Mechanisms of transcriptomic and epigenetic responses to industrial pollutants in fish

Submitted by

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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

Thousands of chemical pollutants enter the environment continuously, each with the potential to cause adverse effects in both terrestrial and aquatic organisms. As a result, organisms are often exposed to a mixture of stressors within their habitat. Populations of fish inhabiting most aquatic environments are exposed to time-varying or repeated pulses of exposure, driven by run-off events or spills, or due to their mobility between polluted and clean waters. Therefore, the sustainability of fish populations is critically dependent on their ability to adapt to frequent changes in their local environment. Despite this, legislation to protect the environment from chemical contamination are generally based on toxicological measurements following exposures to single stressors, conducted under optimal laboratory conditions, and that do not take into account the variation in susceptibility of wild populations, or the potential consequences of exposure for the susceptibility of the population during future exposures, including across generations.

Increasing evidence is suggesting that a number of chemicals may interact with the epigenome, and that differential responses to pollutants may be modulated, at least in part, via epigenetic mechanisms. However, our understanding of the role of epigenetic mechanisms in normal development in fish models or its susceptibility to exposure to environmental stressors is currently very limited.

This thesis aimed to document the mechanisms of genetic and epigenetic responses to industrial pollutants in fish, and to explore the extent to which differential responses can be induced in the lab following exposure during the critical window of embryonic development or in adults. To address these objectives, I performed a series of experiments using both the

zebrafish (*Danio rerio*) and the three-spined stickleback (*Gasterosteus aculeatus*) as fish models.

I first used the zebrafish (*Danio rerio*) model to investigate the sex-specific transcription and DNA methylation profiles for genes involved in the regulation of reproduction and in epigenetic signalling in the livers and gonads. I provide evidence of the sex-specific transcription of genes involved in reproduction and their regulation by epigenetic signalling in this commonly used vertebrate model and highlight important considerations regarding the use of whole tissues comprised of multiple cell types in epigenetic and transcriptomic studies. I then investigated the potential for exposure to Bisphenol A (BPA) to cause adverse effects on reproduction and to disrupt the expression profiles and promotor DNA methylation of target genes important for reproductive function and epigenetic signalling in the zebrafish. To do this, I exposed breeding zebrafish to a range of BPA concentrations over 15 days and found that BPA disrupted reproductive processes in zebrafish, likely via estrogenic mechanisms, but only at high concentrations. Importantly, exposure to environmentally relevant concentrations of BPA resulted in altered transcription of key enzymes involved in DNA methylation maintenance, and caused changes in promoter DNA methylation.

I also conducted a series of repeated exposures to copper in the three-spined stickleback to investigate the extent to which differential susceptibility can be induced in the lab. This work provides evidence that pre-exposure to copper results in differential responses in future exposure scenarios both when the initial exposure occurred in adults and during embryogenesis. For adults, fish appeared to recover completely from the initial exposure following a period of depuration of 30 days, but displayed decreased susceptibility upon re-

exposure. In contrast, for fish exposed during the critical windows of embryonic development when epigenetic reprogramming are hypothesised to occur, differential copper accumulation was maintained throughout life. Importantly, the initial exposure caused increased tolerance in the offspring, which was inherited up to the F2 generation. This work provides valuable information regarding potential critical windows of development which may be more susceptible to effects associated with pre-exposure, highlighting that early life exposure to a low concentration of copper can induce differential responses to copper across generations.

These data highlight the extent of differential responses to chemical stressors likely to be present in wild populations, and point towards the possibility that effective population management will likely require an in-depth understanding of the exposure history of a given population in order to manage restocking initiatives, and to inform conclusions drawn from toxicity testing studies conducted using individuals originating from wild populations. In addition, these data suggest that it is likely that both epigenetic and genetic changes can contribute to the adaptation of individual populations to their local environment. Finally, other vertebrates including humans have been shown to be exposed to the chemicals tested in this thesis. Therefore, this highlights the potential for these chemicals to also cause toxic effects in humans, potentially via (epi) genetic mechanisms, and advocate the testing of the potential for inheritable phenotypes, such as those described in this thesis, to occur in mammalian models.

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Research Papers and Author Declaration

Chapter 2: Research Paper 1

L. V. Laing, J. Viana, E. Dempster, T. M. Uren Webster, R. van Aerle, J. Mill and E. M. Santos. Sex-specific transcription and DNA methylation profiles of reproductive and epigenetic associated genes in the livers and gonads of breeding zebrafish. *Manuscript under review: Epigenetics.*

Chapter 3: Research Paper 2

L. V. Laing, J. Viana, E. L. Dempster, M. Trznadel, L. A. Trunkfield, T. M. Uren Webster, R. van Aerle, G. C. Paull, R. J. Wilson, J. Mill and E. M. Santos (2016). Bisphenol A causes reproductive toxicity, decreases *dnmt1* transcription and reduces global DNA methylation in breeding zebrafish (*Danio rerio*). *Epigenetics; 11; 526-538.*

Chapter 4: Research Paper 3

L. V. Laing, J. A. Fitzgerald, R. van Aerle, N. Bury, J. Mill and E. M. Santos. Pre-exposure to copper caused a reduced transcriptional response and increased copper accumulation upon re-exposure in adult male three-spined stickleback (*Gasterosteus aculeatus*). *Manuscript in preparation, Target Journal: PLOS Genetics.*

Chapter 5: Research Paper 4

L. V. Laing, J. A. Fitzgerald, N. Bury and E. M. Santos. Exposure to copper during embryogenesis caused a differential response to copper in later life and increased tolerance in subsequent generations, in a fish model. *Manuscript in preparation, Target Journal: PNAS.*

Statement of Authors' Contributions:

I, Lauren V Laing, made the following contributions to the research papers presented in this thesis. I carried out all experiments presented in **papers 1 and 2** with the assistance from Laura Trunkfield for the running of the zebrafish exposure. I conducted the RNA and DNA extraction, RT-QPCR, the LUMA assay and pyrosequencing with some technical support from Joana Viana and Emma Dempster. I also performed the data analysis and wrote the manuscript.

For **papers 3 and 4,** I planned and conducted the exposures, sampled the fish and prepared tissue samples for metal analysis. Nic Bury performed the measurements of tissue metal concentrations for **Papers 3 and 4.** For **paper 3** I also prepared the libraries for sequencing, preformed the bioinformatics analysis of sequence data and wrote the manuscript with some bioinformatics support from Ronny van Aerle.

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

List of General Abbreviations

11-KT	11-ketotesterone
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethlcytosine
5mC	5-methylcytosine
Ag	Silver
AS	Air saturation
ATAC-seq	Assay for Transposase-Accessible Chromatin with high throughput sequencing
bp	Base pair
BPA	Bisphenol A
bsRAD-seq	Bisulfite-converted restriction site associated DNA sequencing
Cd	Cadmium
ChIP-seq	Chromatin immunoprecipitation followed by sequencing
CNS	Central nervous system
Co	Cobalt
CpG	5'—C—phosphate—G—3
Cr	Chromium
CTCF	CCCTC-binding factor
CTR	Copper transporter
Cu	Copper
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DMY	DM-domain gene
DNA	Deoxyribonucleic acid
DNA-seq	DNA sequencing
DNMTs	DNA methyltransferases
E2	17β-oestradiol
EDCs	Endocrine Disrupting Chemicals
EE2	17α-ethinylestradiol
ENaC	Putative epithelial sodium channel
ER	Estrogen receptor
EZH2	Enhancer of Zeste Homolog 2
Fe	Iron
GSI	Gonadosomatic index
HDACs	Histone deacetylase
Hi-C seq	Chromosome conformation capture sequenicng
HMTs	Histone methyltransferases
hpf	Hour post fertilisation
HSI	Hepatosomatic index
KBs	Kilobases

lncRNAs	Long non coding RNAs
MAM	Minimum adequate model
MBP	Metal binding proteins
MC	Metallochaperones
meHg	Methylmercury
miRNAs	Micro-RNAs
miRNAs	MicroRNAs
Mn	Manganese
MNK	Menkes protein
MRE	Metal response element
MT	Metallothionein
ncRNAs	Non-coding RNAs
nt	Nucleotides
OECD	Organisation for Economic Co-operation and Development
Pb	Lead
PCBs	Polychlorinated biphenyls
PGCs	Primordial germ cells
piRNAs	Piwi-interacting RNAs
RAD-seq	Restriction site associated DNA markers sequencing
RNA	Ribonucleic acid
RNAPII	RNA polymerase II
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
SAM	S-adenosylmethionine
SEM	Standard error of the mean
SMP	Single methylation polymorphisms
snoRNAs	Small nucleolar RNAs
SNP	Single nucleotide polymorphisms
SOD	Superoxide dismutase
SRY	Sex determining region in the Y chromosome
stRNA	Small temporal RNAs
TBT	Tributyltin
TCDD	2,3,7,8 Tetrachlorodibenzo-p-dioxin
TET	Ten-eleven Translocation
TSS	Transcription start site
ZGA	Zygotic genome activation
Zn	Zinc

List of Species Names

Artemia	*Artemia nauplii*
Atlantic Salmon	*Salmo salar L.*
Brown trout	*Salmo trutta*
Common fruit fly	*Drosophila melanogaster*
Copepoda sp.	*Tigriopus japonicus*
Copepoda sp.	*Tigriopus californicus*
Daphnia sp.	*Daphnia longispina*
Daphnia sp.	*Daphnia magna*
Earthworm	*Aporrectodea tuberculate*
Fathead minnow	*Pimephales promelas*
Goldfish	*Carassius auratus*
Green swordtail	*Xiphophorus helleri*
Guppy	*Poecilia reticulata*
Harbour seal	*Phoca vitulina*
Japanese rice fish	*Oryzias latipes*
Korean rockfish	*Sebastes schlegeli*
Land snail	*Xeropicta derbentina*
Large yellow croaker	*Pseudosciaena croceato*
Least killifish	*Heterandria formosa*
Medaka	*Paralichthys olivaceus*
Mosquito	*Anopheles gambiae*
Nile tilapia	*Oreochromis niloticus*
Nine-spined stickleback	*Pungitius pungitius*
Rainbow trout	*Oncorhynchus mykiss*
Redworm	*Eisenia fetida*
Roundworm	*Caenorhabditis elegans*
Scud	*Hyalella azteca*
Sea Squirt	*Ciona intestinalis*
The Chinese rare minnow	*Gobiocypris rarus*
Three-spined stickleback	*Gasterosteus aculeatus*
Toadflax	*Linaria vulgaris*
Yellow perch	*Perca flavescens*
Zebrafish	*Danio rerio*

Chapter 1

General Introduction

1 Introduction

1.1 Environmental pollutants

Thousands of chemical pollutants enter the environment continuously, and many of these have the potential to cause adverse effects in both terrestrial and aquatic organisms, inducing toxicity via a range of mechanisms [1]. As a result, organisms are often exposed to a mixture of chemical pollutants. Aquatic environments act as a sink where soluble chemical contaminants can persist; therefore organisms in aquatic habitats can be particularly sensitive to environmental chemicals.

Populations of fish inhabiting most aquatic environments are exposed to time-varying or repeated pulses of exposure, driven by run-off events or spills, or due to their mobility between polluted and clean waters [2,3]. Hydrologic dilution and dispersion may contribute to variations in exposure concentration [4], and during periods of depuration from the chemical of interest, detoxification, elimination and recovery may occur within the exposed organism [4,5]. To date, many studies addressing chemical toxicity in the laboratory focus on acute short term exposures due to the relative ease of performing short term toxicity testing [6]. However, repeated and continuous exposures of equivalent dose may not elicit the same degree of toxicity [7]. In addition, organisms are often exposed to a mixture of stressors, including changes in abiotic parameters such as pH, oxygen availability and temperature, in addition to a mixture of chemical pollutants.

The sustainability of fish populations is critically dependent on their ability to adapt to frequent changes in the local environment [8]. Despite this, legislation to protect the environment from chemical contamination are often based on toxicological measurements

conducted under optimal laboratory conditions, and that do not take into account the variation in susceptibility of wild populations, or the potential consequences of toxicant exposure for the susceptibility of individuals during future exposure scenarios, including across generations.

This introduction will first introduce the two chemical pollutants investigated in this thesis, including their occurrence in the aquatic environment and how they affect both mammalian and aquatic organisms at the phenotypic, transcriptomic and epigenetic levels. This introduction will then discuss the epigenetic regulation of genomes, including the modifications identified to date, their role within normal cellular processes, and the potential for these modifications to be altered by environmental cues including chemical pollutants. Finally, the aims and hypotheses of this thesis and a summary of the approach taken to test these hypotheses are presented.

1.1.1 The endocrine system and endocrine disruptors

The endocrine system plays an important role in the regulation of essential processes, including metabolism, development, growth and reproduction. This system is comprised of a network of glands and/or cells that secrete chemical messengers (hormones) which act on target cells, regulating their function. Hormones bind to specific receptors at target cells, where they regulate biological processes via the activation of specific molecular responses [9]. Both natural and synthetic chemical pollutants have been found to cause disruption to many hormone regulated processes by mimicking or antagonising endogenous hormones, disrupting hormone synthesis, storage metabolism or secretion [10,11].

Endocrine Disrupting Chemicals (EDCs) can be defined as natural or synthetic chemicals with the ability to mimic or inhibit endogenous hormone actions and the normal homeostasis of the endocrine system, frequently causing adverse health effects in an intact organism, its progeny or (sub)population [10,12,13]. Studies have linked human EDC exposure with obesity, metabolic syndrome, type 2 diabetes [11], reproductive abnormalities including declines in sperm production [13–17], and the increasing incidence of prostate and breast cancer [18,19].

The endocrine regulation of reproduction is highly conserved in vertebrates, including teleost fish, and exposure to EDCs has been associated with feminising effects in wild fish populations [20–22]. Disruption of normal sex steroid signalling is the most commonly studied form of endocrine disruption. Sex steroids are predominantly produced in the gonads, and play a role in the regulation of not only gonadal development and reproduction, but also more global processes such as metabolism and immune function. Androgens, oestrogens and progestogens represent the three primary classes of steroid hormones involved in the control or reproduction [23,24]. These classes of sex steroids have a number of roles in the regulation of reproduction in the gonads and other distant target tissues, including the brain. In addition to the activation of nuclear receptors in target cells to regulate transcription, sex steroids can also act via membrane bound receptors, eliciting fast responses within the target cell [10,23].

