generally termed oestrogenic and

androgenic chemicals, respectively, and those blocking the action of those hormones are generally referred to as anti-oestrogenic and anti-androgenic chemicals, respectively.

The aquatic environment has been termed the “ultimate sink” for natural and man-made chemicals[85], with EDCs found in the freshwater, estuarine and marine environment, raising the possibility that EDCs impact organisms living in these aquatic environments. This thesis focused on anti-androgenic chemicals as an example of EDCs that have the potential to cause adverse effects in fish, alone or in combination with hypoxia. Therefore, in this section I will discuss the endocrine disruptors with an anti-androgenic mode-of-action.

1.2.3.1 Anti-androgens

Environmental anti-androgens have increasingly been recognised as potential contributing factors in the chemically induced feminisation of wild fish[86]. By competitively binding to the AR and blocking the action of androgens, anti-androgens can create a feminising environment, in turn producing symptoms suggestive of oestrogen exposure[87].

1.2.3.1.1 Occurrence of Anti-androgens in the Aquatic Environment

Studies have identified anti-androgenic activity in the environment[88-90], with known antagonists of the androgen receptor found among pesticides, bactericides and fungicides, for example linuron, fenitrothion, chlorophene and vinclozolin[91]. In 41 of the 43 UK sewage treatment works, anti-androgenic activity was identified in the effluents tested[89], and using models, this was shown to be associated with some of the feminised responses seen in wild roach (*Rutilus rutilus*) living in those rivers[92]. This effect has also been seen in other parts of Europe, including in the River Lambro in Italy, where studies on effluents identified chemicals, such as bisphenol A, iprodione, nonylphenol, p,p′-DDE and 4-tert-octylphenol, which are all known to have anti-androgenic activity[90]. Water extracts were shown to induce feminisation and de-masculinisation in the South African clawed toad (*Xenopus laevis*)[93, 94], and barbel (*Barbus plebejus*) collected from the River Lambo were shown to be intersex[95], further confirming the anti-androgenic activity present in this river. In addition, anti-androgenic activity has been demonstrated using *in vitro*

approaches in produced water from oil production platforms in both Norway[88] and North Italy[90]. In the USA, studies on populations of mosquitofish (*Gambusia affinis*) have suggested that potential anti-androgenic contaminants are responsible for shorter gonopodia and reduced sperm counts in fish from the Lake Apopka, compared to those from reference lakes[96]. Taken together, these examples, and others in the literature, suggest that anti-androgenic activity is widespread in the aquatic environment, with potential consequences for fish populations inhabiting polluted areas.

1.2.3.1.2 Effects of Anti-androgen Exposure in Fish

The effects of exposure to anti-androgens have been studied in a range of fish species and disruption of important reproductive pathways has been observed. Anti-androgens have been shown to decrease testis size and reduce or inhibit spermatogenesis in males in a range of fish species, including guppies (*Poecilia reticulata*)[97,98], rainbow trout[99] and medaka[100]. In females, reduced ovarian growth, altered oocyte development and decreased fecundity were observed in fathead minnows (*Pimephales promelas*)[101,102]. In addition, in juvenile guppies, exposure to the anti-androgen, flutamide, resulted in reduced fish size at maturation and skewed sex ratios[98].

A number of studies have investigated how anti-androgens affect the behaviour of exposed fish. Exposure to flutamide caused a suppression in male behaviours, including nest building and courtship, in adult male three-spined stickleback (*Gasterosteus aculeatus*)[103]. Stickleback exposure to fenitrothion resulted in reductions in both nest building and sexual behaviours[104]. Similarly, in guppies, a reduction in courtship behaviour following exposures to flutamide or vinclozolin was observed[98].

Biomarkers directly responsive to steroid hormones are widely used as indicators of endocrine disruption. The most widely used biomarker of feminisation in fish is the yolk protein, vitellogenin, which is induced in the liver by the circulating female steroid hormone 17β-oestradiol, and has been widely applied to identify xenoestrogens[81]. Analysis of vitellogenin induction or suppression has been used to indirectly study the effects of anti-androgens, where exposure of flutamide resulted in inductions of vitellogenin in the fathead minnow[86,102,105]. However, this is still a non-specific measure of anti-androgenic

activity, and a specific marker of androgen signalling in fish would offer specific advantages for use as a biomarker for both androgenic and anti-androgenic chemicals. In this context, spiggin has been developed as an androgen-specific biomarker in the stickleback. During the breeding season, the kidneys of males undergo hypertrophy and produce spiggin, a glue-like protein, which is used in the building of the nest. The glue is stored in the urinary bladder before being applied as threads to the nest in a characteristic movement[106]. The production of spiggin is androgen dependent, as it disappears after castration and can be induced in both sexes by androgen treatment[84]. Spiggin production has now been validated as a robust biomarker for both androgenic and anti-androgenic activity[107]. Flutamide, fenitrothion, linuron and vinclozolin have all been shown to inhibit spiggin production *in vitro*[108], as well as *in vivo* in the female stickleback[109]. Therefore this is an important endpoint to consider when looking at the effects of anti-androgens, especially considering how widespread and potentially abundant anti-androgenic chemicals are in the aquatic environment.

1.3 Multiple Stressors

Natural and anthropogenic stressors usually do not occur in isolation in aquatic systems, but instead a complex range of stressors are likely to co-occur and interact to cause adverse effects on living organisms. The physicochemical characteristics of water systems fluctuate naturally, as a result of diurnal or seasonal patterns. In addition, stressors of anthropogenic origin can cause changes in these characteristics or in the amplitude or timing of their fluctuation. Many of these systems are also continuously affected by a number of chemical contaminants, resulting in complex exposure scenarios for aquatic organisms. In order to fully understand how stressors affect the physiology of aquatic organisms, it is imperative that we consider how t may be affected by combined exposures of multiple stressors. When exposed to multiple stressors, an organism's sensitivity to each of the individual stressors may be altered in a number of ways, including; exposure to a toxic substance may affect an organism's tolerance range for other environmental factors, or may influence the toxic effect concentration of other compounds[110].

There is a very significant knowledge gap on how stressors may interact to cause harm to both individuals and populations, reflecting the difficulties in conducting studies on multiple stressors. This is particularly relevant when considering the current global environmental changes in ecosystems worldwide, resulting in aquatic systems being increasingly affected by a wide range of stressors acting in combination. Risk assessment procedures use toxicity tests in which organisms are exposed to chemicals under constant and favourable experimental conditions. This is in contrast with natural ecosystems where variable and suboptimal environmental conditions are common, and these fluctuations in environmental conditions need to be taken into account for accurate risk assessment procedures. Therefore, understanding how multiple stressors affect aquatic systems is essential to improve our ability to manage and protect them.

There are significant challenges in conducting multiple stressor assessment that are preventing this from occurring. These challenges range from the difficulties in measuring combined stressor effects to dealing with different qualities of stressors in terms of dose response or spatial scales. A significant research effort to date has focused on predicting the effects of the toxicity of chemical mixtures. A widely used method of predicting additive effects is based on the expectation that the effect of a mixture should be the sum of the effects of its individual components, termed effect summation[111]. However, this method can lead to logical inconsistencies if the stressors cause a sigmoidal dose curve, therefore there are serious doubts as to whether effect summation can be regarded as a reliable method[112,113]. An important issue of multiple stressor research to date is that much of the published work has focused on only one dose level, therefore failing to consider dose–response relationships. It has been shown that using just one concentration can result in false negative results[114], illustrating the importance of considering dose response curves when assessing mixture effects.

Other widely regarded methods for the calculation of expected additive mixture effects are the concentration addition and independent action methods[115-117]. Independent action methods are often used with mixtures that contain constituents with different modes of action. It carries the assumption that chemicals present below zero effect levels are not expected to contribute to the

total mixture effect, producing strong doubts of the use of this method for environmental stressors[113]. Concentration addition has been deemed suitable and a good concept for the prediction of mixture effects of similarly acting agents, for example work with estrogenic chemicals has been shown to correlate strongly with *in vivo* screening of the chemicals[118]. However, its ability to predict mixtures of agents that act via different modes of action is a long standing area of controversy[112].

Ultimately, one of the most significant challenges facing this field is the ability to link the effects seen at the single organism level to effects at the population and ecosystem levels. Populations of diverse species each have their own metabolic capacities for handling stressors, different susceptibilities, susceptible time windows and are exposed to various combinations of biotic and abiotic stressors. Analysing the toxic responses of each species in different time windows to all (mixtures of) pollutants is practically and financially impossible. Ecosystem complexity further encompasses processes such as changes in genetic diversity, the communication between organisms, or the interactions of organisms in the food web. These processes can be influenced by biotic and abiotic stressors and will vary between ecosystems. Currently, ecotoxicology effects assessments make simple assumptions to extrapolate data from the single organism to the population/ecological level, resulting in a reductionist approach, further complicated by uncertainties about if the correct assumptions have been made[119]. It is essential that models improve to help advance this area of multiple stressor research.

An area of research that is gaining more importance is investigating how changes in physicochemical parameters, such as pH, oxygen level or salinity, affect chemical toxicity. For example, Roberts et al showed that exposure of the sediment dwelling crustacean *Corophium valuator* to metal fluxes from contaminated sediments under future pCO_2 levels, resulted in increased mortality and DNA damage[120]. It has also been shown that ocean acidification (decrease pH and increase in pCO_2) significantly increases the toxicity of copper in both the mussel (*Mytilus edulis*) and the sea urchin (*Paracentrotus lividus*)[121]. For both species, the copper-induced damage to DNA and lipids was significantly greater when exposure occurred under ocean acidification (pH 7.71; pCO_2 1480 µatm) compared to control conditions (pH 8.14; pCO_2

470 µatm). However this effect was four times lower in urchins compared to the mussel, which suggested a species-specific response, highlighting the need for a broad range of species to be used to investigate these questions. This area is still very much in its infancy and more research is needed to fully understand the risks to aquatic organisms posed by contaminants with a high likelihood of changes in behaviour and speciation with alterations in physicochemical parameters in the water.

In the following section I review the state of knowledge on research investigating how different oxygen saturations in the water affect the toxicity of chemical pollutants to aquatic organisms. The expansion of hypoxic events, which often overlap with chemical pollution, constitutes a huge threat to aquatic diversity worldwide. Some studies have generated pivotal information on how hypoxia affects chemical toxicity to aquatic organisms, but knowledge remains sparse, highlighting the need for further research.

1.3.1 Hypoxia and Chemical Toxicity

Hypoxia is now considered to be amongst the most pressing and critical problems for the aquatic environment[18], often associated with nutrient rich effluent discharge. These effluents containing nutrients also release a diverse range and large quantities of potentially toxic chemicals into the aquatic environment, resulting in a high likelihood that hypoxia and chemical stressors will co-occur in many water systems. Therefore, it is essential to assess the potential for hypoxia to modify chemical toxicity for aquatic organisms.

To date, studies investigating whether chemical toxicity is modified by the availability of oxygen in the water have considered only a select range of chemicals, including polyaromatic hydrocarbons[122-124], polychlorinated biphenyls[125], phenols[126,127], ammonia[126,128], oestrogenic chemicals[129], pharmaceuticals[130, 131] and toxic metals[71,126,132-136].

Hypoxia has been shown to increase the toxicity of a selection of polyaromatic hydrocarbons (PAHs), including fluoranthene[137], benzo[a]pyrene and benzo[k]floranthene, as well as several mixtures of PAHs[122]. However, hypoxia has also been shown to protect fish embryos from pyrene toxicity[137]. PAHs are aryl hydrocarbon receptor (AhR) agonists, a pathway which is fundamental for

the metabolism of xenobiotics[138]. Xenobiotics, such as PAHs, bind to the AhR, forming an AhR-ligand complex which enters the nucleus. The complex binds to the ARNT protein forming an activated transcription factor which is able to bind to certain segments of DNA resulting in the transcription of CYP proteins in the cell[129] (Fig.4.). The changes in PAH toxicity when exposures occur under hypoxia can be potentially attributed to the fact that the signalling molecule, hypoxia inducible factor (HIF), a key regulator of the cellular response to hypoxia, shares a dimerization partner (aryl hydrocarbon receptor nuclear translocator; ARNT) with the AhR (Fig. 4.). Therefore, combined exposure to hypoxia and PAHs is likely to result in reduction of one or both signalling pathways, due to competition for ARNT.

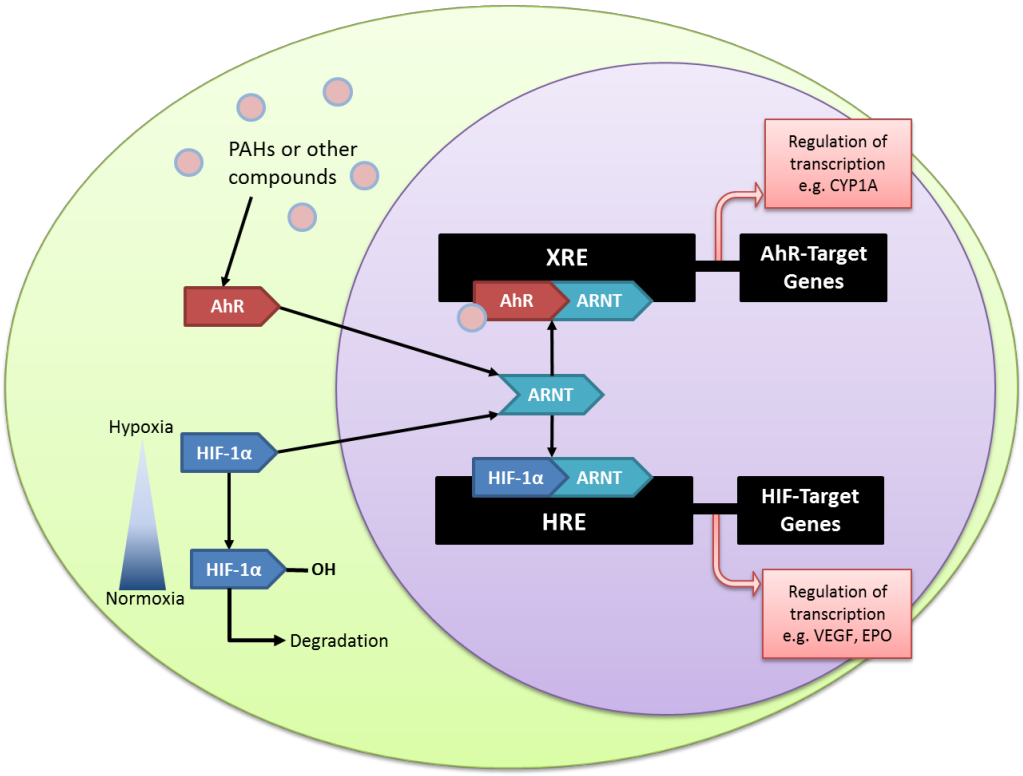

Figure.4. Schematic illustration showing the potential for interactions between the AhR and HIF pathways. Top of the figure: the aryl hydrocarbon receptor (AhR) in the cytoplasm, binds to a xenobiotic chemical (e.g. PAHs) resulting in its activation and translocation to the nucleus. In the nucleus, the activated AhR dimerises with the aryl hydrocarbon receptor nuclear translocator (ARNT), and subsequently binds to xenobiotic response elements (XRE) in the DNA, and regulates the transcription of associated genes. Bottom of the figure: normoxic conditions lead to HIF-1α hydroxylation and degradation. In contrast, a hypoxic environment causes the stabilisation of HIF-1α and translocation to the nucleus. In the nucleus, HIF-1α dimerises with ARNT to induce transcription of the hypoxia response elements (HRE)-

containing genes. Both the AhR and HIF pathways share ARNT as a dimerisation partner, highlighting the potential for competition between both pathways to occur.

Changes in chemical toxicity of PAHs when exposures occur under hypoxia may be associated with the suppression of the chemicals metabolism in the presence of low oxygen. For example, using morpholinos, CYP1A, a key enzyme for xenobiotic metabolism, can be knocked down, mimicking the effects of hypoxia on pyrene and benzo[k]floranthene[122,123], indicating that altered metabolism of these compounds may explain how their toxicity is affected by low oxygen. However, it is also important to consider the impacts of chemical exposure on the organism's ability to cope with low oxygen. If competition for ARNT occurs, it could cause suppression of the activation of the HIF pathway resulting in an increased sensitivity to hypoxia. For example, human cells exposed to TCDD in 5 % O_2 conditions showed reduced HIF-1α stabilisation and HRE-mediated promoter activity[140]. This highlights the importance of studying the interaction of hypoxia and chemical toxicity for chemicals that act via pathways that share key molecular elements with the HIF signalling pathway, of which AhR is a classic example.

Another end product of both the AhR pathway and hypoxia is oxidative stress. Hypoxia induces metabolic changes that may elicit oxidative stress due to the generation of ROS[141]. In addition, during the biotransformation of most pollutants by the cytochrome P450 complex, superoxide anions can be produced, which can result in DNA and protein damage, enzyme inactivation and lipid peroxidation[142]. This has previously been shown to occur for the PAH, benzo[a]pyrene, where exposure of the orange-spotted grouper (*Epinephelus coioides*) to this compound was shown to induce cellular production of ROS and an antioxidant response[143]. Co-treatment with hypoxia and benzo[a]pyrene, not only elevated protein carbonyl content (an indirect measure of ROS, as ROS can catalyse the oxidation of amino acid residues to form carbonyls), but also inhibited SOD activity in the liver, throughout the 21 day exposure. Therefore, chemicals with the potential to cause oxidative stress, should be investigated, as the combined exposure with hypoxia may enhance ROS production and in turn the oxidative stress response in fish.

Phenols are widespread environmental contaminants present in many aquatic industrial wastes, so are highly likely to occur in aquatic environments in association with hypoxia[144]. Exposure of phenol in conjunction with low oxygen levels resulted in increased toxicity, compared to exposure to phenol alone, in the rainbow trout[126], central stoneroller minnow (*Campostoma anomalum*)[127], bronze featherback (*Notopterus notopterus*)[145] and the fathead minnow[146]. Phenol exposure alone was shown to produce a range of histopathological damage to gill tissues, for example shedding of the gill epithelia, loss of blood in the lamellae and increased mucus production[127]. Therefore the increase in toxicity observed in response to exposure to phenol under hypoxia could be as a result of phenol damage to the gill. A common hypoxic response in fish is to increase ventilation rate, in order to increase oxygen uptake, but by doing this, more of the toxicant will likely be taken up due to increased water volume moving across the gill epithelia. In addition, due to the gill damage caused by phenols, oxygen uptake may be compromised, increasing the severity of the effects of hypoxia. This is supported further by a study investigating the effects of exposure to low dissolved oxygen and phenols, where tolerance to the hypoxic conditions was decreased in minnows, as a direct consequence of the gill damage caused by the phenol exposure[127]. This response is not unique to phenols, the same trend was seen for zinc, an essential micronutrient, but which can become toxic when present at high concentrations. Under low oxygen, zinc toxicity increased in the rainbow trout[126] and in carp[134], and this was thought to be as a result of gill damage, rather than increased zinc burden.

Exposures investigating the combined effects of hypoxia and chemical toxicity have also considered behaviour as an endpoint. Kienle *et al*. exposed zebrafish to nickel chloride alone and in combination with low oxygen conditions and assessed the effects of exposure on locomotor behaviour[136]. Nickel chloride exposure alone did not cause any effects on the locomotor behaviour of zebrafish larvae, but exposure to low concentrations of nickel in combination with low oxygen levels resulted in reduced locomotor activity. As there was no effect of nickel chloride alone, this response could be as a result of the organism adjusting to the low oxygen surroundings, as exposure to low oxygen levels alone caused decreased activity. Organisms depress their basal metabolic rate by limiting the use of ATP when exposed to hypoxia, and a key method of limiting ATP use is by reducing movement[27]. For high concentrations

of nickel in combination with low oxygen, Kienle *et al* observed an increase in locomotor activity. This is potentially as a result of the combined exposure to the two stressors eliciting an avoidance/escape response, reflected by higher locomotor activity compared to the single stressors. Therefore this research highlights the importance of selecting an appropriate range of concentrations of the combined stressors, as different concentrations can elicit very different behavioural responses.

Exposure to ammonia in combination with hypoxia in the rainbow trout[126] and the Atlantic salmon (*Salmo salar*) smolts[128] demonstrated increased toxicity compared to exposure to ammonia alone. This was suggested to be as a result of the inhibition of the oxygen binding ability of haemoglobin which is known to result from ammonia exposure. Increasing the affinity of haemoglobin for oxygen is an important response to hypoxia, promoting oxygen transport to target tissues. Therefore, chemicals with the potential to affect blood parameters, including pH, could affect hypoxia acclimation and therefore increase toxicity when exposures occur under reduced oxygen saturation.

Overall there are many potential mechanisms of interaction between hypoxia and chemical toxicity, highlighting the importance of studying their interactions. For the rest of this section I will explore the potential mechanisms of interaction between hypoxia and two model chemicals, copper and anti-androgens, as these interactions form the basis of the research conducted throughout this thesis.

1.3.1.1 Interactions of Copper and Hypoxia

The likelihood of co-occurrence of copper and low oxygen in aquatic environments is high; therefore it is essential to investigate the potential for interactions between these two stressors. Importantly both of these stressors act on some shared toxicological endpoints, including metabolism, oxidative stress and gill function, highlighting the potential for mechanistic interactions to occur. This is therefore an important question to address in order to better manage fish communities in the natural environment.

Previous work investigating the combined effects of copper and hypoxia demonstrated that the copper toxicity was greater at lower oxygen

concentrations in the benthic amphipod (*Corophium volutator*)[147] and in the mayfly (*Ephoron virgo*)[148]. The higher sensitivity to copper under hypoxia in the mayfly was thought to be as a result of increased gill movements at lower air saturations, which in turn, caused a high volume of toxicant-containing water to pass across the gill surface. In fish, increased ventilation rate driven by hypoxia is likely to result in an increase in copper uptake, given that the gills are the main uptake route in fish exposed to copper via the water in freshwater systems. This, in turn, would likely result in increased copper accumulation in the gills and/or in other tissues. This is supported by the observed increased accumulation of copper in the gills of killifish (*Fundulus heteroclitus*) and rainbow trout exposed to copper under hypoxia[149,150]. In addition, exposure to copper under hypoxic conditions via the diet did not result in increased copper accumulation[151], further supporting the hypothesis that hypoxia increased copper uptake in the gills by increasing ventilation rates.

Copper exposure resulted in several histological changes in the gill, including cell proliferation with thickening of gill filament epithelium[152-154]. These responses are considered to be defence mechanisms, as they reduce the branchial area that is in contact with the external environment. However by doing this, fish reduce the surface area of the gill available for oxygen diffusion and increase diffusion distance, therefore limiting the amount of oxygen that the fish can obtain from the water. If oxygen levels in the environment are already reduced, this could add an additional stress and reduce the susceptibility of the fish to the combined stressors. Therefore, this highlights the importance of the gills as a target organ for investigating whether copper toxicity is affected by hypoxia. In marine crabs, a higher gill diffusion barrier thickness was observed following exposure to both copper and hypoxia compared to those exposed to copper or hypoxia alone[154]. In the carp, exposure to copper via the diet under chronic hypoxia induced histopathological changes including fusion of the lamellae and shortening of the secondary lamellae as well as necrotic cells occupying the interlamellar space[151]. However this was thought not to be as a response to copper toxicity, as exposure was via the diet, but as a result of gill remodelling resulting from exposure to hypoxia.

As described above, it is important to consider the oxidative stress pathway when assessing the combined effects of hypoxia and chemical stressors, in

particular for chemicals known to cause oxidative stress, as is the case for copper. Exposure to copper under hypoxia in the pacu (*Piaractus mesopotamicus*) resulted in an increase in the production of ROS [71], and this has also been observed in the carp where combined exposures resulted in increased DNA damage[151]. In response to the increased oxidative stress, changes in antioxidant enzymes have also been observed. Combined exposures to copper and hypoxia have resulted in an increase in SOD and a decrease in glutathione peroxidase and CAT in the pacu[71] and, in the killifish, increased activity of CAT was reported, suggesting an activation of the oxidative stress defence mechanisms[149]. Oxidative stress responses are highly complex and vary over time, across different conditions or cell types, and across different species, potentially explaining the different responses reported in the literature.

Hypoxia survival requires a well-coordinated response to both obtain more O_2 from the depleted environment and to defend against the metabolic consequences of insufficient O_2 at the mitochondria, which limits aerobic ATP production. A mechanism of acclimation to hypoxia involves the adjustment of the organism's metabolism in order to reduce oxygen requirements. However, this process could be affected by co-exposure to copper as the mechanisms of response to copper toxicity are ATP-dependent resulting in increased metabolic rates and consequent increased toxicity. Exposure of copper and hypoxia in the bluegill resulted in greater hyperglycaemia, and lower ATP[132]. In the carp, combined exposure of copper and hypoxia resulted in an inhibition of standard metabolic rate and P_{crit}[156].

The studies published to date have considered a range of organisms (Fig. 5.), the majority of which were fish species with high tolerances to hypoxia, for example the common carp and the killifish. The common carp is highly tolerant to low oxygen, therefore, to induce a response to low oxygen, fish were exposed to levels as low as 20 % AS. These very low levels of air saturation correspond to severe hypoxic events, but will not be common in most aquatic systems. Therefore in order to assure environmental relevance, more sensitive species may be used, and this will further address the question of how common these effects are across different fish species. In addition, to date, no information is available on the effect of these two stressors during early

development for any species, which is an important knowledge gap that needs to be addressed, especially considering that the embryonic life stages are particularly vulnerable to copper toxicity[78].

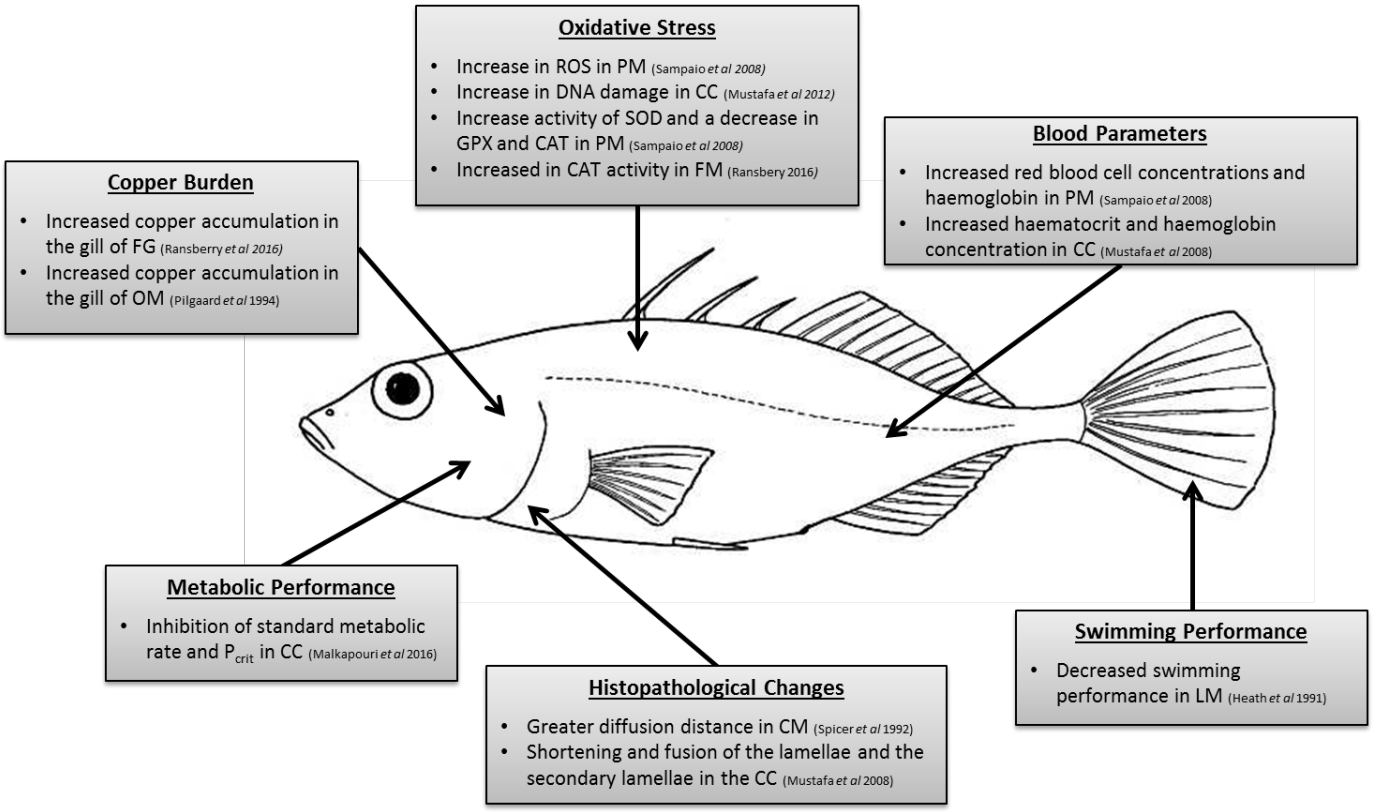

Figure.5. Biological processes affected by the combined exposure of copper and hypoxia in fish, for which interactions between the two stressors have previously been described in the literature. Fish species are: FG – *Fundulus heteroclitus* (killifish), OM - *Oncorhynchus mykiss* (rainbow trout), CC - *Cyprinus carpio* (common carp), PM – *Piaractus mesopotamicus* (pacu), LM - *Lepomis macrochirus* (bluegill), and CM - *Carcinus maenas* (marine crab). Image of fish from www.biology-resources.com.

1.3.1.2 Interactions of Anti-androgens and Hypoxia

To my knowledge, there has been no previous research investigating how low oxygen levels may affect the toxicity of anti-androgens in fish. Considering the widespread presence of anti-androgenic chemicals in the environment and the overlapping effects between hypoxia and this group of chemicals (discussed in section 1.2.1.2 and 1.2.3.1.2), there is the potential for interactions to occur, that

may result in modified risk of exposure for environmental species. For reproductive disruptors, one experiment has been published where interactions between the effects of EDCs and low oxygen were investigated. VTG was used as a biomarker to assess the effects of exposure to a mixture of oestrogenic chemicals under either hypoxia or normoxia in the fathead minnow[129]. The results demonstrated that there was no effect of hypoxia on the VTG response to the mixture of oestrogenic chemicals. This was contradictory with previous *in vitro* work which had shown that hypoxia and oestrogen treatment act together to affect the molecular responses in a human cancer cell line[157]. The differences between the two studies may have been related to a number of factors including changes in chemical uptake and metabolism as a result of the exposure to hypoxia, which could have resulted in modification to the cellular bioavailability of the oestrogenic mixture. Considering the potential for these stressors to co-occur and the overlapping pathways of response, it is essential to carry out more research to investigate the issue of how hypoxia may affect the toxicity of EDCs.

Hypoxia itself has been found to affect reproduction in fish. The reproductive processes affected include steroidogenesis, including decreased activity of the enzymes, oestradiol (E_2) and testosterone (T) concentrations in zebrafish[158] and Atlantic croaker[40,159]. Hypoxia was also shown to impair gonad development, including impaired ovarian growth and oocyte maturation in females[159] and impaired spermatogenesis and testicular development in males, in the Atlantic croaker[160], retarded gonad development in the Gulf killifish (*Fundulus grandis*)[161,162] and in the zebrafish[39]. Despite the clear effects of hypoxia on reproduction, no studies have previously investigated how low oxygen might act in combination with anti-androgenic chemicals, despite many potential hypotheses for interactions between the two stressors.

One of the potential mechanisms of interaction between hypoxia and EDCs occurs at the level of the insulin growth factor-1 (IGF-1) signalling pathway. IGF-1 is involved in the regulation of protein, lipid and carbohydrate metabolism, as well as the differentiation and proliferation of the cells, and ultimately body growth[163]. Therefore this pathway is important in reproduction and crosstalk between IGF signalling and the hypothalamus-pituitary-gonad axis have been described in both males and females. For example, in females, IGF is thought

to induce oocyte maturation and promote germinal vesicle migration and breakdown[164,165], whereas in males, it is known to stimulate spermatogenesis[166]. Insulin-like growth factor binding proteins (IGFBPs) function as carrier proteins in the circulation and contribute to regulation of IGF turnover, transport and half-life of circulating IGFs[163]. In addition, they also modulate IGF availability and biological activity in local tissue by preventing IGFs from binding to their receptor, and in turn can inhibit the biological activity of the IGFs[167]. Hypoxia has been shown to induce IGFBP-1[168], and an HRE element was found in the *igfbp-1* gene in fish[169]. Therefore under hypoxia, up regulation of *igfbp-1* could act to divert energy away from these IGF-controlled processes to other metabolic processes that are a greater critical priority for an organism's survival. An anti-androgen, flutamide, has also been shown to cause an inhibitory effect on IGF mRNA expression in fish[86]. Together, evidence suggests that both hypoxia and anti-androgens affect the IGF signalling pathway, and therefore combined exposure of the two stressors is likely to result in interactions at the level of this pathway, and this is an avenue for further research.

Expression of *star* (steroidogenic acute regulatory), responsible for the import of cholesterol, the precursor for sex steroid biosynthesis, into the mitochondria, was increased in the ovaries of anti-androgen (flutamide) treated fish compared to the control[170]. StAR activity has also been shown to be reduced in bovine cells held under hypoxia[171]. Exposure to hypoxia in the male zebrafish also resulted in a down regulation of 3-hydroxy-3methylglutaryl-coenzyme A reductase (HMGR; a rate-limiting enzyme in cholesterol synthesis) and StAR, the rate limiting step of sex steroid biosynthesis[172]. This impairment of HMGR and StAR expression could consequently result in decreased cholesterol availability for steroid synthesis, and suppress steroidogenesis. However, exposure to hypoxia in association with anti-androgens may have a different effect, as anti-androgens may increase the activity of the steroidogenic pathway, as shown for flutamide above, or a greater decrease may be observed as a consequence of some anti-androgens (linuron) having the potential to suppress the cholesterol biosynthesis pathway[173].

Aromatase activity in the ovary has also been shown to increase in fish exposed to the anti-androgen, flutamide[170]. Hypoxia was reported to affect aromatase

activity in fish, and female Atlantic croaker exposed to hypoxia had reduced aromatase activity in both field and laboratory settings[174]. Similarly, a down regulation in aromatase has also been observed in zebrafish exposed to hypoxia[172,175]. Aromatase in human placental microsomes has also been shown to be inhibited by lipid peroxidation caused by ROS[176] providing a potential mechanism for how hypoxia may affect its expression and mode of action. Therefore, considering that both hypoxia and many anti-androgens are thought to generate ROS, disruption of aromatase could be differentially affected by co-exposure to both stressors.

Reproductive behaviour has also been shown to be disrupted by hypoxia, and failing courtship behaviour in the common carp[177], decreases in frequency of mating displays in the African cichlid[178], as well as increased time spent fanning the eggs for the common goby[179] and the sand goby (*Pomatoschistus minutus*)[180] were observed in fish exposed to hypoxia. This potentially occurs as a result of hypoxia disrupting reproductive signalling pathways in the brain, for example serotonin. Exposure of the Atlantic croaker to hypoxia caused a decrease in serotonin (5-HT) levels, in association with decreases in the rate limiting enzyme for 5-HT synthesis, typtophan hydroxylase[181]. 5-HT regulates gonadotrophin releases, so effects on 5-HT may, in turn, cause disruptions in reproductive function. Anti-androgens are also known to affect reproductive behaviour. For example, suppression in male behaviours, including nest building and courtship, in adult male stickleback were observed after exposure to flutamide[103] and fenitrothion[104], and in the guppy, exposure of juveniles to vinclozolin, p,p′-DDE or flutamide resulted in a decrease of display behaviours[98]. Although the exact mechanisms are not currently known, the observations on the behaviour are considered to occur as a result of the anti-androgens blocking the androgen receptor, and therefore the effects of co-exposure to hypoxia and anti-androgens on reproductive behaviour may be mediated by different molecular pathways. The overlap on downstream effects suggests that interactions between these two stressors at the level of reproductive behaviour would be likely to occur.

Given the high likelihood for hypoxia and anti-androgenic pollution to co-occur, and the overlap between the reproductive effects of these two stressors, there

is a potential for these stressors to interact, causing reproductive disruption. Therefore, their combined effects need to be investigated.

1.4 Fish Models

Fish are particularly useful models for the assessment of water-borne and sediment deposited toxins because they may provide advanced warning of the potential danger of new chemicals and as a result they are used in many Organisation for Economic Co-operation and Development (OECD) test guidelines. Due to the high diversity of fishes, it is impossible to identify any one species as an ideal test model. Therefore, model species need to be selected based on a number of characteristics including the ease of culture, ecological relevance and economic importance. For a given study, it is critical to select a test species that allow the relevant questions to be addressed in full. In this thesis, two model fish species were selected to investigate the effects of low oxygen on chemical toxicity.

1.4.1 Zebrafish

The zebrafish is the fish model of choice for a wide range of research programmes ranging from environmental research to genetics and to human health. This species offers a number of critical advantages including its small size, well documented biology, ease of culture and breeding in the laboratory and cost-effective husbandry and exposure methods[182]. The genome for this species has also been sequenced[183], facilitating mechanistic and molecular studies. Embryonic development is rapid in the zebrafish, with the main body structure established within 24hpf (hours post fertilisation), and within 96hpf most organs are fully developed[184]. This, together with their transparency, allows zebrafish embryos to be easily screened for phenotypic alterations, making them a useful model system in toxicology.

Zebrafish embryos are considered to be relatively tolerant to low oxygen and they are able to survive for up to 24h during early development in anoxia, by undergoing suspended animation[185]. However, extension of exposures past the first 24 hrs of development in anoxic conditions results in 100 % mortality[186]. The threshold for developmental delays to occur has been shown as 0.8 mg of

O_2 /L[186], demonstrating that the zebrafish is relatively tolerant to low oxygen. In addition, the HIF pathway has been shown to be highly conserved in the zebrafish[187], which supports the use of this species as a model organism for studies on hypoxia.

The zebrafish embryo has been used as a model system to investigate how hypoxia affects chemical toxicity. Work focused on chemicals that bind and activate the AhR receptor, due to the high likelihood of cross talk between the HIF and AhR pathway (see section 1.3.1 and Figure.4.). The toxicity of PAH mixtures, including benzo[a]pyrene and 2-aminoanthracene, was increased by hypoxia, resulting in more severe pericardial oedemas and mortality[122]. In contrast, hypoxia deceased the induction of *cyp1a* mRNA by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and caused a reduction in its toxicity, including the formation of oedmas, in exposed zebrafish embryos[188]. Prasch *et al* investigated whether this effect was as a result of the cross talk with the ARNT pathway, but found that mutant embryos lacking ARNT did not develop defects mimicking TCDD toxicity, therefore it was unclear whether the changes in TCDD toxicity under hypoxia were due to the competition of the HIF and AhR pathways for ARNT or through other mechanisms[188]. For the PAHs, fluoranthene, β-naphthoflavone and benzo[a]pyrene, a reduction in toxicity was observed for zebrafish embryos exposed under hypoxia[137]. In addition, for the same study, a 60 % reduction in CYP1A activity was observed for embryos exposed under hypoxia compared to normoxia, but there was no effect on pericardial effusion. This suggests that in addition to interactions between the HIF and AhR pathways, hypoxia may also modify the toxicity of these compounds via other mechanisms. Together these studies highlight how useful the zebrafish is for investigating the combined effects of hypoxia and chemical toxicity. In addition, given that these studies are limited to AhR agonists, a significant knowledge gap exists for how the toxicity of other chemicals is modified by the level of oxygen in the water, and the zebrafish embryo offers a powerful model to undertake these studies.

1.4.2 Three-spined Stickleback

The three-spined stickleback is a model organism found in a wide range of temperate freshwater and marine ecosystems in the Northern Hemisphere[107].

This species is a well-established model organism for environmental toxicology, due to its ubiquity in the environment and ease to obtain and keep in the laboratory, as well as the availability of molecular resources, including a sequenced genome[189].

Studies investigating the effects of hypoxia on the stickleback reported alterations on behaviour, including reduced aggression[190] and loss of inquisitive behaviours and activity[191]. In addition, alteration in biochemical parameters, such as increases in tissue L-lactate[179], and changes in physiological endpoints, including increased gill movement[192] were also observed. To date, no information has been published on the effects of hypoxia on this species during embryonic and larval development. For sticklebacks, this is a particularly interesting question because of the unique nature of parental care by the males in this species. During embryo development, male stickleback invest large amounts of time and energy fanning the embryo bearing nest, in a process thought to be driven by the need to maintain high oxygen concentrations around the embryos[37,193]. Studies have also shown that males increased their fanning tempo in response to low oxygen conditions[194], further suggesting that the embryos are sensitive to decreased oxygen levels. These facts suggest that stickleback embryos may be more sensitive to hypoxia compared to zebrafish at the same stages of development and due to their different tolerances, and life history strategies, they are an interesting species to use as models for studies on combined effects of hypoxia and chemical toxicity.

The fascinating reproductive behaviour displayed by males has made the stickleback a very interesting model for fish reproductive studies. As a result of environmental cues at the start of spring, including increased temperature and increase day length, males exhibit secondary sexual characteristics such as the development of a red throat and blue irises (Fig.6.), and as a result of hormone changes, the male establishes a territory, in which they build a nest using an adhesive protein produced in the kidney (spiggin)[107]. When a gravid female is within a male's territory, he approaches her using a zig-zag dance, where the male jumps back and forth around the female. The female adopts a receptive 'heads up' posture and swims towards the male. Before leading the female to the nest, the male shows a behaviour called dorsal pricking to the under carriage of the female. After this, the male will lead the female to the nest,

showing the female the entrance. She pushes past the male to go into the nest, where she spawns. After she has left the nest, the male creeps through and fertilises the eggs[195]. The androgen-induced glue protein, spiggin (described above), is the only known protein produced in fish strictly as a result of androgen stimulation, thus making it an ideal androgen biomarker for fish[196]. The reproductive behaviour and androgen biomarker are sensitive to chemical and environmental stressors, making this species a good model organism to investigate interactions between low oxygen and anti-androgenic chemicals.

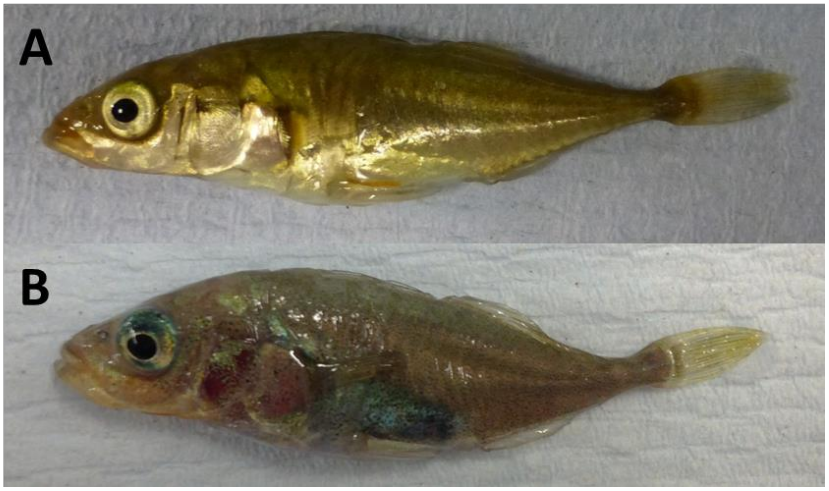

Figure.6. Photos of male three-spined stickleback before (A) and after (B) kidney hypertrophy. Fish after kidney hypertrophy have blue irises and a deep red throat. The red throat of the male attracts the females, and signals to other males their dominant state.

1.5 Aims and Hypothesis of this PhD

The overall aim of this PhD was to investigate if the concentration of oxygen in the water influences chemical toxicity to fish. In this thesis, we formulated the following hypotheses to address this aim:

1. Copper toxicity will be altered by the level of dissolved oxygen in the exposure water
2. The tolerance of a species to hypoxia will influence how hypoxia affects copper toxicity
3. Life stage will influence the interactions between low oxygen and copper toxicity

4. Low oxygen concentrations will alter how fish respond to anti-androgenic chemicals

These hypotheses were addressed through the following experiments, written as individual research papers:

Chapter 2 investigated the interactions between oxygen saturation and copper toxicity during development in the zebrafish (hypotheses 1 and 3). Cumulative mortality curves for copper under normoxia and hypoxia were first conducted to determine if hypoxia affects copper toxicity. Experiments during specific stages of development were then conducted to investigate if the interactions between hypoxia and copper toxicity vary with the developmental stage at which the exposure occurs. The mechanisms responsible for the effects of hypoxia on copper toxicity were investigated by quantifying copper concentrations in exposed embryos and measuring gene expression. In addition, to investigate the role of the HIF pathway on the changes in copper toxicity observed under hypoxia, a chemical inhibitor was used to stabilise HIF in the cytoplasm and mimic cellular hypoxia.

Chapter 3 investigated the effects of reduced oxygen concentrations, alone and combined with copper, on the three-spined stickleback throughout development (hypotheses 1, 2 and 3). Initially, the response of the stickleback to different levels of air saturation during development was determined. This allowed the appropriate level of air saturation to be used in subsequent experiments investigating whether low oxygen affects copper toxicity during early life. Continuous exposures throughout development, as well as exposures in hatched embryos were conducted to assess if stage of development affects the interactions between copper and hypoxia for this species.

Chapter 4 investigated how different levels of oxygen affect copper toxicity in adult sticklebacks (hypotheses 1, 2 and 3). In order to document the tolerance of the stickleback to hypoxia under the experimental conditions used, the P_{crit} was first determined, allowing for the choice of appropriate levels of air saturation to be used in the subsequent exposures. The physiological and molecular responses following exposure to copper and hypoxia and their combinations were determined at the physiological and molecular levels, to investigate the interactions between the two stressors in adult fish.

Chapter 5 investigated the effects of hypoxia on the response of the three-spined stickleback to anti-androgenic chemicals (hypothesis 4). Initially, the effects of three chemicals reported to act via anti-androgenic signalling pathways were assessed by measuring spiggin gene expression in the kidney. Then expression of genes involved in specific pathways hypothesised to be influenced both by hypoxia and anti-androgens were analysed in the liver, in order to generate a mechanistic understanding of how hypoxia modulates the toxicity of these chemicals.

Overall the data presented in this thesis provides the first datasets on how hypoxia influences copper toxicity during embryogenesis for any fish species, how this compares to the effects observed in adults and how hypoxia interacts with environmental chemicals of known anti-androgenic activity, providing a significant advance for this area of research.

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Chapter 2

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

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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

Jennifer A. Fitzgerald,*[†,‡] Hannah M. Jameson,[†] Victoria H. Dewar Fowler,[†] Georgia L. Bond,[†] Lisa K. Bickley,[†] Tamsyn M. Uren Webster,[†] Nic R. Bury,[§] Robert J. Wilson,[†] and Eduarda M. Santos*[†]

[†]Biosciences, College of Life & Environmental Sciences, University of Exeter, Geoffrey Pope Building, Exeter EX4 4QD, United Kingdom

[‡]Centre for Environment, Fisheries and Aquaculture Science, Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB, United Kingdom

[§]King's College London, 83 Franklin-Wilkins Building, London SE1 9NH, United Kingdom

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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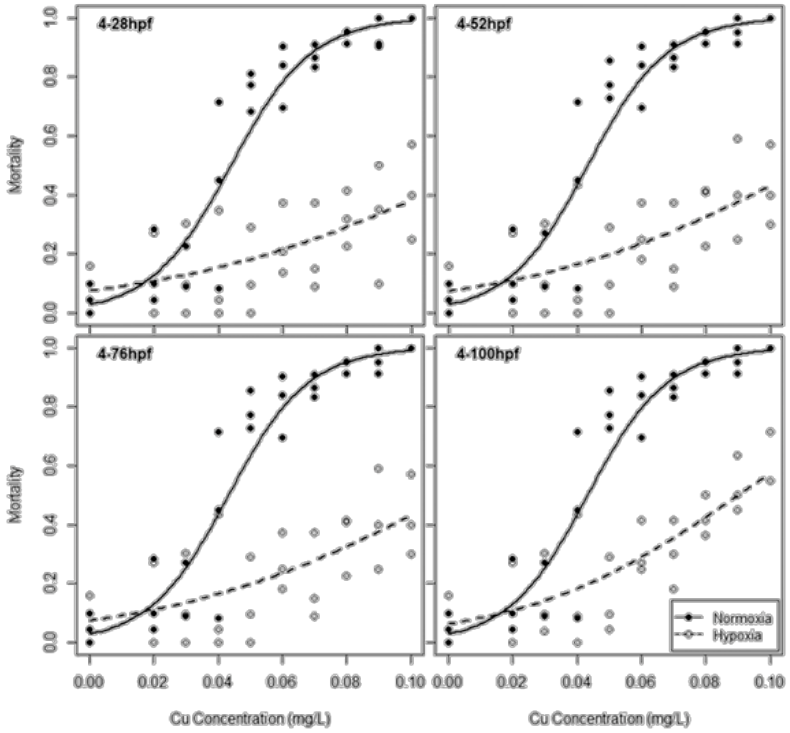

Figure 1. Embryo mortality curves following continuous exposure to copper under normoxia or hypoxia throughout development. Each point on the graph represents the proportion of mortality in an individual replicate tank containing 20 embryos, black and white symbols represent groups exposed to copper 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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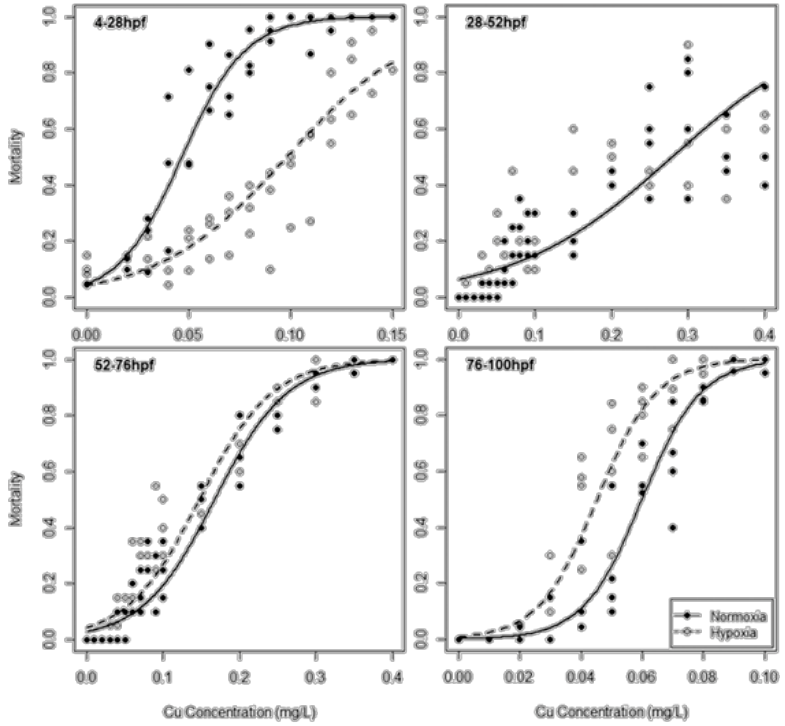

Figure 2. Embryo mortality curves following exposure to copper under normoxia or hypoxia during specific developmental windows. Each point on the graph represents an individual replicate tank containing 20 embryos, black and white symbols represent tanks exposed to copper under normoxia (98.4% ± 0.12 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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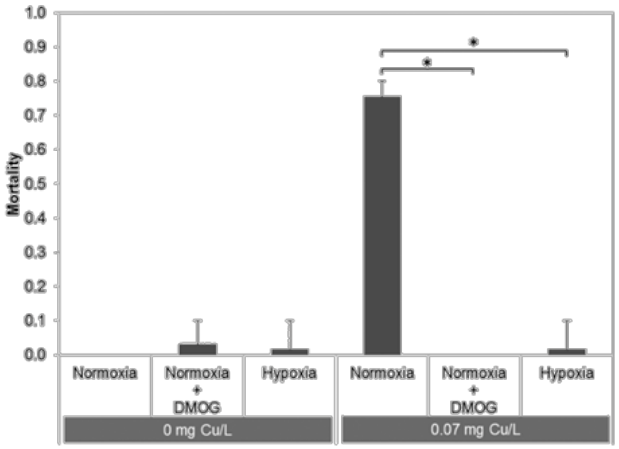

Figure 3. Effects of HIF activation by dimethyloxalylglycine (DMOG) on copper toxicity. Exposures to copper under normoxia, under normoxia in combination with DMOG, and under hypoxia were conducted during the 4–28 hpf developmental period. Six independent tanks containing 10 embryos and 100 mL of exposure 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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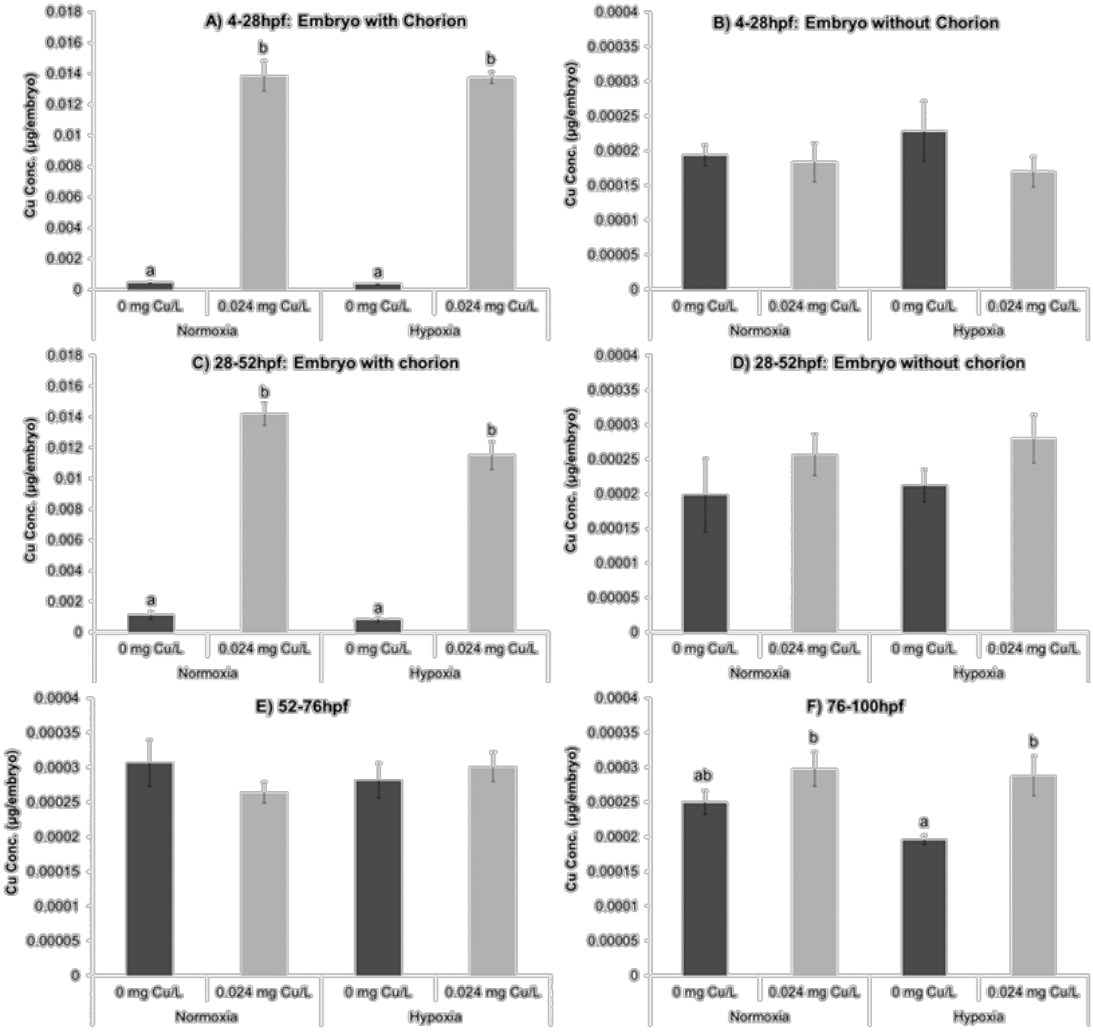

Figure 4. Measured copper concentrations in zebrafish embryos exposed to copper under normoxia or hypoxia. Zebrafish embryos were exposed to 0 or 0.024 mg Cu/L for 24 h during 4 developmental windows (A, B: 4–28. C, D: 28–52. E: 52–76. F: 76–100 hpf), under hypoxia (43.2% ± 0.55 air saturation) or normoxia (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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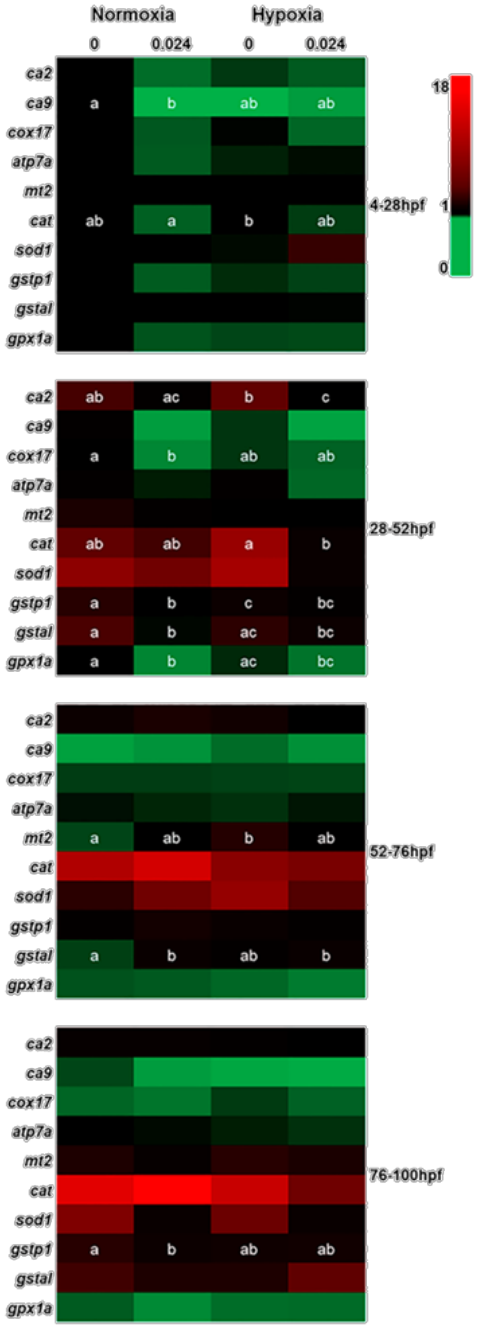

Figure 5. Transcript profiles for selected target genes following exposure to copper under hypoxia and normoxia during specific developmental windows. Embryos were exposed to 0 or 0.024 mg Cu/L under hypoxia (43.2% ± 0.55 air saturation) or normoxia (98.9% ± 0.22 air saturation) during specific 24 h 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 (*gstal*) 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; $P < 0.05$).

Figure 5. continued

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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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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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).

■ AUTHOR INFORMATION

Corresponding Authors

*Jennifer A. Fitzgerald. E-mail: jf277@exeter.ac.uk.
*Eduarda M. Santos. E-mail: E.Santos@exeter.ac.uk.

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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Supplementary Information: Hypoxia suppressed copper toxicity during early development in zebrafish embryos in a process mediated by the activation of the HIF signalling pathway

Jennifer A. Fitzgerald,[,†,‡] Hannah M. Jameson,[†] Victoria H. Dewar Fowler,[†] Georgia L. Bond,[†] Lisa K. Bickley,[†] Tamsyn M. Uren Webster,[†] Nic R. Bury,[§] Robert J. Wilson,[†] Eduarda M. Santos[†,*]*

[†] Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, UK

[‡] Centre for Environment, Fisheries and Aquaculture Science, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, UK

[§] 83 Franklin-Wilkins Building, King's College London, London, SE1 9NH

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TABLES

SI – MATERIAL AND METHODS

Fish source, husbandry and embryo collection

A population of zebrafish (wild-type WIK strain, originally from the Max Planck Institute, Tubingen, Germany) was maintained in the Aquatic Resource Centre at the University of Exeter in mixed sex stock tanks (140L), supplied with aerated synthetic freshwater, at 28±1°C, as previously described [1], with a photoperiod of 12:12h light/dark and a 30 minute gradual transition period at dawn and dusk. Adult fish were fed to satiation every morning with freshly hatched *Artemia nauplii* and with flake food (Tetra; Melle, Germany) every afternoon. Fish were allowed to breed under natural conditions in order to generate fertilised eggs for embryo exposure experiments. Collection of embryos was conducted approximately 1hpf. Embryos were rinsed and incubated in aerated artificial freshwater (according to the ISO-7346/3 guideline, ISO water diluted 1:5 [2]), and unfertilised eggs were identified by visual observation during the blastula stage as described by Kimmel and colleagues [3], using a dissection microscope (Motic DM143, Hong Kong) and removed before the start of the exposures. All experiments were conducted under approved protocols according to the UK Home Office regulations for the use of animals in scientific procedures.

Copper uptake

Embryos were exposed to 0 or 0.024 mg Cu/L (this concentration caused approximately 10% mortality in the continuous copper exposure) for 24h under hypoxic or normoxic conditions, for the 4-28, 28-52, 52-76, 76-100hpf developmental windows, as described in the materials and methods, and each treatment consisted of 4 independent replicate tanks containing 25 embryos. The measured copper concentration in the water at the end of each window of exposure were 68% ±5.4 of the nominal concentrations for exposures conducted under hypoxia, and 71% ±3.8 of the nominal concentration for exposures conducted under normoxia. The pH and ammonia concentrations in the exposure tanks were 7.12±0.01 and 0.19±0.02 mg/L, respectively, and remained constant for all treatments and time points. At the end of the

exposures, two pools of 5 embryos were collected from each exposure tank (8 pools in total for each treatment group), quickly rinsed with HPLC grade water (Fisher, Fair Lawn, USA) and stored in acid-washed 1.5ml centrifuge tubes at -20°C until analysis. For the 4-28 and 28-52hpf time windows, embryos were collected both as whole embryos (with chorion) and after the chorion had been removed (without the chorion and amniotic fluid) and 2 pools of 5 embryos were collected for each exposure tank (8 pools of intact embryos and 8 pools of dechorinated embryos per treatment group).

Prior to analysis of copper concentrations, tissue digestion was conducted by adding 250µl of nitric acid (70%, purified by redistillation, ≥99.999% trace metals basis, Sigma Aldrich) to each tube and incubated at room temperature (20°C) for 48 hours, until all samples had completely digested. The volume of the resulting digested solution was added to ultrapure water, in a 1:10 ratio. The copper content in each sample was measured using a Perkin Elmer NexION 350D instrument running the Syngistix software, v 1.0. at King's College London.

Quantification of gene expression

Transcript profiles were measured in embryos exposed as described above for the copper uptake experiments. Four pools of 5 embryos were collected from each replicate tank, snap frozen in liquid nitrogen and stored at -80°C until analysis. Total RNA was extracted from individual pools using the TRI reagent method (Sigma-Aldrich, UK) according to the manufacturer's instructions. The concentration and purity of the resulting RNA was determined using a NanoDrop-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA), and transcript quantification was conducted in 6 pooled samples per treatment, randomly selected from those where sufficient quality and quantity of RNA was obtained. Total RNA was DNAse treated and reverse transcribed, and quantification of target genes was conducted by RT-QPCR according to previously described methodologies [4]. One µg of total RNA was subjected to DNAse treatment (RQ1 DNase, Promega, Southampton, UK) to remove potential DNA contamination, prior to being converted to cDNA using M-MLV reverse transcriptase (Promega, UK) and primed with random hexamers

(MWG-Biotech), according to the manufacturer's instructions (MJ Research PTC200 Thermal Cycler). cDNA was diluted (1:2), then RT-QPCR was performed using an iCycler iQ Real-time Detection System (BioRad Labatories, Hercules, USA) and SYBR green chemistry (BioRad Labatories, Hercules, USA). Each sample was amplified in triplicate in 96-well optical plates (BioRad Labatories, Hercules, USA) in 15µl reaction volumes. The control gene, *rpl8*, was selected to normalise the transcript profiles for target genes. Efficiency-corrected relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method [5-6]. Samples where amplification of specific fragments was not detected were excluded from analysis.

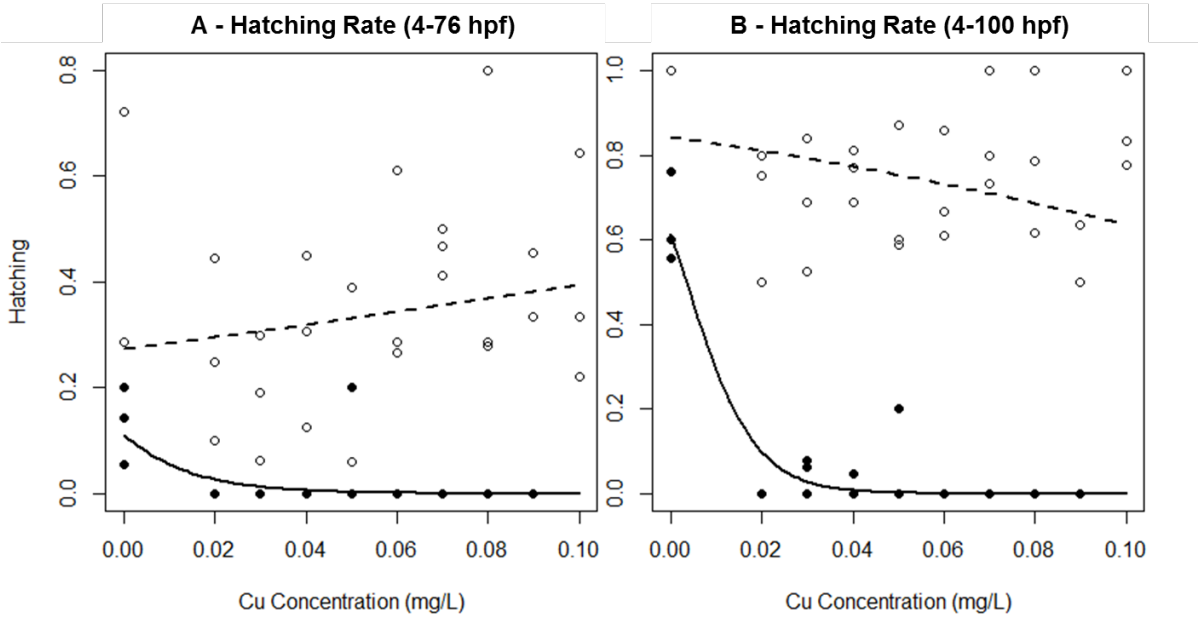

Figure S1. Proportion of hatched embryos exposed to copper under normoxic or hypoxic conditions. Each point on graph represents the proportion of hatched embryos in an individual tank containing 20 embryos. White and black symbols represent hypoxic (45.3% ±0.21 air saturation) and normoxic (98.4% ±0.12 air saturation) treatments, respectively, and the lines represent the best fit line for the data, calculated using a generalized linear model in R (model output summarised in Table S2). (A) Proportion of hatched embryos from 4 to 76hpf from the continuous exposure to copper (n=3 per copper concentration). There was a significant difference in the proportion of hatched embryos exposed to copper under normoxia compared to hypoxia (GLM:$F_{(1,52)}$=4.25, P<0.05) and the slope of the dose response curves also differed (P=0.003). (B) Proportion of hatched embryos at the end of the continuous exposure to copper from 4 to 100hpf (n=3 per copper concentration, except for control where n=9). There was a significant effect of copper on hatching rate under normoxia but not under hypoxia (P<0.01). In addition, there was a significant difference in the proportion of hatched embryos exposed to copper under normoxia compared to hypoxia (P<0.001) and the slope of the dose response curves also differed (P<0.01; Table S2a).

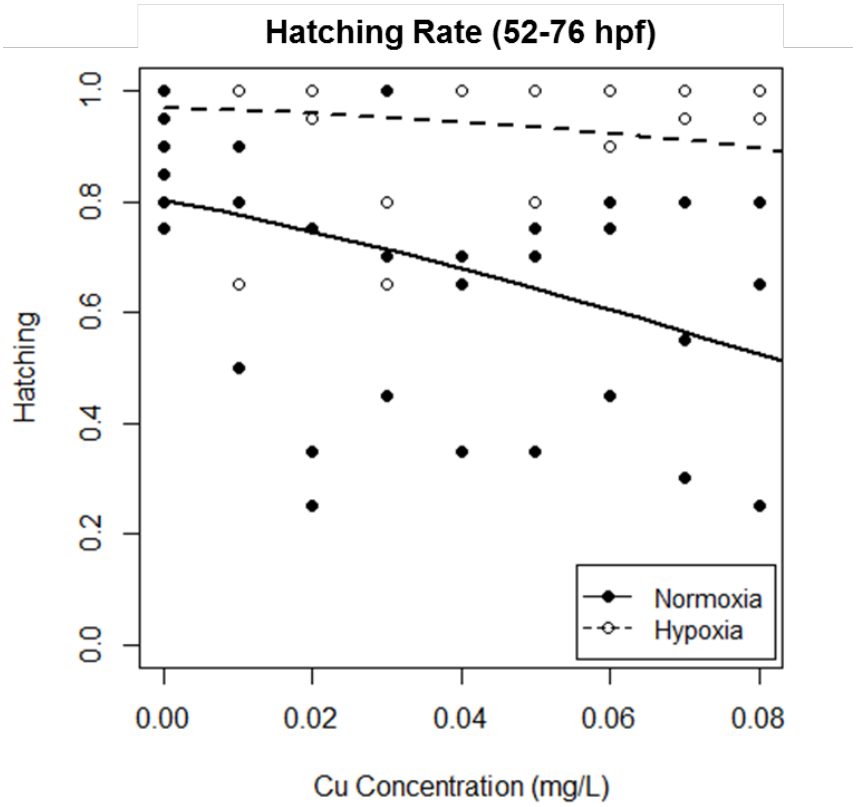

Figure S2. Proportion of hatched embryos exposed to copper under normoxic and hypoxic conditions from 52-76hpf. Each point on the graph represents an individual tank containing 20 embryos (n=3 per copper concentration, except for control where n=9) exposed from 52-76hpf. White and black symbols represent hypoxic (45.3% ±0.21 air saturation) and normoxic (98.4% ±0.12 air saturation) treatments, respectively, and the lines represent the best fit line for the data, calculated using a generalized linear model in R. There was a significant effect of copper under normoxia ($P<0.01$) but not under hypoxia. In addition, there was a significant difference in copper-induced hatching under normoxia compared to hypoxia ($P<0.001$) but the slope of the dose response curves did not differ (Table S2b).

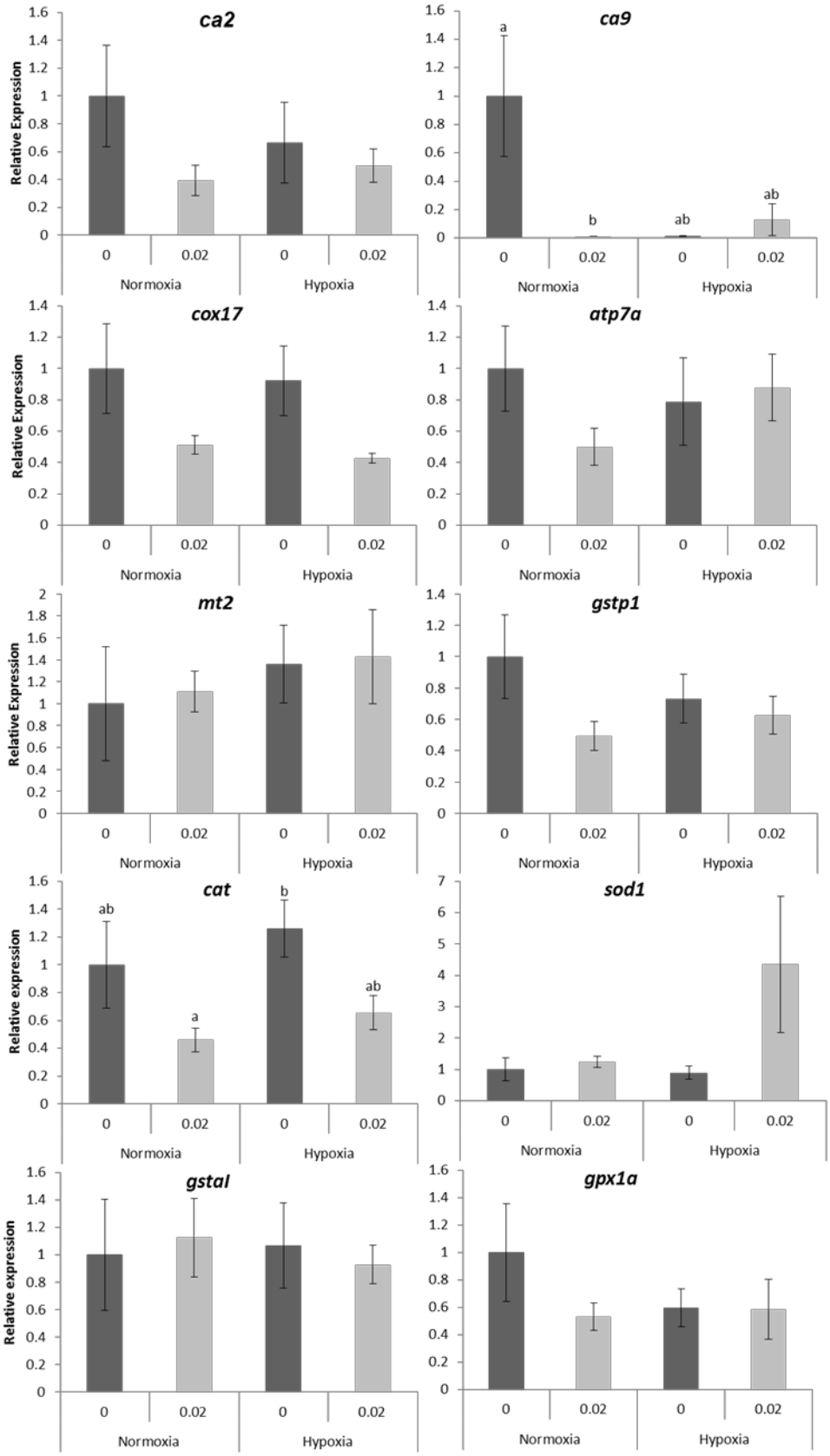

Figure S3. Transcript profiles for target genes in embryos exposed from 4-28hpf. Embryos were exposed to 0 or 0.024 mg Cu/L under hypoxia (43.2% ±0.55 air saturation) or normoxia (98.9% ±0.22 air saturation) in duplicate tanks. Immediately after the exposure period, embryos were sampled and grouped in

pools of 5 embryos per treatment group. 10 target genes were quantified using QPCR: 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-transferease alpha like (*gstal*) and glutathione peroxidase 1 a (*gpx1a*). Six pools of embryos were analysed for each treatment group. Data are presented as averaged relative expression ± standard error of the mean (n=4-6). Letters 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; $P<0.05$).