In males, androgens are predominantly responsible for regulating all stages of spermatogenesis, the process in which spermatogonial stem cells mature into spermatozoa, via mitosis and meiosis [25]. In fish, the principal biologically active androgen is 11-ketotesterone (11-KT). In females, oestrogens play a key role in sexual development and oogenesis, the process in which differentiation of the primordial germ cells results in the

formations of a mature oocyte ready for fertilisation, and the principal oestrogen in fish, as in other vertebrates, is 17β-oestradiol (E2) [23,26]. In oviparous vertebrates such as fish, E2 plays a crucial role in stimulating the production of egg yolk precursor proteins (vitellogenins), in the liver [23,26,27]. Chemicals that may mimic oestrogens or androgens are usually referred to as oestrogenic and androgenic chemicals, respectively. Chemicals which are able to block the normal actions of oestrogens and androgens are termed anti-oestrogenic and anti-androgenic chemicals, respectively.

Exposure to EDCs, and their subsequent interactions with the normal homeostatic processes within the endocrine system, can result in a range of physiological and population relevant effects depending on the mode of action of a given EDC or mixture of chemicals. One of the most commonly studied examples of EDCs is the synthetic oestrogen 17α-ethinylestradiol (EE2) a compound present in the contraceptive pill and found to cause physiological effects in fish. In the fathead minnow (*Pimephales promelas*) an environmentally relevant concentration of EE2 was shown to cause intersex in males and altered oogenesis in females, ultimately leading to the collapse of a population in an experimental lake over a 7 year period [28].

Further examples of EDCs include, tributyltin (TBT) an antifouling agent used in paints, shown to cause masculinisation (imposex) in females of a number of snail species [29]; polychlorinated biphenyls (PCBs) and their metabolites, shown to cause impairment of reproduction in harbour seals (*Phoca vitulina*) when exposure via in the food chain [30] and dichlorodiphenyldichloroethylene (DDE), which has been linked to egg-shell thinning in birds leading to population declines in a number of species of predatory birds in Europe and North America [31]. In addition complex mixtures such as sewage effluents, paper industry

and industrial chemicals have been associated with reproductive effects in a number of fish species [32,33].

An increase in agricultural, urban and industrial activities has led to the discharge of a large variety of potentially hazardous substances into the world's surface waters. Water contamination is considered as one of the main threats to global freshwater biodiversity and also to other organisms, including humans, exposed via drinking water [34–37]. This thesis focusses on Bisphenol A (BPA) as an example of an EDC that has the potential to cause adverse effects across vertebrate species, including humans. Therefore, this section discusses the biological effects associated with exposure to BPA in vertebrate organisms.

1.1.1.1 Bisphenol A

Bisphenol A (BPA) is a commercially important high production chemical and is widely used in the production of epoxy resins, utilised in food and beverage packaging, dental sealants, in some flame retardants, water supply pipes and as a monomer component of polycarbonate plastics [38–40]. With over three million tons produced globally per annum, environmental exposure is common [36] and this chemical is ubiquitous in the environment.

1.1.1.2 Potential for exposure of human populations to Bisphenol A

For humans, the most prominent exposure route for BPA is the ingestion of contaminated food, caused by leaching of BPA from linings of canned goods and polycarbonate packaging; in the USA BPA was measurable in 75% of food products tested [41]. In addition, BPA has also been detected in drinking water at concentrations up to 15 ng/L [42,43]. Inhalation is also

thought to be a plausible secondary route of exposure [36], with BPA found to be present in 86% of domestic dust samples at concentrations ranging from 0.2 to 17.6 µg/g [44]. Evidence for widespread human exposure to BPA has been provided by a range of studies across the world. BPA has been detected in the urine of ~ 95% of adults in the USA and Asia [45,46], and it has also been measured in the serum of adult men and women [47] and in breast milk, foetal plasma and placental tissue, raising concerns about human exposures during critical periods of development [38,48].

1.1.1.3 Occurrence of Bisphenol A in the aquatic environment

BPA is moderately water soluble, entering the environment via direct discharge from BPA production and processing industries, wastewater treatment plants and leachate from landfill sites [49]. Its presence is ubiquitous in the aquatic environment and surface water concentrations have been detected up to the low µg/L range, with peak concentrations reaching up to 21 µg/L [50]. A study exploring the presence of BPA in 38 water systems in the Netherlands detected BPA in 60-80% of the samples collected with mean BPA concentrations shown to be ~ 330 ng/L [51]. Concentrations in landfill leachate have been reported to reach up to 17,200 µg/L [38]. Due to its ubiquitous nature, the potential for environmental exposure in wildlife populations, including fish, is very significant. Levels of BPA reported in fish vary, and 1-11 ng BPA/g dry weight in the muscle and 2-75 ng BPA/g dry weight in the liver have been reported [51].

1.1.1.4 Oestrogenic effects of Bisphenol A exposure

There is extensive evidence in the literature regarding the biochemical and physiological effects of BPA. As a result, BPA has been classified as one of the more potent EDCs with effects reported to have been observed at low doses [52]. BPA dates back to the 1930s, when research was conducted into the potential for BPA to be utilised as a synthetic oestrogen for clinical use [53]. The oestrogenic activity of BPA was demonstrated in rats, but the formulation of a more effective synthetic oestrogen, diethylstilboestrol, at around the same time rendered BPA redundant as a clinical oestrogen [54]. BPA was later utilised as a plasticiser, and the oestrogenic activity of BPA was further demonstrated during a study using MCF-7 breast cancer cells in autoclaved polycarbonate flasks, where BPA was found to leach from the flask causing an increase in the rate of cell proliferation [55].

Molecular studies utilising a number of *in vitro* models have revealed an array of molecular pathways through which BPA may stimulate cellular responses [40]. BPA is known to alter gene expression by binding to nuclear oestrogen receptors (ERs), ERα and ERβ [12,40]. It has been defined as a selective ER modulator, due to its effects being pro-oestrogenic in some tissues or physiological contexts and anti-oestrogenic in others [12,40]. Studies have demonstrated the oestrogen receptor agonistic properties of BPA [56], resulting in BPA binding to oestrogen receptors and subsequently inducing feminising effects [13,57]. The exposure of organisms to environmental oestrogens such as BPA has been associated with alterations in normal sexual functioning including declines in sperm production in humans and other model organisms [13–17], increased incidence of prostate and breast cancer [18,19], and feminising effects in wild populations of fish [20–22].

A study using the human cell line HepG2, found that BPA strongly activated oestrogen receptor 1 (ESR1; previously known as ERα) mediated responses, but did not activate ESR2 (previously known as ERβ), while in the cell line HeLa, BPA was found to activate both ESR1 and ESR2 [56]. In fish, BPA induced *esr1* transcription in the livers of male fathead minnows *(Pimephales promelas)* exposed for 4 days to 10 µg BPA/L, consistent with an oestrogenic mode-of-action [58].

In addition to interacting with the oestrogen receptors, BPA also causes a number of effects upon androgen signalling pathways [40,59–62]. Examples include reductions in testosterone production, reduced sperm motility and velocity and increased transcription of the androgen receptor in a number of vertebrate models, including fish [17,63,64]. BPA also causes further diverse effects on development, including disruption of thyroid hormone function [65–69], effects on differentiation and function of the central nervous system (CNS) [70–72] and influences on the homeostatic control of the immune system [73].

BPA has also been shown to alter the transcriptional profile of steroidogenic enzymes in a time-dependent manner, including aromatase (*cyp19a1a*), which is responsible for the irreversible conversion of androgens into oestrogens in both males and females, and is a key regulator of oestrogen synthesis in the gonads. This enzyme was significantly upregulated in both the ovary and testis of *Gobiocypris rarus* exposed to 15 µg/L BPA for 7 days, followed by suppression after 35 days of exposure [74]. Alterations in aromatase expression affect oestrogen - testosterone ratios, and when these alterations result in significant deviations from the normal oestrogen - testosterone balance, this can affect the functioning of normal reproductive pathways in both male and female fish [75].

Aromatase is encoded by two genes, *cyp19a* known as gonadal aromatase, is regulated by a number of factors including oestrogens, steroidogenic factors and progesterone [76]. The second form of aromatase, *cyp19b,* is predominantly expressed in the brain [77] , and has been shown to be strongly under oestrogen control with several EREs (oestrogen responsive elements) in its promotor [76]. *Cyp19b* is essential for maintaining oestrogen – testosterone balance in the brain, which is in turn essential for male specific behaviours. Oestrogens, such as EE2 [78], nonylphenol (NP) [78,79] and BPA [79] have been shown to strongly up-regulate *cyp19b* gene transcription in a number of fish models, highlighting the potential for *cyp19b* to be a key target of BPA toxicity.

Adverse impacts on reproduction have been observed in several fish models. A multi-generational study in fathead minnow showed that BPA reduced gonadal growth in males and females, reduced hatching in F1 offspring of fish exposed to 640 µg/L and was reported to induce the oestrogen regulated egg yolk protein, vitellogenin, in male fish [80]. Vitellogenin (*vtg*) induction has been recorded in multiple fish species exposed to exogenous oestrogens [81]; *vtg* is an egg yolk precursor protein produced in response to oestrogens in the liver of sexually mature females and deposited in maturing oocytes [82]. Induction of *vtg* at both the transcript and protein level in the liver of male fish is a classic biomarker of oestrogenic exposure [83].

Further multigenerational studies have demonstrated the potential adverse effects associated with exposure to BPA [84,85]. Exposure to BPA in guppies has been associated with reduced sperm quality [15] and the presence of necrotic cells in the seminiferous tubules of *Xiphophorus helleri* was also reported [86]. Together, these studies demonstrate the potential reproductive consequences following exposure to relatively high concentrations of BPA in

fish. Studies such as this provide further support for the need to explore the mechanisms involved in the effects of exposure to EDCs, particularly those induced at environmentally relevant concentrations.

1.1.1.5 Other effects of Bisphenol A exposure

Evidence also exists supporting the involvement of BPA in the etiology of a range of human disease phenotypes, including cardiovascular disease [87], altered behaviour in children [88], prostate cancer [89], and recurrent miscarriage [90]. Low dose effects and non-monotonic dose response curves have been reported [91,92]. In addition, recent studies have begun to highlight the potential for early life exposures to result in the induction of physiological effects later in life, and even across generations, potentially via epigenetic mechanisms such as methylation-meditated promoter silencing [89,93].

1.1.1.6 Epigenetic effects of Bisphenol A exposure

Epigenetic processes are critical for a number of biological functions including tissue differentiation and normal cellular development [94], and a critical discussion regarding the role of epigenetic modifications in development is documented in section 1.2 of this thesis.

DNA methylation patterns are established and maintained by DNA methyltransferases. Recently, increasing evidence suggests that BPA may alter the epigenetic regulation of gene expression; for example, altered DNA methylation patterns have been observed both globally (i.e. changes to the total genomic content of DNA methylation) and at the regulatory regions of specific genes (i.e. locus-specific) in mammals [12,95–98].

The epigenetic potential of BPA was demonstrated in a study by Morgan *et al*., in which BPA caused hypo-methylation of the CpG site upstream of the agouti locus in yellow mice, a classic model for epigenetic studies [98]. A further study found that the use of a methyl donor dietary supplement reversed this effect, strengthening the evidence for BPA exposure to cause changes in the methylation state of specific genes [97]. Further to this, short term exposure of rats to environmentally relevant doses of BPA resulted in elevated expression of the PDE4D4 gene, a molecular marker for prostate cancer, due to hypo-methylation, demonstrating the potential for BPA to interact with the prostate epigenome [89]. A study by Doherty *et al*., found BPA to alter the expression of histone methyltransferases in MCF-7 breast cancer cells resulting in a 2-fold increase in Enhancer of Zeste Homolog 2 (EZH2) mRNA expression, a histone methyltransferase associated with risk of breast cancer [99].

In humans, exposure to BPA in the workplace has been associated with hypo-methylation of LINE-1 in spermatozoa, a marker of global DNA methylation levels in the genome [100]. Understanding the effects of BPA exposure on epigenetic processes, and how these alterations perturb expression of genes that are related to development and reproduction, are important to the evaluation of adverse effects associated with BPA exposure, both in humans and wildlife, particularly for exposures at environmentally-relevant concentrations.

To date, few studies have investigated the potential for BPA to induce epigenetic and transcriptional changes in fish. A study in *Gobiocypris rarus* found BPA exposure to be associated with altered DNA methylation in the 5' flanking region of *cyp19a1a* [74]. In addition, a significant decrease in the expression of DNA methyltransferase 1 (*dnmt1*) in ovarian tissue was reported in these fish, with a significant decrease in global DNA methylation [74].

Given the extensive use and ubiquity of BPA, it is important to understand the mechanisms mediating its toxic effects and the impacts these can have on both wild populations and human health, supporting the need for further studies exploring the potential mechanisms of BPA toxicity in vertebrate models. Studies are required in order to address the phenotypic effects associated with exposure to BPA, specifically population relevant endpoints such as reproductive effects, that may occur immediately after exposure, later in life or in subsequent generations, and to explore the epigenetic and transcriptional changes associated with these effects.

1.1.2 Metals

Metal contamination is widespread in the aquatic environment, driven by sources such as mining, agricultural runoff, household waste and industrial activity. Metals are considered to be particularly hazardous to aquatic life due to their persistence and bioavailability within the aquatic environment. Metals are able to remain in the sediment and the water for long periods of time highlighting the potential for chronic exposure, particularly in benthic organisms. Some metals can also bio accumulate within aquatic organisms where they can become toxic and may result in bio magnification in the food chain.