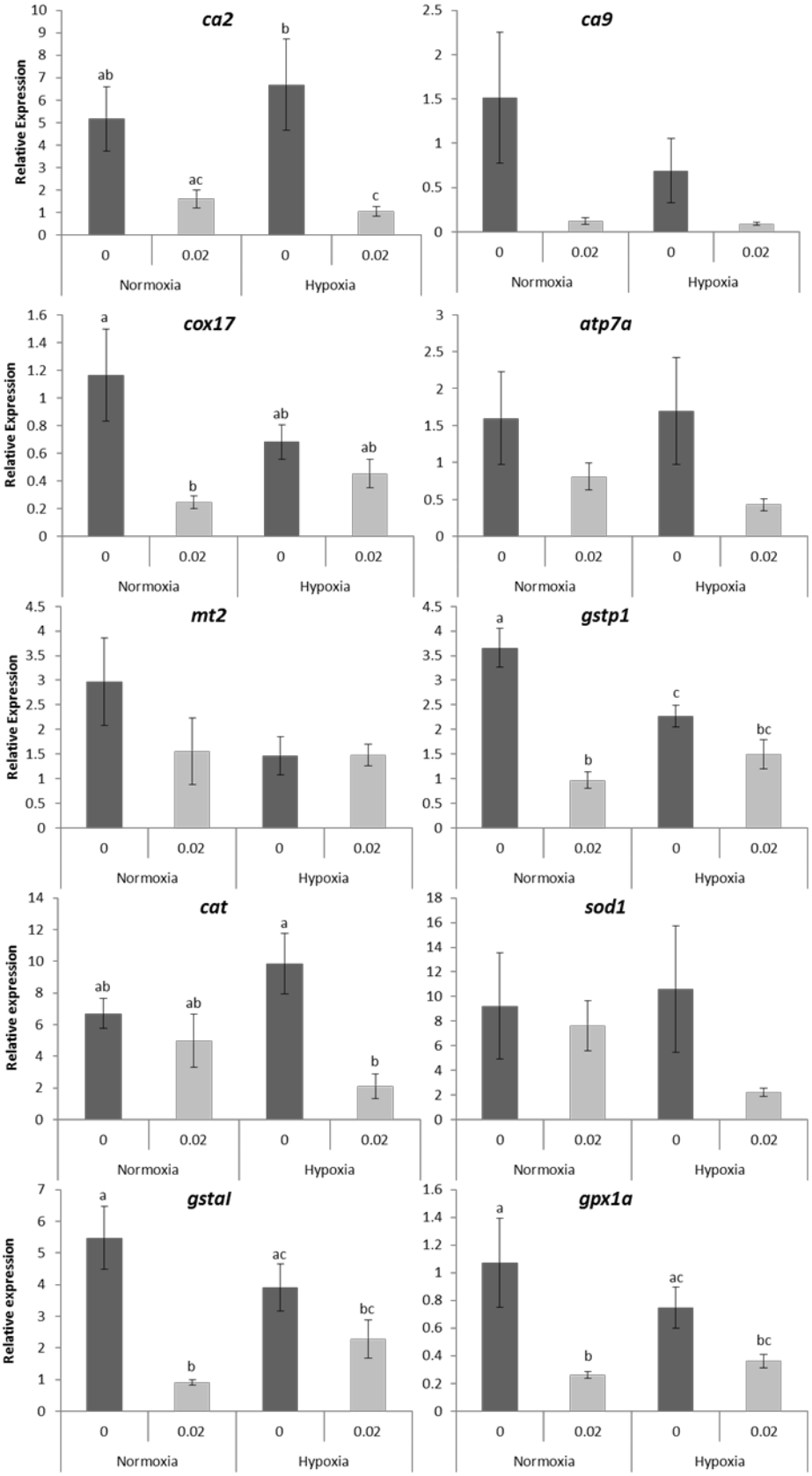

Figure S4. Transcript profiles for target genes in embryos exposed from 28-52hpf. Embryos were exposed to 0 or 0.024 mg Cu/L under hypoxia (43.2% ±0.55 air saturation) or normoxia (98.9% ±0.22 air saturation) in duplicate tanks. Immediately after the exposure period, embryos were sampled and grouped in

pools of 5 embryos per treatment group. 10 target genes were quantified using QPCR: 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-transferease alpha like (*gstal*) and glutathione peroxidase 1 a (*gpx1a*). Six pools of embryos were analysed for each treatment group. Data are presented as averaged relative expression ± standard error of the mean (n=4-6). Letters 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; $P<0.05$).

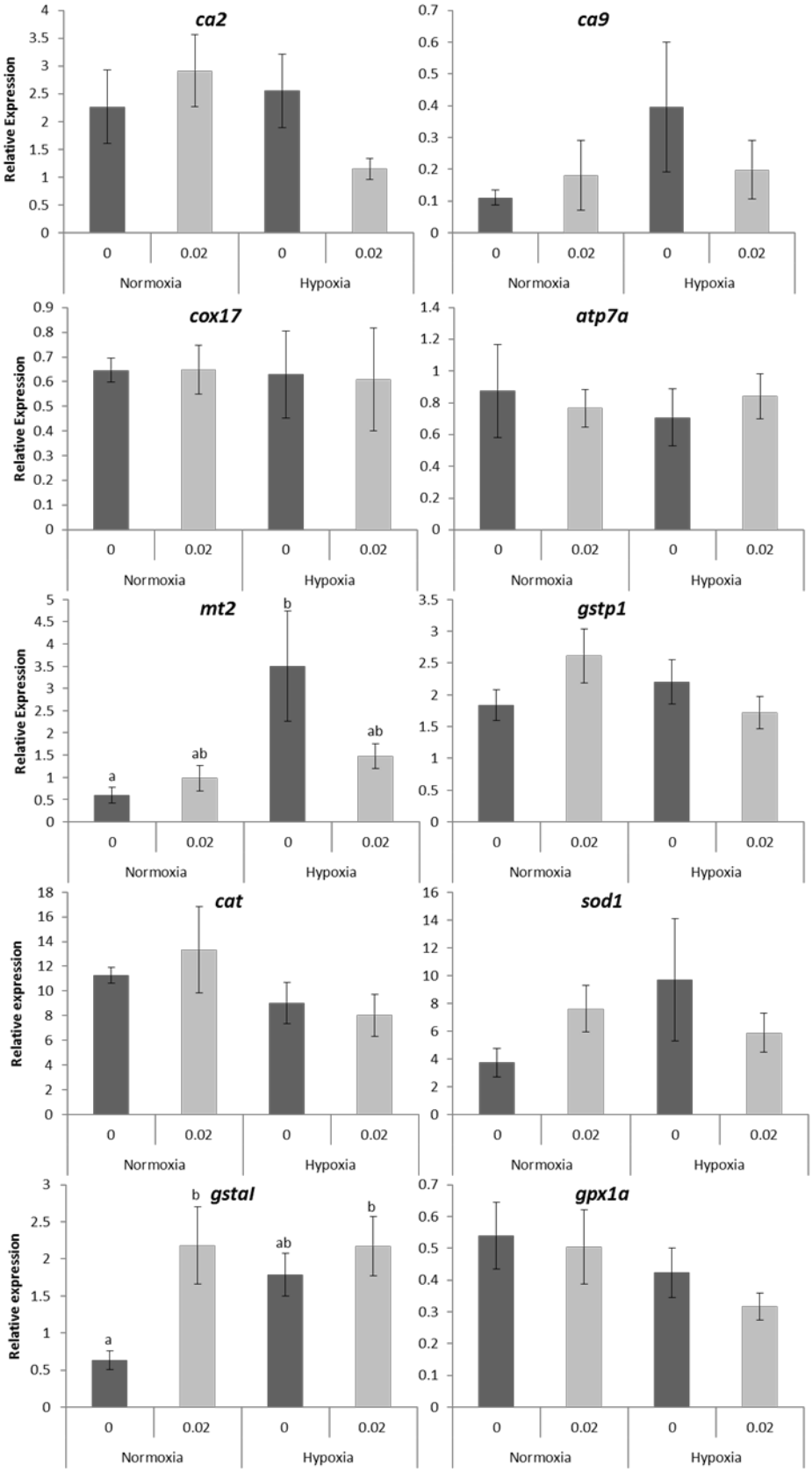

Figure S5. Transcript profiles for target genes in embryos exposed from 52-76hpf. Embryos were exposed to 0 or 0.024 mg Cu/L under hypoxia (43.2% ±0.55 air saturation) or normoxia (98.9% ±0.22 air saturation) in duplicate tanks. Immediately after the exposure period, embryos were sampled and grouped in

pools of 5 embryos per treatment group. 10 target genes were quantified using QPCR: 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-transferease alpha like (*gstal*) and glutathione peroxidase 1 a (*gpx1a*). Six pools of embryos were analysed for each treatment. Data are presented as averaged relative expression ± standard error of the mean (n=4-6). Letters 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; $P<0.05$).

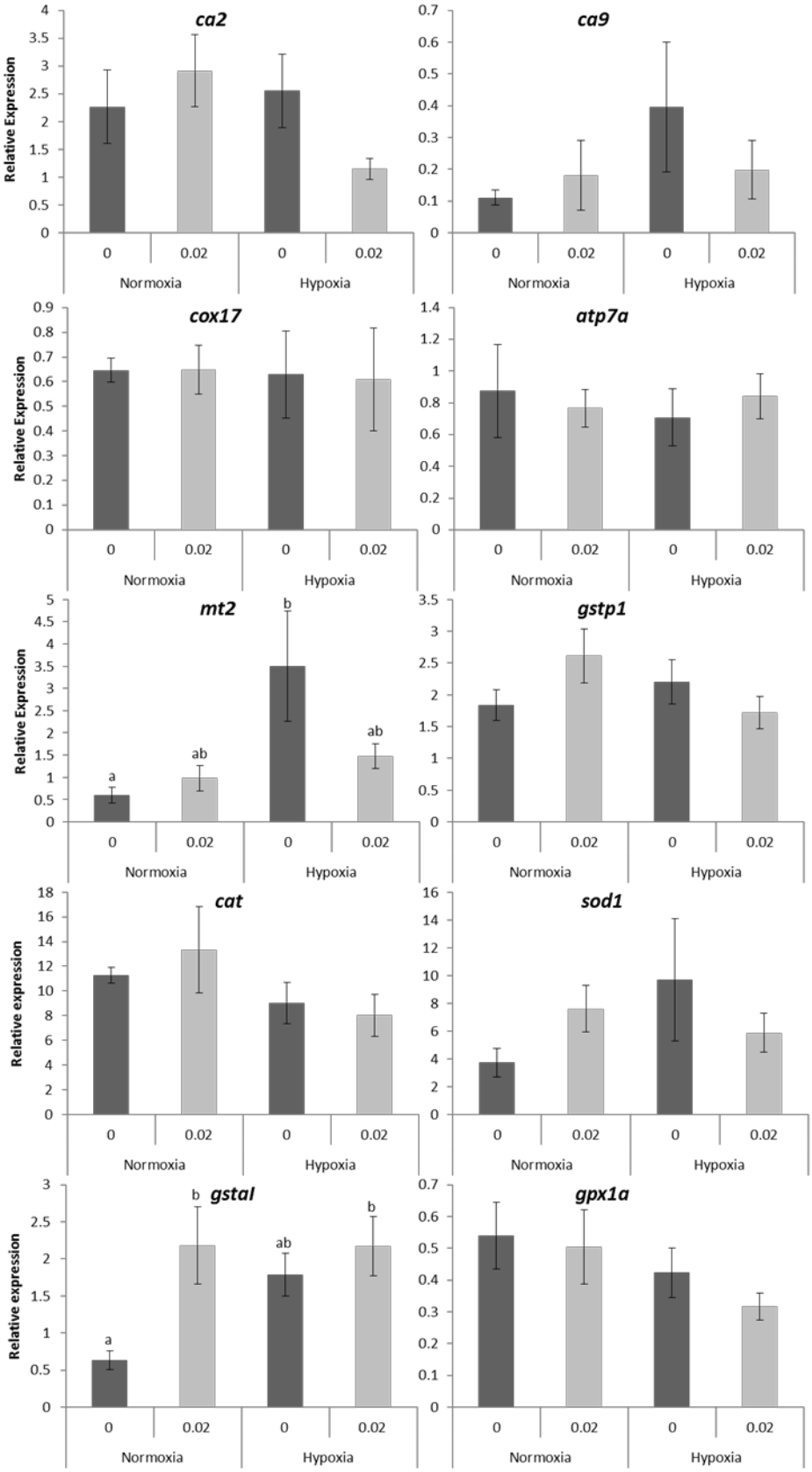

Figure S6. Transcript profiles for target genes in embryos exposed from 76-100hpf. Embryos were exposed to 0 or 0.024 mg Cu/L under hypoxia (43.2% ±0.55 air saturation) or normoxia (98.9% ±0.22 air saturation) in duplicate tanks. Immediately after the exposure period, embryos were sampled and grouped in

pools of 5 embryos per treatment group. 10 target genes were quantified using QPCR: 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-transferease alpha like (*gstal*) and glutathione peroxidase 1 a (*gpx1a*). Six pools of embryos were analysed for each treatment. Data are presented as averaged relative expression ± standard error of the mean (n=4-6). Letters 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; $P<0.05$).

Table S1. Target genes, primer sequences, amplicon product size, annealing temperature and PCR efficiency for the QPCR assays used in this study.

Gene Name	Gene Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Ta(°C)	PCR efficiency
ribosomal protein L8	*rpl8*	CCGAGACCAAGAAATCCAGAG	CCAGCAACAACACCAACAAC	91	59.5	1.95
metallothionein 2	*mt2*	AATGTGAATCTGTTTGTCTACTCC	GCATCGTTTTCCCTCTTTAGC	164	59.0	2.25
carbonic anhydrase II	*ca2*	CACAGGCACATTCAGACTC	CAACCACAGCAAGACCATC	179	59.0	2.19
carbonic anhydrase IX	*ca9*	CTGCTGGGTTTCCTCCTCATC	CTCTCGCTGCTCTTGTCTTCC	86	62.0	2.12
cytochrome c oxidase copper chaperone	*cox17*	GACAGCAGCAGCAGGTTG	CAGGCACAGCAGGGTTTG	174	57.5	1.99
ATPase, Cu^{++}transporting, alpha polypetide	*atp7a*	ATGGTGGCGATAGCAGAC	GAGATGGCAAGACTTCAGC	169	59.0	2.21
catalase	*cat*	AGTTCCCTCTGATTCCTGTG	ATGGCGATGTGTGTCTGG	173	61.0	2.00
superoxide dismutase 1	*sod1*	TTCACTCTCTCACAACTTCTC	GTCACCTTCACTGGCTTC	142	58.0	2.18
glutathione peroxidase 1 a	*gpx1a*	CTGCGTGTTGCCCTTTGAG	GGTGTAATCCCTGACTGTTGTG	189	58.5	1.98
glutathione S-transferase pi 1	*gstp1*	AACGACAGTGAGGCTTCC	GCATTTGAGGTGGTTGGG	141	56.0	1.85
glutathione-s-transferase alpha like	*gstαl*	GGTGGCTCTTGGCTGTTG	GGTGGCTCTTGGCTGTTG	170	61.0	2.03

Table S2. Generalized linear models for the relationships between mortality and copper concentration, O_2 concentration and the copper / O_2 concentrations interaction. Minimum adequate models for proportion of mortality using a quasibinomial error structure are shown. A) Embryo mortality curves following exposure to copper under normoxia or hypoxia throughout development, B) Embryo mortality curves following exposure to copper under normoxia or hypoxia at specific developmental windows. (Significance codes: *** P<0.001, ** P<0.01, * P<0.05)

	Coefficient (± SE)			
	Intercept	**Copper**	**O_2 Concentration**	**Copper / O_2 Conc. interaction**
a)				
4-28 hpf	-2.51 (±0.37)	20.31 (±5.30)***	-1.00 (±0.58)***	59.63 (±10.37)***
4-52 hpf	-2.52 (±0.36)	22.45 (±5.24)***	-0.99 (±0.58) ***	58.78 (±10.52)***
4-76 hpf	-2.52 (±0.36)	22.45 (±5.24)***	-0.99 (±0.58) ***	58.78 (±10.52)***
4-100 hpf	-2.69 (±0.34)	29.87 (±4.92)***	-0.82 (±0.54) ***	51.35 (±9.84)***
b)				
4-28 hpf	-3.11 (±0.27)	31.69 (±2.76)***	0.12 (±0.41) ***	32.47 (±6.16)***
28-52 hpf	-2.70 (±0.148)	9.66 (±0.68)***	NS	NS
52-76 hpf	-3.11 (±0.14)	21.07 (±0.97)***	-0.42 (±0.16)**	NS
76-100 hpf	-4.72 (±0.28)	104.06 (±5.48)***	-1.52 (±0.20)***	NS

Table S3. Generalized linear models for the relationships between hatching and copper concentration, O_2 concentration and the copper / O_2 concentrations interaction. Minimum adequate models for proportion of hatched embryos using a quasibinomial error structure are shown. A) Proportion of hatched embryos at the end of the continuous exposure to copper from 4 to 76 and 4 to 100hpf (signifcance level not shown where O_2 and Cu were retained in the model including an interaction term, if the coefficients of the individual term had P>0.05.), B) Proportion of hatched embryos at the end of the 52-76hpf exposure to copper. (Significance codes: *** P<0.001, ** P<0.01, * P<0.05)

	Coefficient (± SE)			
	Intercept	**Copper**	**O_2 Concentration**	**Copper / O_2 Conc. interaction**
a)				
4-76 hpf	-0.98 (±0.32)	5.52 (±5.56)*	-1.10 (±0.76)***	-81.54 (±50.78)*
4-100 hpf	1.67 (±0.40)	-11.15 (±6.93)	-1.21 (±0.28)***	-122.46 (±37.07)***
b)				
52-76 hpf	3.47 (±0.45)	-16.31 (±5.84)***	-2.06 (±0.41)**	NS

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Chapter 3

Contrasting effects of hypoxia on copper toxicity during development in the three-spined stickleback (*Gasterosteus aculeatus*)

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Contrasting effects of hypoxia on copper toxicity during development in the three-spined stickleback (*Gasterosteus aculeatus*)

Jennifer A. Fitzgerald[1,2*], *Ioanna Katsiadaki*[2], *Eduarda M. Santos*[1*]

[1] Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, UK

[2] Centre for Environment, Fisheries and Aquaculture Science, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, UK

Corresponding authors:

Jennifer A. Fitzgerald, Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, UK. E-mail: jf277@exeter.ac.uk.

Eduarda M. Santos, Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, UK. Phone: 00 44 (0)1392 264607; Email: e.santos@exeter.ac.uk.

Abstract

Hypoxia is a global problem in aquatic systems and often co-occurs with pollutants, despite this, little is known about the combined effects of these stressors on aquatic organisms. The objective of this study was to investigate the combined effects of hypoxia and copper, a toxic metal widespread in the aquatic environment. We used the three-spined stickleback (*Gasterosteus aculeatus*) as a model because of its environmental relevance and amenability for environmental toxicology studies. We focused on embryonic development as this is considered to be a sensitive life stage to environmental pollution. We first investigated the effects of hypoxia alone on stickleback development in order to generate the basic information required to design subsequent studies. Our data showed that exposure to low oxygen concentrations (24.7 ±0.9 % air saturation;AS) resulted in strong developmental delays and ultimately caused mortality, whereas mild hypoxia (75.0 ±0.5 %AS) resulted in premature hatching. Stickleback embryos were then exposed to a range of copper concentrations under hypoxia (56.1 ±0.2 %AS) or normoxia (97.6 ±0.1 %AS), continuously, from fertilisation to free swimming larvae. Hypoxia caused significant changes in copper toxicity throughout embryonic development. Prior to hatching, hypoxia suppressed the occurrence of mortalities, but after hatching, hypoxia significantly increased copper toxicity. Interestingly, when exposures were conducted only after hatching, the onset of copper-induced mortality was delayed under hypoxia compared to normoxia, but after 48h copper was more toxic to hatched embryos under hypoxia. This is the second species for which the protective effect of hypoxia on copper toxicity prior to hatching, followed by its exacerbating effect after hatching is demonstrated, suggesting the hypothesis that this pattern may be common for teleost species. Our research highlights the importance of considering the interactions between multiple stressors, as understanding these interactions is essential to facilitate the accurate prediction of the consequences of exposure to complex stressors in a rapidly changing environment.

Keywords: oxygen, toxic metal, freshwater, combined stressors, fish

Capsule Copper toxicity is modified by the level of oxygen in the environment in a developmental stage specific manner in fish.

Introduction

For most aquatic organisms, oxygen is essential for life but its levels in the environment can vary widely, and when dissolved oxygen concentrations drop below the levels required to sustain aerobic life (hypoxia), changes in ecosystem dynamics are likely to occur (Diaz and Breitburg, 2009). Areas of low oxygen can occur naturally in both freshwater and marine ecosystems, and evidence has shown that freshwater systems are more prone to hypoxia and anoxia (Jenny et al., 2016). In temperate freshwater lakes, for example, hypoxia can occur when factors affect vertical mixing, such as wind and temperature, or if a lake is covered by snow or ice preventing reaeration (Richards, 2009). In tropical freshwater systems, oxygen concentrations can vary depending on the amount of rainfall; during the dry season oxygen concentrations drop as the water becomes stagnant, whereas rain promotes oxygenation of the water through increasing flow and mixing (Richards, 2009).

Hypoxia has increased rapidly in recent years and is a growing threat to aquatic ecosystems worldwide (Gewin, 2010). The causes of the increase in frequency, duration and severity of hypoxic events have been attributed to excessive anthropogenic nutrients entering water bodies (Gamenick et al., 1996), observed in both marine (Assessment, 2005; Diaz and Rosenberg, 2008; Friedrich et al., 2014) and freshwater systems (Jenny et al., 2016; Richards, 2009). Excess nutrients in aquatic systems cause increased proliferation of algae at the surface and decomposition of organic materials in deep waters (eutrophication) resulting in oxygen depletion. Other factors, including changes in temperature, can affect oxygen solubility, stratification of water bodies and metabolism of organisms, and contribute to the changing oxygen budget in the aquatic environment.

Periods of lowest oxygen concentrations often coincide with the presence of the highest contaminant concentrations (van der Geest et al., 2002), so it is fundamental to understand how hypoxia influences chemical toxicity. To date, studies investigating whether hypoxia modifies chemical toxicity in fish have considered endocrine disrupting chemicals (Brian et al., 2008), metals (Chun et al., 2000; Hattink et al., 2005; Hattink et al., 2006; Heath, 1991; Lloyd, 1961; Kienle et al., 2008; Mustafa et al., 2012; Sampaio et al., 2008), polyaromatic hydrocarbons (Prasch et al., 2004) and pharmaceuticals (Prokkola., 2015;

Lubiana et al., 2016). Together, these studies suggest that the effects of hypoxia on chemical toxicity can vary widely as a function of the chemicals considered, the species and its life stage. This highlights a critical research need to understand how the effects of a stressor are influenced by the presence of other stressors, in order to accurately predict the consequences of exposure to combined stressors in the aquatic environment.

Among the contaminants in aquatic systems, copper is an important chemical of concern, due to its widespread distribution and high toxicity at concentrations found in some of the most polluted environments. Copper concentrations vary naturally in freshwater systems, however as a consequence of both contemporary and historical mining activities, industrial processes, and urban and agricultural runoff, many water bodies have become significantly contaminated (Batty et al., 2010). Examples of highly contaminated aquatic systems include Watarase Basin, Japan, where levels reached over 70 µg Cu/L (Ohmichi et al., 2006), the catchment of the River Lee in the UK where measured concentrations were 124 µg Cu/L (Snook and Whitehead, 2004) and the River Hayle River (UK), where concentrations reached up to 193 µg Cu/L (Uren Webster et al., 2013). Copper was also identified as the metal causing the greatest threat to the aquatic environment in the UK, based on its current measured concentrations and toxic effects (Donnachie et al., 2014).

It has been widely documented that decreased water hardness dramatically increase copper toxicity to fish, and copper bioavailability is usually higher in freshwater than seawater (Flemming and Trevors, 1989). As a result, copper is particularly toxic to freshwater fish species. Excess copper can cause toxicity by affecting osmoregulation (Chowdhury et al., 2016; Grosell and Wood, 2002; Laurén and McDonald, 1987; Wood et al., 2012), causing disturbances of metabolic processes as a result of inhibition of respiratory enzymes, and compromising gas exchange by inducing gill damage (Beaumont et al., 2003; Chowdhury et al., 2016; McDonald and Wood, 1993). In addition, copper can also induce oxidative stress, via increased reactive oxygen species, resulting in DNA damage and lipid peroxidation (Craig et al., 2007; Nawaz et al., 2006; Sanchez et al., 2005; Sevcikova et al., 2011). In fish, the most sensitive stages of development are thought to be the embryo-larval or early juvenile stages (Flemming and Trevors, 1989). Observed effects during early life in zebrafish

(*Danio rerio*) included the reduction in yolk sac utilisation and decreased body length in exposed larvae, suggesting that copper may delay development. In addition, exposure to copper caused a significant reduction in neuromasts in zebrafish embryos, potentially affecting their ability to survive due to lateral line dysfunction and behaviour impairment (Hernández et al., 2006; Johnson et al., 2007; Linbo et al., 2006).

In our previous work we demonstrated that copper toxicity strongly decreased under low dissolved oxygen conditions during the first 24 hours of development in zebrafish embryos, but in contrast, hypoxia significantly increased copper toxicity after hatching (Fitzgerald et al., 2016). In the common carp (*Cyprinus carpio*) copper toxicity was increased under hypoxia compared to normoxia in adults (Malekpouri et al., 2016; Mustafa et al., 2012). Based on this evidence, we hypothesise that the combined effects of hypoxia and copper on fish are dependent on life stage, with critical switch points occurring during development. To test this, more studies are needed to elucidate the interactions between copper and hypoxia for a range of representative model species, in particular during development, a life stage particularly vulnerable to environmental stressors and that has received little attention to date.

The three-spined stickleback (*Gasterosteus aculeatus*) was used as a model organism for this study because of its ecological relevance to temperate freshwater and marine ecosystems in the Northern hemisphere and its well established role as a model species for environmental toxicology (Katsiadaki et al., 2007). Studies investigating the responses of this species to hypoxia have reported behavioural effects (fewer aggressive acts (Sneddon and Yerbury, 2004) and loss of inquisitive and active behaviour (Leveelahti et al., 2011)), physiological responses (increased rate of gill movements (Jones, 1952)) and biochemical effects (increases in tissue L-lactate (Sneddon and Yerbury, 2004)). During development sticklebacks are deemed to be sensitive to low oxygen, a characteristic associated with the parental behaviour of adult males (Green and McCormick, 2005; Pollock et al., 2007). Male sticklebacks invest significant time and energy resources to fanning their embryo-bearing nest, resulting in an increased oxygen concentration around the embryos. Under hypoxic conditions increased fanning tempo was observed resulting in increased oxygenation of the nest (Reebs et al., 2007). However the effects of

hypoxia on embryo development have not been previously documented. This information is essential to facilitate the use of stickleback embryos as models to investigate the combined effects of hypoxia and chemical stressors.

The aim of this study was to investigate the effects of reduced oxygen concentrations, alone and combined with copper, on the stickleback throughout development. We first established the responses of stickleback to various concentrations of oxygen during development and determined the sensitivity of this species to hypoxia during early life stages. These data informed the design of experiments investigating whether mild hypoxia affect copper toxicity during early life. To do this, embryos were exposed continuously throughout development until they reach the stage of free feeding larvae (which is 48h after hatching at 19 °C), or only after hatching, to a range of concentrations of copper under hypoxia and normoxia. Dose response curves for mortality and hatching rates were compared to determine the differences in copper toxicity when exposures were conducted under normoxia or hypoxia.

Material and Methods

Fish source, culture and husbandry

A population of freshwater three-spined stickleback (originating from the River Erme, Devon, United Kingdom, kindly provided by the University of Plymouth) was maintained in the Aquatic Resource Centre at the University of Exeter in mixed sex stock tanks (120 L), supplied with aerated synthetic freshwater. Before it was supplied to each aquarium, mains tap water was filtered by reverse osmosis (Environmental Water Systems (UK) Ltd) and reconstituted with Analar-grade mineral salts to standardized synthetic freshwater (final concentrations resulting in a conductivity of 300 mS), aerated, and heated to 19 ±1 °C in a reservoir. Throughout the experimental period all adults were maintained under summer conditions which corresponded to a temperature of 19 ±1 °C and a photoperiod of 18:6 h light/dark (with a 30 minute dawn/dusk transition period). Fish were fed to satiation every day with blood worm (*Chironomus sp.*; Tropical Marine Centre, Chorleywood, UK).

The stickleback embryos included in this study were obtained via artificial fertilisation (method adapted from Barber and Arnott (2000)). Unfertilised eggs were identified by visual observation as described by Swarup (1958), using a dissection microscope (Motic DM143, Hong Kong) and removed. Fertilised embryos were incubated in aerated artificial freshwater (according to the ISO-7346/3 guideline, ISO water, diluted 1:5 (International Organization for Standardization, 1996)), in experimental exposure tanks, from 1hr post fertilisation, set up as described below. All fish were maintained under approved protocols, according to the UK Home Office regulations for the use of animals in scientific procedures.

Embryo exposures to hypoxia

Stickleback embryos were exposed to different levels of air saturation (AS) to determine the effects to hypoxia on development.

To produce the required percentage of AS, water was aerated to produce 100 % AS, or bubbled with nitrogen for 1hr to remove all dissolved oxygen, then allowed to equilibrate to 19 ±1 °C for at least 1 hour before the start of the exposure. Water was then mixed at the appropriate proportion to obtain 100, 80, 60, 40 and 20 % AS.

Exposures were initiated at 1 hour post fertilisation (hpf; corresponding to the one cell stage; blastodermic cap), when 20 fertilised eggs were randomly allocated to 600ml acid-washed glass tanks and sealed with a glass lid, to prevent re-oxygenation, and all treatments were run in triplicate. To avoid changes in the water characteristics by the metabolic activity of the embryos, a large volume of water (30 ml per embryo) was used. After 24 hrs of exposure, the percentage of AS was measured in each exposure tank using an optical dissolved oxygen meter (Mettler SevenGo Pro OptiOx, U.S.) and was found to be within 5 % of the nominal AS (measured AS were 96.5 ±0.3 %, 75.0 ±0.5 %, 57.6 ±0.6 %, 40.9 ±0.5 % and 24.7 ±0.9 %, for the 100 %, 80 %, 60 %, 40 % and 20 % treatments, respectively). Individual embryos were observed using a dissection microscope (Motic DM143, Hong Kong) and the proportion of mortalities, hatched embryos, the stage of development and any developmental abnormalities were recorded. From each dish, 1 embryo was removed, anesthetised using tricane (Sigma Aldrich) and mounted on a slide using

methylcellulose (2 %; Sigma Aldrich) for imaging. Photographs were taken using a compound microscope (Nikon SM21500, Japan) equipped with a digital camera, to allow visualisation of any developmental effects resulting from the exposure to low oxygen concentrations. A complete water change was carried out at each 24-hour period, as described above, and the experiment was maintained until 217 hpf. Up until this time point under our experimental conditions, sticklebacks have yolk sacs and as such are not considered to be free feeding; this has regulatory implications as under UK legislation, fish embryos become protected under the Animals (Scientific Procedures) Act from the free feeding stage onwards.

Copper exposures under normoxia and hypoxia

Embryos were continuously exposed to a range of copper concentrations (copper sulphate; Fisher, Fair Lawn NJ), 0, 0.015, 0.025, 0.05, 0.0625, 0.075, 0.0875, 0.1, 0.125, 0.15 mg Cu/L, throughout development (1-217 hpf). The range of concentrations was selected to encompass concentrations causing 0 % and 100 % mortalities, based on preliminary experiments. Based on the hypoxia experiments described above, 50 % AS was selected as the nominal level of AS for the combined copper and hypoxia exposures, because this was the lowest level that did not result in observable developmental delays. The measured levels of AS were 97.6 ±0.1 % and 56.1 ±0.2 % for exposures conducted under normoxia and hypoxia, respectively. For each treatment, 20 embryos were randomly allocated to 600 ml acid washed glass tanks, with water prepared as above, containing the appropriate concentration of copper and at the appropriate AS. After each 24 h exposure period, the percentage of AS was immediately measured and the proportion of mortalities, hatched embryos, the stage of development and any developmental abnormalities were recorded for each exposure tank. Water was then completely replaced with freshly made exposure water at the appropriate AS and copper concentration, as described above. All exposures were conducted in triplicate.

Copper exposures under normoxia and hypoxia to hatched embryos

Additional exposures were conducted during the post hatch embryo stage (169-217 hpf). Embryos were randomly allocated to exposure tanks as described above and incubated under control conditions (97.6 ±0.1 % AS, 0 mg Cu/L) up

to the start of the exposure period (169 hpf). Embryos were then exposed to a range of copper concentrations (from 0-0.15 mg/L) under normoxia and hypoxia (97.6 ±0.1 % and 56.1 ±0.2 %, respectively), as described above. Similarly to the continuous exposure, oxygen levels were measured and mortality and developmental delays monitored before water was replaced after 24 h of exposure, and were maintained until 48 hours post exposure. All combined exposures to copper and hypoxia were conducted in triplicate.

Statistical analysis

Statistical analyses were conducted in R (Team, 2014). Effects of AS on the survival and hatching success of embryos after the exposure to different AS levels was analysed using a general linear model. Differences between the proportion of mortality and hatching following exposure to copper under hypoxia or normoxia were also identified using generalised linear models. A separate model was used for each time period after fertilisation, 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 (Crawley, 2012). A similar approach of model simplification of generalized linear models with quasibinomial error structure was used to test for the effects of the exposures on the proportion of hatched embryos following exposure to copper under hypoxia or normoxia and their interaction. F tests reported refer to the significance of removing terms from the models. All data was considered statistically significant when $p<0.05$.

Results

Effects of oxygen concentrations on stickleback development

Embryos exposed to the lowest oxygen concentration (24.7 ±0.9 % AS) showed the strongest adverse response (Fig. 1; Fig. 2), including an increased rate of mortality (Fig. 2a; Table S3). In addition, hatching and developmental delays occurred at this level of AS (Fig. 1; Fig. 2b; Table S3), with embryos observed to be smaller and having less developed pigmentation compared to the controls

(Fig. 1). Delays in development were also observed at 40 % AS, with embryos appearing to be smaller than the 100 % control, although no significant effects on hatching or mortalities were observed at this oxygen concentration (Fig. 1; Fig. 2). For concentrations of oxygen above 60 % AS, no developmental delays were observed in our study (Fig. 1; Fig. 2). However embryos exposed to 80 % AS (measured: 75.0 ±0.5 % AS), hatched on average 24 hours earlier than the controls (Fig. 1; Table S3).

Effects of hypoxia on copper toxicity throughout development

Copper caused mortalities to stickleback embryos under both hypoxic and normoxic conditions after 49 hpf, and the proportion of mortalities increased progressively throughout development, (Fig. 3). The effects of hypoxia on copper toxicity were dependent on the stage of development. For the first 48 h of exposure, there was no significant difference between the two different AS treatments ($P=0.0609$; Fig. 3). From 73-145 hpf, copper was more toxic to embryos under normoxia than under hypoxia ($P<0.01$; Fig. 3). During the period when hatching occurs (from 145-193 hpf), there was no significant difference in copper-induced mortalities under hypoxia compared to normoxia (1-169 hpf: $P=0.1554$, 1-193 hpf: $P=0.3591$; Fig. 3). In contrast, during the final 24 hours of exposure, copper caused significantly more mortalities to stickleback embryos under hypoxia compared to normoxia ($P<0.01$; Fig. 3).

Copper significantly delayed hatching during the periods of development when hatching occurs (169 hpf: $P<0.001$, Fig. 4a; 193 hpf: $P<0.001$, Fig. 4b; 216 hpf: $P<0.001$, Fig. 4c). Hypoxia had no effect on the proportion of embryos that had hatched following exposure to copper at 169 hpf ($P=0.411$; Fig. 4a) and 193 hpf ($P=0.730$; Fig. 4b). However, by 217 hpf, the delay in hatching caused by copper exposure was more severe under normoxia compared to hypoxia ($P=0.0305$; Fig. 4c).

Effects of hypoxia on copper toxicity to hatched embryos

Copper was significantly more toxic to hatched embryos under normoxia than under hypoxia during the first 24 h of exposure ($P<0.01$; Fig. 5). Maintaining the exposure for a further 24 h resulted in a dramatic change, with copper

significantly more toxic to hatched embryos under hypoxia than under normoxia ($P<0.05$; Fig. 5).

Discussion

Impacts of hypoxia on stickleback development

Hypoxia caused a range of developmental effects on stickleback development, with their severity proportional to the levels of oxygen depletion. The stickleback population used in this study was found to be comparatively sensitive to hypoxia when compared with some cyprinid species (goldfish (*Carassius auratus* (Bickler and Buck, 2007)), crucian carp (Nilsson and Renshaw, 2004) and zebrafish (Padilla and Roth, 2001)), or the annual killifish (*Austrofundulus limnaeus* (Podrabsky et al., 2007)), which have been shown to tolerate very low concentrations of oxygen or even anoxia during development or as adult fish, but greater than other teleost species, for example salmon (Bickler and Buck, 2007), who's high metabolic demands result in high sensitivity to hypoxia.

For the rainbow trout (*Oncorhynchus mykiss*), developmental delays and mortalities where observed when embryos were exposed to 40 % AS and lower (Roussel, 2007), a similar result to what was observed with the stickleback, which showed developmental delays at 40 % AS or below, but mortalities only for 20 % AS. In the environment, hypoxia-sensitive species such as the rainbow trout and the stickleback have adopted ecological strategies to avoid low oxygen conditions during development, reducing the risk of adverse effects when developing in hypoxic environments. Rainbow trout spawn in fast flowing, well oxygenated rivers and streams, resulting in constant high oxygen concentrations in the water surrounding the embryos (Ciuhandu et al., 2007). Stickleback, however, are typically found in still or slow flowing water of lakes, ponds, lowland streams and sheltered coastal bays, associated with their slow swimming behaviour (Wootton, 1984). To avoid the costs of low oxygen on development, stickleback males have evolved behaviours associated with parental care, and nurture embryos as they are incubated in nests throughout development. The male commits large amounts of time and energy to maintaining the nest, including vigorously fanning the nest and removing decaying embryos (Van Iersel, 1953; Wootton, 1984). Lipid and glycogen levels

have been shown to fall by 43 % and 37 %, respectively, during the reproductive season in breeding males, and the protein concentration in the carcass of breeding males was shown to fall by 70 % of pre-breeding levels (Chellappa et al., 1989), demonstrating the high energetic cost of parental care in breading males during the reproductive season. Male sticklebacks trade-off time and energy to ensure embryo survival and avoid the reduced growth and developmental delays which would result in a longer incubation time, reducing the predation risk and ensuring the fitness and survival of the embryos (Ostlund-Nilsson et al., 2006).

No developmental delays were observed in embryos exposed to concentrations of oxygen of 60 % AS and above. Oxygen concentrations in stickleback nests were shown to vary depending on whether the male is attending the nest or not. When parental fanning occurred, AS levels were measured at 95-100 % AS, but dropped to a minimum of 67 % when the male abandoned the nest (Green and McCormick, 2005). This corresponds to the AS range where no developmental delays were observed in our study, indicating that for AS levels occurring naturally within nests, normal development would occur.

For embryos exposed to 80 % AS, embryos hatched on average 24 hours earlier than the controls. The metabolic rate of the embryo is known to increase throughout development, causing an increase in their oxygen demands (Green and McCormick, 2005). For the 80 % AS treatment there was no sign of either delayed or accelerated development, suggesting they were at the same stage of development and in turn required the same oxygen concentrations as the control, despite less environmental oxygen available. Premature hatching may have occurred to compensate for the insufficient diffusion of oxygen through the chorion as previously seen for the Atlantic salmon (*Salmo salar*) and the rainbow trout (Hamor and Garside, 1976; Wu, 2009).

Combined effects of copper and hypoxia

The effects of hypoxia on copper toxicity were strongly dependent on the stage of development with contrasting effects observed prior to and after hatching. During early development, hypoxia significantly supressed copper toxicity, but during the hatching period this effect was no longer present and hypoxia did not alter copper toxicity. To our knowledge, there is only one other dataset

investigating the effects of hypoxia on copper toxicity during embryonic development in fish. In that study hypoxia strongly modified copper toxicity to zebrafish embryos, in a process dependent on developmental stage, and hypoxia protected embryos from copper toxicity during early development (Fitzgerald et al., 2016). In contrast to the stickleback where copper at the concentrations tested had no toxic effect until 49 hpf, in zebrafish the majority of mortalities occurred during the first 24 hours of exposure (Fitzgerald et al., 2016). The differences in tolerance between these species may have been as a result of the chorion in sticklebacks providing a greater barrier to copper in comparison to the zebrafish (Hartmann and Englert, 2012; Hosemann et al., 2004), as this would likely affect the amount of copper reaching embryonic tissues.

In contrast to what occurred during early development, after hatching hypoxia increased copper toxicity to stickleback embryos. This is similar to that observed for the zebrafish, where copper toxicity was greater under hypoxia after hatching (Fitzgerald et al., 2016). This suggests that independently of the species and its tolerance to hypoxia, after hatching hypoxia results in an increase in copper toxicity. This is supported by the results of exposures conducted in adult fish, which reported increased copper toxicity under hypoxia. For example, Mustafa *et al.* (2012) showed that carp were more sensitive to copper under hypoxia compared to normoxia, as combined exposures induced a significantly higher level of oxidative DNA damage. Hypoxia has also been shown to exacerbate the impacts of copper on the metabolic capabilities of the common carp, compared to copper exposure alone (Malekpouri et al., 2016). The similar trends in toxicity between hatched embryos and adult fish may be explained by the mode of uptake, which in both cases involve direct contact of fish with copper contaminated water and uptake via the skin and/or gills (Grosell and Wood, 2002; Zimmer et al., 2012; Zimmer et al., 2014). In contrast, prior to hatching, embryos are protected by the chorion, which will likely modify the exposure dynamics and how much copper reaches the embryonic tissues.

A second hypothesis explaining the hypoxia induced changes in copper toxicity before and after hatching may be due to different molecular responses to hypoxia in early embryos compared to later life stages. Around hatching there is a dramatic change in the expression, at the transcript and protein levels, of the

hypoxia inducible factor (HIF) alpha molecules in embryos (Köblitz et al., 2015; Rytkönen et al., 2013; Rytkönen et al., 2014), with consequent shifts in the molecular and physiological responses to hypoxia. These changes may result in alterations in the physiological processes regulated by HIF, including red blood cell formation and oxygen transport, changes in energy metabolism, suppression of cell growth and proliferation, and oxidative stress response (Lushchak, 2011; Nikinmaa and Rees, 2005; Richards, 2009). It has been shown in the zebrafish that hatching is a critical developmental period for the transcription and protein expression of the HIF alpha molecules (Kopp et al., 2011; Rytkönen et al., 2014), and it is possible that similar changes in the dynamics of which HIF isoforms are expressed, and the physiological changes they regulate, also take place in the three spined stickleback, potentially explaining the opposing effects of hypoxia on copper toxicity as development progresses. Further work, including studies in other fish species, is essential for confirmation of these hypotheses and to document the mechanistic basis of the shift in the effects of hypoxia on copper toxicity, from a protective effect during early development to an increase in toxicity in hatched embryos.

Copper significantly delayed hatching both under hypoxia and normoxia and this may be as a result of copper disrupting the activity of chorionase, an enzyme involved in the process of hatching (Dave and Xiu, 1991; Jezierska et al., 2009; Johnson et al., 2007). There was no effect of AS level on the hatching rate from 169 to 193hpf, however by 217 hpf, the delay in hatching was greater under normoxia compared to hypoxia. At this time point (217 hpf), copper also caused greater proportion of mortality under hypoxia compared to normoxia, therefore the increase in hatching rate observed in exposures conducted under hypoxia could be associated with the observed increase in copper toxicity.

Effects of hypoxia on copper toxicity to hatched embryos

The concentration of oxygen in freshwater systems is very variable, with oxygen levels potentially dropping to levels as low as 50 % overnight due to maintenance of cellular respiration in the absence of photosynthesis (Tengberg et al., 2006), and vary rapidly over time in the course of days due to changes in temperature, precipitation and water mixing. It is important, and environmentally relevant, to consider the effects of hypoxia on chemical toxicity during short

developmental windows. Post-hatch embryos are considered to be the most sensitive stage of development for sticklebacks (Ostlund-Nilsson et al., 2006), marking this developmental stage critically sensitive to the combined effects of copper and hypoxia. Therefore, we investigated the effect of hypoxia on copper toxicity during this life stage, in the absence of pre-exposure to these stressors, to determine if pre-exposure during development affected the sensitivity of hatched embryos.

During the first 24 hrs of exposure of hatched embryos, copper was significantly more toxic under normoxia compared to hypoxia, but by 48 h copper was significantly more toxic under hypoxia than under normoxia. The data for this later time point concurs with what was observed for embryos exposed continuously throughout development and discussed above, with hypoxia increasing copper toxicity after hatching. We hypothesise that hypoxia may delay the uptake of copper into the embryos resulting in a later onset of toxicity, and/or that the cellular responses to hypoxia may initially protect from the toxic effects of copper, for example by the activation of oxidative stress defence mechanisms, a known effect of the activation of the HIF signalling pathway (Ransberry et al., 2016). Further work will be required to test these hypotheses across a representative range of aquatic species, in order to facilitate the prediction of how species will respond to combinations of hypoxia and copper toxicity.

Mortality was greater when embryos were exposed only after hatching compared to that measured at the same developmental stage for embryos exposed continuously throughout development. For example, for the continuous exposure throughout development, 0.1mg Cu/L caused 68 % and 87 % mortalities under normoxia and hypoxia, respectively (Fig. 3), while when embryos were exposed only after hatching the same copper concentration resulted in 97 % and 100 % mortality under normoxia and hypoxia, respectively (Fig. 5). This may be as a result of the embryos in the continuous exposure having a period where acclimation to copper and hypoxia may have occurred. Although comparable results for developmental exposures in fish have not been previously reported, evidence exists for a protective effect of pre-exposure on subsequent tolerance to copper in other organisms. Herkovits and Pérez-Coll (2007) showed that pre-exposure of copper resulted in a transient beneficial

effect on embryo survival when re-exposed to the same metal in *Bufo arenarum* (Herkovits and Pérez-Coll, 2007). Similarly, pre-exposure to arsenic decreased its toxicity during subsequent exposures in killifish (Shaw et al., 2007). This may be as a result of the activation of protective response mechanisms, including increased expression of metal binding proteins (such as metallothionein) or oxidative stress response mechanisms. Furthermore, pre-conditioning to hypoxia has also been shown to increase tolerance during subsequent exposures in a number of organisms, including in the zebrafish during embryogenesis (Manchenkov et al., 2015).

It is more likely that hatched embryos will experience hypoxia compared to those prior to hatching. Following hatching, stickleback males reduce fanning of the nest possibly because the presence of a current caused by fanning would add a high metabolic stress on the free swimming embryos (Garenc et al., 1999), reducing the flow of oxygenised water. If exposed to copper, the likelihood of the embryo surviving would be reduced as hatched embryos were most sensitive to the combined effects of copper and hypoxia during this developmental stage, both when exposures were conducted only after hatching and for exposures initiated immediately after fertilisation. Therefore, hatched embryos are a critical developmental stage at risk from the adverse effects of exposure to combinations of copper and hypoxia in the natural environment.

Conclusions

We report evidence for significant effects of hypoxia on copper toxicity during embryonic development for a teleost species. Similarly to that reported for the zebrafish, during the early stages of development, hypoxia protected embryos from copper toxicity, but in contrast, after hatching, hypoxia increased copper toxicity to hatched embryos. We suggest that this pattern of interaction between copper and hypoxia during development may be common across teleost species, and further data across a range of families of fish will be required to verify this hypothesis. Considering the very significant increase in the incidence, prevalence and severity of hypoxic events in both marine and freshwater systems worldwide, the likelihood of aquatic organisms being exposed to hypoxia during development is very significant. Therefore, taking into account the combination of chemical stressors and hypoxia is important to generate

environmentally relevant information on chemical effects on aquatic organisms, to support appropriate regulatory and management decisions. For example, our data demonstrate that the evaluation of the toxicity of either copper or hypoxia alone would have under-estimated the effects of these stressors when they occur in combination, for hatched embryos. This highlights a critical research need in order to be able to predict the consequences of world-wide expansion in oxygen depletion to fish communities challenged by anthropogenic toxicants.

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Figures

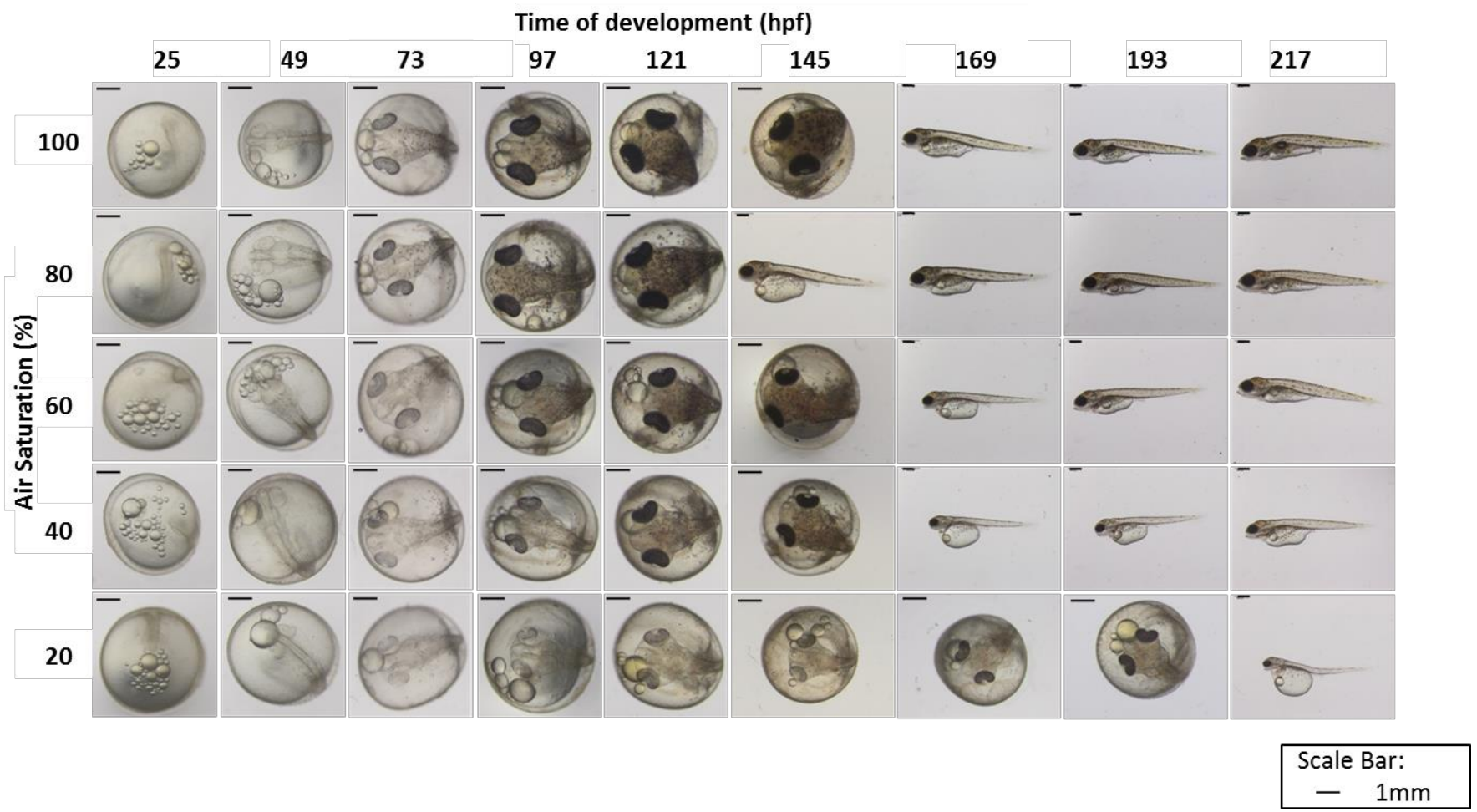

Figure 1. Stickleback embryos exposed to different air saturations (AS) throughout development. Video-captured images (in a Nikon SM21500, Japan) of embryos from 1-217 hpf, exposed to 100 %, 80 %, 60 %, 40 % and 20 % AS (measured average AS: 96.5 ±0.3 %, 75.0 ±0.5 %, 57.6 ±0.6 %, 40.9 ±0.5 % and 24.7 ±0.9 %, respectively). Developmental delays were observed

in embryos exposed to 20 % and 40 % AS, with embryos appearing to be smaller and with less pigmentation than the controls. Hatching occurred 24 hours earlier in the 80 % AS treatment compared to the control.

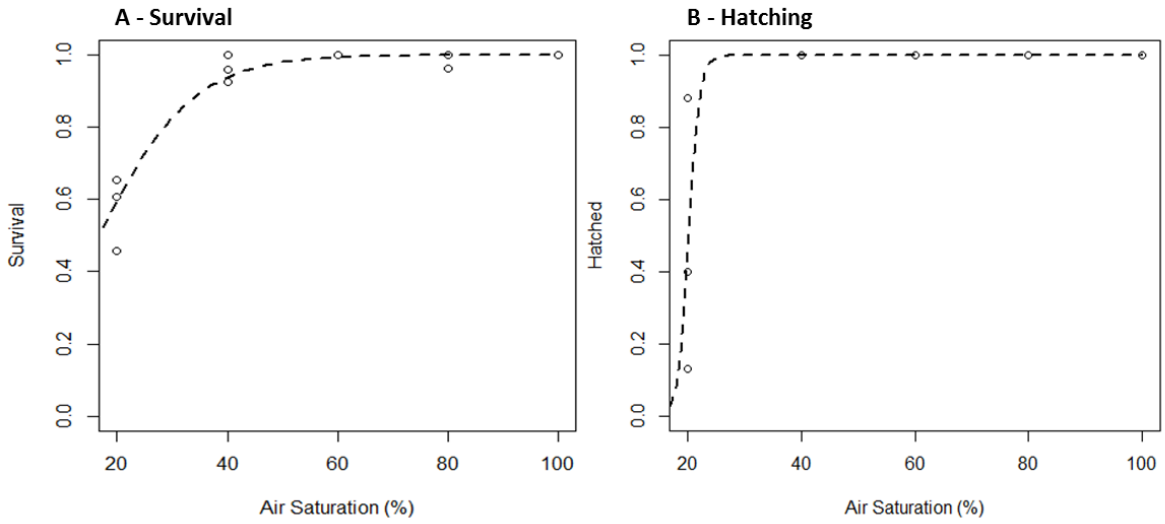

Figure 2. Cumulative proportion of embryos that have A) survived and B) hatched at the end of the experiment (217 hpf), following exposure to different air saturations (AS), continuously throughout development. Each point on the graph represents an individual replicate 600 ml tank containing approximately 30 embryos, and the lines represent the best fit model for the data, calculated using generalized linear models in R. For the conditions tested, increases in the percentage of AS resulted in an increase in survival and in the proportion of hatched embryos ($P<0.001$).

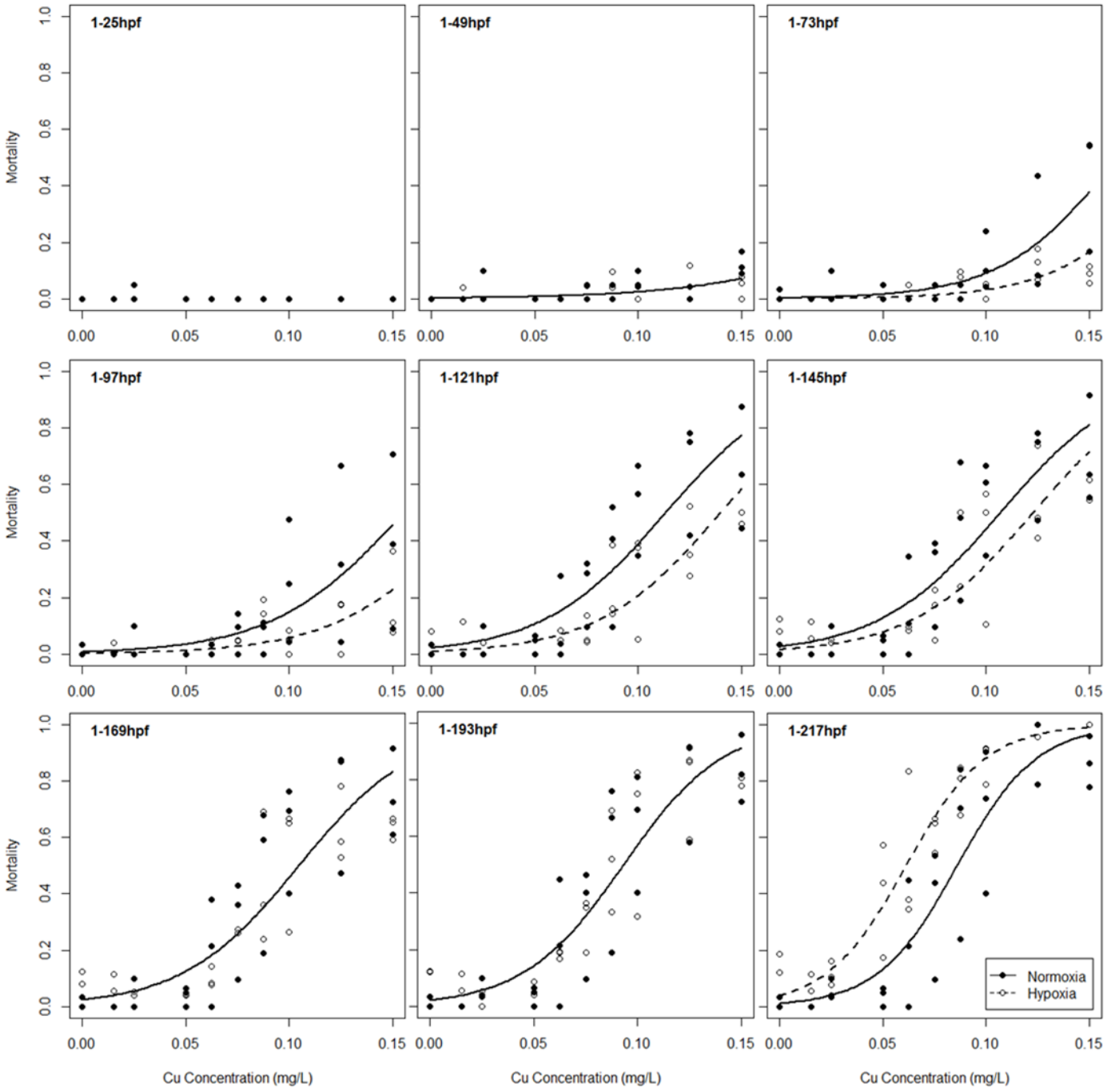

Figure 3. Embryo mortality curves following continuous exposure to copper under normoxia or hypoxia throughout development. Each point on the graph represents the proportion of mortality in an individual replicate tank containing 20 embryos, black and white symbols represent groups exposed to copper under normoxia (97.6 ±0.1 % air saturation (AS)) or hypoxia (56.1 ±0.2 % AS), respectively, and the lines represent the best fit model for the data, calculated using generalized linear models in R (model output summarised in Table S2a).

From 1-25 hpf, there was no significant effect of AS or copper on the mortality ($P=0.31$). For all other time periods, copper caused an increase in mortality both under hypoxia and normoxia ($P<0.001$). There was a significant increase in copper-induced mortality under normoxia compared to under hypoxia from 73-145 hpf (1-73 hpf, $P<0.01$; 1-97 hpf, $P<0.01$; 1-121 hpf, $P<0.001$; 1-145 hpf, $P<0.05$). For the 1-49, 1-169 and 1-193 hpf periods, there was no significant difference in mortalities between the embryos exposed to copper at different AS. Copper toxicity was significantly greater under hypoxia compared to normoxia for the final time stage, 1-217 hpf ($P<0.01$).

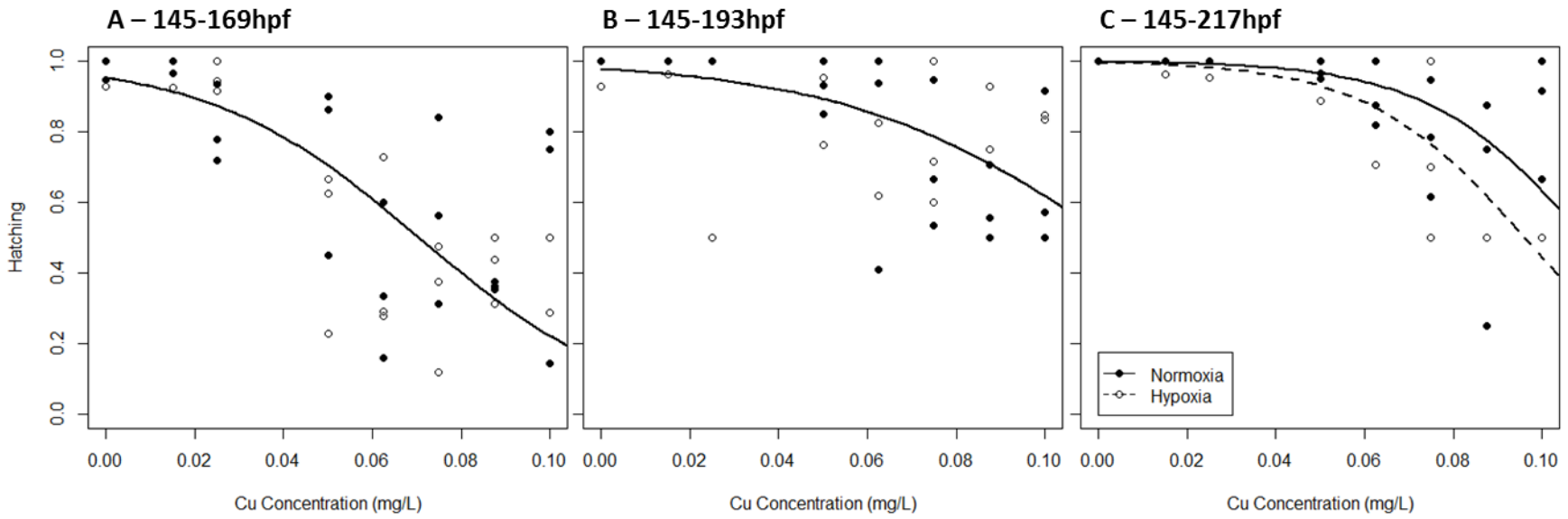

Figure 4. Proportion of hatching in embryos exposed to copper under normoxic or hypoxic conditions, for the time stages; 145-169, 145-193 and 145-217 hpf. Each point on the graph represents the proportion of hatched embryos in an individual tank containing 20 embryos. White and black symbols represent hypoxic (56.1 ±0.2 % air saturation (AS)) or normoxic (97.6 ±0.1 % AS) treatments, respectively, and the lines represent the best fit model for the data, calculated using a generalized linear model in R (model output summarised in Table S3). There was a significant effect of copper on hatching rate under normoxia and hypoxia for all time periods ($P<0.001$). For 145-169 and 145-193 hpf, there was no significant difference in the proportion of hatched embryos exposed to copper under normoxia compared to hypoxia (145-169 hpf, $P=0.411$; 145-193 hpf, $P=0.730$). For 145-217 hpf, there was a significant increase in the proportion of hatched embryos exposed to copper under normoxia compared to hypoxia ($P=0.0305$).

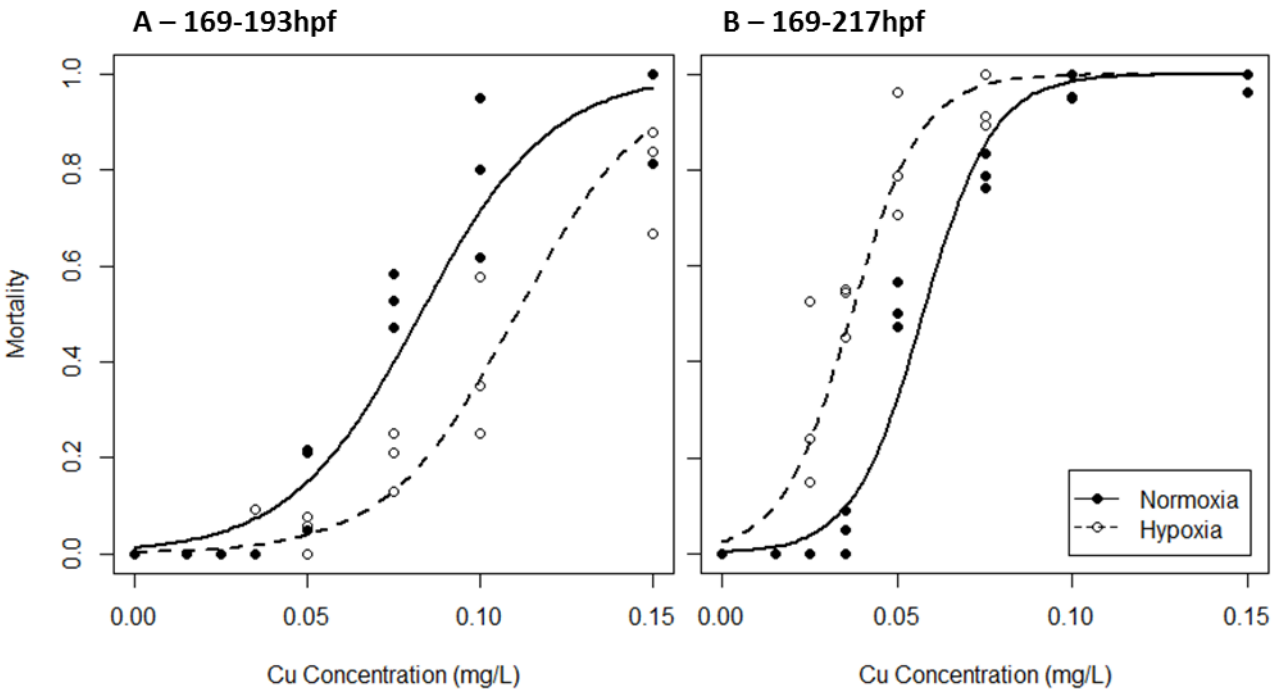

Figure 5. Cumulative mortality curves following exposure to copper under normoxia or hypoxia in hatched embryos, A) 24 hours after exposure (169-193 hpf) and B) 48 hours after exposure (169-217 hpf). Each point on the graph represents the proportion of mortality in an individual replicate tank containing 20 embryos, black and white symbols represent groups exposed to copper under normoxia (97.6 ±0.1 % air saturation (AS)) or hypoxia (56.1 ±0.2 % AS), respectively, and the lines represent the best fit model for the data, calculated using generalized linear models in R (model output summarised in Table S4). Copper caused an increase in mortality both under hypoxia and normoxia over the whole exposure period ($P<0.001$). There was a significantly greater mortality under normoxia compared to under hypoxia, after the first 24 hours of copper exposure ($P<0.001$). After 48 hours of exposure, copper toxicity was significantly greater under hypoxia compared to normoxia ($P<0.001$).

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Supplementary Information: Contrasting effects of hypoxia on copper toxicity during development in the three-spined stickleback (*Gasterosteus aculeatus*)

Jennifer A. Fitzgerald, Ioanna Katsiadaki, Eduarda M. Santos

Pages

TABLES

Table S1. Proportion of embryo survival and hatching following exposure to a range of air saturation (AS) levels during development.

AS (%)	Hours post fertilisation (hpf)								
	25	**49**	**73**	**97**	**121**	**145**	**169**	**193**	**217**
a) Mortality									
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
80	1.00	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
60	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
40	1.00	1.00	1.00	0.99	0.99	0.98	0.96	0.96	0.96
20	1.00	0.98	0.98	0.96	0.95	0.94	0.83	0.76	0.61
b) Hatching									
100						0.08	1.00	1.00	1.00
80						0.96	1.00	1.00	1.00
60						0.03	1.00	1.00	1.00
40						0.01	0.92	1.00	1.00
20						0.01	0.05	0.28	0.47

Table S2. Generalized linear models for the relationships between mortality with copper concentration, air saturation and the copper/air saturation interaction. Minimum adequate models for proportion mortality using a quasibinomial error structure are shown. A) Embryo mortality curves following exposure to copper under normoxia or hypoxia throughout development, B) Mortality curves following exposure to copper under normoxia or hypoxia during the larval stage of development. (Significance codes: *** P<0.001, ** P<0.01, * P<0.05)

	Coefficient (± SE)			
	Intercept	**Copper**	**Air Saturation**	**Copper /Air Saturation Interaction**
a)				
1-25 hpf	-7.24 (±1.08)***	NS	NS	NS
1-49 hpf	-5.98 (±0.72)***	22.86 (±6.18)***	NS	NS
1-73 hpf	-7.02 (±0.70)***	36.17 (±5.09)***	1.12 (±0.37)**	NS
1-97 hpf	-5.92 (±0.61)***	31.41 (±4.57)***	1.05 (±2.98)**	NS
1-121 hpf	-4.69 (±0.41)***	33.55 (±3.4)***	0.89 (±3.50)***	NS
1-145 hpf	-4.14 (±0.37)***	33.78 (±3.38)***	0.54 (±0.25)*	NS
1-169 hpf	-3.72 (±0.33)***	35.60 (±3.49)***	NS	NS
1-193 hpf	-3.86 (±0.34)***	41.43 (±0.34)***	NS	NS
1-217 hpf	-3.18 (±0.33)***	52.04 (±4.51)***	-1.29 (±0.26)***	NS
b)				
145-169 hpf	-5.86 (±0.50)***	53.00 (±4.57)***	1.48 (±0.33)***	NS
145-217 hpf	-3.70 (±0.88)***	100.44 (±22.24)***	-2.06 (±0.85)***	NS

Table S3. Generalized linear models for the relationships between hatching with copper concentration, air saturation and the copper/air saturation interaction. Minimum adequate models for proportion hatching using a quasibinomial error structure are shown. Data from the continuous embryo exposure, the proportion of hatched embryos from 145 to 169 hpf, 145- 193 hpf and 145-217 hpf. (Significance codes: *** P<0.001, ** P<0.01, * P<0.05)

	Coefficient (± SE)			
	Intercept	**Copper**	**Air Saturation**	**Copper / Air Saturation Interaction**
145-169 hpf	2.99 (±0.38)***	-36.18 (±6.07)***	NS	NS
145-193 hpf	3.76 (±0.61)***	-32.39 (±8.81)***	NS	NS
145-217 hpf	5.38 (±0.58)***	-56.06 (±7.76)***	0.77 (±0.34)*	NS

Chapter 4

Copper suppresses physiological responses to hypoxia in the three-spined stickleback (Gasterosteus aculeatus).

Manuscript in preparation

Copper suppresses physiological responses to hypoxia in the three-spined stickleback (*Gasterosteus aculeatus*).

Jennifer A. Fitzgerald[1,2], Mauricio. G. Urbina[1,3], Nicholas J. Rogers[1], Nic R. Bury[4], Ioanna Katsiadaki[2], Rod W. Wilson[1], Eduarda M. Santos[1*]*

[1] Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, UK

[2] Centre for Environment, Fisheries and Aquaculture Science, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, UK

[3] Centre Departamento de Zoología, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile

[4] Kings College London, Franklin Wilkins Building, 150 Stamford Street, London, SE1 9NH

**** Corresponding author:***

Jennifer Fitzgerald, Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, UK. E-mail: jf277@exeter.ac.uk.

Eduarda Santos, Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, UK. Phone: 00 44 (0)1392 264607; E-mail: e.santos@exeter.ac.uk.

Abstract

Copper is widespread in freshwater and marine environments and can be toxic to aquatic organisms if environmental levels become too high. In these environments, low oxygen levels (hypoxia) are also likely to occur; despite this our understanding of the interactions between copper and hypoxia is limited. We have investigated this question in adult male three-spined sticklebacks (*Gasterosteus aculeatus*), a species occupying a broad range of freshwater and marine habitats. The critical oxygen level (P_{crit}) was determined as 48.9 ±2.7 % air saturation (AS), under our experimental conditions (temperature= 15°C; freshwater). Fish were then exposed to copper (20 µg/L) and different AS (97.3 ±0.3 %, 76.8 ±0.3 % and 55.2 ±0.9 %) over a 4 day period. Using physiological and molecular endpoints, the combined exposures to copper and hypoxia were found to result in a decreased tolerance to hypoxia. Fish were able to lower their P_{crit} in response to low oxygen conditions when exposed to hypoxia alone but not when exposed to hypoxia in combination with copper. This was despite an increased ventilation rate observed for combined copper and hypoxia exposure, compared to the single stressors or the controls. Our findings provide strong evidence for interactions to occur in fish exposed to hypoxia and copper, with copper becoming more toxic when exposures occurred at lower oxygen levels (55.2 % AS). Given the ubiquity and increasing prevalence of aquatic hypoxia and copper pollution, our data sets an important precedent for environmental risk assessments globally.

Keywords

Teleost, dissolved oxygen, toxic metals, freshwater, combined stressors, critical oxygen level

Introduction

Copper occurs naturally in the environment and is essential for life, acting as a co-factor for a wide range of enzymatic reactions including oxygen transport, cellular metabolism and energy production[1]. However if the concentrations in the environment exceed physiological thresholds, copper can become highly toxic to living organisms. Copper concentrations in the aquatic environment are of particular concern because organisms are continuously exposed to contaminated water via the skin and other respiratory surfaces, as well as via the diet, including ingestion of contaminated water or sediment[2]. Copper is a particular threat to freshwater fish when water hardness is low. Water hardness in the environment depends on the concentration of OH^- and carbonate ions present, the harder the water the greater the concentration of ions[3]. Copper toxicity is lower in hard waters due to the competition of copper with hardness cations (Ca^{2+} and Mg^{2+}) at the active ion transporters in the gill[4]. Therefore, copper toxicity is inversely proportional to water hardness. As a consequence of both contemporary and historical mining activities, industrial and urban chemical production and agricultural runoff, copper concentrations currently reported in the aquatic environment often reach those known to be toxic to fish[5]. Studies have shown that copper can adversely affect a number of biological processes including branchial ion transport, haematopoiesis and enzyme activities, as well as causing immune suppression and oxidative stress[6]. In addition, fish exposed to copper have been observed to lose the ability to sense environmental oxygen levels[2]. This raises a significant concern as hypoxic aquatic systems can also contain high concentrations of copper[7,8].

In the natural environment oxygen levels fluctuate, but when the oxygen concentrations are sufficiently low to become a stressor, hypoxia occurs[8, 9]. Areas of low oxygen occur naturally in aquatic ecosystems, but in the last decade hypoxic areas have increased rapidly likely as a result of anthropogenic pressures, forming extensive hypoxic zones. This is now a major problem affecting aquatic systems worldwide[10]. Fish respond to hypoxia through a wide range of biochemical, physiological, anatomical and behavioural changes, and the tolerance to hypoxia vary among species, life stages and habitats[11]. Aquatic organisms attempt to maintain blood oxygen supply by increasing gill ventilation, resorting to air breathing or gill reconstruction[12]. Fish can also adapt

to hypoxia by lowering their metabolism, reducing activity, feeding and growth. In addition, fish employ a number of behavioural adaptations to environmental hypoxia including avoidance of hypoxic zones, changes in activity level and use of aquatic surface respiration[6]. At the molecular level, the activation of the hypoxia-inducible factor (HIF) pathway plays an important role in co-ordinating the molecular responses to hypoxia. HIF is a transcriptional complex that plays a key role in cellular response to low oxygen by regulating the transcription of genes involved in cellular and systemic responses to hypoxia[13]. For example, erythropoietin (EPO) transcription has been shown to increase following HIF activation under low oxygen conditions, causing increased red blood cell production and promoting oxygen transport to target tissues[14].

Discharge of waste from industrial and agricultural activities can contaminate aquatic environments with excess nutrients, resulting in eutrophication and hypoxia[15]. These effluents also contain a wide range of contaminants including toxic metals, resulting in a high likelihood for the combination of hypoxia and heavy metals to occur. Studies to date investigating the combined effects of copper and hypoxia have shown that copper toxicity increased under hypoxic conditions for hatched zebrafish (*Danio rerio*) larvae[16]. The combined exposure to these two stressors has also been shown to cause increased oxidative stress compared to the controls, along with histopathological changes in blood parameters in the killifish (*Fundulus heteroclitus*)[17], pacu (*Piaractus mesopotamicus*)[18] and carp (*Cyprinus carpio*)[19]. In addition, for the common carp, a reduction in standard metabolic rate and critical oxygen level (P_{crit}) was seen after combined exposure to copper and hypoxia[8]. The previous studies to date are limited as these species are considered to be relatively able to adapt to low oxygen levels, so there is currently a knowledge gap to investigate how these combined stressors affect species that have lower tolerances to hypoxia.

In this study we used the three-spined stickleback (*Gasterosteus aculeatus*) to investigate how different levels of oxygen affect the response of adult fish to copper. In order to do this, we first determined the tolerance of our stickleback population to hypoxia by determining their P_{crit} under the experimental conditions used for subsequent exposures. This information was used to identify appropriate levels of air saturation to be used during the chemical exposures. Both the physiological and molecular responses following exposure

to copper, hypoxia and their combinations were then determined to document potential interactions between these two stressors.