Of the 94 naturally occurring elements, 70 are metals [101], and in terms of their biological relevance, these metals can be further categorised into essential and non-essential metals. Essential metals are unconditionally required for a number of biological processes including as cofactors in metabolic reactions, and therefore these metals are essential to sustain life [101]. Examples of essential metals include copper, iron, manganese, zinc, chromium and cobalt (Cu, Fe, Mn, Zn, Cr, and Co respectively) [102]. For non-essential metals, no known

biological functions have been identified [101] and examples include silver, cadmium and lead (Ag, Cd, and Pb respectively).

For essential metals, organisms have evolved specific mechanisms to ensure their uptake from the environment. However, although classified as essential, when in excess these metals can become highly toxic. While the modes of action of metals are variable, chronic metal exposure has been associated with a number of toxic effects in fish, including impaired growth, immunity, osmoregulation, locomotion and reproduction. Metal exposure has also been shown to impact on the genetic diversity in wild populations, potentially leading to adaptation [103–106].

Metal toxicity is significantly influenced by a number of processes that affect their bioavailability within aquatic environments including solubility, complexation and speciation. These physical properties are strongly modulated by the chemical properties of each aquatic system, for example the salinity, pH and dissolved organic matter of a water body [101,107].

In this thesis, copper has been selected as a metal pollutant of interest; the section below will discuss its functional role in biological systems, known environmental impacts and toxic effects.

1.1.2.1 Copper

Occurring naturally in the aquatic environment, copper is a particularly interesting trace metal due to its role as an essential element for life. Copper is necessary as a co-factor in a number of enzymatic reactions, including energy production, cellular metabolism, iron

acquisition and oxygen transport [108], however, when found in excess copper is a potent toxicant [101]. Aquatic organisms are particularly sensitive to copper toxicity, due to their continual exposure to contaminated waters via the skin and the gills, as well as via the diet, due to the ingestion of contaminated water or sediment [101]. In addition, although there are no significant differences in the solubility of copper between freshwater and marine systems (added as $CuSO_4$) [109], freshwater fish species are at significantly increased risk to copper toxicity when compared to marine species. This is due to the fact that decreased water hardness has been shown to cause an increase in copper toxicity, likely related to the greater bioavailability of the free metal ion at lower salinity conditions [110,111]. Copper uptake via the gills is characterized by competition with other ions for transport, therefore in freshwater systems, where fewer competing ions are present, copper uptake via the gill is proportionally increased. In addition, in freshwater systems fish are required to actively transport ions from the surrounding water into the blood by chloride secretory cells in the gills in order to mitigate the passive movement of water into the body and maintain osmotic balance [112], further increasing the likelihood of copper being transported into the organism.

1.1.2.2 Occurrence of copper in the aquatic environment

Copper is relatively abundant on earth and is present in a wide variety of rocks and minerals, resulting in its widespread distribution in terrestrial and aquatic environments [110,113]. In freshwater systems, copper concentrations are generally reported to be up to 30 µg/L [113]. As a result of increased industrial and agricultural activity, copper concentrations reported to date often reach levels known to be toxic to fish [114], and in a recent analysis copper has been identified as the most significant metal pollutant in UK waters [115]. The

economic importance of copper has driven the need for mining and subsequent entry into the aquatic environment [101,116]. Copper is utilised in a number of products, such as electrical equipment, fertilisers, pesticides and antifouling paints [117]. In areas historically impacted by mining activity concentrations can reach remarkably high levels. For example, copper concentrations as high as 417 µg/L have been reported for the River Hayle in the south west of England [106,118], and in the River Lee concentrations have been reported to reach 193 µg/L, both well above the lethal concentrations for many freshwater fish species, demonstrating a need to understand the toxic effects of copper exposure in aquatic organisms in order to inform policy and management practices [106].

1.1.2.3 Copper uptake and storage

Due to its importance as an essential element and its risks as a toxicant, mechanisms of homeostatic control have evolved at the organismal and cellular level in order to tightly regulate copper acquisition and storage [101]. In most organisms copper uptake occurs predominantly via the diet, however, in aquatic organisms copper uptake also occurs via the gill epithelium [119].

Although copper uptake via the gill makes up a small proportion of the overall copper intake in fish under control conditions, there is evidence from aquatic toxicity studies that the gill can contribute considerably to copper uptake when fish are exposed to elevated copper in the water or dietary deficiency [101,120]. Usually, copper most likely enters the gills via either a putative epithelial sodium channel (ENaC) or copper transporter 1 (CTR1) [119]. Metallochaperones (MC) such as COX17, SCO1 and ATX1 [121], are thought to bind copper, guiding it to the Golgi network (GN) [119]. At the GN copper is transported into the Golgi

lumen via a Menkes protein (MNK)-`like' Cu^{+} -ATPase, and then incorporated into metal binding proteins (MBP) within the GN [119]. GN vesicles are then responsible for trafficking copper to the basolateral membrane for release via exocytosis. Other ATPases on the basolateral membrane exporting copper (i.e. Ag^{+}/Cu^{+} - ATPase) may also be present and play a role in copper transfer across the membrane [119] (Figure 1). However, when in excess, copper can also enter the gill on the basis of competitive interactions with sodium via apical Na^{+} channels [122].

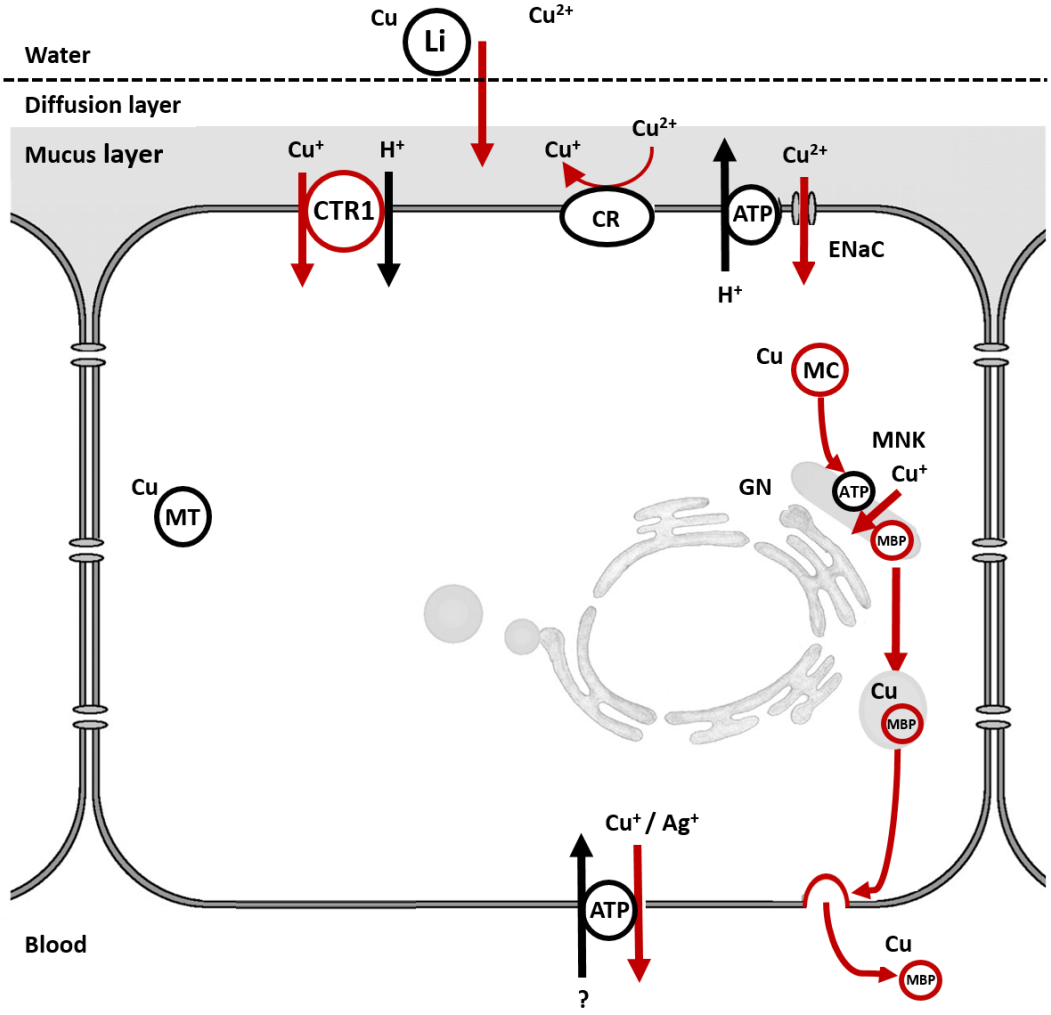

Figure 1. A hypothetical representation of copper uptake pathways in fish, combining data from gill and intestine. Figure adapted from [119]. Cupric copper is probably reduced to Cu^{+} and enters the cell via a putative epithelial sodium channel (EnaC) or a copper transporter 1 (CTR1). Cu^{+} is then guided to the Golgi network (GN) bound to Metallochaperones (MC). At the GN, Cu^{+} is transported into the Golgi lumen via a MNK-'like' Cu^{+} -ATPase. Here Cu^{+} is incorporated into metal binding proteins (MBP) within the GN. Copper is then transported to the basolateral membrane via GN vesicles for release via exocytosis. Other ATPases present on the basolateral membrane may also contribute to exporting copper (i.e. Ag^{+}/Cu^{+} -ATPase). It is important to note, that these processes may not all occur simultaneously within the same cell. Li, aquatic ligand; MNK, Menkes Cu^{+}-ATPase; CR, copper reductase.

The liver is the main organ involved in copper homeostasis [123,124], and is the main site of production for the most abundant copper containing protein in the body, ceruloplasmin [119]. Ceruloplasmin is secreted into the blood, where it acts as a source of copper to distant tissues beyond the liver. The main site of copper excretion in teleost fish is via the bile, but the gills have also been implicated in copper excretion [101].

Metallothionein (MT) is a metal binding protein that plays an important role in the transport, storage and detoxification of toxic metals, through sequestering and therefore reducing the amount of the free metal ions [125]. The definite function of metallothionein is still unknown, but it is generally accepted that the primary function of these proteins is the homeostatic metabolism of the essential metals such as copper and zinc [125]. Metallothionein in fish is most abundant in the liver, gill, kidney and intestine, and its expression has also been detected in the blood [125–128].

Exposure to sub-lethal concentrations of a number of essential and non-essential metals including copper, mercury, zinc and cadmium, has been shown to stimulate metallothionein expression [118,125,129,130], via binding to metal response elements in its promotor region and stimulating transcription [125,131]. Due to the role of metallothionein in homeostatic metabolism of a number of metals in key tissues involved in copper uptake and storage, and its responsiveness to exposure to metals, this protein is now used as a key biomarker of toxic metal pollution [101].

1.1.2.4 Effects of copper exposure in fish

Exposure to toxic levels of copper are known to disrupt a number of biological processes including branchial ion regulation, locomotion, enzyme activities, immune function and to

cause oxidative stress [101,132,133]. In addition, copper has been shown to disrupt metabolic processes through causing inhibition of respiratory enzymes, impair gaseous exchange by causing gill structural damage and disrupt osmoregulation through the inhibition of gill Na^+-K^+-activated ATPase [101,134]. The Na^+/K^+-ATPase, is located in the gill at the basolateral region, where it plays a key role in the transport of sodium in and out of the organism. Here, copper competes with sodium ions at the gill membrane, and can result in competition with and eventually damage of the sodium pump, leading to a reduction in internal sodium concentration and may subsequently lead to osmotic stress [122,135,136]. Copper can also impair the function of olfactory organs, this is associated with changes in blood chemistry, enzyme activity and corticosteroid metabolism and affect mechanoreceptors of lateral lines [101,137].

Exposure to sub-lethal concentrations of copper have been shown to cause increased levels of reactive oxygen species (ROS) and subsequently oxidative stress owing to copper's high reactivity with H_2O_2 and ability to undergo redox reactions, forming ROS, in a process known as the Fenton reaction [138,139]. ROS production can lead to lipid peroxidation, DNA damage and protein carbonyl production [138,140–142]. Oxidative stress can also be induced through the inhibition of antioxidant enzymatic activity and alterations in the mitochondrial electron-transfer chain leading to ROS production [142]. Diverse responses to copper have been reported for a number of antioxidant enzymes; for example the gene expression and enzymatic activity of catalase has been reported to increase or decrease within a few days of exposure in gills, liver, and kidney of freshwater fish [143–145]. Superoxide dismutase (SOD) expression has been shown to be increased in response to copper exposure in gill, liver, and kidney [146], and copper exposure has also been reported to result in increases in mRNA expression of glutathione peroxidase in the brown trout (*Salmo trutta*) [101].

Copper has the potential to alter the behaviour of fish, likely facilitated through interactions with the olfactory epithelium. The ability of fish to respond to olfactory cues is critical for survival and whole population health. These responses are vital for predator avoidance, prey identification and successful reproduction, therefore alterations in the olfactory system have the potential to result in important population relevant effects [101,147–149].

High concentrations of copper have been associated with alterations in the genetic diversity of populations, and studies in *Daphnia longispina* populations showed that differential resistance to copper was associated with genetic adaptation [150–153]. In addition, a study in *Daphnia magna* reported that organisms exposed to 0.5 to 100 µg/L copper over three generations, demonstrate a two-fold increase in acute copper tolerance [154,155].

For metals, extreme cases exist of fish populations that are able to survive in highly contaminated waters, where concentrations of metals far exceed their LC50 [106,118,156]. The LC50 can be defined as the lethal concentration required to kill 50% of a population. Well studied examples include the lakes in North America; despite being highly contaminated with metals (particularly copper, cadmium and nickel) as a result of local industrial and mining activity, yellow perch (*Perca flavescens*) populations have been found to inhabit these lakes [103–105,157–159]. For these populations, evidence suggests that genetic diversity is negatively correlated with elevated copper contamination in the livers of these fish [103].

Perhaps the most remarkable example of adaptation to chronic metal exposure in freshwater fish has been reported in brown trout (*Salmo trutta*), inhabiting the River Hayle in Cornwall (Southwest England), where fish are chronically exposed to elevated metal concentrations (particularly zinc, iron and copper), entering the river via run off from the surrounding historic mines after peaks in rainfall [118,160]. Genetic analysis revealed

differences between fish populating the River Hayle and nearby clean references [106], and physiological studies report that Hayle trout contain the highest metal concentrations in their tissues ever reported for wild fish [118].