Material and Methods

Fish source, culture and husbandry

A population of freshwater three-spined stickleback (originating from the River Erme, Devon, United Kingdom, kindly provided by the University of Plymouth) was maintained in the Aquatic Resource Centre at the University of Exeter in mixed sex stock tanks (112 L), supplied with aerated synthetic freshwater[20]. Before it was supplied to each aquarium, mains tap water was filtered by reverse osmosis (Environmental Water Systems (UK) Ltd) and reconstituted with Analar-grade mineral salts to standardized synthetic freshwater (final concentrations to give a conductivity of 300 mS: 122 mg/L $CaCl_2 2H_2O$, 9.4 mg/L $NaHCO_3$, 50 mg/L $MgSO_4 7H_2O$, 2.5 mg/L KCl, 50 mg/L, Tropic Marin Sea Salt), aerated, and maintained at 15 °C in a reservoir. Prior to and during the experimental period, fish were maintained at a temperature of 15 ±1 °C and a photoperiod of 18:6 light/dark (with a 30 minute dawn/dusk transitional periods) and fed to satiation every day with blood worms (*Chironomus sp.*; Tropical Marine Centre, Chorleywood, UK).

Determination of the P_{crit}

The P_{crit} was determined in 7 adult male stickleback by semi-close respirometry. Individual fish where placed in an experimental glass chamber of 500 ml at 15 °C, with each chamber sealed with a rubber bung containing inlets and outlets for two pump controlled systems. In the first system, dissolved oxygen was continually assessed using a flow-through cell (Firesting OXFTC) connected to a FSO2-4 optical oxygen meter (Firesting OXFTC) during set periods of time when oxygen consumption was determined. Water was pumped past the sensor at 50 ml/min, with the sensor measuring the pO_2 (partial pressure of oxygen) every 2 seconds. Oxygen electrodes were calibrated daily with fully aerated water (100 % oxygen saturation) and a saturated sodium sulfite solution (0% oxygen saturation)[21]. The second system was a loop to periodically flush the chamber in order to reset the water to the desired pO_2.

After 15 minutes of continuously measuring the drop in oxygen concentration for determination of metabolic rate (MO_2), the chambers were flushed with water from a stock tank at the desired pO_2. MO_2 measurements were conducted four times at each of the selected levels of air saturation; 100 %, 80 %, 60 %, 40 %, 30 %, 20 %, 15 % and 10 % (measured level: 100 ±0.1 %, 83 ±0.9 %, 61 ±0.5 %, 43 ±0.5 %, 34 ±0.3 %, 24 ±0.5 %, 17 ±0.4 % and 14 ±0.1 %, respectively). To achieve each air saturation level, pO_2 was controlled by continuous bubbling with a pre-set gas mixture of O_2, N_2 and CO_2 (constant atmospheric level; 0.04 %), achieved by controlling the proportional gas flow rates from cylinders of each gas, using precision gas flow controllers (MC Series Mass Flow Controllers, Qubit Systems Inc., Ontario, Canada). The mass flow controllers were connected to a PC running a gas mixing software (C960 Gas Mixing Software, Qubit Systems Inc., Ontario, Canada) in order to set the gas mixtures. To ensure water within each respirometry chamber was well mixed, chambers were placed on a stir plate (Thermo scientific, imareci Poly Komet). A magnetic stir bar was placed at the bottom of each chamber covered by mesh and weighed down by marbles, to prevent the fish from becoming distressed or knocking the stir bar during the exposure. Fish were moved into chambers 24 hours before measurements started, to allow for fish to acclimate to the chambers, and return to a stable resting metabolic rate. Throughout the acclimation and experimental periods, fish were not fed.

After the experiment, the P_{crit} was determined by the conversion of each 15 minute measurement into the MO_2. MO_2 was calculated using the slope of oxygen consumption over time (continuous measurement over 15 min), divided by the mass of fish. Regression lines were drawn on the graphs using regression analysis in Excel, and the breakpoint (P_{crit}) when there was a sudden change in the response variable (Y: MO_2) as a function of the independent variable (X: average external pO2) was determined. P_{crit} was obtained using the intersection of the two linear regressions[22, 23]. To ensure no human bias from analysis, the P_{crit} was additionally calculated using the Yeager free software[24], both methods reaching similar results (P=0.74), and the P_{crit} is stated throughout using the first method of determination. The P_{crit} was determined to be 48.9 ±2.7 % AS for our population of stickleback. To ensure our system was measuring the correct P_{crit}, and stress from the set up was not affecting the results, a further closed respirometry test was carried out where oxygen levels

inside the chambers were reduced by the oxygen consumption of the fish. Dissolved oxygen was continuously assessed using a needle-type fibre optic sensor (Firesting OXR 230) connected to a FSO2-4 optical oxygen meter, with the results 51.76 ±2.4 % AS validating those obtained with the open system.

Combined exposures to copper and varying oxygen concentrations

Adult stickleback males were exposed to 0 or 20 µg/L of copper (added as copper sulphate, Sigma Aldrich), under three different oxygen concentrations: normoxic treatment at 100 % AS, a moderate oxygen concentration (75 % AS) and hypoxia, corresponding to the P_{crit} of this species (50 % AS). pO_2 was controlled by continuous bubbling with a pre-set gas mixture of O_2, N_2 and CO_2 (0.04%) as described above. Chemical exposure was conducted via a flow through system for 4 days, with each treatment group comprised of three exposure tanks (40 L) containing 5 fish per tank (n=15 fish per treatment). Due to the equipment needed to control the oxygen concentration, the different oxygen treatments could not be run simultaneously and had to be run on consecutive weeks. To ensure that this had no effect on the experimental parameters, an additional control tank (100 % AS and 0 µg Cu/L) was run each week to confirm that no changes occurred across time.

The concentration of oxygen in each tank was measured twice daily to ensure they were kept at the correct level. Daily measurements of conductivity and pH where also conducted. Fish were not fed over the exposure period. On day 1, 2 and 3 of the exposure period, fish were monitored for behavioural effects by recording the position of the fish in the tank. The position of each fish in every tank was scored over a period of an hour, with 6 scores taken per tank. Ventilation rates were also measured on day 1, 2 and 3 of the exposure period, for every fish in each tank, by counting the number of times each fish opens its mouth/moves its operculum within a 1 minute period.

All fish were sacrificed on day 4 of the exposure period by lethal dose of benzocaine followed by destruction of the brain, in accordance with the UK Home Office regulations. Wet weight and fork length were recorded and the condition factor (k= (weight (g) x 100)/(fork length (cm^3)) was calculated for each individual fish. Gill and liver samples were taken from each fish, for analysis of gene expression (snap frozen in liquid nitrogen and stored at -80

°C), and for metal analysis (placed in an acid washed tube, weighed to determine wet mass of the tissue, then stored at -20°C).

Metal analysis in water and tissue samples

Water samples were collected from each tank on days 1 and 3 of the exposure period and stored at -20 °C prior to chemical analysis. Prior to analysis of total copper concentrations, samples where acidified by adding nitric acid (70 %, purified by redistillation, ≥99.999 % trace metals basis, Sigma Aldrich) to a final concentration of 0.01 % to each falcon tube. Tissue samples were initially freeze dried to determine the dry mass of the samples before acid digestion. Tissue digestion was conducted by adding 500 µl of nitric acid (70 %, purified by redistillation, ≥99.999 % trace metals basis, Sigma Aldrich) to each tube and incubated at room temperature for 48 hours, until all samples had completely digested. 0.1% hydrogen peroxide (Fisher; Hydrogen Peroxide, 100 volume >30%w/v) was added for breakdown and removal of the fatty material. All samples were covered with in Parafilm to prevent evaporation. The resulting digested solution was then was the diluted in a 1:10 ratio with ultrapure water. The copper content in each water and tissue sample was measured by ICP-MS using a Perkin Elmer NexION 350D instrument running the Syngistix software, v1.0. at King's College London.

Transcript profiling

Real-time quantitative PCR (RT-QPCR) was used to quantify the transcript profiles in the liver and gills of exposed fish (n=12 per treatment group) for a number of target genes including: metallothionein 1 (*mt1*) a metal binding protein and important biomarker for metal exposure; catalase (*cat*) a gene involved in the response to oxidative stress, and hypoxia inducible factor 1 α (*hif1α*) a gene involved in the response to hypoxia. Three control genes were included in this study: ribosomal protein l8 (*rpl8*), ubiquitin (*ubi*) and beta-tubulin (*tubb4*). Ct values for each sample where plotted, and regression analysis was carried out to identify the most appropriate control gene, classified as the gene that remained stable across all exposures and had the smallest distribution range, which resulted in the selection of *rpl8* for the analysis. Quantitative RT-QPCR assays for each target gene were optimised as previously described[25] (detailed information of assays can be found in Supplementary Table 1).

Total RNA was extracted from tissues samples using the Tri reagent method (Sigma-Aldrich, UK) according to manufacturer's instructions. The concentration and purity of the resulting RNA was determined using a NamoDrop-1000 Spectrophotometer (NanoDrop technologies, Wilmington, USA). One µg of total RNA was subjected to DNase treatment (RQ1 DNase, Promega, Southampton, UK) to remove potential DNA contamination, prior to being converted to cDNA using M-MLV reverse transcriptase (Promega, UK) and primed with random hexamers (MWG-Biotech), according to the manufacturer's instructions (MJ Research PTC200 Thermal Cycler). cDNA was diluted (1:2), then RT-QPCR was performed using an iCycler iQ Real-time Detection System (BioRad Laboratories, Hercules, USA) and SYBR green chemistry (BioRad Laboratories, Hercules, USA). Each sample was amplified in duplicate in 96-well optical plates (BioRad Laboratories, Hercules, USA) in 15 µl reaction volumes. Efficiency-corrected relative expression levels were determined using the $2^{-\Delta\Delta}$CT method[26, 27]. Samples where amplification was not detected were excluded from analysis, resulting in n=8-12 per treatment group.

P_{crit} determination in fish after exposure to copper at varying oxygen concentrations

Using the method described above, P_{crit} was measured after exposure to copper in combination with different oxygen levels. Eight fish where exposed to either 100 % or 50 % air saturation in combination with 0 or 20 µg/L of copper, as described above. On day 3 of exposure, fish where moved into individual glass respirometry chambers containing the same water conditions as the exposure tanks, for acclimation. Oxygen consumption rate were measured from the beginning of acclimation, to ensure that the fish had settled, and throughout day 4 over 20 minute periods, followed by decreasing the oxygen levels after 80 minutes intervals (i.e. 4 separate measurements of oxygen consumption for each fish at each oxygen level). After exposure, tissues were sampled as described above, and blood was sampled for haemocrit (relative volume of red blood cells (RBC) compared to whole blood) and haemoglobin measurement[15]. Haemoglobin was measured by adding 2 µl of blood extracted from each fish to 500 µl of Drabkin's reagent (Sigma), absorbance of samples were read at 540 nm on a spectrophotometer (Teacon i control). Additional control fish (that had not been placed under exposure conditions, either copper or different air

saturations) were sampled for tissue and blood to confirm that the hypoxia that the fish experienced during the P_{crit} measurement had no effect on the results.

Statistics

Gene expression data was first scrutinised by Chauvenets criterion to detect outliers for each gene and these were subsequently removed before analysis took place[28]. Data that did not meet the normality (Sharpo-Wilko test) and equal variance (Bartlett) test was log transformed before analysis. All data was analysed using an analysis of variance model in R[29]. A separate model was used for each dataset, to test for effects of exposure to copper, air saturation (categorical variables) and the interaction between these variables. Minimum adequate models were derived by model simplification using F tests based on analysis of deviance[53]. Tests reported refer to the significance of removing terms from the models. When a significant effect of interaction was identified, pairwise comparisons to determine which groups differed were conducted using Tukey's HSD post hoc test. All data was considered statistically significant when $p<0.05$.

Results

Water chemistry and morphometric parameters

For both the main exposure and the P_{crit} determination, the mean measured concentration of copper in the tank water was 22.14 ±0.42 µg/L for copper treatments and 0.47 ±0.01 µg/L for treatments without copper and detailed breakdown is presented in Supporting Information Table 1. The mean measured air saturation for treatment tanks for 100 %, 75 % and 50 % were 97.3 ±0.3 %, 76.8 ±0.3 % and 55.2 ±0.9 %, respectively. The average conductivity (229.66 ±0.50 µs) and pH level (7.48 ±0.02) measured in the tanks throughout exposure remained stable and were not affected by the treatment.

Throughout the exposure, fish remained in good condition and no mortalities were recorded. The mean body weight, length and condition factor of the fish where 1.58 ±0.03 g, 5.55 ±0.03 cm and 0.92 ±0.01, respectively, and there were no significant differences between treatment groups.

Effects of the exposures on the behaviour and ventilation rates

There was no significant effect of treatment on the fish behaviour, with the majority of fish occupying primarily the bottom third of the tank. It is interesting to note that fish exposed to 75 % air saturation in the absence of copper appeared to be more active, although this difference was not significant (Fig S1.).

With decreasing oxygen levels both in the presence and absence of copper, there was a significant increase in ventilation rate ($P<0.001$; Fig.1; Table S3.A.). At all air saturations, fish exposed to 20 µg Cu/L had a significantly increased ventilation rate compared to fish exposed to the same level of air saturation in the absence of copper ($P<0.001$; Fig.1).

Copper content in fish tissues

In the gill, there was an approximately 20fold increase in the tissue copper concentration in fish exposed to copper, compared to non-exposed controls ($P<0.001$; Fig. 2A; Table S3.B.), and this was not affected by the level of air saturation ($P=0.120$). In addition, there was no effect of AS level or copper exposure on the copper content measured in the liver of exposed fish ($P=0.938$; Fig. 2B; Table S3.B.).

Transcript profiling

The metal binding protein, *mt1*, was significantly up-regulated in the gill of fish exposed to copper for all air saturations tested ($P<0.001$; Fig. 3; Table S3.C.). In addition, there was a significant effect of air saturation on the expression of *mt1* in the gill ($P<0.001$). In the liver, a similar trend was observed but the level of *mt1* was approximately 5-fold lower than the in the gill (Fig. 3). *mt1* expression was significantly affected by copper exposure ($P<0.001$) but not by air saturation ($P=0.087$). In contrast, for *cat*, no changes associated with any of the exposure conditions were observed in either of the tissues analysed (Gill: $P=0.569$, and Liver: $P=0.051$; Fig. 3; Table S3.D.).

For *hif1α*, significant effects were observed in the liver and gill of exposed fish (Fig. 3). In the gill of fish exposed to copper, there was significant effect of the different air saturations ($P=0.02$; Fig.3.; Table S3.E.) and copper exposure ($P<0.001$; Fig. 3.; Table S3.E.). In the liver, *hif1α* was significantly upregulated

in fish exposed to copper compared to the controls (P<0.001; Fig. 3). There was also a significant effect of air saturation on *hif1α* expression (P<0.001).

Effects of exposure to copper and hypoxia on P_{crit}

Exposure to 50 % AS in the absence of copper resulted in a decreased P_{crit} compared to fish maintained under normoxia (P<0.003; Fig. 4). Conversely, in the presence of copper, exposure to 50 % AS did not result in a decrease in P_{crit} as observed in the absence of copper (P= 0.941; Fig. 4).

There was no significant difference between haematocrit in the normoxic fish that had their P_{crit} measured and the control fish, so the process of measuring the P_{crit} was not long enough to alter haematocrit (P=0.061). There was a significant increase in the haematocrit in fish exposed to hypoxia compared to fish exposed to normoxia (P=0.004; Fig. 5A), however there was no effect of copper on the haematocrit levels (P=0.773).There was no significant effect of oxygen or copper treatment on haemoglobin levels (P=0.240; Fig. 5B).

Discussion

The objective of this study was to determine the influence of oxygen availability on the effects of copper exposure in the three-spined stickleback. Fish were able to lower their critical oxygen tension in response to low oxygen conditions but this ability to acclimate to hypoxia was no longer present in fish exposed to copper. This suggests that adult fish may be at an increased risk of adverse effects resulting from the combined exposures to copper and hypoxia compared to the risk predicted based in data for each stressor alone.

Response of the stickleback to copper exposure

Copper content was strongly increased in the gills of fish exposed to copper compared to fish maintained under control conditions for all air saturations tested. These data were mirrored by the induction of *mt1*, a key biomarker for metal toxicity. *mt1* is a metal binding protein that detoxifies and stores metal ions through binding and removal of their redox potential. Metallothioneins account for a major proportion of the cellular storage of copper in fish[2, 30], with increases in *mt* expression shown to occur in response to metals, in both short

and long term exposures and in chronically exposed wild fish[31-33]. A single *mt* isoform is predominately induced by metals in most fish species, which bind to metal response elements in its promotor region, stimulating transcription[2].

No significant changes in *cat* expression were observed in the gill of the fish exposed to copper. The lack of changes observed may be as a result of intracellular copper being bound to metallothionein and not in its biologically active form. With metallothionein binding copper and removing its redox potential, copper is detoxified within the cell, so its toxic effects are no longer seen. In a previous exposure to copper in three-spined stickleback, glutathione levels and antioxidant enzymes in the liver returned back to a basal level despite copper accumulation as a result of metallothionein binding capabilities[34], supporting the observations made in our study for *cat*.

The gills are the main site of copper uptake from the ambient water. Copper is exported from the gill cells to the blood and transported to the liver, a key organ for copper homeostasis. In the liver, copper is incorporated into ceruloplasmin transcuprein and also bound to albumin to be exported and used by the body[35]. Alternatively, copper in the liver can be sequestered by metal chaperone proteins, such as MTs, for detoxification and storage, and ultimately to be excreted via the bile[35]. In our study, there was no observable increase in copper content measured in the liver. However, there was an induction of *mt1* in the liver of fish exposed to copper compared to the controls, suggesting that the variation of the liver data may have been too great to distinguish potential changes in copper concentrations, especially as copper levels in the liver were significantly higher than in the gill. This is further supported by the changes in *cat* expression after copper exposure in the liver. Although not significant, there was a strong trend towards *cat* being induced under copper exposure and with a greater response with decreasing air saturations.

The hypoxia inducible factor (HIF) pathway is essential for the response to low oxygen within the cell and has been shown to be induced following some chemical exposures. Under normoxic conditions, the HIF-1α subunit is modified by oxygen sensitive-enzymes called HIF-PHDs (prolyhydroxylases)[36], allowing the protein-ubiquitin ligase complex to recognise HIF-1α, resulting in the degradation of HIF-1α by the proteasome[37]. However, under low oxygen concentrations, the activity of PHD is inhibited, resulting in the stabilisation of

HIF-1α. The accumulated HIF-1α translocates to the nucleus where it dimerises with the aryl hydrocarbon receptor nuclear translocator, ARNT (also known as HIF-1β)[38]. The HIF-1α-ARNT complex acts as a transcription factor that binds to hypoxia responsive elements (HRE) resulting in the regulation of transcription of a wide range of hypoxia response genes[39]. An increase in *hif-1α* expression was observed as a result of exposure to copper, but this induction was absent in fish exposed to low oxygen alone. It has previously been demonstrated that exposure to copper in rainbow trout (*Oncorhynchus mykiss*) induced HIF-1α protein in their gills[40]. Work by Martin *et al.* 2005 demonstrated that copper can stabilise the nuclear HIF-1α under normoxic conditions, through PHD/HIF/HRE-dependent gene regulation, serving as a sensory system not only for oxygen but also for copper[41]. Using *in vitro* hydroxylation assays, copper inhibited prolyl-4-hydroxylation independently of iron and oxygen concentration. The authors suggested that this could mean that increased concentration of free copper regulates the expression of its binding protein, ceruloplasmin, in the liver via a HIF-dependent mechanism[41]. This was further supported by observations *in vivo* showing an increase in HIF target genes as a result of copper exposure, including plasma ceruloplasmin, after copper was administered intravenously to copper-deficient pigs[42] and the increase in VEGF at wound sites treated with copper sulphate[43]. Therefore, it is likely that in our study copper is regulating *hif-1α* transcription, whereas hypoxia is regulating the HIF activity at the protein level. This induction of *hif-1α* may also suggest that some copper in the liver may be in an unbound state.

Hypoxia increased the adverse effects of copper

In this study, as oxygen level decreased, the ventilation rate of the fish increased. Hyperventilation is a common response to low oxygen in fish, as it allows the maintenance of the same rate of delivery of oxygen to the gills despite declining oxygen concentration in the inspired water. This is an important physiological response as increases in ventilation volume driven by changes in breathing frequency or amplitude help maintain arterial oxygen levels which in turn delay the switch from aerobic to anaerobic respiration[44]. However, this is not common to all species, as some species, such as the Japanese eel (*Anguilla japonica*) and white sturgeon (*Acipenser transmontanus*) have been shown to decrease their ventilation rate with

decreasing oxygen level[45, 46], which is thought to be linked to their ability to use alternative respiratory strategies, such as skin breathing, aquatic surface respiration or air-breathing.

Copper significantly increased the ventilation rate of exposed fish for each air saturation tested. This has been seen in other species, including in the trout, where exposure to copper resulted in reduced swimming activity and increased ventilation rates[47], and in the flying barb (*Esomus danricus*) where exposure to copper, resulted in an increase in ventilation frequency[48]. Increased ventilation rates are considered to be linked to the physiological stress induced by copper toxicity. For example, copper exposure can induce mucous secretion over the gills, which may increase barriers for gas diffusion, requiring a compensatory increase in ventilation rate.

Importantly, the combined exposure to copper and reduced oxygen resulted in a greater increase in ventilation rate compared to that observed following exposure to the individual stressors alone, for both 75 % and 50 % AS. Increasing the ventilation rate incurs a metabolic cost and there is usually a limit for how much a fish may increase its ventilation rate before reaching a threshold where this adaptation is no longer beneficial[44]. Therefore, compared to exposure to each stressor individually, this supports the hypothesis that combined exposures to copper and hypoxia result in even greater adverse effects and greater metabolic costs.

Copper reduced the stickleback's ability to acclimate to hypoxia

The P_{crit} is a useful measure of hypoxia tolerance in fish because it reflects the capacity to maintain aerobic metabolism in low pO_2 environments. It is defined as the point at which an organism transitions from being an oxyregulator to an oxyconformer[49]. Recently a study has put together a comprehensive database of P_{crit} values, from 96 published studies, covering 151 species of teleost, highlighting the importance of this measure when investigating hypoxia tolerance[50]. In this study we used this measure to assess if exposure to copper and hypoxia, either alone or in combination, would affect oxygen tolerance in adult sticklebacks.

When sticklebacks were maintained under hypoxia for 4 days, a decrease in their P_{crit} was observed, reflecting their ability to acclimate to the reduced oxygen in the water. Upon exposure to hypoxia, immediate survival is dependent on the fish's ability to maintain metabolic function through the modification of existing physiological and biochemical pathways. In general, the first response to hypoxia involves attempting to maintain oxygen delivery (by increasing water flow over the gills or by increasing the diffusion capacity through the gill), and the conservation of energy expenditure (through the reduction in general metabolism, down regulation of protein synthesis, and/or through the reduction of locomotor activities)[12]. If the fish are successful with the immediate responses to the hypoxic conditions, they might have the opportunity to acclimate. Acclimation can involve changes in gene expression, resulting in functional changes of hypoxia specific pathways that will help the fish to survive and function under low oxygen conditions.

An important element in the process of acclimating to hypoxia involves increasing oxygen transport, which involves the modification of haemoglobin-oxygen binding affinity and blood haemoglobin content. In our study, a significant increase in blood haematocrit levels was observed in fish maintained under hypoxic conditions compared to normoxia, but copper did not affect haematocrit. However, there were no significant alterations in the haemoglobin levels as a result of hypoxia or copper exposure, despite the increase in red blood cell levels. This is likely to be as a result of the red blood cells absorbing more water and swelling under hypoxic conditions, and not as a result of an increase production of red blood cells into the circulation. This effect is a well-known stress and hypoxia response in fish[51]. Increases in cell volume as a result of hypoxia exposure are thought to be a knock-on effect of erythrocyte pH regulation due to the activation of sodium/proton exchange across the membrane[52]. This increases cellular Na^+ content, drawing in water by osmosis, whilst simultaneously elevating red blood cell pH and thereby the haemoglobin-oxygen affinity. This allows the blood to bind oxygen more effectively at the gills and therefore helps in acclimating to the low oxygen surroundings.

Exposure to copper prevented the fish from being able to reduce their P_{crit} in response to simultaneous exposure to hypoxia waters. To our knowledge this is the first time that copper has been demonstrated to affect the ability of the fish

to acclimate to low oxygen conditions. Previous work by Malekpouri *et al*. 2016 reported a different trend when they investigated the effects of the combined exposure to copper and hypoxia on the metabolic capabilities of the crucian carp[8]. They found that the interaction of the two stressors caused the maximum metabolic rate and aerobic scope to increase after a chronic 7 day exposure but the P_{crit} after the exposure to the two stressors was reduced. The crucian carp is commonly found in a wide range of freshwater habitats with low oxygen levels and has been shown to be extremely tolerant to hypoxia, being able to survive very low oxygen levels and even anoxic conditions for extended periods of time[53]. To survive such low levels of oxygen, the carp has developed adaptive strategies, including the ability to produce ethanol as the end-product of anaerobic metabolism, adjustment of the gill morphology to help supply its oxygen needs, haemoglobin with extremely high oxygen affinity and mechanisms of neural depression[53]. Therefore it is perhaps unsurprising that carp are able to reduce their P_{crit} even when exposed to combinations of copper and hypoxia, as they have such an elegant array of mechanisms of adaptation to low oxygen levels, especially compared to the stickleback that is considered a relatively intolerant species to hypoxia (also supported by the relatively high P_{crit} found in the present study). This emphasises why it is important to study fish with different tolerances to hypoxia when investigating how fish respond to combination of hypoxia with other stressors, as this natural ability to adapt to hypoxia could be a strong determinant of their ability to respond to combined exposures, in particular for chemicals that share pathways of effect with hypoxia.

Gills are the first target for waterborne pollutants due to the high exchange rates with the external environment, as well as the main site of copper uptake. Fish exposed to copper show several histological alterations, namely lamellar epithelium lifting, epithelium proliferation, lamellar axis vasodilation, oedema in the filament, fusion of lamellae and lamellar aneurisms[54]. Cell proliferation with thickening of gill filament epithelium has also been reported in fish exposed to copper [55-57], which could lead to lamellar fusion[54]. These responses are considered defence mechanisms that reduce the branchial area in contact with external environment. However by doing this, fish reduce the surface area of the gill available for oxygen diffusion and increase diffusion distance, therefore limiting the amount of oxygen that the fish can obtain from the water. This is

also supported by the increased ventilation rates found in the group of fish exposed to copper in our study. In an environment where the oxygen levels are already reduced, this could add additional stress and reduce the ability of the stickleback to adapt to low oxygen in the surrounding water. In addition, teleosts have been shown to reversibly remodel their gill in response to low oxygen. Studies have shown that the lamellae of crucian carp are embedded in a cell mass during normoxic conditions, while much of this cell mass is lost under hypoxic conditions resulting in much larger respiratory surface area for gas exchange[58]. If this is the case, competition between copper and hypoxia response in fish may prevent acclimation, hence the limited ability to adapt to low oxygen surroundings when copper is present.

Interestingly, the data for *hif-1α* and *mt1* appeared to have a greater molecular response to copper under 75 % AS compared to 50 % AS. This is an interesting response, as it seems that moderate reductions in ambient oxygen actually resulted in a greater molecular response to the combined exposures, compared to more pronounced reduction in oxygen. 50 % AS corresponded to the critical oxygen level (P_{crit}) for this population, so the fish were likely to be under significant stress and energetic demands to maintain their oxygen supply in an oxygen depleted environment, which may have resulted in a reduced ability to increase gene transcription in these fish.

Conclusions

We have demonstrated that that copper increases the adverse effects of low oxygen by reducing the ability of the stickleback to acclimate to low oxygen in the surrounding water. To our knowledge, this is the first time it is shown that a toxic metal (copper) can elicit the expected decrease in P_{crit} after acclimation to hypoxia. It is important to consider different species when investigating the effects of hypoxia on chemical toxicity, as species with different tolerances to hypoxia are likely to respond differently to these stressors both alone or in combination, in particular for chemicals that share pathways of effect with hypoxia. Considering the strong likelihood that hypoxic events could co-occur with metal pollution, it is important to consider the combination of stressors on aquatic organisms to support regulatory and management decisions.

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Figures

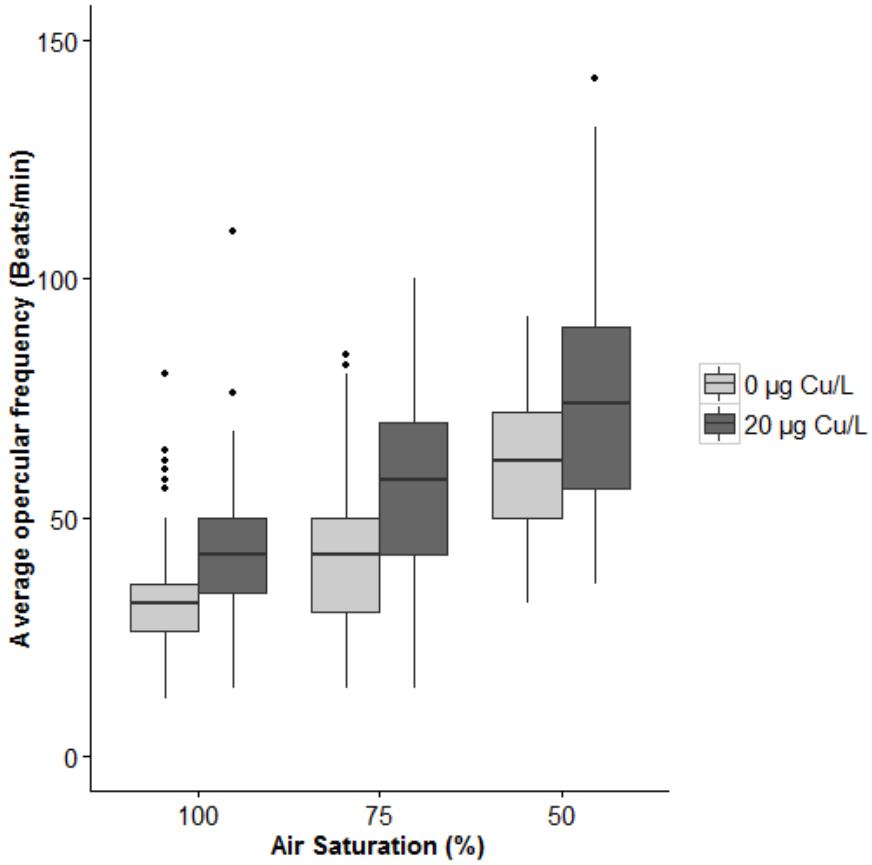

Figure 1. Measured ventilation rates in fish exposed to copper under different levels of air saturation. Male sticklebacks were exposed to 0 or 20 µg Cu/L for 4 days under different levels of air saturation (97.3 ±0.3 % AS, 76.8 ±0.3 % AS and 55.2 ±0.9 % AS). Each treatment consisted of 3 tanks containing 5 fish, over the first 3 days of exposure, with the number of beats of the operculum over one minute measured for individual fish. Statistics carried out using a minimum adequate model (analysis of variance model, R; Table S3.A.), showed an significant effect of air saturation ($P<0.001$) and copper concentration ($P<0.001$) but there was no interaction between the variables.

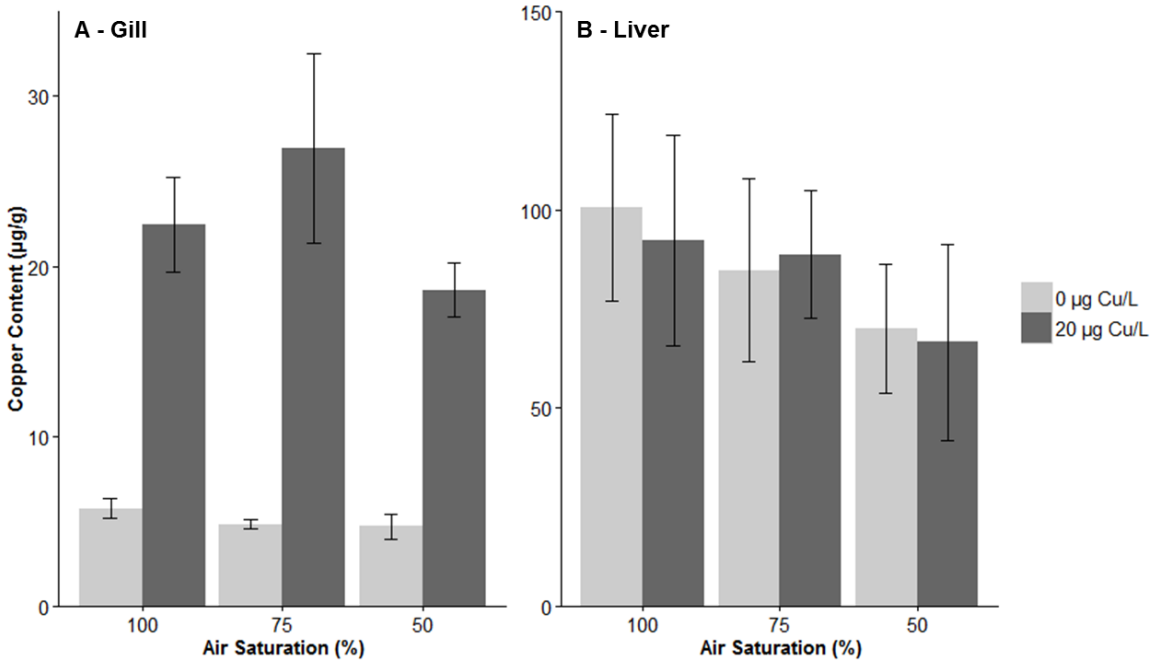

Figure 2. Measured copper concentrations in tissues of the stickleback exposed to copper under different levels of air saturation. Copper content were measured in the A) Gill and B) Liver of male sticklebacks that were exposed to 0 or 20 µg Cu/L for 4 days under different levels of air saturation (97.3 ±0.3 % AS, 76.8 ±0.3 % AS and 55.2 ±0.9 % AS) by ICPMS (n=7/8 fish for each treatment group). Data is presented as mean µg Cu/g ± standard error mean. Statistics carried out using accepted minimum adequate models (analysis of variance model, R; $P<0.05$) with model details reported in Table S3.B. For the gill, there was a significant effect of copper exposure ($P<0.001$).

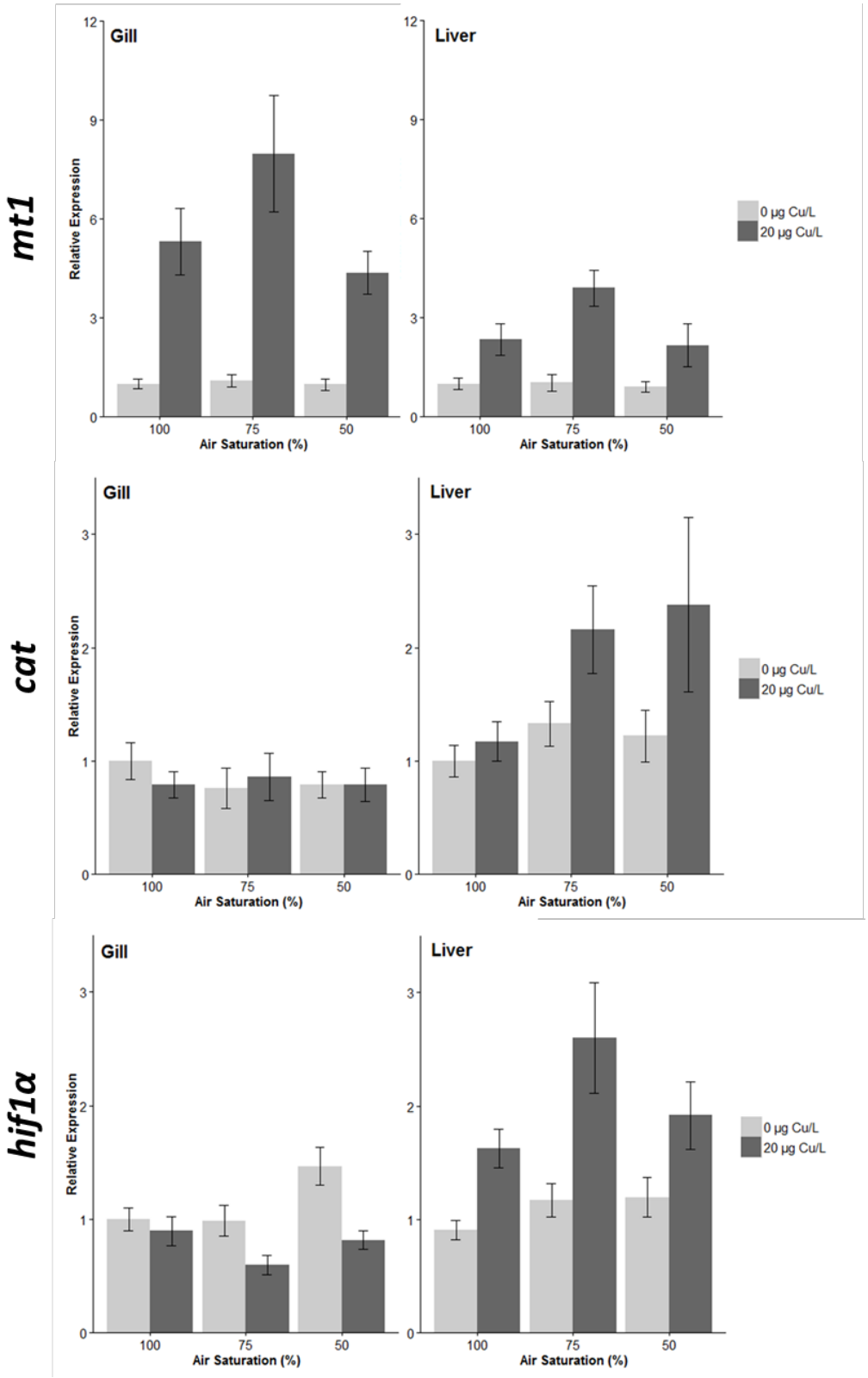

Figure 3. Transcript profiles for selected target genes following exposure to copper under different levels of air saturation. Male sticklebacks were exposed to 0 or 20 µg Cu/L for 4 days under different levels of air saturation (97.3 ±0.3 % AS, 76.8 ±0.3 % AS and 55.2 ±0.9 % AS).Transcript profiles were determined

using RT-QPCR for the gills and livers. Genes analysed included: metalothionein 1 (*mt1*), catalase (*cat*), and hypoxia inducible factor 1α (*hif1α*). Data are presented as average relative expression (normalised 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=8-12 fish per treatment group. Statistics carried out using accepted minimum adequate models (analysis of variance model, R; $P<0.05$) with model details reported in Table S3.C.D.E. For *mt1*, there was a significant effect of copper treatment in both the gill and the liver ($P<0.001$ and $P<0.001$, respectively), and in the gill there was also a significant effect of air saturation ($P<0.001$). For *hif-1α*, there was no significant interaction, but a significant effect of copper treatment and air saturation in both the gill and the liver ($P<0.001$ and $P<0.001$, respectively).