1.1.2.5 Differential responses to copper exposure

Organisms exposed to non-lethal concentrations of pollutants may be able to develop an increased tolerance to subsequent exposure scenarios driven through acclimation rather than genetic selection, and examples exist where the toxicological responses of fish inhabiting relatively un-polluted waters vary significantly [136,161,162]. Furthermore, organisms could become differentially sensitive to a toxicant, driven by prior exposure [163]. As a result, it is critically important to consider exposure history of a given population in order to draw meaningful conclusions from toxicity testing, and to understand the possible mechanisms driving changes in organism responses to a pollutant upon re-exposure [164].

A study exploring the occurrence of copper acclimation in the least killifish (*Heterandria Formosa*) found that fish pre-exposed to 15 µg/L copper for seven days, followed by exposure to a lethal concentration (150 µg/L copper) had a significantly longer time to death compared to the control fish [136]. In addition, Grosell *et al*., report that copper acclimated rainbow trout (*Oncorhynchus mykiss*) clear a single bolus of injected copper from their plasma more effectively than non-acclimated fish, reporting a four-fold increase in hepatobiliary copper excretion in copper acclimated fish [165]. These data suggest that pre-exposure to an environmentally relevant sub-lethal concentration of copper can induce an acclimation response in two fish species and highlight the importance of understanding the factors that drive tolerance in order to better protect and manage local populations.

1.1.2.6 Epigenetic effects of copper exposure

DNA methylation and histone acetylation are epigenetic mechanisms which regulate aspects of cell and tissue specific gene expression [166]. There is now increasing evidence that exposure to toxic metals, including copper, can directly modify the epigenetic state of the genome [166,167].

In mammalian models, a number of examples exist where copper exposure has been associated with changes in DNA methylation. In the Jackson toxic milk mouse model of Wilson's disease, changes in hepatic copper concentration have been associated with alterations in global DNA methylation [168]. Wilson's disease is caused by mutations in the ATP7B gene, which is responsible for copper excretion, resulting in copper accumulation in hepatic tissue, with progressive liver damage to cirrhosis [168]. Studies using the Cohen diabetic sensitive rats, also associated changes in global DNA methylation with copper supplementation via the diet, specifically hyper global DNA methylation in placental tissue, in response to copper supplementation [169]. The concentration chosen in this study was selected due to the lack of clinical signs of toxicity, therefore highlighting the potential for dietary concentrations of copper acting as nutritional supplementations to influence DNA methylation patterns.

Copper exposure has also been associated with changes to epigenetic marks within chromatin. Exposure of HL-60 human leukaemia cells to copper has been shown to cause a concentration dependant decrease in histone acetylation, attributed to Cu-induced oxidative stress [166,170]. These findings are concordant with previous findings from Kang *et al*., who found copper exposure of Hep3B cells to cause inhibition of histone acetylation both at toxic and non-toxic concentrations [167,171].

The potential for exposure to elevated concentrations of copper to cause alterations in the epigenome has rarely been explored in fish. Of the studies in fish where the potential for copper to influence DNA methylation is considered, many do not measure DNA methylation itself, or look only at changes in global DNA methylation, a measure which does not allow the resolution to explore changes in DNA methylation at the gene level, which could include both hyper and hypo-methylating effects. For example, Dorts *et al.,* report that exposure of zebrafish embryos to 325 µg Cu/L from fertilization (<1 hpf) to 4 hpf resulted in significant upregulation of the expression of *de novo* DNA methyltransferase genes (*dnmt3*), however, no changes in mean global DNA methylation were observed [172].

Exposure of the Nile tilapia (*Oreochromis niloticus*) to soluble fraction of industrial solid waste (including metals such as copper, zinc, lead, nickel and chromium) was associated with a significant increase in global DNA methylation [173]. In addition, a toxic metal mixture containing copper, zinc, lead and cadmium was found to cause a significant increase in hepatic global DNA methylation in the goldfish (*Carassius auratus*) [174].

The sparsity of studies evaluating the impact of copper on epigenetic signalling represents a substantial knowledge gap and highlights a significant research need to document the potential epigenetic effects of copper in fish. This is important to inform of the potential mechanisms by which fish populations acclimate to metal toxicants in their environment and whether exposures may have long term consequences across generations.

1.2 Introduction to epigenetics

The term 'epigenetics' was introduced by the embryologist Conrad Waddington in 1942 to define the emerging division of biology that studies the 'causal interactions between genes and their products which bring the phenotype into being' [175,176]. The words "epi", meaning "above", was fused with the word "genetic" to name the contributions of non-inherited factors to embryonic development [177] The first molecular feature identified was DNA methylation in the 1970s [178], followed by histone modifications in the 1990s [179].

Modern definitions are hotly debated, however in general definitions refer to epigenetics as the study of changes in gene expression arising without changes to the DNA sequence itself, which can be mitotically inherited, and account for the phenotypic variability sometimes observed between genetically identical individuals [96,180]. More recently researchers have argued that the defining aspect of epigenetic change is not whether DNA is modified [181], but the ready reversibility of epigenetic change contrasting directly with changes such as nucleotide substitutions, insertion of foreign DNA sequences and other genetic events which alter the genetic code itself [177]. Therefore, epigenetics can also be defined as the study of readily reversible mitotically and/or meiotically heritable changes.

Epigenetic processes are critical for tissue differentiation, normal cellular development, X-chromosome inactivation [182,183], genomic imprinting [184,185], and play a key role in the regulation of gene function and expression [94]. Epigenetic changes include DNA and histone modifications, and non-coding RNAs. This introduction will now explain each of these in further detail below.

1.2.1 DNA modifications

DNA methylation is the most widely studied and well characterised epigenetic mechanism to date [186,187]. In eukaryotes CpG methylation is tissue-specific and species-specific, and plays a fundamental role in the regulation of transcript expression, usually associated with transcriptional repression [187].

DNA methylation has been found in the genomes of a diverse array of organisms, including both prokaryotes and eukaryotes. DNA methylation occurs on both cytosine and adenine bases in prokaryotes; however in eukaryotic species DNA methylation has generally been considered to be confined predominantly to cytosine bases within CpG sites [186,188,189]. Due to methylated cytosines being more liable to spontaneous deamination than unmethylated cytosines, CpG sites are less common in the genome than would generally be predicted by chance. Methylation is only found on approximately 1% of all cytosine residues within the mammalian genome [190]. However, recently researcher have identified methylation at non-CpG sequences such as CpHpG and CpHpH, where H can be A, C or T [189].

When considering CpG methylation alone, it is interesting to note that CpGs are not found randomly throughout the genome, instead CpGs are generally clustered in what is referred to as CpG islands [190]. CpG islands are short (up to1 kb) genomic regions which are highly enriched at or near promoter regions and remain mostly unmethylated in somatic cells, suggesting they may play a role transcriptional regulation [190,191].

A smaller proportion of the CpG sites have been located instead within gene bodies and these sites are often extensively methylated [192,193]. The role of gene body methylation is less well understood, it has been proposed that due to exons being more highly methylated than introns, and the existence of transitions in the degree of methylation occurring at exon–

intron boundaries, it is possible that gene body methylation may play a role in regulating splicing [194]. A study has suggested that a DNA-binding protein, CCCTC-binding factor (CTCF), which is known to regulate DNA methylation, results in the pausing of RNA polymerase II (RNAPII) and, as the kinetics of RNA polymerase II movement influences alternative splicing, this suggests that DNA methylation may be associated with the regulation of splicing [195].

In the animal groups studied to date, the array of methylation levels present and patterns within the genome is very broad. At the low end of the spectrum is the nematode worm *Caenorhabditis elegans*, whose genome lacks detectable cytosine methylation and does not encode any conventional DNA methyltransferases [196]. Also, the insect *Drosophila melanogaster*, originally thought to be completely lacking in methylation, has a DNA methyltransferase-like gene [197] and is reported to contain very low methylation levels [198,199], although mostly in the CpT dinucleotide rather than in CpG. Most other invertebrate genomes studied have moderately high levels of CpG [196], for example the *Ciona intestinalis* genome is extremely highly methylated, with levels ranging from 70.5% in muscles cells to 88% in the intestine, and almost all CpG sites in the testis have been reported to be methylated in 90% to 100% of the cells [196,200].

DNA methylation has been shown to be essential for viability in a mice, where targeted deletion or partial inactivation of DNA methyltransferase enzymes including *dnmt1*, *dnmt3a* and *dnmt3b* resulted in lethality [186,201,202]. In many cases correlations have been reported between transcriptional activation and demethylation, however, despite evidence to suggest the important role of methylation within a number of cellular processes, causation has not been demonstrated to date [203].

1.2.1.1 DNA methyltransferases and TET proteins

In eukaryotic species, DNA methylation involves the transfer of a methyl group to the 5th position of the cytosine pyrimidine ring (Figure 2). This reaction requires S-adenosylmethionine (SAM) as a methyl donor, and is catalysed by DNA methyltransferases (DNMTs) [186].

Cytosine DNA methyltransferase enzymes fit into two general groups. The *de novo* methyltransferases, referred to in both mammalian and most fish species as DNMT3a and DNMT3b are mainly responsible for introducing cytosine methylation at previously unmethylated CpG sites. DNMT1, the maintenance methyltransferase, plays a key role in copying pre-existing methylation patterns onto the new DNA strand during DNA replication [186]. In mammalian species, a fourth DNA methyltransferase, DNMT2, has been identified. This DNA methyltransferase has weak methyltransferase activity *in vitro* [204], and targeted deletion of the DNMT2 gene in embryonic stem cells results in no detectable effect on global DNA methylation, therefore suggesting that DNMT2 has relatively little involvement in establishing and maintaining DNA methylation patterns [186,205].

Traditionally DNA methylation was thought to be stable, but recently DNA methylation has been shown to undergo both active and passive demethylation [206–208]. The gain, loss and maintenance of the methyl groups on the cytosine in a CpG site is the result of the relative contribution of three interrelated pathways; the addition of new methylation marks by *de novo* methylation, the maintenance of existing methylation patterns during DNA replication and the active replication-independent removal of DNA methylation [209]. The mechanism by which this DNA demethylation is achieved has only recently been described. TET proteins have been shown to remove methyl groups by sequential oxidation of DNA methylation

(5mC), resulting in multiple intermediate forms of CpG modifications, including 5-hydroxymethlcytosine (5hmC), 5-formylcytosine (5fC) and finally 5-carboxylcytosine (5caC) which is then removed by thymine DNA glycosylase (Figure 2) [190,210–213]. Three TET enzymes (TET1, TET2 and TET3) have been identified in vertebrate models, including in fish [212,214], which demonstrate varying tissue specificity and all of which can catalyse the conversion of 5mC to 5hmC. TET3 has been shown to be strongly expressed in human oocytes and zygotes and has been shown to play an important role in early development, including epigenetic reprogramming and the regulation of tissue specific gene expression [215,216].

Initially, 5hmC, 5fC and 5caC were thought to be intermediate by-products of the processes involved in active 5mC demethylation, but recent evidence has suggested that these species may play an important role in regulatory and developmental processes themselves [217–220]. In particular, there has been a significant research effort into the potential for 5hmC to play an important role in early life development, ageing, disorders of the human brain, and studies have found 5hmC to be preferentially enriched in the gene bodies of expressed genes, and positively correlated with RNA expression for protein coding genes [221–224]. To date, few studies have explored the role of 5hmc in fish, with most studies being performed in the zebrafish [225].

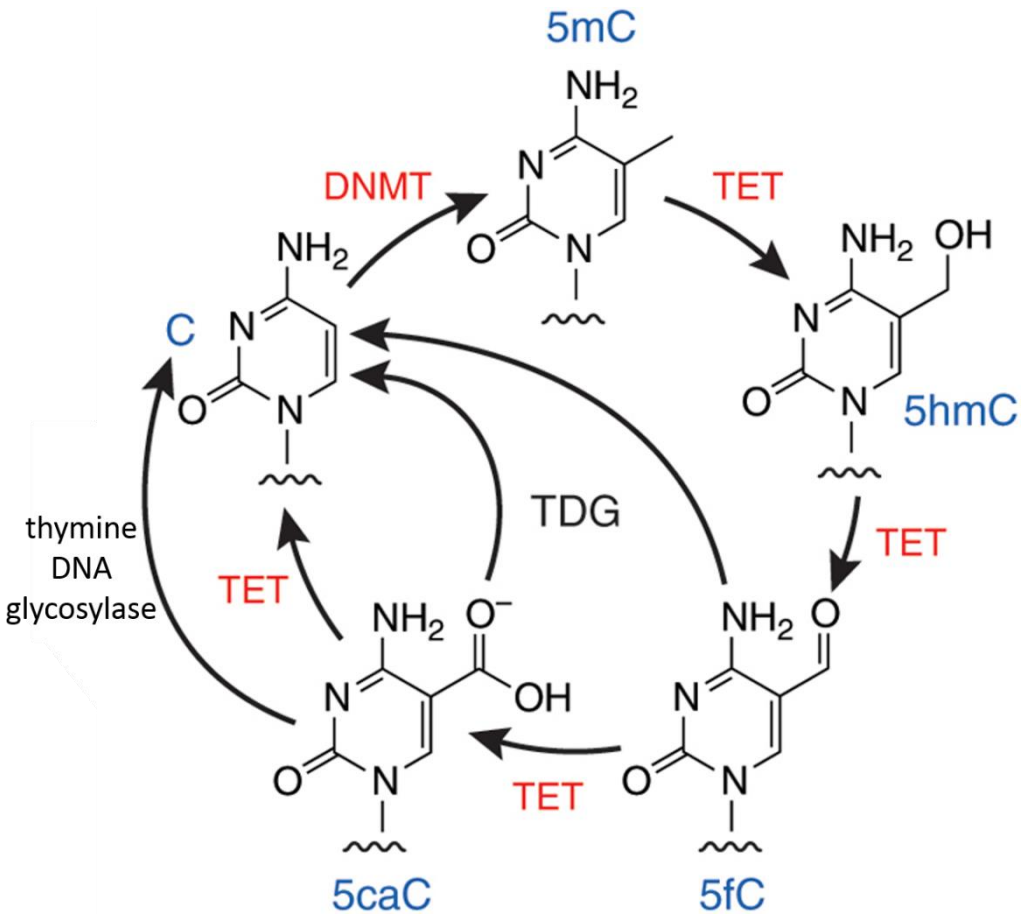

Figure 2. The cycle of DNA methylation and demethylation by DNA methyltransferases (DNMTs) and TET proteins. DNMTs are mainly responsible for introducing cytosine methylation at previously unmethylated CpG sites (DNMT3A and DNMT3B), and play a key role in copying pre-existing methylation patterns onto the new DNA strand during DNA replication (DNMT1) [186]. Ten-eleven translocation (TET) proteins have been shown to remove methyl groups by sequential oxidation of 5-methlcytosine DNA methylation (5mC), resulting in multiple intermediate forms of CpG modification, including 5-hydroxymethlcytosine (5hmC), 5-formylcytosine (5fC) and finally 5-carboxylcytosine (5caC) which is then removed by thymine DNA glycosylase. Figure adapted from [226].

Histone modifications

In the nuclei of all eukaryotic cells, histone and non-histone proteins package and order the highly folded genomic DNA in a dynamic structure called chromatin [227]. Regions of the chromosome which are not actively being transcribed are maintained highly condensed within the chromatin structure. The basic repeating unit of chromatin, the nucleosome, is comprised of DNA wrapped roughly twice around an octamer core of histone proteins, forming the structure shown in Figure 3.

Within the nucleosome's core, four types of histone protein combine (H2A, H2B, H3 and H4) [228]. Chromatin structure plays an important role in the regulation of a number of signalling pathways [227]. It is the N-terminal tails of the histones which extend from the nucleosome and are available for post-synthesis modifications [228]. These covalent modifications include histone acetylation, phosphorylation, methylation, sumoylation, deamination, proline isomerization and ubiquitylation, each contributing to the 'histone code' which plays a role in the regulation of gene expression via alterations in chromatin structure [227,229,230].

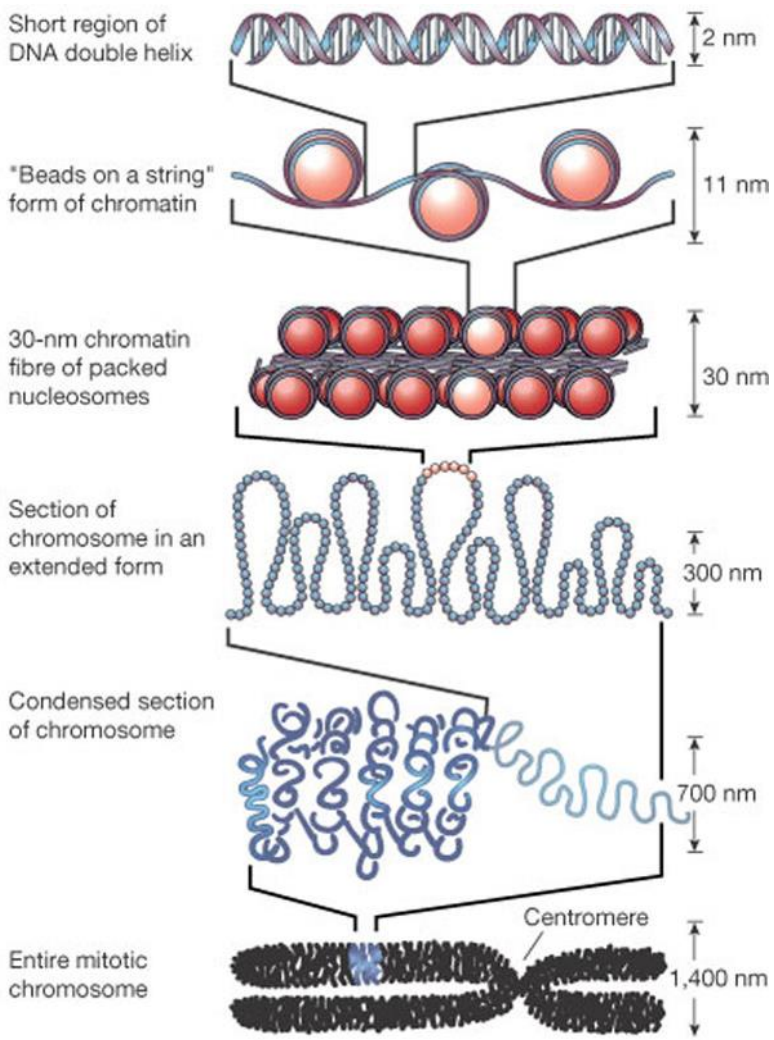

Figure 3. Chromatin structure. Figure adapted from [231]. The basic repeating unit of chromatin, the nucleosome, is comprised of DNA wrapped roughly twice around an octamer core of histone proteins. Nucleosomes are connected to one another by short stretches of linker DNA.

The accessibility of genomic DNA for the transcriptional machinery is modulated by these covalent modifications. In an open chromatic state (euchromatin), the transcriptional machinery is able to access the DNA and drive transcription, while in the condensed chromatin state (heterochromatin), transcription is repressed by blocking the access of transcription factors and other co-factors to binding sites in the DNA [230].

Histone modifications are dynamic, controlled by the combined action of both histone methyltransferases (HMTs) and histone deacetylase (HDACs), in addition to proteins that read histone marks [232]. HMTs for example, include demethylases such as PAD4/PADI4, BHC110/LSD1, and JmjC domain-containing demethylases [233]. HDACs deacetylate histones and certain transcription factors, and are classified into three classes based on sequence homology to different yeast HDACs [234].

Histone modifications have also been shown to play a fundamental role in epigenetic reprogramming [184]. While the maternal genome appears to be generally epigenetically static, at fertilisation the paternal genome exchanges histones for protamines before undergoing global DNA demethylation and acquiring histone modifications [184]. During preimplantation development, further reorganisation of histone modifications occurs. These changes to the histone code are thought to be important for totipotency and the initiation of embryonic gene expression [184]. Interestingly, during ZGA in zebrafish, histone modifications have also been reported to increase, however literature regarding histone modifications during epigenetic reprograming in fish is limitted [235].

1.2.2 Non-coding RNA

Genomic studies have shown that as much as 90% of the mammalian genome is transcribed indicating that a substantially larger proportion of the genome is transcribed than can be accounted for by annotated genes [236–238]. Non-coding RNAs (ncRNAs) are products of genes that are transcribed but not translated into proteins. The size of these non-coding RNAs can range from as small as 18-22 nucleotides (nt) to tens of kilobases (KBs). A large number of ncRNAs have been reported to have important regulatory functions within the cell [190].

There are a diverse array of different ncRNAs all with different associated functions, for example micro-RNAs (miRNAs), small temporal RNAs (stRNA), piwi-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), and long ncRNAs (lncRNAs) [239,240].

MiRNAs are the most well studied class of small ncRNAs, and have been shown to regulate gene expression by sequence specific post-translational gene silencing. Researchers have estimated that miRNAs may regulate 74–92% of all protein-coding mRNAs [241]. miRNAs function through the recruitment of a protein complex, RISC (RNA-induced silencing complex), to the targeted gene transcripts. This leads to silencing via the degradation of the messenger RNA or by preventing its translation [190]. In the zebrafish miRNAs have been reported to play an important role in the regulation of gene expression during embryonic development [242]. In addition, toxicity studies have reported that a number of toxicants, including triazophos, fipronil and their mixture can alter the expression of miRNAs [243].

1.2.3 Transcriptional regulation and the epigenetic landscape

The combination of DNA and histone modifications, transcription factors, chromatin regulators and ncRNAs are often referred to as the epigenetic landscape. The epigenetic landscape and the complex interactions between the epigenetic modifications which encompass its elements are established during development and maintained down the cell lineage, and in turn influence cellular transcriptional activity. A number of examples of interactions between elements of the epigenetic landscape have been reported, for example ncRNAs have also been shown to interact with certain histone modifications [244], and lncRNAs have been shown to function as modular scaffolds for histone modifying complexes [245]. In addition, recruitment of histone deacetylases (HDACs) by DNMTs and methyl-CpG binding protein 2 (MeCP2) has been associated with gene expression silencing and chromatin remodelling [246]. It is clear that no single epigenetic modification acts upon transcriptional activity alone, however, the extent to which these modifications interact is still poorly understood, representing a significant research need [247].

1.2.3.1 Epigenetic reprogramming

During development, different cells and tissues within multicellular organisms acquire different programmes of gene expression, thought to be controlled, at least in part by differing epigenetic patterns such as DNA methylation [184,196]. Therefore, each cell has its own epigenetic signature which reflects the genotype, developmental history and environmental influences, and ultimately this epigenetic information is reflected in the transcriptional activity and phenotype of the cell [184]. In general, these epigenetic marks are considered to be fixed after cellular differentiation. However, studies have shown that some

cells undergo major ”reprogramming” events in terms of their epigenome during normal development and during the onset of disease phenotypes. These reprogramming events involve the removal of epigenetic marks such as cytosine methylation within the nuclei, followed by re-establishment of different marks, controlled by *de novo* methyltransferases in the case of DNA methylation [184,248–250].

1.2.3.1.1 Epigenetic reprogramming in mammals

Epigenetic reprogramming events are well studied in mammalian species. In mammalian embryos two major cycles of epigenetic reprogramming of the genome have been described; during pre-implantation development and during germ cell development. Gametic DNA methylation marks have been shown to be erased and replaced with embryonic marks required for early embryonic development and toti- or pluripotency after fertilisation [184]. In addition, major epigenetic reprogramming has also been shown to occurs in primordial germ cells (PGCs) during which parental imprints are erased and totipotency is restored [184].

1.2.3.1.1.1 Reprogramming in early embryos

Reprogramming in early embryos has been shown to occur both by active and passive mechanisms [249]. DNA demethylation occurs at the one-cell stage, preferentially affecting the male pronucleus [235]. The sperm genome undergoes remodelling of paternal chromatin through the removal of protamines which are replaced by (acetylated) histones, immediately followed by genome wide de-methylation [251,252], and this process is complete

before DNA replication begins. It is interesting to note, that some sequences in the mammalian paternal chromosomes are protected from this de-methylation event at fertilisation, including the imprinted genes [249]. During the subsequent divisions, passive de-methylation takes place because Dnmt1 is excluded from the nucleus, however, Dnmt1 knockout mice have demonstrated the crucial role of Dnmt1 in the maintenance of methylation for imprinted genes [253]. This DNA methylation reprogramming event of the paternal genome in the zygote appears to be conserved in eutherian mammals [249]. It is hypothesized that during this process, sequences that escape reprogramming may be involved in epigenetic inheritance.

1.2.3.1.1.2 Reprogramming in germ cells

The genomes of mature sperm cells and oocytes in mammals are highly methylated when compared to the methylation of somatic cells [249]. During the early development of primordial germ cells in mice, genome wide de-methylation of these cells has been shown to occur [249], and demethylation is completed by embryonic day 13 to 14 in both male and female germ cells [250]. It is not yet known if de-programming occurs passively or via active demethylation. Re-methylation takes place several days later, this appears to occur earlier in the male germ line, beginning at the prospermatogonia stage around embryonic days 15 to 16 and onwards [184,249]. Re-methylation in the female germ line however, takes place after birth during the growth of oocytes. Reprogramming of germ cells is required for the resetting of genomic imprints. However, whether this process only occurs in species shown to have imprinting is not known, because comparative data have yet to be generated for other vertebrates (e.g. amphibians or birds) in which imprinting is thought to be absent [249].

It is also thought, that this re-programming event may have a function in the removal of acquired epigenetic modifications as a result of environmental factors or individual genetic variation [254–256].

1.2.3.1.2 Epigenetic reprogramming in fish

In comparison to mammalian models, few studies exist in fish exploring the role or reprogramming in development and most studies have been performed in the zebrafish.

The zebrafish genome is 1.5 GB in size and is partitioned into 25 chromosomes. In addition, the zebrafish genome lacks a defined sex chromosome, and has ~24,800 nuclear genes and one mitochondrial chromosome [235]. In comparison to other species the zebrafish nuclear genome is GC poor (37%), compared with the *Xenopus*, mouse and or human genomes which have 40%, 42% and 42% GC content, respectively. Despite this, the zebrafish genome has more than 2-fold more CpG sites than the mouse or human genomes [235]. Zebrafish have been shown to have similar methylation patterns compared to mammals and show conservation of DNA methylation and other epigenetic pathways[225] .

The fundamental enzymes for epigenetic regulation (DNMT1, DNMT3A/B and TET family proteins), have been shown to be conserved across vertebrates and are present in the zebrafish, but this species lacks parental imprinting [257]. Zebrafish perform moderate DNA de-methylation after fertilisation, followed by re-methylation to levels comparable to that of somatic cells. This processes occurs prior to zygotic genome activation (ZGA), at the ~1000 cell blastula stage (~3 hours post fertilisation) [235,257]. It is interesting to note, that ZGA occurs much earlier in mice (~2 cell stage) and humans (~4-8 cell stage) [235,258]. Also in

zebrafish, despite the large number of cell divisions occurring prior to reprogramming, this process is complete prior to ZGA, similar to that occurring in mammals.

During this re-programming event, DNA methylation reaches a minimum at the 64-cell stage, with re-methylation occurring by the 256-cell stage, and methylation levels are restored by the sphere stage [235]. Potok *et al*., report that once these reprogramming events are complete, and the developing embryo reaches the ZGA stage, transcription start site (TSS) loci within the oocyte have been "reprogrammed" to the same state observed in the sperm genome [235]. For zebrafish, as for other vertebrates, embryogenesis is therefore considered the most vulnerable stage to environmental exposures due to the complex DNA methylation and chromatin patterning taking place during this period [97,186].

A second wave of DNA methylation reprogramming in primordial germ cells such as that described for mammalian species (described above) has not been confirmed in zebrafish. However, the DNA methylation patterns of sperm and oocytes in zebrafish have been reported to differ significantly [235], suggesting that DNA methylation reprogramming events may also occur during primordial germ cell development and gametogenesis in zebrafish [259].