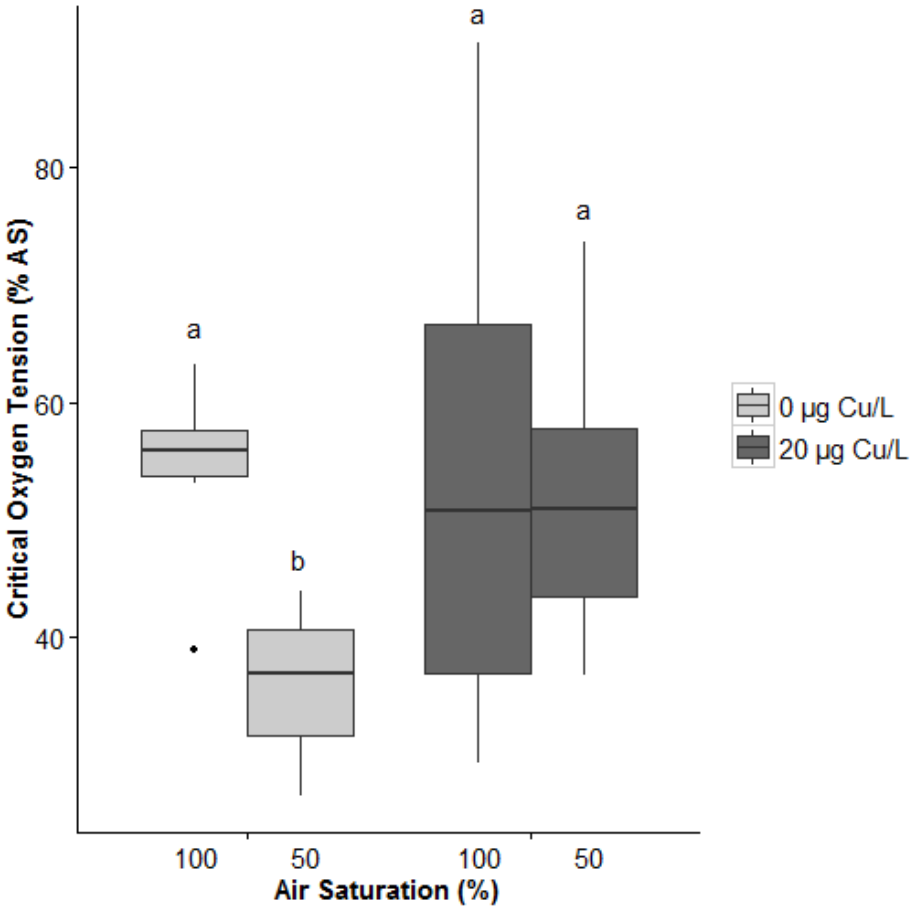

Figure 4. The critical oxygen tension (P_{crit}) measured for stickleback following exposure to copper under different levels of air saturation. Stickleback were exposed to 0 or 20 µg Cu/L for 4 days under 100 % (97.3 ±0.3 % AS) or 50 % (55.2 ±0.9 % AS) before determination of the critical oxygen tension (n=8 fish per treatment). Statistics carried out using accepted minimum adequate models (analysis of variance model, R; $P<0.05$). Letters above each bar indicate significant differences between treatment groups when there was a significant interaction from the model (Tukey's HSD post hoc test; $P<0.05$)

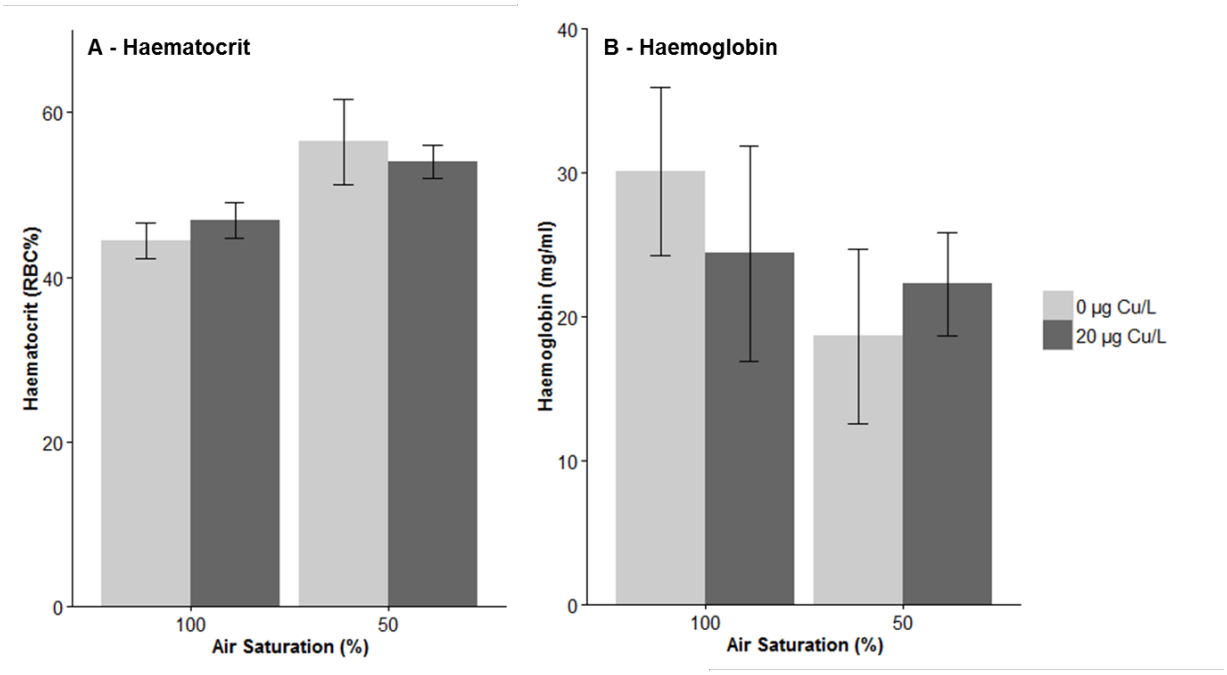

Figure 5. Measured haematocrit (A) and haemoglobin levels (B) from stickleback exposed to copper under different levels of air saturation. Stickleback (n = 8 fish for each treatment group) were exposed to 0 or 20 µg Cu/L for 4 days under 100 % (97.3 ±0.26 % AS) or 50 % (55.2 ±0.85 % AS) following determination of the critical oxygen tension. Statistics carried out using accepted minimum adequate models (analysis of variance model, R; $P<0.05$) with model details reported in Table S3.G. For haematocrit, there was a significant effect of air saturation ($P=0.002$).

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Supplementary Information: Copper suppresses physiological responses to hypoxia in the three-spined stickleback (*Gasterosteus aculeatus*)

Jennifer A. Fitzgerald, Mauricio. G. Urbina, Nicholas J. Rogers, Nic R. Bury, Ioanna Katsiadaki, Rod W. Wilson, Eduarda M. Santos

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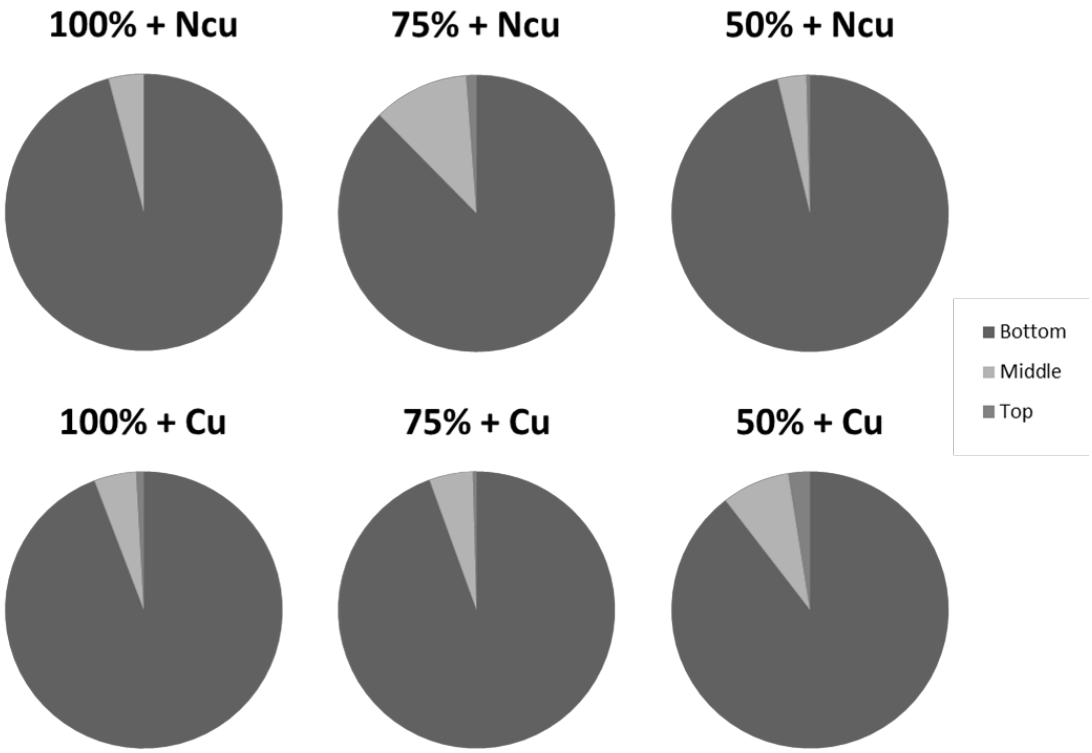

Figure S1. Pie charts documenting the position in the tank of fish over the exposure period. Male sticklebacks were exposed to 0 or 20µg Cu/L for 4 days under different levels of air saturation (97.3 ±0.26 % AS, 76.8 ±0.33 % AS and 55.2 ±0.85 % AS). Each treatment consisted of 3 tanks containing 5 fish, and the position in the tank of each individual fish for the first 3 days of exposure was recorded.

Table S1: Target genes, primer sequences, amplicon product size and annealing temperature and PCR efficiency for the RT-QPCR assays.

Gene Name	Gene Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Ta (°C)	PCR Efficiency
Ribosomal protein L8	*rpl8*	GGTGCGTCCCT CCTGATG	GCGTGGTGTG GCTATGAAC	93	60.5	2.059
Beta tubulin	*tubb4*	TCTTCAGACCA GACAACTT	CTCCTTCCTCA CCACATC	119	60.0	2.025
Ubiquitin	*ubc*	GGAGGGCAGT AAAGTGATT	CAAGGCAGGA GATTCAGTT	161	57.0	1.967
Metallothionein 1	*mt1*	CCCCTGCTGCC CGACTG	TGTTCAAACTG CCGCCATCTC	137	63.0	2.119
Catalase	*cat*	CCAGAAGCGTA ATCCTCAA	GAACAAGAAAG ACACCTGATG	100	59.0	2.071
Hypoxia inducible factor 1 alpha	*hif1α*	GGCAATGGAAG ACTTGGA	TGGACTGGAGA ACCTTGA	135	60.5	2.083

Table S2: Measured concentrations of copper in the exposure water. Concentrations were measured for three replicate treatment tanks on days 1 and 3 using ICP-MS and are presented as mean values ± SEM.

AS Treatment	**100 %**		**75 %**		**50 %**	
Nominal Concentration	**0 µg Cu/L**	**20µg Cu/L**	**0 µg Cu/L**	**20µg Cu/L**	**0 µg Cu/L**	**20µg Cu/L**
Day 1	0.432 ± 0.024	21.634 ± 0.206	0.4801 ± 0.022	22.943 ± 0.371	0.430 ± 0.041	21.302 ± 1.163
Day 3	0.544 ± 0.026	21.837 ± 0.247	0.493 ± 0.034	22.359 ± 0.763	0.446 ± 0.030	22.533 ± 1.532
Mean	**0.488 ± 0.027**	**21.735 ± 0.151**	**0.487 ± 0.019**	**22.651 ± 0.401**	**0.440 ± 0.023**	**22.071 ± 1.017**

Table S3: Analysis of variance models for the relationships between copper concentration, air saturation and the copper concentration/air saturation interaction. Minimum adequate models (F value) for the each A) ventilation rate, B) metal content, C) *mt1* transcription, D) *cat* transcription, E) *hif1-α* transcription, F) P_{crit} and G) Blood parameters are shown (Significance codes: *** P<0.001, ** P<0.01, * P<0.05)

		df	Minimum Adequate Model		
			Copper Conc.	Air Saturation	Copper Conc. / Air Sat. Interaction
A) Ventilation Rate		311	37.44***	85.46***	NS
B) Metal Content	**Gill**	42	212.63***	NS	NS
	Liver	NS	NS	NS	NS
C) *mt1* transcription	**Gill**	72	63.42***	15.06***	NS
	Liver	79	26.94***	NS	NS
D) *cat* transcription	**Gill**	NS	NS	NS	NS
	Liver	NS	NS	NS	NS
E) *hif-1α* transcription	**Gill**	76	12.61***	4.07*	NS
	Liver	63	12.48***	8.07***	NS
F) P_{crit}		23	0.84	6.14*	10.01**
G) Blood Parameters	**Haematocrit**	30	NS	10.01**	NS
	Haemoglobin	NS	NS	NS	NS

Chapter 5

Effects of combined exposure to anti-androgenic chemicals and low air saturation in the three-spined stickleback (*Gasterosteus aculeatus*)

Manuscript in preparation

Effects of combined exposure to anti-androgenic chemicals and low air saturation in the three-spined stickleback (*Gasterosteus aculeatus*)

Jennifer A. Fitzgerald[1,2*], *Maciej Tznadel*[1], *Ioanna Katsiadaki*[2], *Eduarda M. Santos*[1*]

[1] Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, UK

[2] Centre for Environment, Fisheries and Aquaculture Science, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, UK

**** Corresponding authors:***

Jennifer Fitzgerald, Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, UK. E-mail: jf277@exeter.ac.uk.

Eduarda Santos, Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, UK. Phone: 00 44 (0)1392 264607; E-mail: e.santos@exeter.ac.uk.

Abstract

Hypoxia is often linked with excess nutrients entering water systems as a result of sewage effluent discharges and agricultural runoff. These sources of nutrients also contain complex mixtures of chemicals, many of which cause adverse effects on reproduction. In this study we exposed male three-spined stickleback (*Gasterosteus aculeatus*), during the winter to spring transition, to a model anti-androgen (flutamide; 250 µg/L) and two chemicals reported to have anti-androgenic activity, linuron (250 µg/L) and fenitrothion (150 µg/L), under either 100 % or 50 % air saturation (AS). We assessed the effects of each chemical, alone and in combination with reduced air saturation, by measuring the transcription of spiggin in the kidney, as a marker for the activation or inhibition of the androgen signalling pathway. In addition, we measured the transcription of 11 genes involved in molecular pathways suspected of being affected by the experimental conditions in the liver, to further elucidate the mechanisms of effect of each chemical and their interactions with low oxygen conditions. Each chemical had a unique transcriptional response alone and in combination with reduced oxygen saturation. Spiggin transcription was strongly inhibited by exposure to flutamide, under both AS conditions. In contrast, exposure to fenitrothion did not result in a significant effect on spiggin transcription. Interestingly, for linuron, a strong inhibition of spiggin was observed under 100 % AS, but this effect was lost under low air saturation, likely via interactions between the hypoxia inducible factor (HIF) and the aryl hydrocarbon receptor (AhR) pathways. Finally, a strong induction of *hif-1α* was observed following exposure to all three chemicals, but the mechanisms or implications of this finding remain unclear. This work illustrates the potential for interactions between reduced oxygen and chemical toxicity to occur, especially for AhR agonists, and provides a mechanistic understanding of these interactions.

Keywords

fish, endocrine disrupting chemicals, freshwater, combined stressors, hypoxia

Introduction

Hypoxia occurs naturally in aquatic ecosystems, but in recent decades there has been an exponential increase in its occurrence worldwide[1]. This has principally been associated with nutrients increasingly entering water bodies, originating from land run-off and sewage discharges[2]. These sewage effluents also contain complex cocktails of chemicals, many of which have endocrine activity[3-8] and have been shown to cause adverse effects on fish at the individual and population levels[3,9-11]. Similarly, agricultural run-off, also contains complex mixtures of natural hormones[12] and pesticides (including linuron[13], fenitrothion[14], glyphosate[15], atrazine[16] and others), many of which have been shown to cause their reproductive disruption.

Hypoxia has been found to directly affect reproduction in fish. Disruption of the reproductive system at the level of the brain has been reported in the Atlantic croaker (*Micropogonias undulates*), where exposure to hypoxia caused a decrease in serotonin (5-HT) concentrations associated with decreases in the activity of the rate limiting enzyme for 5-HT synthesis, typtophan hydroxylase[17]. 5-HT regulates gonadatrophin release, which play fundamental roles in gamete development and maturation. Hypoxia has also been shown to affect sex steroid concentrations, and exposures have caused decreased estradiol (E_2) and testosterone (T) concentrations in zebrafish (*Danio rerio*)[18] and Atlantic croaker[17,19]. In addition, effects on gonad development have also been reported, including impaired oocyte maturation and ovarian growth in females[19] and impaired spermatogenesis and testicular development in Atlantic croaker males[20], and stunted gonadal development in the Gulf killifish (*Fundulus grandis*)[21,22] and the zebrafish[23]. At the behavioural level, hypoxia was shown to cause alterations in reproductive behaviour, including impairment of courtship behaviour in the common carp (*Cyprinus carpio*)[24], decreased frequency of mating displays in the African cichlid[25], and increased time spent fanning the eggs in the common goby (*Pomatoschistus microps*)[26], sand goby (*Pomatoschistus minutus*)[27] and three-spined stickleback (*Gasterosteus aculeatus*)[28].

To date, only one study investigated the effects of EDCs in combination with low oxygen conditions[29]. Male fathead minnows were exposed to a mixture of estrogenic chemicals under either hypoxic or normoxic conditions, and plasma

concentrations of vitellogenin (VTG) were monitored as a biomarker of reproductive disruption in exposed fish. The results demonstrated that air saturation did not affect VTG induction in fathead minnows exposed to the mixture of estrogenic chemicals. Considering the very strong evidence for the effects of hypoxia on reproduction at all levels of the brain-pituitary-gonadal axis, and the scarcity of data on interactions between hypoxia and reproductive disrupting chemicals, it is a research priority to consider this question.

Here, we address this knowledge gap by investigating the combined effects of hypoxia and three anti-androgenic chemicals. We hypothesised that interactions between hypoxia and anti-androgenic chemicals are likely to occur, in particular for male fish, because of the known inhibitory effects of hypoxia on male reproduction, which overlaps with the effects of anti-androgenic chemicals. We selected 3 chemicals because of their well-established anti-androgenic mechanisms of action (flutamide) and their potential environmental relevance (linuron and fenitrothion).

Flutamide is a potent androgenic receptor (AR) antagonist that compete with endogenous androgens at the androgen receptor hormone binding site and prevents AR-DNA binding and transcription of androgen-dependent genes[30, 31]. Although it is of low environmental concern, the anti-androgenic effects of this chemical have been extensively documented because of its proven clinical efficiency in the treatment of androgen-dependent prostate cancer[32], and its extensive use as an anti-androgenic reference chemical in endocrine disruption assays[33].

The pesticide, linuron, was selected for study because of its biological activity as a weakly competitive AR antagonist. Linuron was shown to induce positive response in both the stickleback and the Hershberger assay, via suppression of androgen dependent gene expression[31,34,35]. This chemical is used as an herbicide applied to suppress broad leaf and grassy weed growth, resulting in its entering surface waters in agricultural runoff, particularly in association with sediment, where it is moderately persistent[36]. Linuron, as a result, has been detected in drinking water and in food residues[37,38]. In addition to its anti-androgenic activity, linuron has been shown to activate the aryl hydrocarbon receptor (AhR) signalling pathway[39], an important pathway in the cellular metabolism of toxicants. The AhR and hypoxia signalling pathways have

previously been shown to interact[40], which likely occur as a result of both AhR and the hypoxia inducible factor (HIF; the transcription factor responsible for intracellular oxygen sensing) sharing a dimerization partner, aryl hydrogen receptor nuclear translocator (ARNT). This provides an additional hypothesis for the potential interaction mechanism between hypoxia and linuron.

The last chemical studied was fenitrothion, an organophosphate insecticide that was widely used in the EU until its withdrawal in 2008, although it can still be used in non-agricultural settings. Fenitrothion has structural similarities with both flutamide and linuron[14], but studies have shown that its anti-androgenic potency is far greater[31,41,42].

The three-spined stickleback was used as a model organism in this study because of its useful traits for studying anti-androgens[43]. Male sticklebacks produce spiggin, a glue protein produced in the kidney of mature fish, and used in nest building[44]. Spiggin production is under the control of androgens and has been shown to be suppressed upon exposure to anti-androgenic chemicals[31]. Suppression of spiggin protein has been validated as a useful biomarker to detect chemicals able to inhibit androgen signalling[31,44], including at regulatory levels[45].

In this study, we exposed male stickleback to anti-androgenic chemicals under 100 % or 50 % air saturation (AS). The chemical concentrations used for this study were chosen based on their known anti-androgenic activity and ability to cause inhibition of spiggin production[31]. The aim of the study was to facilitate a mechanistic analysis of the interactions between these chemicals and reduced oxygen in the water on male stickleback. Initially, spiggin transcription in the kidney was measured to determine whether exposure to the chemicals alone or in combination with reduced air saturation inhibited the spiggin increase expected to occur in males as they transition into breeding season during the spring. We then measured the transcription of 11 genes in the liver, chosen as markers for signalling pathways hypothesised to be regulated by both hypoxia and the anti-androgenic compounds selected. These pathways included androgen and oestrogen signalling, oxidative stress response, hypoxia signalling, cholesterol biosynthesis and insulin growth factor signalling.

Material and Methods

Fish source, culture and husbandry

Freshwater three-spined sticklebacks (originating from the River Erme, Devon, United Kingdom) were kindly provided by the University of Plymouth, and bred in the Aquatic Resource Centre at the University of Exeter. Fish were maintained in mixed sex stock tanks (112 L), at a temperature of 12 ±1 °C and a photoperiod of 8:16 light/dark (with a 30-minute transitional dawn/dusk period). Water supplied to tanks was aerated synthetic freshwater[46], which was made from tap water filtered by reverse osmosis (Environmental Water Systems (UK) Ltd) and reconstituted to standardised synthetic freshwater using Analar-grade mineral salts. Fish were fed to satiation every day with blood worm (Chironomus sp.; Tropical Marine Centre, Chorleywood, UK).

Sex identification

An allozyme of isocitrate dehydrogenase (IDH) was found to be sexually dimorphic in the three-spined stickleback[47] and can be used to differentiate males and females at the genetic level, prior to secondary sex characteristics becoming evident. Initially, fish were selected for morphological parameters as described by De Kermoysan *et al*.[48]. Spine clips (second dorsal spine) from fish perceived to be males were collected, DNA was extracted using the HotSHOT method[49], and then amplified in PCR reactions using the *idh* primers (5′-GGGACGAGCAAGATTTATTG-3′ and 5′- TTATCGTTAGCCAGGAGATGG-3′). The amplified products were then visualised on a 2 % agarose gel, as described in Peichel *et al*[47]. The primers amplified a 302 bp fragment in females and two (302 bp and 271 bp) fragments in males, allowing the identification of the males. After successful identification, males were maintained in a high density tank in winter conditions to prevent sexual maturation.

Exposure to anti-androgens and varying oxygen concentrations

Male sticklebacks were exposed to either flutamide (250 µg/L; Sigma Aldrich), linuron (250 µg/L; Sigma Aldrich) or fenitrothion (150 µg/L; Sigma Aldrich), under 100 % or 50 % AS, along with appropriate controls. The lower level of AS was selected to correspond with the critical oxygen level (P_{crit}) for this population of stickleback under the experimental conditions employed during

the chemical exposures (this was determined in Fitzgerald *et al*, in prep; Chapter 4). The oxygen concentration was controlled by continuously bubbling with a pre-set gas mixture of O_2, N_2 and CO_2, achieved by controlling the proportional flow rate from cylinders of each gas, using precision gas flow controllers (MC Series Mass Flow Controllers, Qubit Systems Inc., Ontario, Canada), connected to a PC running gas mixture software (C690 Gas Mixing Software, Qubit Systems Inc., Ontario, Canada). The chemical solutions were supplied by a flow-through system to the experimental tanks, using ethanol (0.001 %) as a solvent. An absolute control and a solvent control receiving the same concentration of ethanol as the chemical exposures were also included, both under 50 % and 100 % AS. For each treatment there were 4 tanks, with one fish per tank. The experiment was carried out twice over two consecutive weeks, resulting in a replication of n=8 fish, each housed in individual tanks, per treatment.

On day 0 of exposure, tanks were spiked with the appropriate amount of chemical to achieve the desired test concentrations and connected to a flow through system. Flow rates were monitored daily to ensure that the chemical concentrations remained consistent and working stock solutions were replaced every 48 h. The level of air saturation was measured twice daily to ensure that it was maintaining at the correct level. Throughout the exposure, daily measurements of pH, conductivity and temperature where also conducted. All fish were fed the same amount of food daily (4 % body weight), and starved 24 h prior to sampling.

To encourage the maturation of the stickleback under the exposure conditions, the photoperiod was raised by 2 hr/day, over the first 5 days of exposure. For the final 2 days of exposure, photoperiod was maintained at 18:6 light/dark. Gravel was placed in a petri dish on one side of the tank to provide environmental enrichment and some substrate to encourage nest building.

All fish were sacrificed on day 7 of the exposure period by lethal dose of benzocaine followed by destruction of the brain, in accordance with the UK Home Office regulations. Wet weight and fork length were recorded and the condition factor (k=(weight (g) x 100)/ (fork length (cm^3)) was calculated for each individual fish. Tissues were collected and weighed, and the heaptatosomatic index (HSI: (liver weight (mg)/total weight (mg)) x 100),

gonadosomatic index (GSI: (gonad weight (mg)/total weight (mg))x100) and the nephrosomatic index (NSI: (kidney weight (mg)/total weight (mg))x100) were calculated. The kidney and liver samples were taken from each fish for molecular analysis, snap frozen in liquid nitrogen and stored at -80 °C.

Water chemistry

Water samples from each experimental tank were taken on day 0, 1 and 6 of the exposure for chemical analysis. Samples containing flutamide were mixed with methanol (HPLC grade, Fischer chemical) and 0.2 % formic acid (Sigma Aldrich), linuron samples were mixed with methanol and fenitrothion samples were mixed with acetonitrile (HPLC gradient, Fischer), all in a 1:1 ratio. For each solvent and water control tanks, 3 water samples were taken and each treated in the same way as samples containing each of these chemicals. The concentration of each anti-androgen was measured using a LC-MS method, and detailed description of how each chemical was measured is given in the Supplementary Information.

Transcript Profiling

Real-time quantitative PCR (RT-QPCR) was used to quantify the transcription of a number of target genes in the kidney and the liver. In the kidney, spiggin transcription was measured to determine if the experimental conditions inhibited the increase in spiggin expected to occur in males as they transition from winter to summer conditions. In the liver, transcription of androgen receptors (*ar1* and *ar2*), estrogen receptors (*esr1*, *esr2a* and *esr2b*), cytochrome P450, family 1, subfamily A (*cyp1a*), hypoxia inducible factor 1α (*hif1α*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), isopentenyl-diphosphate delta isomerase 1 (*idi*), catalase (*cat*) and insulin-like growth factor-binding protein 1b (*igfbp1b*) were determined. Three control genes were used in this study: ribosomal protein l8 (*rpl8*), ubiquitin (*ubi*) and beta-tubulin (*tubb4*). To identify the most appropriate control gene for data normalisation, ct values were plotted and regression analysis was conducted. For the kidney and liver, *ubi* and *rpl8*, respectively, were identified to be the most stable genes, and were selected as control genes for the subsequent data analysis.

Spiggin primers were designed over a conserved generic region from all annotated *spg* genes in order to measure the transcription of all isoforms, simultaneously. Beacon Designer 3.0 software (Premier Biosoft International, Paulo Alto, CA) was used to design the primers for all other genes. Assays were optimised and standard curves were generated for each transcript as previously described[15]. Single amplification of products, at the expected melting temperature, confirmed primer specificity. For each case, the linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was >0.99, and efficiency values were between 1.8-2.1. The primer sequences, annealing temperatures and PCR product sizes for each primer pair are shown in Table S1.

RNA was extracted from the kidney and liver of 8 male fish from each treatment group using the TRI reagent method (Sigma-Aldrich, UK), according to the manufacturer's instructions. After extraction, a NanoDrop-1000 Spectrophotometer (NanoDrop technologies, Wilmington, USA) was used to assess the concentration and purity of the resulting RNA. DNase treatment (RQ1 DNase, Promega, Southampton, UK) was carried out on one µg of RNA to remove potential DNA contamination, prior to conversion to cDNA using M-MLV reverse transcriptase (Promega, UK) and primed with random hexamers (MWG-Biotech), according to the manufacturer's instructions (MJ Research PTC200 Thermal Cycle). The resulting cDNA was diluted (1:2) before RT-QPCR was performed. RT-QPCR was carried out using an iCylcer iQ Real-time Detection System (BioRad Laboratories, Hercules, USA) and SYBR green chemistry (BioRad Laboratories, Hercules, USA). Each sample was run in duplicate in 96-well optical plates (BioRad Laboratories, Hercules, USA) in 15µl reaction volumes. Efficiency-corrected relative expression levels were determined prior to normalisation to a control gene, using the $2^{-\Delta\Delta}$CT method[50]. For *esr1*, due to low expression levels, neat cDNA (instead of 1:2 diluted) was used, with the control gene also run in neat samples for appropriate normalisation. Efficiency values for the standard curves starting from neat cDNA were 2.13 and 2.11 for *esr1* and *rpl8*, respectively.

Statistics

Comparison of solvent control and water control was performed using a t-test. Gene expression data was first scrutinised by the Chauvenets criterion to detect outliers for each gene and these were subsequently removed before analysis took place[51]. Data that did not meet the normality (Sharpo-Wilko test) and equal variance (Bartlett) test was log transformed before analysis. All data was analysed using an analysis of variance model in R[52]. A separate model was used for each gene, to test for effects on gene expression of the exposure to solvent or chemical, and 100 % or 50 % AS (categorical variables) and the interaction between these variables. Minimum adequate models were derived by model simplification using F tests based on analysis of deviance[53]. Tests reported refer to the significance of removing terms from the models. When a significant effect of interaction was identified, pairwise comparisons to determine which groups differed were conducted using Tukey's HSD post hoc test. All data was considered statistically significant when $p<0.05$.

Results

Water and Morphometric Parameters

The mean measured chemical concentrations in the tank water, expressed as percentages of the nominal concentrations, were 102 ±1.4 % for flutamide and 109 ±1.6 % for linuron and are presented in Table S3. For fenitrothion, results are currently unavailable.

The mean measured air saturation for the 100 % and 50 % treatment tanks were 97.4 ±0.1 % and 54.9 ±0.1 %, respectively. The average conductivity (323.35 ±2.10 µs) and pH (7.42 ±0.01) measured in the tanks throughout exposure remained stable over time and were not affected by the treatment.

Throughout the exposure, fish remained in good condition and there were no mortalities attributed to the exposure. The mean body weight, length and condition factor of the fish were 1.41 ±0.03 g, 5.3 ±0.03 cm and 0.94 ±0.01, respectively, and there were no significant differences between treatment groups (body weight: P=0.146 ; length: P=0.247; condition factor: P=0.247). There was no effect of treatment on the GSI (0.68 ±0.02; P=0.318), NSI (0.84 ±0.05; P=0.366), and HSI (3.60 ±0.07; P=0.315).

Transcript profiling in the kidney

There were no differences in gene expression between the solvent control and the water control under 100 % or 50 % AS for any gene tested (Fig. S1 and Fig. S2, respectively), therefore all gene expression data was compared to the solvent control.

Flutamide exposure resulted in a significant down-regulation of *spg* transcription in the kidney (P<0.001, Fig.1.A.; Table S3), but this was not affected by the level of air saturation (P=0.316). Exposure to linuron resulted in decrease *spg* expression under 100 % AS compared to the solvent control (P=0.021, Fig.1.B.; Table S4), but this effect was not present under 50 % air saturation (P=0.992). Fenitrothion had no effect on *spg* expression under 100 % or 50 % AS (P=0.234, Fig.1.C.; Table S5).

Transcript profiling in the liver

The expression levels of the androgen receptors were not affected by the exposures to flutamide (*ar1*: P=0.713 and *ar2*: P=0.520; Fig.2.; Table S3), linuron (*ar1*: P=0.171 and *ar2*: P=0.303; Fig.3.; Table S4) or fenitrothion exposure (*ar1*: P=0.983 and *ar2*: P=0.188; Fig.4.; Table S5). The estrogen receptors were not significantly affected by exposure to either flutamide (*esr1*: P=0.107, *esr2a*: P=0.440 and esr2b: P=0.651; Fig.2.; Table S3) or linuron (*esr1*: P=0.986, *esr2a*: P= 0.058and esr2b: P=0.324; Fig.3.; Table S4). However, exposure to fenitrothion significantly decreased expression of *esr2a* (P= 0.027; Fig.4.; Table S5), but no significant effect was observed for *esr1* (P=0.969) and *esr2b* (P=0.098). For *esr2b*, a similar trend to *esr2a* was apparent.

The oxidative stress response gene, *cat*, was significantly down-regulated in the liver following exposure to flutamide under 100 % AS (P=0.037; Fig.2.; Table S3), but not under 50 % AS (P=0.970). In comparison, *cat* was not affected by the exposure to linuron (P=0.405; Fig. 3.; Table S4) or fenitrothion (P=0.550; Fig. 4.; Table S5), for both 50 % and 100 % AS.

cyp1a expression was not affected by exposure to flutamide (P=0.134; Fig. 2.; Table S3) or fenitrothion (P=0.903; Fig. 4.; Table S5) for both 50 % and 100 % AS. However, this transcript was significantly up-regulated in fish exposed to linuron under 100 % AS (P=0.001; Fig. 3.; Table S4). In contrast, linuron did not

affect *cyp1a* expression under 50 % AS (P=0.215) and there was no difference between the 100 % and 50 % AS groups (P=0.843).

For the genes encoding selected cholesterol biosynthesis enzymes, *hmgcs* and *idi*, there was no effect of exposure for flutamide (P=0.905 and P=0.464, respectively; Fig.2.; Table S3), linuron (P=0.417 and P=0.09, respectively; Fig.4.; Table S4) or fenitrothion (P=0.594 and P=0.701, respectively; Fig.4.; Table S5). However, there was a significant effect of air saturation on *idi* expression for flutamide (P=0.011). No significant response was observed for the insulin growth factor binding protein, *igfbp1b*, after exposure to flutamide (P=0.362; Fig.2.; Table S3) or linuron (P=0.771; Fig. 3.; Table S4). Exposure to fenitrothion caused a suppression in *igfbp1b* expression (P=0.001; Fig.4.; Table S5), but there was no significant effect of air saturation (P=0.446).

Reduced air saturation did not affect the transcription of *hif-1α* (flutamide: P=0.584, linuron: P=0.509 or fenitrothion: P=0.054; Fig. 5.). Interestingly, there was a strong and very significant up regulation of this transcript under both 100 % and 50 % AS following exposure to flutamide (P<0.001; Fig. 5A.; Table S3), linuron (P<0.001; Fig. 5B.; Table S4) and fenitrothion (P=0.04; Fig. 5C.; Table S5), but the induction of *hif-1α* transcription was not influenced by AS.

Discussion

The objective of this study was to determine the influence of reduced oxygen concentrations on the effects of chemicals with an anti-androgenic mode of action. The interactions between low oxygen and each of the anti-androgens was unique to each chemical, and this was illustrated by their contrasting spiggin responses. Spiggin transcription was strongly inhibited by exposure to flutamide, under normoxic and hypoxic conditions. For fish exposed to linuron, spiggin transcription was dependent on the level of air saturation that the sticklebacks were exposed to. In contrast, exposure to fenitrothion did not result in a significant effect on spiggin. The differences in how fish responded to each chemical, as assessed by the level of spiggin induction, are also reflected in their transcriptional responses in the liver.

Flutamide inhibits spiggin production independently of the level of air saturation

Spiggin expression was significantly down regulated in fish exposed to flutamide compared to those maintained under control conditions, but this was not affected by low oxygen. This is consistent with previous studies reporting that exposure of adult stickleback males to flutamide resulted in reduced spiggin production in the kidney and significant decreases in the number of nests built[54]. Similarly co-exposure to flutamide and a model androgen (17α-methyltestosterone) in female sticklebacks resulted in a suppression of the androgen-induced spiggin production, confirming its anti-androgenic mode of action for this species[31]. Kidney hypertrophy which is associated with spiggin production is controlled by 11-ketotestosterone (11-KT), the most important male androgen in fish[55], in a process induced via an AR-mediated pathway[56]. Spiggin induction by 11-KT was shown *in vitro* to be blocked by the androgen receptor agonist, flutamide[57]. Flutamide acts as a potent androgen receptor (AR) antagonist via competition with androgens preventing AR-DNA binding and transcription of androgen-dependent genes[58]. There was no effect of flutamide exposure on the expression of androgen receptors, in either this dataset or in the experiment conducted by Olsson *et al.*, which suggests that auto-regulation of the AR is not involved in differential ligand activation[59].