1.2.3.2 Epigenetic memory and transgenerational inheritance?

A key question in epigenetics is how stable epigenetic marks are over time and if these marks can be transferred from one generation to another in the absence of continued exposure. An important property of epigenetic memory is its dynamic nature and particularly its ability to be erased [260]. Epigenetic reprogramming events occur during germline and pre-implantation in mammalian development, and have been shown to occur in the zebrafish post fertilisation and prior to zygotic genome activation (ZGA). These

reprogramming events are followed by the establishment of new chromatin configurations [261,262]. Therefore one of the focal areas of research is this area is investigating the possibility for epigenetic marks, induced by exposure to environmental cues, to be maintained during epigenetic reprogramming events, leading to epigenetic inheritance.

The theory of transgenerational epigenetic inheritance has received a lot of attention within the scientific community, but debate still continues regarding how much transgenerational inheritance actually takes place, and ultimately the impact this may have on individual organisms and populations [263].

An array of environmental factors have been proposed to cause epigenetic transgenerational inheritance of disease or phenotypic variation [264,265], and are listed in Table 1. There are a number of examples of transgenerational epigenetic inheritance in plant species [266], where changes in DNA methylation, without changes to the genomic sequence itself, resulted in transgenerational inheritance. For example, in toadflax (*Linaria vulgaris*), changes in DNA methylation of the CYCLOIDEA gene, which is responsible for the control of formation of dorsal petals, resulted in radially symmetric flowers, rather than the usual bilaterally symmetric phenotype and this was maintained over several generations [267].

In mammals however, very few, and often controversial cases of transgenerational epigenetic inheritance have been reported, and often these studies have been highly criticised for their experimental design [263]. In addition, criticism is frequently focused on the fact that these studies often concern inter- rather than transgenerational epigenetic effects, and seldom exclude DNA sequence changes as a possible underlying cause for heritability [268].

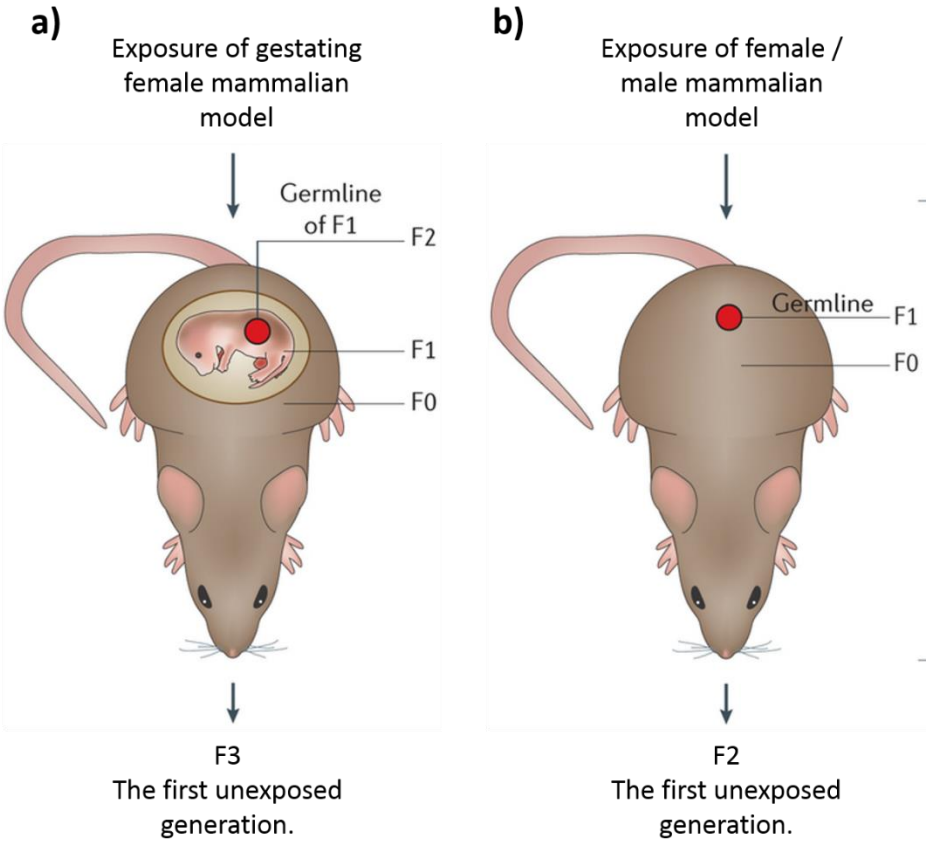

Figure 4. Schematic representation of environmental exposure and affected generations in a mammalian model. Figure adapted from [269].

Intergenerational epigenetic inheritance or parental effects can be defined as the passage of an epigenetic mark, such as DNA methylation, to future generations, but not to the unexposed generation. Transgenerational effects are characterised by the passage of an epigenetic modification to the unexposed generation in the absence of re-exposure. It is important to distinguish between transgenerational epigenetic effects and parental effects or intergenerational effects. Many parental effects (or in the case of mammalian species for example, grandparental effects) result from direct exposure of individuals or the germ cells that will form a future generation and are not epigenetically inherited [263]. When designing these studies to explore transgenerational epigenetic inheritance considerable care must be taken to ensure that any induced heritable phenotype is truly dependent on passage of epigenetic signatures through the germline to the unexposed generation [262]. When considering environmentally induced effects, transgenerational epigenetic inheritance can only be inferred if the effects last over multiple generations without subsequent re-exposure to the environmental cue of interest. For example, in a pregnant mammal, during environmental exposures, not only is the mother (F0) and foetus (F1) under exposure, but also the foetus's primordial germ cells, which will form the animal's grandchildren (F2). Therefore, in order to conclude that a phenotype or epigenetic mark is transgenerationally inherited, this needs to be observed at least in the F3 generation (Figure 4a) [262]. For fish and non-pregnant mammalian models on the other hand, the initial exposure only results in the exposure of the F0 adult, and its gametes (F1), therefore, in order to demonstrate transgenerational inheritance the effects or marks must be observed in the F2 generation (Figure 4b and Figure 5).

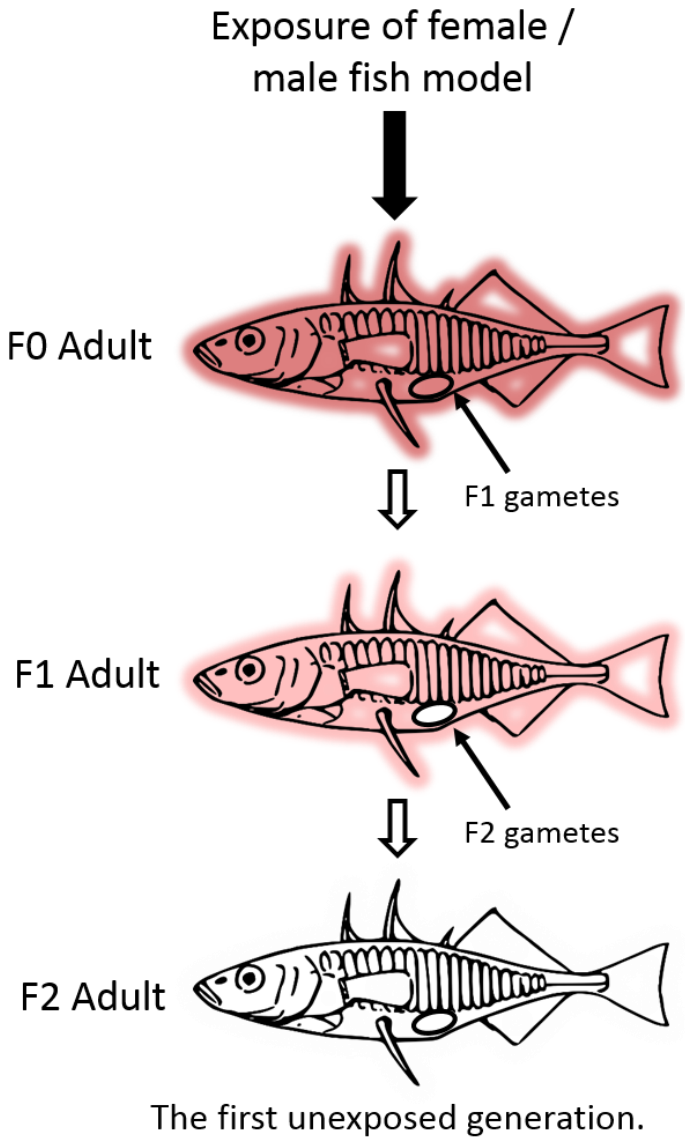

Figure 5. Schematic representation of environmental exposure and affected generations in a fish model.

A key model for exploring epigenetic inheritance is found in the study of the expression of the methylation-sensitive, metastable agouti viable yellow (A^{vy}) allele, which regulates mouse coat colour and shows epigenetic inheritance meiotically modulated by the animal's diet [270]. The specific diet regime fed to pregnant female mice can alter the inheritance pattern over two generations, however this change is not maintained in the third generation, which is essential to constitute transgenerational inheritance [263,271]. Therefore, although still likely mediated at least in part through epigenetic mechanisms, the effects observed are explained only by parental and grandparental effects (direct exposure of an organisms or its germ cells).

In addition, a further study in male rats fed a high fat diet has shown that these sires produce female offspring with a subtle pancreatic phenotype in addition to changes in the expression of a number of genes involved in insulin regulation and glucose metabolism compared with male rats fed a normal diet [272]. The sires were only kept in the same cage as the dams for a few days, and therefore the opportunity for effects to passed to offspring via alternative processes other than the gametes was considered to be minimal [262]. These effects were also associated with a change in DNA methylation at a single CpG site in close proximity to the TSS of the interleukin 13 receptor alpha 2 (*Il13ra2*) in the pancreas and a significant increase in its expression was observed [262]. However, effects were only measured in the F1 offspring, and DNA methylation changes were not reported in the sperm.

It has been proposed that transgenerational epigenetic inheritance is a good candidate mechanism to explain male-line transgenerational effects [261,263,273,274]. For example, a study by Anway *et al*., exposed outbred female rats during pregnancy at the stage of gonadal differentiation to the fungicide vinclozolin, and as a result male offspring were found to

possess abnormal spermatogenesis [93]. This phenotype was reported to pass down the male germline, and epigenetic changes were reported in the sperm, namely 52 different regions with statistically significant altered DNA methylation in promoter regions in sperm cells [275]. This study represents a key example which have been heavily criticised within the scientific community. Anway *et al*., did not test for the possibility that genetic variation could be responsible for the observed changes in spermatogenesis related phenotype, and no effects were observed when this study was repeated in another strain of inbred rats [276]. However, it is important to note, that studies performed in order to replicate this study by Anway *et al*., did not use the same experimental setup, including different rats strains and differing concentrations of vinclozolin.

A further classic example of the potential for transgenerational epigenetic inheritance to explain the passage of a specific phenotype across generation is the Dutch famine [277]. Studies investigating the role of maternal undernutrition during gestation and the associated phenotypic effects including increased metabolic and cardiovascular disease in subsequent generations found that undernutrition of the foetus during critical windows of development may increase the risk of diseases in later life [278].

Studies exploring the role of transgenerational epigenetic inheritance in the passage of a given phenotype across multiple generations in mammalian models, expose females throughout gestation in order to ensure that embryos are exposed during the most sensitive developmental window, for example to target post fertilisation and germ cell epigenetic reprogramming. In fish however, external exposures of eggs directly after fertilisation is possible, therefore the experimental design required to include reprogramming events during exposures is simplified compared to that for mammalian species [259].

There still remains concern regarding adequate experimental design and the elimination of genetic factors in determining a gold standard dataset describing true epigenetic transgenerational inheritance [264,279]. Intergenerational effects (such as parental effects) have been widely reported to occur in a number of models, including fish and mammals. However, although the possibility that epigenetic marks may play a role in transgenerational epigenetic inheritance is an attractive concept, the molecular mechanisms that could mediate these processes remain unclear [280]. It is important the exact mechanism of epigenetic inheritance is defined, and that other potential mechanisms such as genetic variation are excluded, in order to validate that epigenetic inheritance has occurred. In both mammals and fish, efficient epigenetic reprogramming events have been identified, which may leave little chance for the inheritance of epigenetic marks. Therefore, it is likely that if true epigenetic inheritance is identified in mammals or fish, this is likely to be rare [268]. In addition, it is likely that if epigenetic inheritance is viable, critical windows of development, such as during epigenetic reprogramming, will be more sensitive to alterations in epigenetic marks, which may have a consequence for future generations [281].

Table 1. Examples of chemicals reported to result in exposure induced transgenerational epigenetic inheritance. Adapted from [264].

Toxicants	Species	Generation	Proposed mechanism	References
Vinclozolin (agricultural fungicide)	Rat and mouse	F4	DNA methylation	93,275,282
Methoxychlor (agricultural pesticide)	Rat	F4	DNA methylation	93,283
TCDD/dioxin (industrial contaminant)	Rat, mouse, fish	F3	DNA methylation	284–286
Plastics (Bisphenol A, phthalate-DEHP and DBP)	Rat	F3	DNA methylation	285,287,288
Jet fuel [JP8] (hydrocarbon mixture)	Rat	F3	DNA methylation	289
Permethrin and DEET: pesticide and insect repellent	Rat	F3	DNA methylation	290
DDT (pesticide)	Rat	F4	DNA methylation	291
Methylmercury (MeHg)	Fish	F3	DNA methylation	292
Bisphenol A (BPA) (plastic toxicant)	Rat, mouse, fish	F3	DNA methylation	293–295
Phthalates (plastic toxicant)	Rat, zebrafish	F3	DNA methylation	259,296
Tributyltin (industrial toxicant)	Rat	F3	NA	297
High fat diet (nutrition)	Mouse and rat	F2, F3	DNA methylation	298
Caloric restriction (nutrition)	Human, rat, pig, worm, flies	F2, F3	DNA methylation	277,299–303
Smoking (health)	Human	F2, F3	NA	300,304
Increased temperature	Flour moth	F20	piRNAs	305

1.3 Fish models

Model organisms are non-human species that are used in the laboratory and represent vital tools utilised in a broad range of research areas including in ecotoxicology, fundamental biology and human health research. Fish represent a useful model for the assessment of water-borne and sediment deposited pollutants, acting as an advanced warning of the potential risk associated with environmental exposure both for other fish species and other vertebrate organisms. This is evidenced by the fact that they are used in many Organisation for Economic Co-operation and Development (OECD) test guidelines.

Fish are particularly useful models for the study of transgenerational effects, due to the fact that in fish a phenotype or epigenetic mark must only be observed to be maintained to the F2 generation or later, while in mammals it is necessary to observe these effects in the F3 generation (Figures 4 and 5). In addition, embryos can be directly exposed to the stressor of interest during critical periods of development, avoiding the exposure of pregnant mothers. This facilitates the control of the exposure conditions and interpretation of the results.

Each individual fish model presents its own advantaged and disadvantages, therefore model species need to be selected based on a number of key attributes and the specific requirements of the research question being addressed. These may include the ease of culture, ecological relevance, economic importance and existing genomic tools. In this thesis, two model fish species were selected to investigate the transcriptomic and epigenetic mechanisms of chemical toxicity in single, repeated and multigenerational exposure scenarios.

1.3.1 Zebrafish

The zebrafish has been used as a model for a broad range of research, including environmental research and genomics, vertebrate development, disease, behaviour, physiology and as a model for human health research [306]. The zebrafish is one of the most popular model organisms due to its small size, well documented biology, ease of culture, cost-effective husbandry and applicability for environmental toxicity testing [307]. Embryonic development is fast in the zebrafish with the main body structure established within 24 hpf (hours post fertilisation), and within 96 hpf most organs are fully developed [307]. This, together with their transparency, allows zebrafish embryos to be easily screened for phenotypic alterations, making them a useful model system in toxicology.

For epigenetic studies, experiments comprising 3 generations can be established in just 9 months, however, the fast development can cause practical problems for ensuring that the initial exposure begins prior to epigenetic reprogramming. The genome for this species has been sequenced [307], and this is one of the only fish models for which there is considerable epigenomic information in the scientific literature [235,308,309].

Despite the popularity of zebrafish as a model organism, significant knowledge gaps remain regarding the primary mode of sex determination, differentiation and maintenance, and to date no true conserved sex chromosomes have been identified for laboratory strain zebrafish [77,306,310], despite reports that sex is genetically controlled [311]. This is particularly interesting, considering that zebrafish are a popular model for the testing of oestrogenic compounds, which have the potential to cause endocrine disrupting effects. Evidence thus far demonstrates that zebrafish sex determination is polygenic and may require female-dominant genetic factors but genes influencing sex determination may vary depending on

the strain or environmental condition [311,312]. Although the triggers of sex determination in zebrafish are poorly understood, the sex differentiation process and molecular factors regulating this are fairly well studied and are conserved across vertebrates [313–316]. Given its extensive use as a model organism, information in the literature about the sex-specific transcription patterns and promoter DNA methylation of reproductive and epigenetic related genes is relatively scarce. This information is highly relevant to inform on the design and interpretation of studies investigating how reproduction is regulated and how this process is disrupted by environmental stressors.

1.3.2 Three-spined Stickleback

The three-spined stickleback is an environmentally relevant model, inhabiting a wide range of temperate freshwater and marine ecosystems in the Northern Hemisphere [317]. This species is a well-established model organism for environmental toxicology and evolutionary biology, due to its ubiquity in the freshwater and marine temperate environment in the Northern Hemisphere and ease to obtain and maintain in the laboratory. Stickleback are frequently utilised in chemical testing [318]. In addition, there are a number of molecular resources established for the three-spined stickleback, including a sequenced genome and part of the stickleback methylome has been sequenced (using reduced representation bisulfite sequencing) [319,320].

The three-spined stickleback make a good model for transgenerational effect testing, due to the fact that development is slow enough to ensure chemical exposure is conducted prior to epigenetic reprogramming, by starting the exposure at the 1 cell stage (1 hpf). In addition, there are a number of studies already conducted in the stickleback documenting the effects

of copper exposure on both molecular and phenotypic endpoints [129,321–323] that informed the experimental design for the work presented in this thesis.

1.4 General aims and hypotheses of this thesis

Increased understanding of the role of gene function and epigenetic regulation in normal cellular development, particularly in mammalian models, has led to a greater appreciation of the potential for environmental pollutants to mediate their toxic effects via disruption of epi (genetic) processes. Initial data suggests that a number of environmental pollutants may interact with epigenetic processes, and that their disruption could contribute towards toxicity and may modulate differential responses in future exposure scenarios. Evidence also suggests that certain windows of development are particularly sensitive to chemical toxicity mediated via epigenomic modifications, for example during epigenetic reprogramming in early life.

To date, our knowledge about normal epigenetic processes in fish, the potential for chemical pollutants to interact with the epigenome and the potential for exposure to sub-lethal concentrations of chemical pollutants to modulate susceptibility in subsequent exposure scenarios is limited, and these areas represent significant research needs.

The overall aims of this thesis were to investigate the epigenetic and transcriptional regulation of reproduction in fish, and to establish how environmental oestrogenic chemicals (BPA) alter these processes. In addition, this thesis aimed to determine the effects of previous exposure on the susceptibility of fish upon re-exposure both later in life and in subsequent generations, and both when exposures occur during the embryonic

development or in mature fish. These aims were addressed throughout the thesis in individual research papers or manuscripts in preparation with the following specific aims:

1. Empirical chapter 1 (**Chapter 2**): *Sex-specific transcription and methylation profiles for reproductive and epigenetic genes in the livers and gonads of breeding zebrafish.*

The aim of this chapter was to investigate the sex-specific transcription and methylation profiles for reproductive and epigenetic genes in the livers and gonads of breeding zebrafish, an important model organism frequently used in studies of vertebrate development, disease, behaviour, physiology, and as a model for human health research. I hypothesised that transcription and methylation profiles for key genes known to play a role in reproductive processes and in epigenetic pathways may be sexually dimorphic. In this work the transcription of genes involved in epigenetic signalling and reproductive function were quantified, together with global and locus-specific DNA methylation in the livers and gonads of mature males and females.

2. Empirical chapter 2 (**Chapter 3**): *Bisphenol A causes reproductive toxicity, decreases dnmt1 transcription and reduces global DNA methylation in breeding zebrafish.*

The aim of this chapter was to investigate the effects of BPA on reproduction in the zebrafish model and identify epigenetic and transcriptional changes associated with BPA exposure. I hypothesised that BPA is likely to result in disruption to normal reproductive processes, likely via oestrogenic mechanisms. In addition, I hypothesized that exposure to

BPA may be associated with the disruption of epigenetic processes. In this study breeding groups of zebrafish were exposed to BPA for 15 days to determine if reproduction was affected by the exposure. The concentrations tested included environmentally relevant concentrations found world-wide (0.01 mg/L) and at point sources (0.1 mg/L). The highest concentration tested (1 mg/L) has only been reported in landfill leachate and is unlikely to occur in surface waters, but it was included to enable a mechanistic analysis of BPA toxicity. The transcription of genes involved in epigenetic signalling and reproductive function were quantified, together with global and locus-specific DNA methylation in exposed fish.

3. Empirical chapter 3 (**Chapter 4**): *Pre-exposure to copper caused a reduced transcriptional response and increased copper accumulation upon re-exposure in adult male three-spined stickleback (Gasterosteus aculeatus).*

The aim of this chapter was to investigate if prior exposure to copper results in differential susceptibility to subsequent exposures in adult male stickleback. I hypothesised that prior exposure to copper in fish may significantly alter responses in subsequent exposure scenarios, potentially via epigenetic mechanisms. In this chapter, RNA-seq analysis was combined with measures of tissue metal concentration in order to explore the molecular mechanisms underpinning differential responses to copper in the three-spined stickleback. The stickleback was chosen because of its environmental relevance to temperate freshwaters and used for both this chapter and chapter 5 in order to enable the critical comparison of the role of critical windows of development in modulating differential responses to copper in subsequent exposure scenarios.

4. Empirical chapter 4 (**Chapter 5**): *Exposure to copper during embryogenesis caused a differential response to copper in later life and increased tolerance in subsequent generations, in a fish model.*

The aim of this chapter was to investigate if early life exposure to copper results in differential responses in adult stickleback, and differential susceptibility in subsequent generations. I hypothesised that prior exposure to copper during early life may significantly alter responses in later life and in subsequent generations, and that early life exposure may be more likely to result in long term changes in the physiology of the exposed fish compared to adult fish (tested in chapter 4). If this is the case, I hypothesise that such effects may be due to epigenetic alterations during susceptible developmental windows, including during reprogramming. In this chapter, stickleback were exposed to an environmentally relevant concentration of copper from 1-217 hpf in order to ensure that exposure was conducted during critical processes including during epigenetic reprogramming (if present in this species) and ZGA. Fish were them maintained in clean water until the F2 generation. Toxicity tests were performed on a subset of F1 and F2 embryos, and metal accumulation was measured in F0 embryos and F0 adult fish to assess the effects of the developmental exposure on the responses of the same individuals or their progeny upon re-exposure.

As a whole, this thesis addresses critical knowledge gaps regarding the extent to which environmental pollutants may interact with epigenetic signalling pathways, and the

potential for altered epigenetic marks to play a role in differential responses observed in later life, and subsequent generations.

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Chapter 2

Sex-specific transcription and DNA methylation profiles of reproductive and epigenetic associated genes in the livers and gonads of breeding zebrafish.

Under Review
Epigenetics

Sex-specific transcription and DNA methylation profiles of reproductive and epigenetic associated genes in the livers and gonads of breeding zebrafish.

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Abstract:

Reproduction is an essential process for life and is regulated by complex hormone networks and environmental factors. To date, little is known about the contribution of epigenetic mechanisms to the regulation of reproduction, particularly in lower vertebrates. We used the zebrafish (*Danio rerio*) model to investigate the sex-specific transcription and DNA methylation profiles for genes involved in the regulation of reproduction and in epigenetic signalling in the livers and gonads. We found evidence for associations between DNA promotor methylation and transcription for *esr1* (gonads and female livers), *amh* (gonads) and *dnmt1* (livers). In the liver, *esr1* was shown to be significantly over-expressed in females compared to males, and its promoter was significantly hypo-methylated in females compared to males. In the gonads, genes involved in epigenetic processes including *dnmt1, dnmt3 and hdac1* were over-expressed in the ovary compared to the testis. In addition, *dnmt1* and *dnmt3* transcription in the testis was found to be strongly correlated with global DNA methylation. These data provide evidence of the sex-specific epigenetic regulation and transcription of genes involved in reproduction and epigenetic signalling in a commonly used vertebrate model.

Keywords: LUMA assay, pyrosequencing, teleost, gamete, hepatic, germ cells.

Introduction:

Reproduction is essential for species proliferation and a variety of sexual and asexual reproductive strategies have evolved in multicellular organisms. Vertebrates generally reproduce sexually and, for gonochoristic species, undifferentiated gonads differentiate into either ovaries or testis during development. Compared to mammals, where sex determination is controlled by a cascade of molecular events associated with the presence or absence of the sex determining region in the Y chromosome (SRY) [1], many fish species, including the zebrafish, display a greater degree of plasticity with regards to sexual determination and differentiation [2,3]. Recent studies in medaka have shown that *DMY* (the DM-domain gene on the Y chromosome), an SRY homologue, is the key initiator of male masculinisation [4,5]. However, to date this has been the only SRY homologue found in fish, despite evidence for genetic sex determination and differentiation [6,7]. More recently, several studies have demonstrated the involvement of autosomal regions in the involvement of sex determination [5] and downstream pathways regulating sex differentiation and reproductive function are conserved across vertebrates [8–11]. At the level of the gonads, large numbers of transcripts were reported to be differentially expressed between ovaries and testis in a range of vertebrate species including the zebrafish [12,13], rainbow trout (*Oncorhynchus mykiss*) [14] and mouse [15]. Several of these genes have been linked to gonad differentiation including anti-mullerian hormone (*amh*) and sry (sex determining region Y)-box 9a (*sox9a*), both predominantly expressed in the testis, and aromatase (*cyp19a1*), predominantly expressed in the ovaries [13,16].

Although less pronounced, sexual dimorphism in gene transcription has also been reported in other tissues for both mammals and fish, including in the brain, liver, kidney, muscle and

adipose tissue [3,17–20]. In the liver of fish (and other oviparous vertebrates), vitellogenin (*vtg*) and zona radiata proteins (*zrp*) are strongly over-expressed in females and their expression are regulated by oestrogens [21,22]. In addition oestrogen receptor 1 (*esr1*) transcription was found to be greater in the livers of females compared to males in the fathead minnow (*Pimephales promelas*), but in contrast oestrogen receptor 2b (*esr2b*) was significantly higher in males [23].

Besides being regulated by molecular and endocrine factors, sexual differentiation in fish is sensitive to environmental cues, including temperature and oxygen saturation and can be disturbed when fish are exposed to altered environmental conditions resulting in altered sex ratios in fish populations [24,25]. The role of epigenetic processes on both the endogenous and environmental regulation of reproduction is still poorly understood [26], but evidence from a range of vertebrates have demonstrated its importance in some circumstances including in animals exposed to environmental chemicals [27] and increased temperature [3,28].

In mammalian species the SRY gene is normally epigenetically silenced and activated during specific developmental windows [29], and DNA methylation has been associated with sex-linked differential expression of sex steroid hormone related genes in conjunction with histone modifications in human cells [30,31]. In addition, a sex difference in DNA methylation of the oestrogen receptor – alpha (*esr1*) promotor region has been reported in rats, with males exhibiting a greater proportion of DNA methylation in the preoptic area of the brain [32].