Exposure to flutamide resulted in a down regulation of catalase compared to the solvent control under 100 % AS. This has also previously been observed in both cell lines and in an *in vivo* mice model, where catalase activity was increased under testosterone stimulation but with the addition of flutamide this activity was supressed[60,61]. Catalase is an antioxidant enzyme in fish and an important indicator of oxidative stress. Oxidative stress is the result of an imbalance between the production of reactive oxygen species (ROS) and anti-oxidant defences[62]. Under normal conditions, mitochondrial respiration causes a constant generation of superoxides, such as hydrogen peroxide (H_2O_2), and hydroxyl radicals, which are a major source of ROS[63]. Cells are adapted to scavenge the excess free radicals through their antioxidant defence mechanisms, ensuring a fine balance between reactive oxygen species and antioxidant enzymes. However, if the levels of ROS in the cell become too high, this equilibrium can be lost, resulting in damage to cellular DNA, lipid and proteins. The first cellular response to this imbalance, is the enhancement of the expression of superoxide dismutase (SOD) converting the superoxide radical to H_2O_2 which is further converted to H_2O by glutathione peroxidase and

catalase[64,65]. When the androgen receptors are blocked, a marked increase in the ROS generation occurred[61], probably by inhibition of the testosterone-induced catalase expression. Therefore, in our experiment, flutamide may have reduced catalase expression via its anti-androgenic activity. In contrast, exposure to flutamide under 50 % AS resulted in no effect on catalase expression. Decreases in oxygen concentrations have also been shown to induce oxidative stress in fish[66]. The mechanisms by which hypoxia induces oxidative stress are not yet known, but suggestions have included greater electron leakage the electron transfer chain under low oxygen, forming more ROS[66]. Exposure to hypoxia was shown to result in inductions of antioxidant response enzymes, for example exposure to low oxygen resulted in increased SOD and catalase activity in the liver of the goldfish (*Carassius auratus*)[67] and the common carp[68]. In addition, exposure to hypoxia resulted in increased activities of catalase and glutathione peroxidase in the freshwater clam (*Corbicula fluminea*)[69]. Therefore, we hypothesise that the increase in antioxidant defence systems as a result of the exposure to 50 % AS could prevent the suppression of catalase transcription observed when fish were exposed to flutamide under normoxic conditions.

Hypoxia reduces the anti-androgenic effects of linuron via inhibition of the AhR pathway

Exposure of male sticklebacks to linuron resulted in a decrease in *spg* expression compared to the solvent control under 100 % AS. Linuron is a weak competitive AR antagonist *in vitro* and has been shown to suppress androgen-dependent gene expression[34,35]. Exposure to linuron was shown to inhibit androgen-induced spiggin protein production in the female stickleback model[56,70] but at the transcription level, *spg* was not shown to decrease under linuron exposure in mature male stickleback[71]. This difference in findings is likely to be as a result of different exposure time (Hogan *et al*. exposed males for only 3 days, whereas in this study fish were exposed for 7 days) and experimental conditions (males in this study were stimulated to mature during the experiment by progressively increasing the photoperiod, to induce kidney hypertrophy, whereas the exposure by Hogan *et al*. was carried out under a fixed winter temperature and light regime). Similarly to the study by Hogan *et al*., exposure to linuron in our study did not result in changes in either of the

androgen receptors mRNA levels[71]. This lack of effect could be due to a lack AR auto-regulation, but more work is needed to clarify this further.

Exposure to linuron under 100 % AS resulted in a strong increase in the expression of *cyp1a* in the liver. The same finding was reported for brown trout exposed to linuron over 4 days, where an environmentally relevant concentration of linuron (1.7 µg/L) caused a moderate induction of *cyp1a* transcription and exposure to 225.9 µg/L dramatically increased the transcription of this gene by 560 fold[39]. *cyp1a* is involved in the detoxification and/or metabolic activation of a number of xenobiotics, and is among the most readily induced cellular proteins[72]. This gene is primarily regulated via the AhR signalling pathway, which is fundamental for the metabolism of xenobiotics[73]. Xenobiotics that have entered the cell bind to the AhR, forming an AhR-ligand complex which then translocates to the nucleus. The complex binds to the ARNT protein forming an activated transcription factor which is able to bind to xenobiotic responsive elements, resulting in the transcription of CYP proteins[74]. The induction of *cyp1a* at both the gene and protein levels has been used extensively as a measure of exposure to a number of xenobiotics including planar aromatic hydrocarbons (PAH), and PCBs[75]. Linuron has been reported to be one of the most potent activators of the AhR in both fish and mice models[39,76,77].

The increase in *cyp1a* transcription observed following exposure to linuron under 100 % AS was absent when exposures occurred in the presence of 50 % AS. We hypothesise that under low air saturation competition occurs for the dimerization partner (ARNT), which is shared by both the oxygen-sensitive HIF pathway (discussed in section 4.4) and the AhR pathway[78]. This competition for ARNT may have resulted in a reduction of AhR-ARNT dimerization, and, consequently, a suppression of linuron-induced *cyp1a* transcription.

Under reduced oxygen, in addition to the suppression of *cyp1a*, the effects of linuron on spiggin were also absent. The exact mechanism why this response is observed is currently not known, due to a lack of literature on linuron biotransformation and degradation, and how the parent compounds, as well as its metabolites, interact with androgen receptors and/or other molecules part of the androgen signalling pathway. However, due to the strong effect of linuron on *cyp1a* transcription, we speculate that linuron may be required to undergo bio-

activation by CYP1A before acting as an anti-androgen, which in turn would cause the suppression of spiggin. Under hypoxia, due to the suppression of the activity of the AhR pathway, the strong induction of *cyp1a* is reduced, lessening the bio activation of linuron, potentially explaining why spiggin is no longer affected by the exposure. Currently, there is no information available about linuron's metabolism by the cytochrome P450 enzymes, therefore we can only speculate at this stage. However, this mechanism has previously been suggested for the PAH, pyrene; a study showed that a delay in pyrene toxicity occurs in CYP1A-morphants, which strongly suggests that a metabolite of pyrene is responsible for its toxicity, and not pyrene itself[79]. Similar work for linuron would allow our hypothesis to be tested.

Fenitrothion had no effect on spiggin production

Exposure to fenitrothion under both 100 % and 50 % AS caused no significant effect to the expression of spiggin. Previous work has reported varied results with regards to the anti-androgenicity of fenitrothion. Research by Sohoni *et al* observed no conclusive anti-androgenic effects of fenitrothion in either intact or castrated male rats[80] and Turner *et al.* did not identify any significant alterations of androgen-dependent sexual differentiation in male rates after fenitrothion exposure[81]. However, fenitrothion has been reported to act as an anti-androgen, *in vitro*, in HepG2 human hepatoma liver cells and *in vivo*, using the Hershberger test in male rats[14]. Katisadaki *et al.* also demonstrated that fenitrothion is a potent anti-androgen *in vivo,* in fish, as simultaneous exposure of female three-spined stickleback to 17α-methyltestosterone and 150 µg/L fenitrothion completely inhibited spiggin production, and 15 µg/L caused a significant reduction in spiggin production[31]. It has also been shown that fenitrothion exposure causes a concentration-dependent inhibition of spiggin induction in DHT-primed female sticklebacks[70]. *In vitro*, fenitrothion has been clearly demonstrated to act as a direct antagonist of the androgen receptor[14,82], with potency much greater than flutamide and approximately 8- to 35- fold greater than the environmental anti-androgens *p,p DDE* and linuron, respectively[41,42]. However, at high concentrations (above 10^{-5} M), fenitrothion was shown to have AR agonist activity *in vivo* in HepG2 human hepatoma liver cells[14]. Therefore, in our exposure the concentration of fenitrothion may have been above the threshold for androgenic activity to be dominant, causing

fenitrothion to act as an androgen receptor agonist instead of an antagonist. However, in the absence of water chemistry, it is hard to conclude whether this is actually as a result of varying concentrations of fenitrothion during the exposure.

This hypothesis is supported by the data for *esr2a*, where a significant decrease in transcription in fish exposed to fenitrothion was observed, independent of air saturation. Studies have previously shown that increased levels of androgens decrease the expression of estrogen receptor mRNA[83,84]. Therefore, the suppression of *esr2a* transcription further indicates that fenitrothion may be acting as an androgen agonist.

Exposure to fenitrothion resulted in a down regulation of *igfbp1b* in fish exposed to fenitrothion compared to the controls. Insulin growth factor-1 (IGF-1) is involved in the regulation of protein, lipid and carbohydrate metabolism, as well as cell differentiation and proliferation, and ultimately body growth[85]. IGF-1 circulates in the blood tightly bound to specific binding proteins (IGFBPs) that differ in site of origin as well as biological functions. IGFBPs function as carrier proteins in the circulation and regulate IGF, transport and half-life of circulating IGFs[85]. In addition, they also modulate IGF availability and biological activity in local tissues, by preventing receptor binding and, in turn, they can inhibit the biological activity of the IGFs[86]. In humans, testosterone treatment has been shown to increases IGF-1 transcription in the skeletal muscle[87,88], but this increase has been shown to be suppressed by flutamide[89]. In this study, fenitrothion appears to be acting as an androgen receptor agonist potentially increasing *igf* via androgen receptor activation. Suppression of *igfbp1b*, which can prevent IGF binding to its receptor, may in turn further contribute to increasing IGF activity. This further supports the idea that fenitrothion may have acted as an androgen agonist at the concentration tested in our experimental setting.

Interaction between anti-androgenic chemicals and the hypoxia-inducible factor pathway

There was a strong up-regulation of *hif-1α* expression in fish exposed to all three chemicals tested both under 100 % and 50 % AS. HIF is a key oxygen sensing molecule and transcription factor regulating the cellular response to

hypoxia, which results in the adaptive response of maintaining oxygen homeostasis under low environmental oxygen by the stimulation of the transcription of genes that promote a series of processes including vasodilation, erythropoiesis and angiogenesis, as well as energy production via anaerobic glycolysis[90]. Under normal oxygen levels, the HIF-1α subunit is modified by the oxygen sensitive enzymes, PHDs (prolylhydroxlases), allowing the protein-ubiquitin ligase complex to recognise HIF-1α, resulting in its degradation by the proteasome pathway[91]. However, under low oxygen concentrations, HIF-1α is stabilised, due to the inhibition of PHD. HIF-1α accumulates in the cell and translocated into the nucleus where it forms a dimer with ARNT. The HIF-1α-ARNT dimer acts as a transcription factor that binds to hypoxia responsive elements causing the regulation of a wide range of genes involved in the hypoxia response. There is now accumulating evidence that HIF-1α responds to non-hypoxic stimuli, including hormones such as insulin, growth factors, coagulation factors, vasoactive peptides and cytokines[92-98], but to our knowledge, there is no evidence in the literature for very significant *hif-1α* induction following exposure to organic xenobiotics. In our study *hif-1α* is clearly induced by three chemicals with a known anti-androgen mode of action, however the mechanism by which these chemicals induce *hif1-α* at the transcription level is not known. We hypothesise that intracellular oxygen may have become depleted as a result of metabolism of these chemicals by the P450 cytochrome monooxygenases. Fish detoxify xenobiotics through the reduction, oxidation or hydrolysis of compounds to more water soluble products, which, in turn, allows their excretion to occur via the bile[99]. During this process, the xenobiotic substrate will bind to the hydrophobic site on the cytochrome P450 (CYP) enzyme[100], where the iron present in the enzyme is oxidised. Oxygen interacts with the binding site on the CYP molecule causing a conformational change, and a reduction in iron by an electron transfer via cytochrome P450 reductase. More oxygen is added, before addition of another electron via the same pathway to create a short term intermediate complex. This is rapidly protonated, releasing a water molecule, whilst the other oxygen atom is inserted into the substrate complex to make it hydroxylated[100,101]. This process uses up cellular oxygen, so may produce intracellular hypoxia in hepatocytes, which in turn could cause the up regulation in *hif-1α* observed in our study. Further studies are essential to investigate the mechanisms by which

these three compounds cause transcriptional changes in *hif-1α*, and whether this is common to other chemicals.

Conclusions

We have demonstrated that reduced oxygen in the water can suppress the anti-androgenic activity of linuron, likely via competition between the HIF and AhR pathways. In addition, we demonstrated that hypoxia did not modify the effects of flutamide and fenitrothion on spiggin transcription, at least for the transcripts considered in this study and for the chemical concentrations tested. Despite all three chemicals being considered to be anti-androgenic, they each had a very different molecular response alone and in combination with hypoxia both at the level of the kidney and the liver, highlighting the need for further research on how exactly these chemicals affect fish in real environmental scenarios. In addition, further work investigating how these chemicals, alone and in combination with reduced air saturation, affect the reproductive output and behaviour of fish species is required to fully appreciate the implications of the effects seen for fish populations in contaminated environments. Although each chemical is unlikely to be found in the aquatic environment alone at the concentrations used in these exposures, the mixtures of different anti-androgens that enter the aquatic environment can reach anti-androgenic activity similar to that tested in this study, suggesting that our findings may be environmentally relevant. The lack of concordance between the responses of these three reported anti-androgenic chemicals highlights the need to consider how the concentration of oxygen in the environment affects the toxicity of a wide range of environmental relevant xenobiotics with the potential to adversely impact fish populations, in order to better protect wild fish populations.

Acknowledgements

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Figures

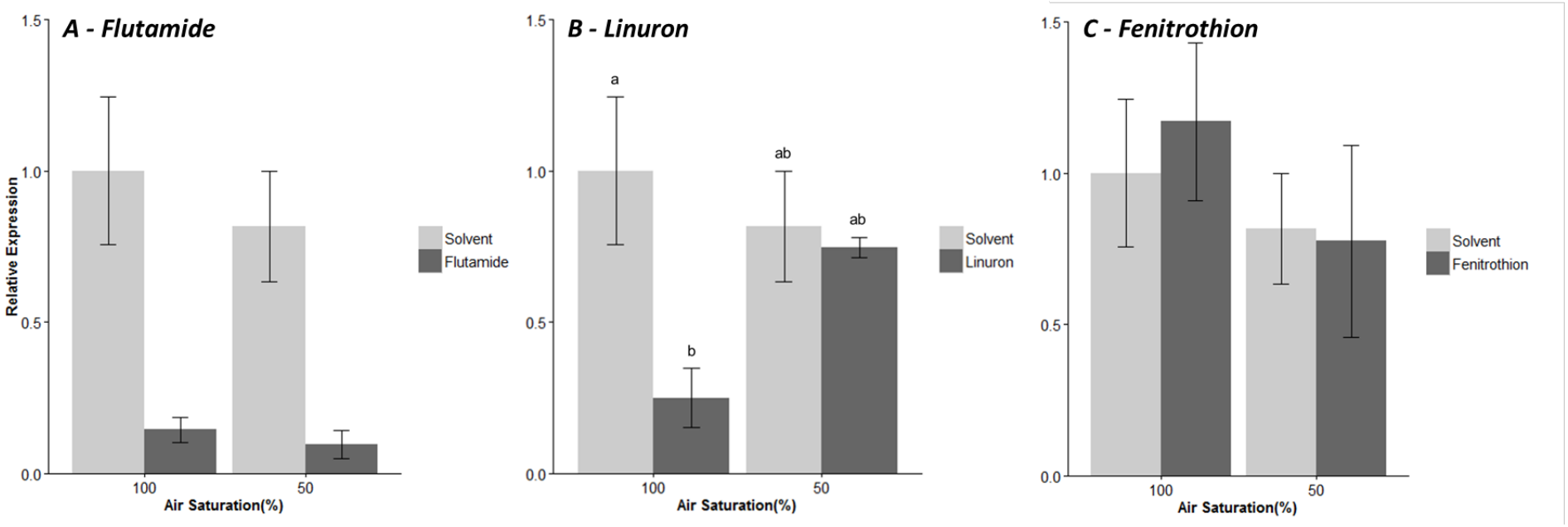

Figure 1. Transcript profiles for spiggin following exposure to three anti-androgenic chemicals under different levels of air saturation. Male sticklebacks were exposed to A: 0 or 250 µg Flutamide/L, B: 0 or 250 µg Linuron/L or C: 0 or 150 µg Fenitrothion/L for 7 days under 97.4 ±0.1 % AS and 54.9 ±0.1 % AS. Spiggin transcript levels in the kidney were determined using RT-QPCR and normalised to the control gene *ubi*. 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=6-8 fish per treatment group. Plotted data is translated (+1) and then LOG10 transformed. Statistics carried out using accepted minimum adequate models (analysis of variance model, R; $P<0.05$) with model details reported in Table S3, 4, and 5. Letters above

each bar indicate significant differences between treatment groups when there was a significant interaction from the model (Tukey's HSD post hoc test; $P<0.05$). There was a significant effect of flutamide exposure on gene expression ($P<0.001$).

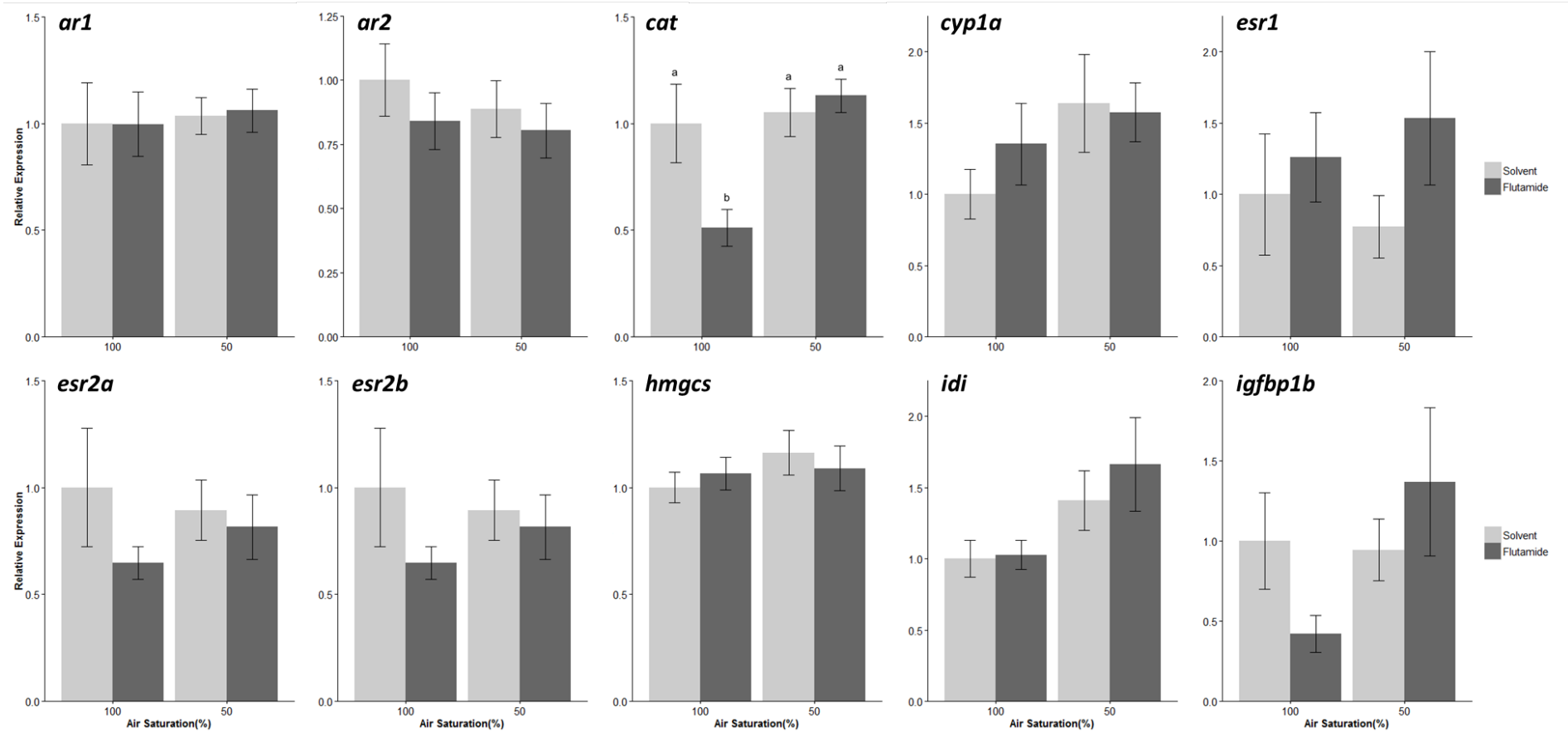

Figure 2. Transcript profiles for selected target genes following exposure to flutamide under different levels of air saturation. Male sticklebacks were exposed to 0 or 250 µg flutamide/L for 7 days under 97.4 ±0.1 % AS and 54.9 ±0.1 % AS. Transcript profiles were determined using RT-QPCR. Genes analysed included: androgen receptors (*ar1* and *ar2*), catalase (*cat*), cyctochrome P450, family 1, subfamily A (*cyp1a*), estrogen receptors (*esr1*, *esr2a* and *esr2b*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), isopentenyl-diphosphate delta isomerase 1 (*idi*) and insulin-like growth factor-binding protein 1b (*igfbp1b*). Plotted data

are presented as average relative expression (normalised 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=6-8 fish per treatment group. Statistics carried out using accepted minimum adequate models (analysis of variance model, R; $P<0.05$) with model details reported in Table S3. Letters above each bar indicate significant differences between treatment groups when there was a significant interaction from the model (Tukey's HSD post hoc test; $P<0.05$). For *idi* and *igfbp1b*, there was a significant effect of air saturation on gene expression ($P=0.011$ and $P=0.001$, respectively).

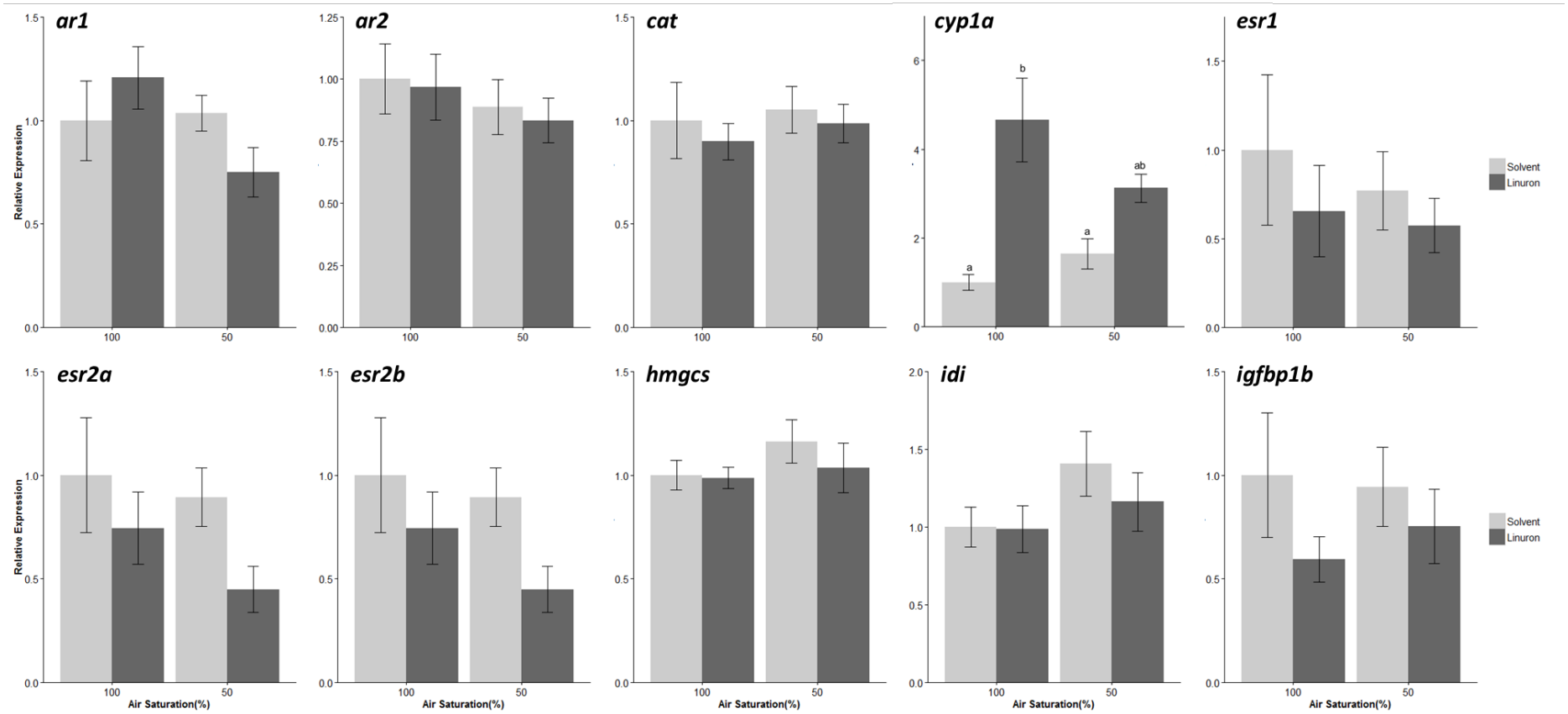

Figure 3. Transcript profiles for selected target genes following exposure to linuron under different levels of air saturation. Male sticklebacks were exposed to 0 or 250 µg linuron/L for 7 days under 97.4 ±0.1 % AS and 54.9 ±0.1 % AS. Transcript profiles were determined using RT-QPCR. Genes analysed included: androgen receptors (*ar1* and *ar2*), catalase (*cat*), cyctochrome P450, family 1, subfamily A (*cyp1a*), estrogen receptors (*esr1*, *esr2a* and *esr2b*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), isopentenyl-diphosphate delta isomerase 1 (*idi*) and insulin-like growth factor-binding protein 1b (*igfbp1b*). Plotted data are

presented as average relative expression (normalised 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=6-8 fish per treatment group. Statistics carried out using accepted minimum adequate models (analysis of variance model, R; $P<0.05$) with model details reported in Table S4. Letters above each bar indicate significant differences between treatment groups when there was a significant interaction from the model (Tukey's HSD post hoc test; $P<0.05$)

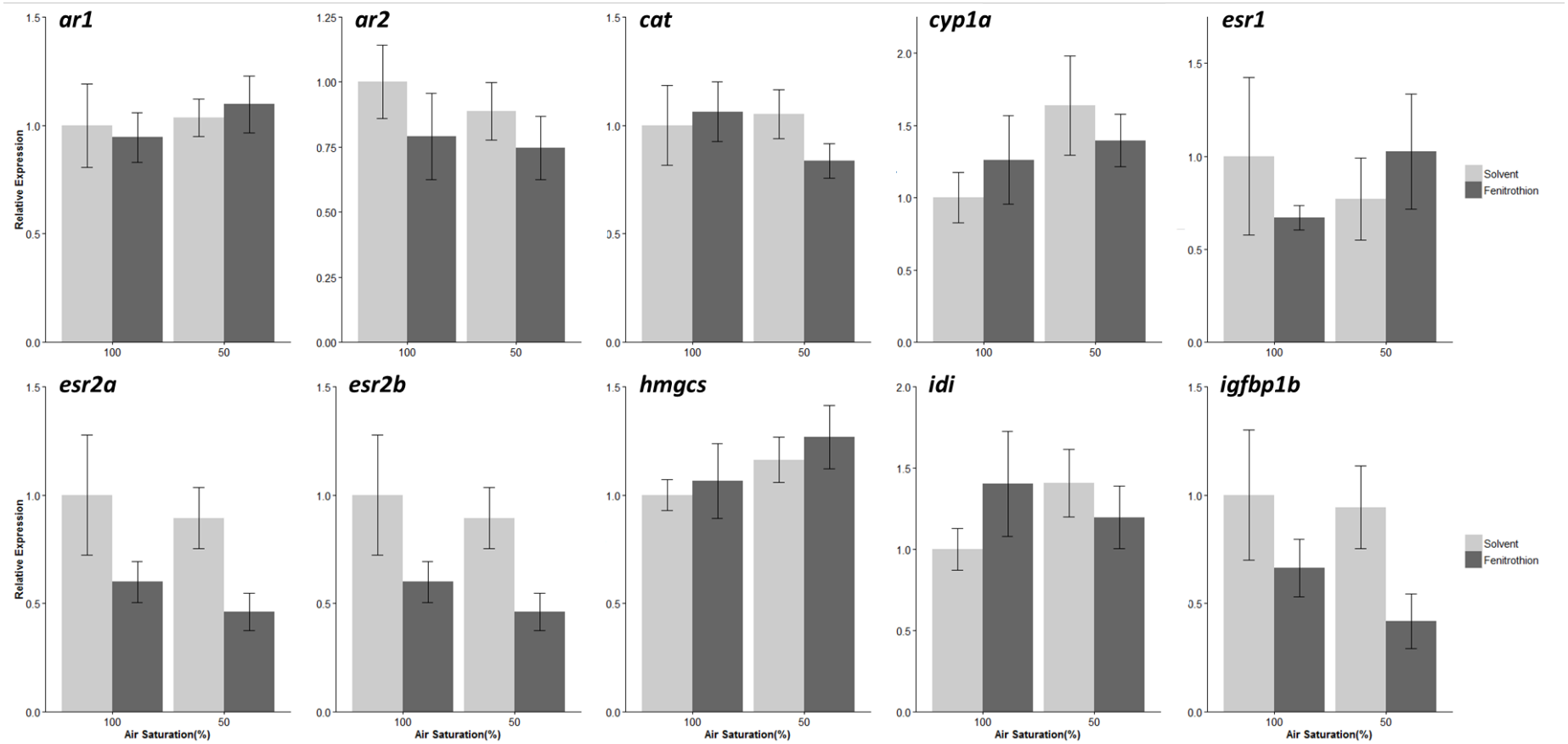

Figure 4. Transcript profiles for selected target genes following exposure to fenitrothion under different levels of air saturation. Male sticklebacks were exposed to 0 or 150 µg fenitrothion/L for 7 days under 97.4 ±0.1 % AS and 54.9 ±0.1 % AS. Transcript profiles were determined using RT-QPCR. Genes analysed included: androgen receptors (*ar1* and *ar2*), catalase (*cat*), cyctochrome P450,

family 1, subfamily A (*cyp1a*), estrogen receptors (*esr1*, *esr2a* and *esr2b*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), isopentenyl-diphosphate delta isomerase 1 (*idi*) and insulin-like growth factor-binding protein 1b (*igfbp1b*). Plotted data are presented as average relative expression (normalised 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=6-8 fish per treatment group. Statistics carried out using accepted minimum adequate models (analysis of variance model, R; $P<0.05$), with model details reported in Table S5. Letters above each bar indicate significant differences between treatment groups when there was a significant interaction from the model (Tukey's HSD post hoc test; $P<0.05$). For *esr2a* and *igfbp1b*, there was a significant effect of fenitrothion exposure on gene expression ($P=0.03$ and $P=0.001$, respectively).

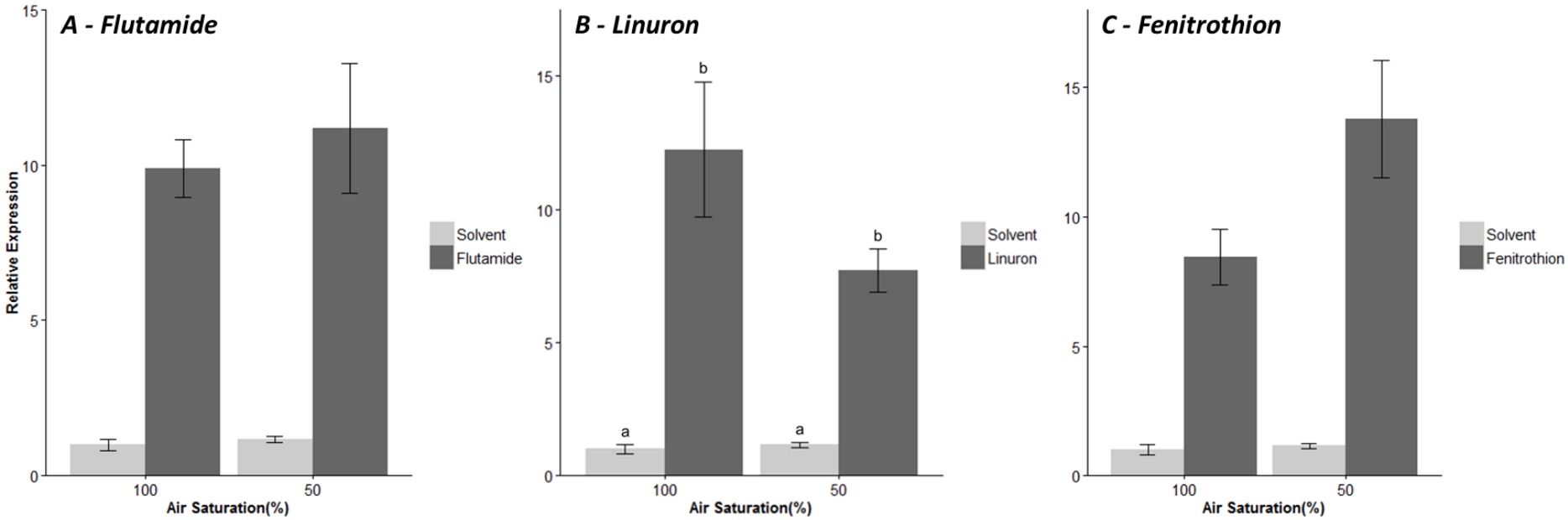

Figure 5. Transcript profiles for *hif-1α* following exposure to three anti-androgenic chemicals under different levels of air saturation. Male sticklebacks were exposed to A: 0 or 250 µg Flutamide/L, B: 0 or 250 µg Linuron/L or C: 0 or 150 µg Fenitrothion/L for 7 days under 97.4 ±0.1 % AS and 54.9 ±0.1 % AS. Expression levels in the liver were determined using RT-QPCR and normalised to 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=6-8 fish per treatment group. Statistics carried out using accepted minimum adequate models (analysis of variance model, R; $P<0.05$), with model details reported in Table S3, 4, and 5. Letters above each bar indicate significant differences between treatment groups when there was a significant interaction from the model (Tukey' HSD post hoc test; $P<0.05$). For flutamide and fenitrothion, there was a significant effect of anti-androgen treatment on gene expression ($P<0.001$ and $P=0.04$, respectively).

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Supplementary Information: Effects of combined exposure to anti-androgenic chemicals and low air saturation in the three-spined stickleback (*Gasterosteus aculeatus*)

Jennifer A. Fitzgerald, Maciej Tznadel, Ioanna Katsiadaki, Eduarda M. Santos

Pages

SI – MATERIAL AND METHODS

Water chemistry analysis

Analyses were performed using Surveyor MS Pump Plus HPLC pump with HTC PAL autosampler coupled to TSQ Vantage triple quadrupole mass spectrometer equipped with heated electrospray (HESI II) source (all ThermoFisher Scientific, Hemel Hempstead, UK). Chromatographic separation was achieved using reversed-phase, 3 µm particle size, C18 Hypersil GOLD column 50 mm × 2.1 mm i.d. (Thermo Scientific, San Jose CA, USA). Analytes were separated using a linear gradient of (A) Water and (B) Methanol, both containing 0.1 % of Formic Acid. Solvent B increased from 20 % to 100 % in 1.5 min and was maintained for 1.5 min before returning to the initial condition for 2 min. The flow rate was 500 µL/min. Temperature of autosampler was set at 8 ˚C, while column was kept at room temperature. HESI probe was operating in both negative and positive mode; an ion-spray voltage of -2.75 kV was applied for analysis of flutamide and +3.75 kV for linuron. The heated capillary temperature was set at 270 °C and the vaporizer temperature was 350 °C. Nitrogen was employed as sheath and auxiliary gas at a pressure of 60 and 2 arbitrary units, respectively. The argon CID gas was used at a pressure of 1.5 mTorr and the optimum collision energy (CE) for each transition was selected. Quantification of the target compounds was performed by monitoring two characteristic multiple reaction monitoring (MRM) transitions (Table below)

Compound	Parent ion (*m*/*z*)	Product ion (*m*/*z*)	CE (V)
Linuron	249.0	160.1	17
		182.1	16
Flutamide	275.1	202.1	25
		205.1	24

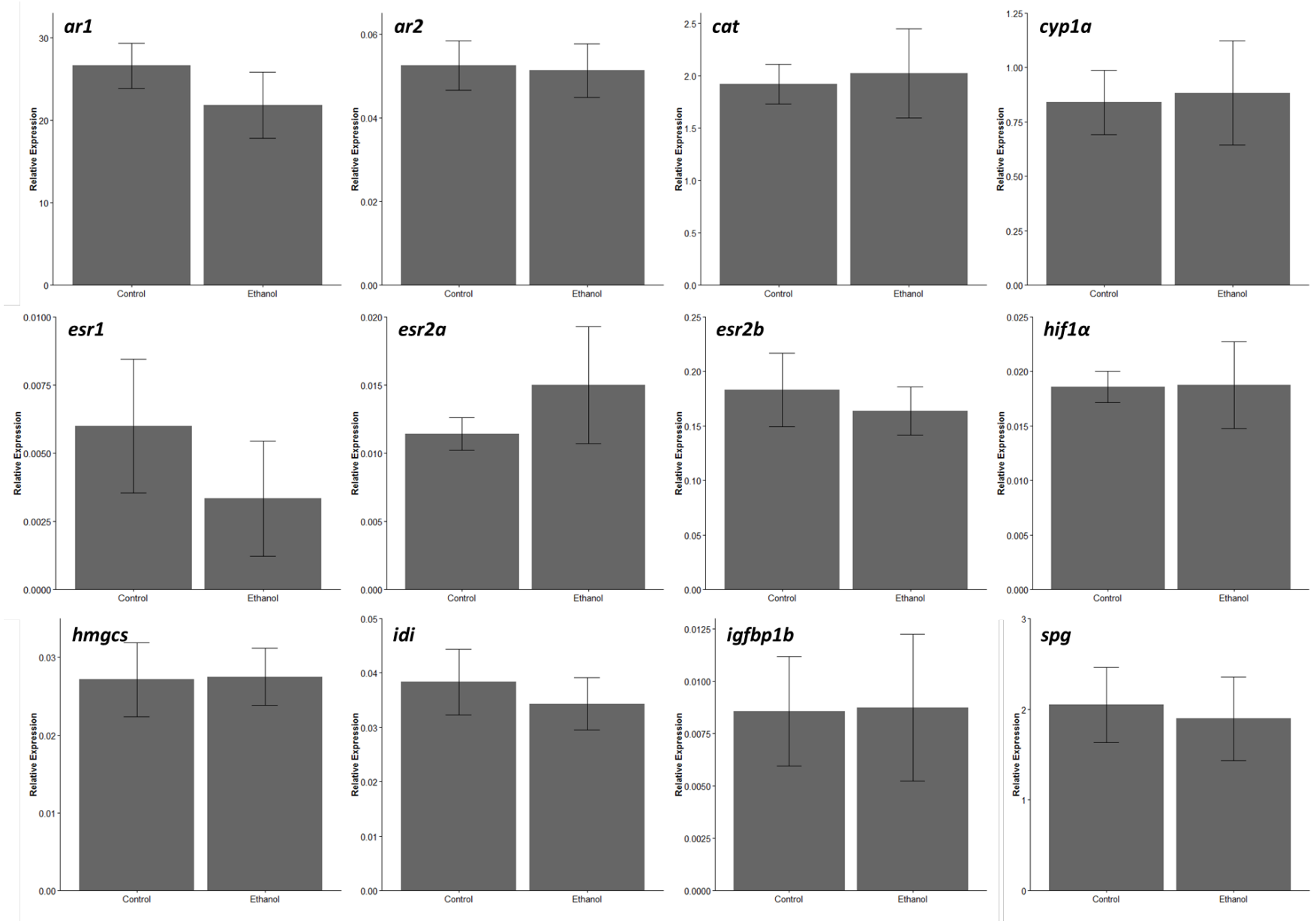
ar1
ar2
cat
cyp1a
esr1
esr2a
esr2b
hif1α
hmgcs
idi
igfbp1b
spg
Relative Expression
Control
Ethanol

Figure S1. Comparison between transcript profiles for control and solvent control treatments under 100 % for selected target genes. Male fish kept in either water alone or water containing 0.001 % ethanol for 7 days under 97.4 ±0.10 % AS are compared. Transcript profiles were determined using RT-QPCR. Genes analysed included: androgen receptors (*ar1* and *ar2*), catalase (*cat*), cyctochrome P450, family 1, subfamily A (*cyp1a*), estrogen receptors (*esr1*, *esr2a* and *esr2b*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), isopentenyl-diphosphate delta isomerase 1 (*idi*) and insulin-like growth factor-binding protein 1b (*igfbp1b*). Plotted data are presented as average relative expression. Points for which the expression was below the detection limit of the assay were excluded from the analysis, resulting in a replication of n=6-8 fish per treatment group. There was no significant difference between the water control and ethanol for any of the genes tested (*ar1*: P=0.358, *ar2*: P=0.792, *cat*: P=0.871, *cyp1a*: P=0.889, *esr1*: P=0.797, *esr2a*: P=0.936, *esr2b*: P=0.607, *hif1α*: P=0.961, *hmgcs*: P=0.900, *idi*: P=0.424, *igfbp1b*: P=0.477 and *spg*: P=0.808).

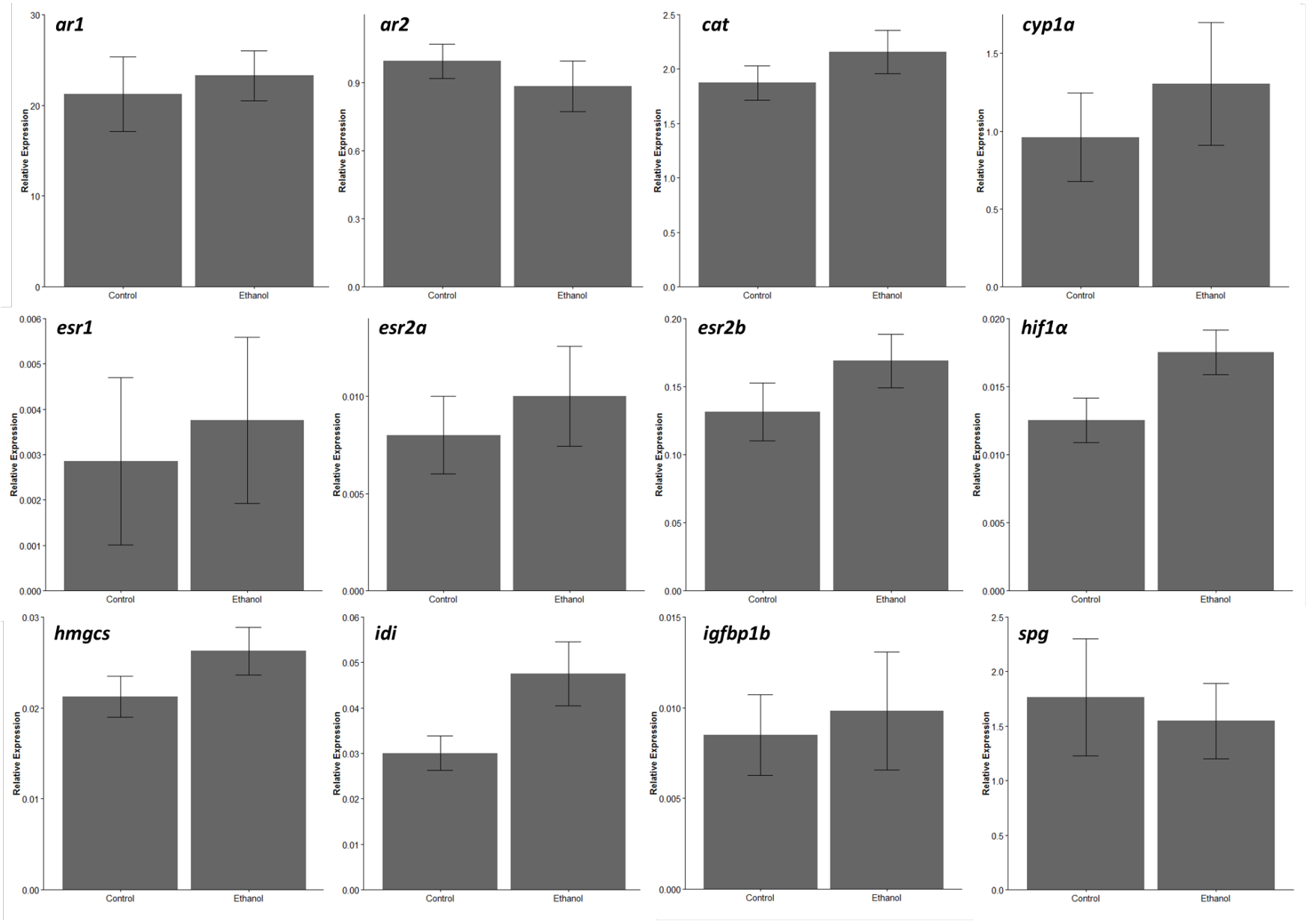
ar1
ar2
cat
cyp1a
esr1
esr2a
esr2b
hif1α
hmgcs
idi
igfbp1b
spg
Relative Expression
Control
Ethanol

Figure S2. Comparison between transcript profiles for control and solvent control treatments under 50 % for selected target genes. Male fish kept in either water alone or water containing 0.001% ethanol for 7 days under 54.9 ±0.14 % AS are compared. Transcript profiles were determined using RT-QPCR. Genes analysed included: androgen receptors (*ar1* and *ar2*), catalase (*cat*), cyctochrome P450, family 1, subfamily A (*cyp1a*), estrogen receptors (*esr1*, *esr2a* and *esr2b*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), isopentenyl-diphosphate delta isomerase 1 (*idi*) and insulin-like growth factor-binding protein 1b (*igfbp1b*). Plotted data are presented as average relative expression. Points for which the expression was below the detection limit of the assay were excluded from the analysis, resulting in a replication of n=6-8 fish per treatment group. There was no significant difference between the water control and ethanol for any of the genes tested (*ar1*: P=0.692, *ar2*: P=0.910, *cat*: P=0.282, *cyp1a*: P=0.481, *esr1*: P=0.334, *esr2a*: P=0.265, *esr2b*: P=0.224, *hif1α*: P=0.063, *hmgcs*: P=0.115, *idi*: P=0.053, *igfbp1b*: P=0.127 and *spg*: P=0.741).

S1 Table: Details of primer sequences and assay conditions for the QPCR assays

Gene Name	Gene Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Ta (°C)	PCR Efficiency
Ribosomal protein L8	*rpl8*	GGTGCGTCCCTC CTGATG	GCGTGGTGTGG CTATGAAC	93	60.5	2.059
Beta tubulin	*tubb4*	TCTTCAGACCAG ACAACTT	CTCCTTCCTCAC CACATC	119	60.0	2.025
Ubiquitin	*ubc*	GGAGGGCAGTAA AGTGATT	CAAGGCAGGAGA TTCAGTT	161	57.0	1.967
Spiggin	*spg*	GCTTTGAAAACA GCCAGAGCATCT	TGGACAGGAACA GGTTTCAGTGAG T	212	58.5	2.001
Androgen receptor 1	*ar1*	CATACACTCTCA CTAACA	GTTCATACATACT GGAAAC	90	56.0	2.008
Androgen receptor 2	*ar2*	CGGAAGGCAAAC AGAAATAC	CGACAGGATGGA CAGTTC	85	59.5	1.959
Estrogen receptor 1	*esr1*	TTGGAATAGAGG CAGGAG	GGAGTGGAGAC GAGTATC	85	62.0	2.130
Estrogen receptor 2a	*esr2a*	GCCTCTCAGAAA TCTTTG	CAGACATACTCC TCTCTC	82	56.0	2.108
Estrogen receptor 2b	*esr2b*	CAAGAACCGACG CAAAAG	TACACCGCACTT CATCAT	76	59.0	1.926
Cyctochrome P450, family 1, subfamily A	*cyp1a*	CCTTCGCCATTC TTCATTC	GACCTGCCACTG ATTGAT	121	59.0	2.029
Hypoxia inducible factor 1 alpha	*hif1α*	GGCAATGGAAGA CTTGGA	TGGACTGGAGAA CCTTGA	135	60.5	2.083
Catalase	*cat*	CCAGAAGCGTAA TCCTCAA	GAACAAGAAAGA CACCTGATG	100	59.0	2.003
3-hydroxy-3-methylglutaryl-coenzyme A synthase	*hmgcs*	GGTTTTGACAGA CTTGGACTTG	GGTTGATGGAGC GGAATGG	95	62.0	1.959
Isopentenyl-diphosphate delta isomerase 1	*idi*	AGAGCGTTCAGC GTGTTC	TCCGTGTGTAGA GGGTGAC	125	60.0	2.041
Insulin-like growth factor-binding protein 1b	*igfbp1b*	CCCAACTGCGAC AAACAC	TTCTTGCCGTTC CAGGAG	104	61.0	2.098

S2 Table: Measured concentrations for the three anti-androgenic chemicals in the exposure water. Concentrations were measured for 8 replicate treatment tanks on days 0, 1 and 6 and are presented as mean values ± SEM.

Anti-Androgen	**Flutamide**				**Linuron**			
AS Treatment	**100%**		**50%**		**100%**		**50%**	
Nominal Concentration	**0µg/L**	**250µg/L**	**0µg/L**	**250µg/L**	**0µg/L**	**250µg/L**	**0µg/L**	**250µg/L**
Day 1	<0.98	257 ± 9.4	<0.98	262 ± 10.5	<0.98	260 ± 5.7	<0.98	270 ± 6.0
Day 2	<0.98	242 ± 7.1	<0.98	239 ± 6.4	<0.98	280 ± 7.6	<0.98	286 ± 5.6
Day 6	<0.98	268 ± 8.4	<0.98	264 ± 6.5	<0.98	297 ± 8.9	<0.98	257 ± 15.2
Mean	**<0.98**	**256 ± 5.1**	**<0.98**	**255 ±5.1**	**<0.98**	**278 ±5.2**	**<0.98**	**271 ± 6.1**

S3 Table: Analysis of variance models for the relationships between chemical exposure, air saturation and the chemical/air saturation interaction for the gene expression profiles measured in fish exposed to flutamide exposure under 100 % or 50 % AS. Minimum adequate models (F value) for the relative expression are shown for the genes analysed: androgen receptors (*ar1* and *ar2*), catalase (*cat*), cyctochrome P450, family 1, subfamily A (*cyp1a*), estrogen receptors (*esr1*, *esr2a* and *esr2b*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), hypoxia inducible factor 1-α (*hif-1α*), isopentenyl-diphosphate delta isomerase 1 (*idi*), insulin-like growth factor-binding protein 1b (*igfbp1b*) and spiggin (*spg*). (Significance codes: * P<0.05, ** P<0.01, *** P<0.001)

	df	Minimum Adequate Model		
		Flutamide	Air Saturation	Flutamide / Air Sat. Interaction
ar1	NS	NS	NS	NS
ar2	NS	NS	NS	NS
cat	26	10.38***	2.71	5.80*
cyp1a	NS	NS	NS	NS
esr1	NS	NS	NS	NS
esr2a	NS	NS	NS	NS
esr2b	NS	NS	NS	NS
hif1α	27	294.82***	NS	NS
hmgcs	NS	NS	NS	NS
idi	27	NS	6.39*	NS
igfbp1b	26	NS	4.22*	NS
spg	27	22.03***	NS	NS

S4 Table: Analysis of variance models for the relationships between chemical exposure, air saturation and the chemical/air saturation interaction for the gene expression profiles measured in fish exposed to linuron exposure under 100 % or 50 % AS. Minimum adequate models (F value) for the relative expression are shown for the genes analysed: androgen receptors (*ar1* and *ar2*), catalase (*cat*), cyctochrome P450, family 1, subfamily A (*cyp1a*), estrogen receptors (*esr1*, *esr2a* and *esr2b*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), hypoxia inducible factor 1-α (*hif-1α*), isopentenyl-diphosphate delta isomerase 1 (*idi*), insulin-like growth factor-binding protein 1b (*igfbp1b*) and spiggin (*spg*). (Significance codes: * P<0.05, ** P<0.01, *** P<0.001)

	df	Minimum Adequate Model		
		Linuron	Air Saturation	Linuron / Air Sat. Interaction
ar1	NS	NS	NS	NS
ar2	NS	NS	NS	NS
cat	NS	NS	NS	NS
cyp1a	20	1.53	20.90***	3.98
esr1	NS	NS	NS	NS
esr2a	NS	NS	NS	NS
esr2b	NS	NS	NS	NS
hif1α	26	226.58***	0.45	4.73*
hmgcs	NS	NS	NS	NS
idi	NS	NS	NS	NS
igfbp1b	NS	NS	NS	NS
spg	26	0.63	5.78*	4.02

S5 Table: Analysis of variance models for the relationships between chemical exposure, air saturation and the chemical/air saturation interaction for the gene expression profiles measured in fish exposed to fenitrothion exposure under 100 % or 50 % AS. Minimum adequate models (F value) for the relative expression are shown for the genes analysed: androgen receptors (*ar1* and *ar2*), catalase (*cat*), cyctochrome P450, family 1, subfamily A (*cyp1a*), estrogen receptors (*esr1*, *esr2a* and *esr2b*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), hypoxia inducible factor 1-α (*hif-1α*), isopentenyl-diphosphate delta isomerase 1 (*idi*), insulin-like growth factor-binding protein 1b (*igfbp1b*) and spiggin (*spg*). (Significance codes: * P<0.05, ** P<0.01, *** P<0.001)

	df	Minimum Adequate Model		
		Fenitrothion	Air Saturation	Fenitrothion / Air Sat. Interaction
ar1	NS	NS	NS	NS
ar2	NS	NS	NS	NS
cat	NS	NS	NS	NS
cyp1a	NS	NS	NS	NS
esr1	NS	NS	NS	NS
esr2a	25	4.84*	NS	NS
esr2b	NS	NS	NS	NS
hif1α	28	219.00***	4.19	NS
hmgcs	NS	NS	NS	NS
idi	NS	NS	NS	NS
igfbp1b	25	6.73*	NS	NS
spg	NS	NS	NS	NS

Chapter 6

Discussion

6.1 Overview of findings

The aim of this thesis was to investigate whether hypoxia modifies the toxicity of environmental chemicals in fish. Two fish models were selected and used in a series of experiments investigating whether hypoxia affects the toxicity of copper and three selected chemicals with an anti-androgenic mode of action. For both embryos and adult fish, hypoxia was found to strongly influence copper toxicity, and these effects were dependent on the stage of development. For zebrafish embryos, during early development hypoxia strongly suppressed copper toxicity in a process mediated by the activation of the HIF signalling pathway, whereas after hatching this effect was reversed and copper toxicity increased under hypoxia in a process likely related to increased copper uptake. This switch in toxicity around hatching was also observed for another fish model, a more hypoxia sensitive species, the three-spined stickleback, suggesting that the combined effects of copper and hypoxia during development may be conserved across fish species. For stickleback adults, the combined exposure to copper and hypoxia resulted in the suppression of the fish's ability to acclimate to reduced oxygen availability in the water. This suggested that after hatching and for adults fish are at greater risk of adverse effects when exposed to copper in combination with hypoxia. Finally, the effects of exposure to hypoxia combined with anti-androgenic chemicals were investigated. Each anti-androgenic chemical had a unique transcriptional response, for example reduced oxygen strongly suppressed linuron's anti-androgenic effect in a process potentially mediated by the activation of the aryl hydrocarbon receptor pathway and its competition with the hypoxia inducible factor pathway.

Due to factors associated with climate change and human population growth, in particular around coastal areas, the progressive increase in the occurrence, frequency and severity of hypoxic events in aquatic systems worldwide will likely continue[1]. In parallel, the amount of chemical contamination into aquatic systems is projected to continue to increase, resulting in the increased likelihood of co-exposure to hypoxia and chemical pollutants. My findings demonstrate the importance of considering the concentration of oxygen in the environment when defining the impact of chemical exposures on aquatic organisms. To better protect aquatic ecosystems, it is fundamental to generate an understanding of the interactions between hypoxia and groups of chemicals likely to occur in the environment for a range of teleost

species with varying hypoxia tolerances. This information will, in turn, facilitate accurate predictions of the consequences of worldwide expansion in oxygen depletion to fish populations challenged by anthropogenic toxicants.

This discussion will give a detailed view of how the research undertaken in this thesis helps in the understanding of this issue and provides a critical synopsis of research priorities within this topic.

6.2 Choice of model species to use for research on multiple stressors

Choosing fish models for multi-stressor experiments is challenging, as these species need to represent environmental reality and allow for predictions to be made across other teleost species. In addition, model species are also required to have characteristics that facilitate experimentation in the lab and/or that allow for field based or field realistic studies to be conducted [2]. This would allow the datasets generated to be more informative and in turn support appropriate regulatory and management decisions.

The research presented in this thesis demonstrated that for studies investigating how hypoxia affects copper toxicity during embryogenesis, similar effects were observed for two species with different tolerances to low oxygen. However, to ensure that this effect is conserved across teleosts, more species need to be studied. Research considering species with different levels of tolerance to low oxygen in their environment will allow the research community to build a better understanding of how hypoxia may modulate chemical toxicity. This in turn could allow for extrapolations to be made across species with similar characteristics, avoiding the need to test the many thousands of fish species that require environmental protection in aquatic ecosystems.

The two species used in this thesis, although with different tolerances, are within a narrow range, so additional studies should consider species with extreme tolerances to hypoxia. Examples of species that could be studied include the common carp or the goldfish, both well known for their extreme tolerance to hypoxic and even anoxic environments[3] Previous work has been conducted for the adult life stage of the carp, investigating the effects of combined exposures to hypoxia and copper[4, 5], but other critical life stages, including embryonic development, have not been considered. In

addition, the mechanisms of adaption to low oxygen have been well characterised in the carp but only for adults[3], and little is known about the responses to hypoxia during development for this species. So it would be interesting to investigate if these mechanisms are conserved throughout all stages of development, and how hypoxia influences copper toxicity during early life. On the other hand, it is also important to consider how hypoxia modifies chemical toxicity in hypoxia sensitive species. Work to date has mainly focused on tolerant species, e.g. the carp or the killifish[5-7], therefore additional studies in intolerant species, for example salmonids, is essential.

It is also important to consider how research conducted under controlled laboratory conditions is representative of the natural environment. The questions to be considered include whether the fish models used in a study are representative of the wild populations found in the environment, and whether the conditions in the laboratory are aligned with environmental reality. In order to improve the relevance of laboratory studies, the use of fish from wild populations, or the use of mesocosms or transplantation experiments could aid in ensuring the relevance of the results obtained with the model laboratory strains. However, confounding issues may arise when using natural populations of fish, including the fact that they may have already been pre-exposed to a number of stressors, potentially affecting their responses during subsequent exposures. Although this is an interesting and important question, it is important to establish first how fish reared in control conditions respond to combined stressors, and what interactions may be occurring, before establishing if wild populations may respond differently as a result of their exposure history.

6.3 Selection of chemicals and conditions for research on multiple stressors

When considering realistic scenarios in the environment, there is a high likelihood that a wide range of chemicals and other materials will co-occur in waters depleted in oxygen (reviewed in chapter 1 of this thesis). However, for the majority of chemicals, the potential for hypoxia to modify their toxicity, or the potential for the hypoxia tolerance of exposed organisms to be affected by chemical exposures has never been considered. My studies on flutamide, linuron and fenitrothion were the first to investigate interactions between these chemicals and hypoxia, therefore representing a significant contribution to addressing this knowledge gap. In addition, my studies on combined exposures to copper and hypoxia were the first to document

the effects on early developmental stages and a protective effect of hypoxia on copper toxicity. My contributions to this field illustrate the potential for hypoxia to alter chemical toxicity and highlight the need for many more chemicals to be considered as a priority for future research.

In this thesis chemicals for study were selected based on the identification of hypotheses for how they interact with hypoxia. This strategy should be used to help prioritise chemicals for study in the future, and to guide the design of experiments and identification of appropriate endpoints to measure. Some examples of chemical groups that may be prioritised for further research include chemicals that cause metabolic inhibition such as some metals[8], pesticides[9] or flame retardants[10]. Under hypoxia, metabolic inhibition and decreased ATP production also occurs[11], so combined exposures would likely result in changes in chemical toxicity. Given the abundant evidence for hypoxia to affect reproduction and the high likelihood for hypoxia and EDCs to co-occur, more research is needed to investigate the potential effects of these combined stressors on fish. Hypoxia has been shown to affect many different reproductive processes; including germ cell migration, sex determination, steroidogenesis and gonadotrophin signalling[12], so any chemicals that interact with these pathways are also priorities to be studied.

In this thesis I report some key mechanisms of interaction between hypoxia and chemical toxicity. For linuron, evidence from my studies suggested that the suppression in its anti-androgenic effects under hypoxia were due to cross-talk between the AhR pathways (induced by linuron) and the HIF pathway (induced by hypoxia), possibly due to competition for their shared dimerization partner (ARNT). I also demonstrated for the first time that during early embryo development, in zebrafish, combined exposure to copper and hypoxia resulted in a large decrease in chemical toxicity compared to exposure to copper alone. Using a prolyl-4-hydroxylase inhibitor, dimethylglycine (DMOG), I demonstrated that the mechanism responsible for the strong reduction in copper toxicity observed was the activation of the HIF pathway. Considering that it is not realistic to test all the many thousands of man-made chemicals that are present in aquatic environments worldwide, information from studies, such as those included in my thesis, in conjunction with information about the mechanisms of toxicity of individual chemicals and their environmental concentrations, can help to prioritise chemicals for further study and

generalise the conclusions obtained to other chemicals with the same mode of action.

As well as considering different chemicals, different environmental stressors will also be present in hypoxic waters. A known factor associated with the increase in hypoxia in aquatic systems is climate change. Other environmental variables are also changing rapidly in association with this global phenomenon, including changes in pCO_2, salinity, temperature and pathogens[13]. Therefore to protect species in environmentally relevant conditions, studies should include more stressors acting simultaneously, allowing the investigation of how they interact with each other. This is a very significant challenge, and experiments to address this will not be easy to perform. However, by understanding the mechanisms underpinning interactions between pairs of these stressors, research can be advanced, and may aid in the development of mathematical models that could help in the prediction of how organisms living in these complex systems will respond to further change.

6.4 Different environments to consider for multiple stressor scenarios

To date, most studies investigating the combined effects of hypoxia and chemical toxicity have focused on freshwater systems. Little work has been directed to addressing the question of how hypoxia affects the biological response of aquatic organisms to chemicals and other stressors in marine environments, or in artificial environments, such as in aquaculture settings.

For copper, and also for other metals, the importance of considering a range of aquatic environments with differing salinities, is particularly evident. Copper causes greater toxicity to fish in freshwater, due to its competition with hardness cations (Ca^{+} and Mg^{2+}) at the active gill ion transporters[14], resulting in greater uptake in freshwater compared to in marine environments. In this thesis, all experiments were conducted in freshwater systems, where both copper pollution and hypoxia are environmentally relevant threats to fish species. Previous research investigating how hypoxia affects copper toxicity has, to my knowledge, only focused on freshwater fish species, including the common carp[5], the pacu[15] and the killifish[7]. For invertebrate species, the effects of hypoxia in combination with copper on the benthic amphipod (*Corophium volutator*) were investigated, and an increase in

mortalities for combined exposures was reported[16]. This is similar to what was observed in this thesis for post-hatched embryos and adult fish. It would be interesting to study how hypoxia affects copper toxicity in fishes in marine systems, to determine if similar trends occur to those reported for freshwater species in this thesis.

Direct comparison between marine and freshwater environments is not strictly possible because chemicals interact with environmental conditions affecting their speciation, uptake routes and bioavailability. For example, the uptake route of a water soluble chemical in marine environments could occur via the digestive system as well as the gills. This is due to the fact that marine fish drink large amounts of water to maintain osmotic balance. This contrasts to freshwater systems, where the main route of chemical uptake usually is the gills, as freshwater fish do not drink. Given the environmental relevance of considering combined stressors in marine systems, as well as the very significant differences in the routes of uptake, speciation and toxicity for environmental chemicals in freshwaters and marine waters, considering how chemical toxicity is affected by hypoxia in the marine environment should be a priority for further research. Sticklebacks could be a good model to investigate this as they can live in both freshwater and marine environments facilitating direct comparisons between the two environments using the same fish models.

For artificial aquatic environments, such as aquaculture systems, there is a significant likelihood that stressful conditions, including hypoxia, may occur. For example, scallops farmed in Peru were reported to be exposed to hypoxia for up to 70% of the time, and this was shown to have a significant negative effect on the species metabolism, with the potential to harm the performance of the culture[17]. Atlantic salmon cultured in sea cages can also experience periodic hypoxia, and it was shown that they were unable to acclimate to the frequent drops in oxygen[18]. In addition to this, copper sulphate is often used for controlling macrophytes and algae in aquaculture[19], suggesting a high likelihood that hypoxic conditions may co-occur with elevated copper in this aquaculture environment. The data presented in this thesis could inform on best practice for the aquaculture industry, and the data presented in this thesis may inform on safety thresholds for use in aquaculture settings where hypoxia can occur.

Aquaculture settings also use a wide range of pharmaceuticals to combat and prevent disease. Some pharmaceuticals are known to activate the AhR pathway and to be metabolised by CYPs. In chapter 5, we showed how linuron and hypoxia interact, likely due to the cross talk between the AhR and the HIF pathways. Therefore, there is a high likelihood that pharmaceuticals metabolised via the cytochrome P450 enzymes could interact with hypoxia in these aquaculture environments, potentially modifying their pharmacological effects to the target fish species. These environments are also affected by other changes in water chemistry besides hypoxia, including increased CO_2 production as a result of respiration, and associated reduced pH. More work is required to study how species respond to the combined stressors that are likely to occur in aquaculture settings in order to better manage culture conditions and to reduce their impact on the surrounding aquatic environment.

6.5 Methodological approaches for studying multiple stressors

Methods currently used to study multiple stressors have varied widely. For example, limited reasoning for the selection of the concentration of chemicals or stressors is a key issue in the published literature, as they prevent comparisons across studies and hinder the interpretation of the findings. In this section I explain how the different methodologies I used in this thesis are useful and could benefit the future design of multiple stressor experiments.

For exposures conducted during embryonic development, the strategy used to determine whether hypoxia influences copper toxicity in this thesis was to generate dose-response curves and compare how these curves differ for each developmental stage. This strategy was used for both the zebrafish and the stickleback and proved to be extremely successful in accurately determining the occurrence of interactions between copper and hypoxia or if there are only significant individual effects of the treatments. In addition, consideration of the various stages of development individually allowed the identification of contrasting effects of hypoxia during early and late development for both model species tested. This, in turn, can help to understand whether or not a different mechanism or action is present for the oxygen concentrations tested. Embryos and *in vitro* systems are ideal models to use in this type of experimental design, as a high number of replicates are needed, so ethically

it would not be appropriate to use adult fish. This method is more informative than conducting experiments on single concentrations for the chemical of interest and should be employed whenever possible to evaluate the effects of environmental conditions on chemical toxicity. This is supported by the current thinking in the field of chemical mixtures, where use of experimental designs based on single doses have compromised in some cases the identification of mixture effects, resulting in false negatives[20]. However, it is important to remember the issue of cost and ethics of dose response curves, which is why this was not used as a method in chapter 4, for exposure to the adult life stage of the three-spined stickleback. Throughout the thesis, QPCR was used as a methodology to investigate the changes in transcription for target genes hypothesised to be involved in the responses to both hypoxia and the chemicals being tested (Chapters 2, 4 and 5). QPCR is a commonly used method in ecotoxicology, as assays for genes of interest can be designed and accurately validated, and it can be relatively quick and inexpensive to perform large experiments. This targeted approach is ideal for investigating specific hypotheses about the mechanisms of toxicity of a given chemical or mixture. For example, *mt1*, a well-established biomarker for metal exposure was measured to determine the effects of copper exposure and whether hypoxia affected this response (Chapter 4).

Another approach that could be useful for investigating the effect of hypoxia on chemical toxicity would be to explore the genome-wide changes in transcription, for example using RNA-Seq. Compared to targeted approaches, global transcriptome profiling allows a more comprehensive assessment of the molecular mechanisms of toxicity of a given chemical or mixture, including the potential for revealing novel or unexpected effects. For example, although we were able to determine that exposure to anti-androgenic chemicals results in a strong induction of *hif-1α* transcription, genome-wide sequencing may help in deducing the mechanisms mediating this effect, or how other pathways may be contributing to the effects seen. Generation of more mechanistic data could also help in the development of predictive models, which could help in identifying which chemicals should be prioritised for study. Predictive models have been stated in the literature as in important method of helping in risk assessment and environmental protection, especially when considering population/ecological level effects. The generation of more mechanistic data, will inform on the generation of adverse outcome pathways, a novel modelling

concept that has been proposed to support environmental management[21], potentially leading to the improvement of the accuracy of current model approaches.

Once the potential mechanisms of interaction have been identified using gene expression studies, the exact mechanisms responsible for interactions should be validated. This can be done through chemical manipulation of the pathways of interest, by either activating or inhibiting them. For example, in chapter 2, I used DMOG to chemically activate the HIF pathway, to determine if the activation of this pathway was responsible for the decrease in copper toxicity observed under hypoxia during early development. There are also other methods to validate proposed mechanisms including using genetic manipulation of the pathways of interest. This can be carried out using a number of technologies including morpholinos to suppress gene expression, transgenic or mutant models, or more recently developed methods such as CRISPR/Cas9 targeted genome editing, which allows researchers to probe the function of genes and gene regulators.

In Chapter 5, QPCR was used to investigate hypotheses about the potential mechanisms of interaction between hypoxia and anti-androgenic chemicals. For example, I hypothesised that exposure of linuron under low oxygen would affect *cyp1a* transcription as a result of competition of the dimerization partner ARNT. This hypothesis was confirmed by the observations that exposure to linuron under hypoxia resulted in a loss of *cyp1a* up-regulation. This effect was mimicked by the results for the expression of spiggin, as linuron lost its anti-androgenic effect when exposure occurred under low oxygen. Production of spiggin is essential for reproductive function in sticklebacks and in under strict androgen regulation[22]. Disruptions of androgen signalling could result in effects on behaviour and differences in reproductive output[23], and if this occurred in the environment it could have a knock on effect at the population level. Therefore, it would be interesting to see if these chemicals affect how fish respond physiologically and behaviourally in response to the combined exposure to anti-androgens and hypoxia in order to determine if these effects could have population level consequences.

Throughout this thesis the experiments were designed to ensure that the fish were exposed to the appropriate levels of each stressor. For example, in chapter 4, the critical oxygen level (P_{crit}) of the population of sticklebacks under the experimental

conditions was determined to inform on experimental design. This then allowed appropriate air saturations to be selected to coincide with a mild and a more severe reduction in air saturation, but where the fish are still able to oxyregulate (just above their critical oxygen level). In addition, for chapter 3, stickleback embryos were initially exposed to different levels of air saturation, to determine the sensitivity of the population to hypoxia so that the level of air saturation selected was just above levels that cause developmental delays, allowing for comparisons of how this level of hypoxia affected copper toxicity, for the same developmental stages in both exposure scenarios. Generally, studies that have previously investigated how hypoxia affects chemical toxicity have not justified their choice of air saturation. Therefore, it is not possible to deduce if the level of air saturation is well below the P_{crit} and therefore leading to significant stress, or above the P_{crit} allowing the fish to maintain homeostasis, as it is expected that these differences would have an effect on the results and conclusions drawn from the studies. Therefore, it is essential that studies justify the choice of air saturation in the context of the physiology of the species used. This will, in turn, help in allowing better comparison between datasets and across different exposure scenarios.

Exposure of adult sticklebacks to copper in combination with 75% AS resulted in greater inductions of *mt1* and *hif-1α* compared to fish exposed to copper under 100% or 50%AS. This is an interesting response, as it seemed that a moderate reduction in ambient oxygen actually resulted in a greater ability of the fish to elicit a molecular response to the combined stressors. In addition, in chapter 3, stickleback embryos exposed to 80% AS hatched 24 hours earlier than those incubated in the presence of 100% AS. This premature hatching could have been as a result of insufficient diffusion of oxygen across the chorion, to satisfy the needs of the developing embryo. An alternative hypothesis is that the optimal level of air saturation for these embryos is not 100% air saturation, but instead embryos of this species are adapted to a lower level of oxygen. Evidence from both these datasets potentially suggests that a moderate reduction in ambient air saturation is actually beneficial and may correspond to the optimal level of air saturation for this species. This raises the question of what actually an “environmentally relevant normoxic environment” is for sticklebacks. All experiments for chemical testing aim to maintain air saturation at ~100%, but potentially this could be considered hyperoxia for some fish species,

causing adverse effects on their physiology. Data in the literature is limited over the natural range of air saturation in aquatic environments, but collaboration with monitoring agencies (such as the Environment Agency) and analysis of existing historical data on air saturations in freshwater rivers and lakes, as well as physiological studies on the effects of hypoxia in species of interest across their life cycle is essential for clarification of this issue. This is especially important as current regulations for fish embryo testing by the Organisation for Economic Co-operation and Development (OECD)[24], require that the dissolved oxygen measured at the end of the exposure to be ≥80% AS, and includes hatching as an endpoint of consideration. Therefore, there may be a need for more specific guidelines on oxygen levels for regulatory testing to avoid the level of oxygen acting as a confounding factor.

Ultimately, to help in the understanding and correct identification of how environmental factors can affect chemical toxicity, standardised testing guidelines should be introduced to ensure that studies are designed correctly. This would allow for studies to be directly compared, and would, in turn, help in the control and regulation of chemical toxicity. To do this it would be important to include at least two species for comparison within the testing guidelines, a tolerant vs an intolerant species, to ensure that the information needed is gathered to appropriately access interactions between factors and support extrapolations across species. I would also suggest that the embryo/larvae developmental stage could be used as an important life stage to consider, especially as a result of its ethical advantages for test development. When considering interactions between copper and hypoxia, the larval stage of development had similar responses to the adult, so this could be used a proxy for how the adult may respond while avoiding ethical issues of using adult vertebrates for testing. However, further work is needed to ensure that this response is similar for different species and chemicals before the development of testing guidelines. Overall, introducing testing guidelines could facilitate more accurate comparison between studies to help predict the effects of combined stressors.

6.6 Future perspectives on multiple stressor research

The aquatic environment is changing at rapid rate due to climate change and as a result of human activities. Therefore, it is essential to understand how ecosystems are affected and changing over time, and how regulators can develop management strategies to respond to these changes and minimise their impact on living systems. To do this future research needs to focus on increasing the research depth, monitoring the current changes, and modelling predicted impacts. There is a need for more laboratory and field research studies to provide information on how individual species and communities respond to multiple stressors. To date, studies on multiple stressors have focused primarily on describing the responses of organisms to those stressors, however to truly understand the impact of multiple stressors and to be able to develop predictive modelling, mechanisms responsible for how stressors interact will be beneficial and this should be a priority for the future in this field.

Modelling provides a quantitative assessment of the processes involved in the responses to stressors and helps to build accurate estimations of the overall impacts populations and ecosystems. Modelling of multiple stressor scenarios needs to be improved, and more specifically models that help in the prioritisation and management of chemicals that may increase toxicity when physiochemical parameters change. Also, there should be a greater emphasis on scaling up current models to include ecosystem level impacts, in order to support the management decisions required to protect aquatic ecosystems now and in the future. In addition, more monitoring of physical, chemical and biological parameters in the environment will be important to provide more information on how aquatic systems are changing and the seriousness of the threats to those ecosystems.

6.7 Conclusions

In this thesis, I have investigated how hypoxia can affect chemical toxicity and in turn discussed how this issue is important for the protection of aquatic environments which are readily affected by complex combinations of stressors. To help in the correct environmental management, studies should ensure that employ suitable methodologies, appropriate model organisms, well justified level of air saturation for the organisms tested and well-designed exposures ensuring that the questions being

investigated are fully addressed. This, in turn, could contribute to future legislation and help in the protection of aquatic environments affected by complex mixtures of stressors of anthropogenic origin.

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