Examples of epigenetic regulation of genes involved in reproduction have also been reported in fish species. In the Japanese Flounder (*Paralichthys olivaceus*), the expression of doublesex and Mab-3-related transcription factor 1 (*dmrt1*), a transcription factor involved in sex determination and differentiation in fish, was reported to be 70 times higher in the

ovary compared to the testis in mature fish [33]. The *dmrt1* promotor was found to be predominantly unmethylated in testis cells, while in the ovary the 13 CpG sites measured were found to be relatively hyper-methylated (57.69%). In the same study the expression of *cyp19a1,* an enzyme responsible for catalysing the irreversible conversion of androgen to oestrogens, was found to be 40 times higher in the ovary than in the testis, with the *cyp19a1* promoter being notably hyper-methylated on average across 12 CpG sites in the testis compared to the ovary [33]. This sex related pattern of *cyp19a1* transcription and promoter DNA methylation has also been described in the European sea bass (*Dicentrarchus labrax*) [28]. In this study, elevated temperature resulted in the masculinization of females and was associated with an increase in *cyp19a1* promotor DNA methylation and a decrease in gene expression, suggesting that the temperature-dependent masculinization process involves DNA methylation-mediated control of the *cyp19a1* gene.

The zebrafish is an important model organism frequently used in studies of vertebrate development, disease, behaviour, physiology and as a model for human health research [6]. Given its extensive use as a model organism, information about the sex-specific transcription patterns and promoter DNA methylation of reproductive and epigenetic related genes is highly relevant to inform on the design and interpretation of studies investigating how reproduction is regulated and how this process is disrupted by environmental stressors. To date, little is known regarding the primary mode of sex determination, differentiation and maintenance in the zebrafish, and no true conserved sex chromosomes have been determined for lab strains [6,34,35]. Evidence thus far suggests that zebrafish sex determination is polygenic and may require female-dominant genetic factors,

and genes influencing sex determination may vary depending on the strain or environmental condition [36,37].

The present study aimed to investigate the sex-specific transcription and DNA methylation profiles for reproductive and epigenetic genes in the livers and gonads of breeding zebrafish. To achieve this, we quantified the transcription of genes involved in epigenetic signalling and reproductive function, together with global and locus-specific DNA methylation in the gonads and livers of mature males and females.

Results:

Zebrafish breeding groups of 4 males and 4 females were allowed to breed naturally and reproduction (egg output and proportion of fertilization) was quantified for 25 days. During this period, fish reproduced normally as demonstrated by consistent egg production and fertilization rates (Supplementary Information Figure S1). The gonadosomatic index (GSI; the ratio of gonad weight to body weight) was significantly lower in male fish compared to female fish (0.92 and 6.75 respectively; $P \leq 0.001$; Supplementary Information Figure S2A). The mean hepatosomatic index (HSI; the ratio of liver weight to body weight) in males was significantly lower than for females (1.04 and 3.87 respectively; $P \leq 0.001$; Supplementary Information Figure S2B). There were no significant differences in the condition factors of male and female fish in this study (0.97 and 1.08 respectively; $P = 0.093$).

Sex-Specific Transcription and DNA Methylation Levels in the Gonads

We measured the transcription of target genes of interest using quantitative PCR, locus specific methylation using bisulfite pyrosequencing in selected loci (chosen based on their proximity to the transcription start site (TSS) and binding sites for transcription factors of recognised importance for reproductive function; Figure 1) and global methylation using the LUMA assay.

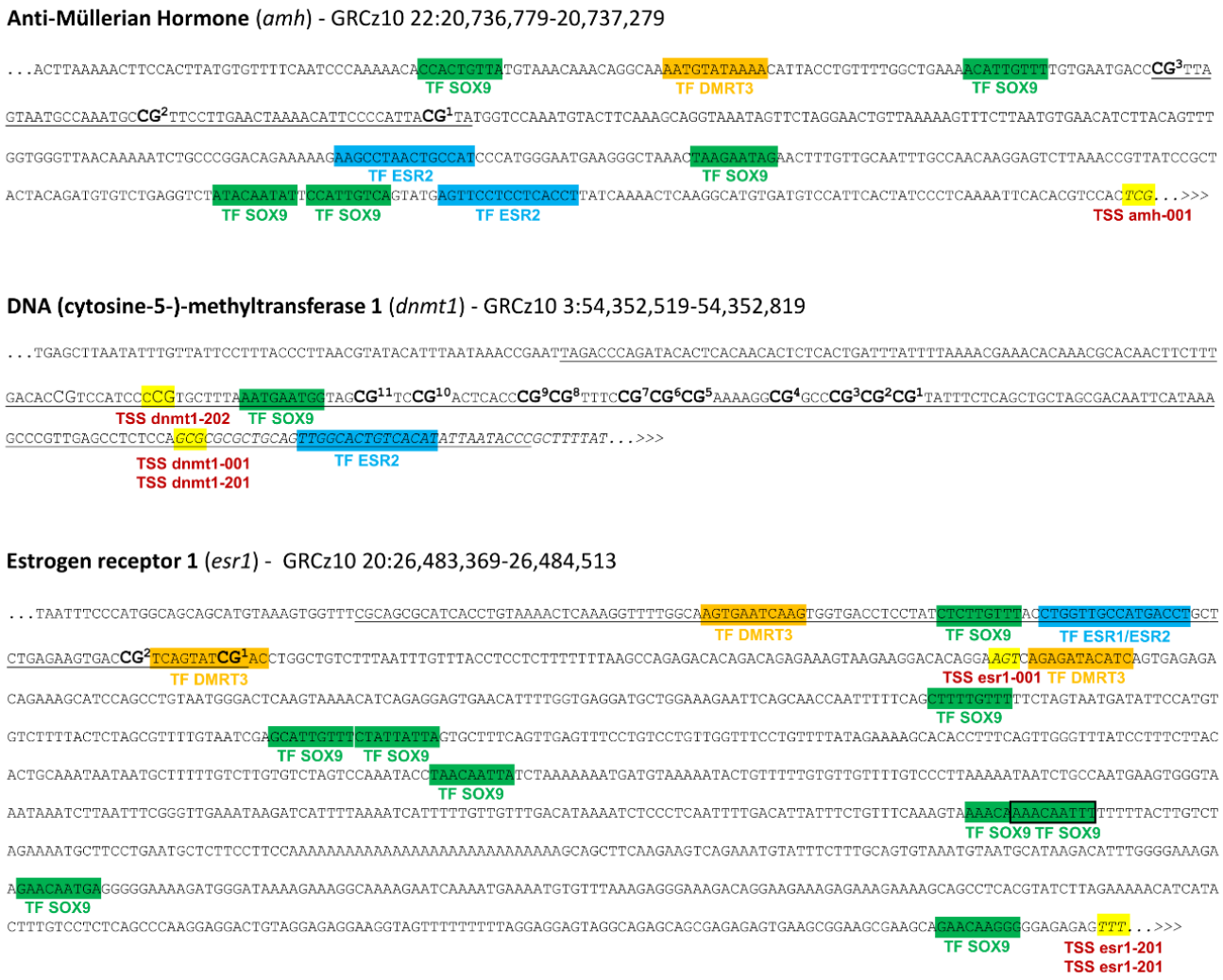

Figure 1. Promoter regions of *amh*, *dnmt1* and *esr1*, showing the location of the CpG sites (indicated in bold), the target sequences used for pyrosequencing (underlined) and putative transcription factor binding sites (EREs, DMRT3, and SOX9; highlighted in blue, green and orange, respectively) in relation to the transcription start sites (TSSs; highlighted in red). The sequences shown were derived from Ensembl Zv9 (release 83; assembly GRCz10) and correspond to the following genomic positions: chr22:20,736,779-20,737,279 (*amh*), chr3:54,352,519-54,352,819 (*dnmt1*) and chr20:26,483,369-26,484,513 (*esr1*).

In the gonads, sex-specific patterns of transcription were identified for oestrogen receptor 1 (*esr1*; 4.66 fold; P = 0.031), *esr2b* (14.75 fold; P = < 0.001), *amh* (21.71 fold; P = < 0.001) and *cyp19a1a* (46.47 fold; P = 0.032) but not for oestrogen receptor 2a (*esr2a*) or the androgen receptor (*ar*; Figure 2A). *esr1* and *cyp19a1a* were predominantly expressed in the ovary, while *esr2b* and *amh* were found to be predominantly expressed in the testis. For transcripts involved in epigenetic regulation, sex-specific patterns of transcription were observed for *dnmt1* (45.46 fold; P = 0.031), *hdac1* (2.96 fold; P = 0.035), *dnmt3* (30.13 fold; P = 0.022), *mecp2* (7.22 fold; P = 0.006), *mbd2* (4.90 fold; P = 0.028) and *mbd3a* (24.10 fold; P = 0.033), where the relative mRNA transcription level for each of these gene was found to be greater in the ovary compared to the testis (Figure 2A).

Mean global DNA methylation in the gonads was significantly higher in males (87.76%) compared to females (82.78%; P ≤ 0.001, Figure 3). Furthermore, in the testis *dnmt1* and *dnmt3* transcription were strongly inversely correlated with global DNA methylation (correlation coefficient = -0.919, P = 0.010 and correlation coefficient = - 0.927, P = 0.003 respectively; Table 1).

In the gonads, sex-specific DNA methylation levels were identified in the promoter region of *esr1* (male = 93.13%, female = 18.95%, P ≤ 0.001, Figure 2B) and *amh* (male = 75.14%, female = 50.88%, P = < 0.001, Figure 2B; DNA methylation levels are reported as an average percentage methylation across all CpG sites measured). However, there was no significant difference in the sex-specific DNA methylation levels for *dnmt1* (Figure 2B) despite the differences in *dnmt1* transcription observed between males and females.

The *esr1* gene transcription was significantly inversely correlated with promoter DNA methylation in the ovary (positions 1 and 2; Table 1) and in the testis (CpG position 1; Table

1). For the *amh* gene, transcription levels were strongly inversely correlated with promotor DNA methylation in the ovary (CpG position 1; Table 1) and the testis (CpG positions 2 and 3; Table 1). The *dnmt1* transcription did not correlate with promotor DNA methylation in the ovary or testis.

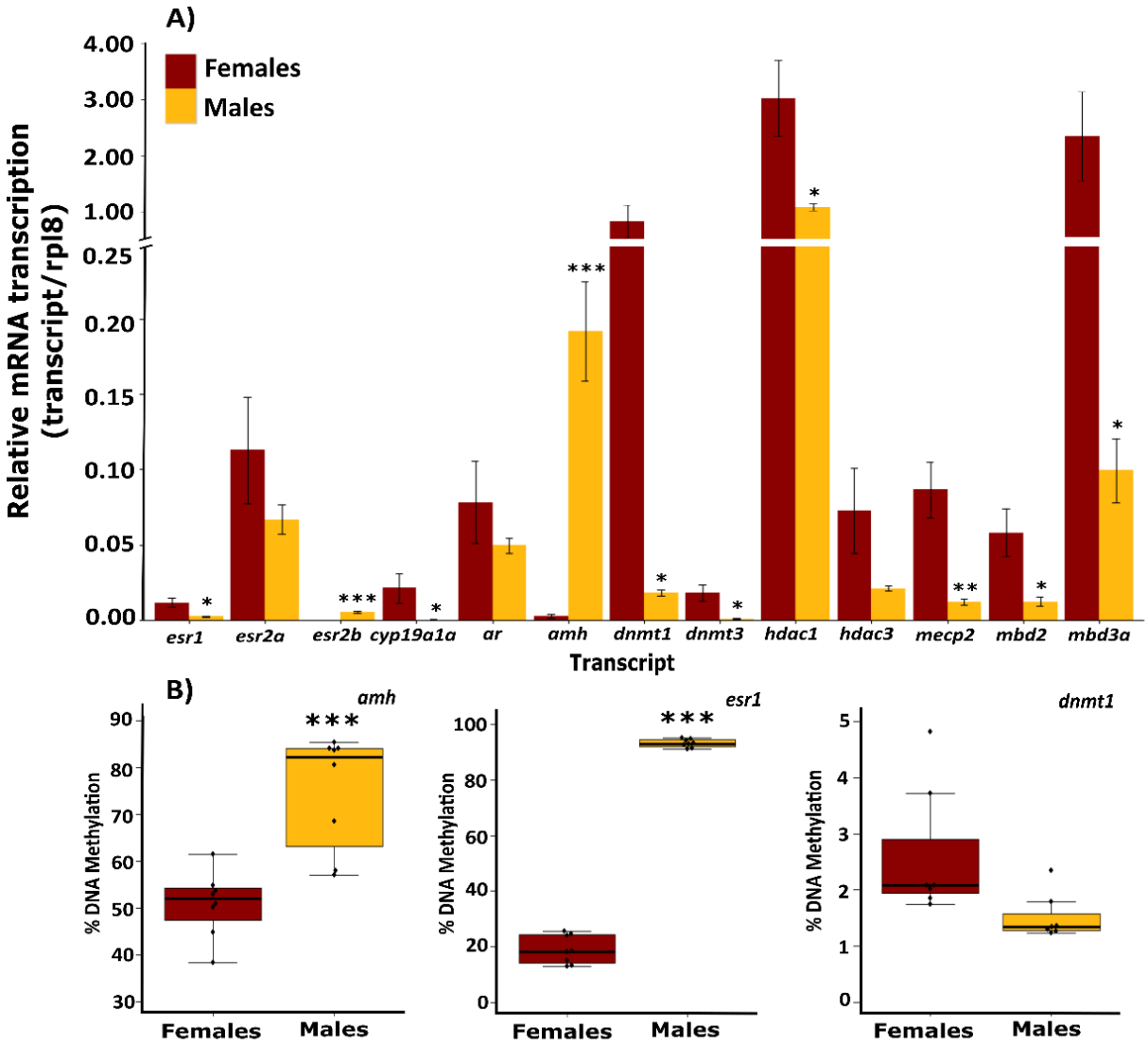

Figure 2. A) Comparison of the relative transcript profiles (target gene transcription/*rpl8* transcription) between females and males in adult zebrafish gonads (n= 6-8 for each group). **B**) Comparison of gene specific average promoter methylation between females and males in adult zebrafish gonads. Data for individual CpG sites are presented in supplementary figure 3. Asterisks indicate significant differences between males and females (Student's t-Test; *P<0.05 **P<0.01 ***P<0.001)

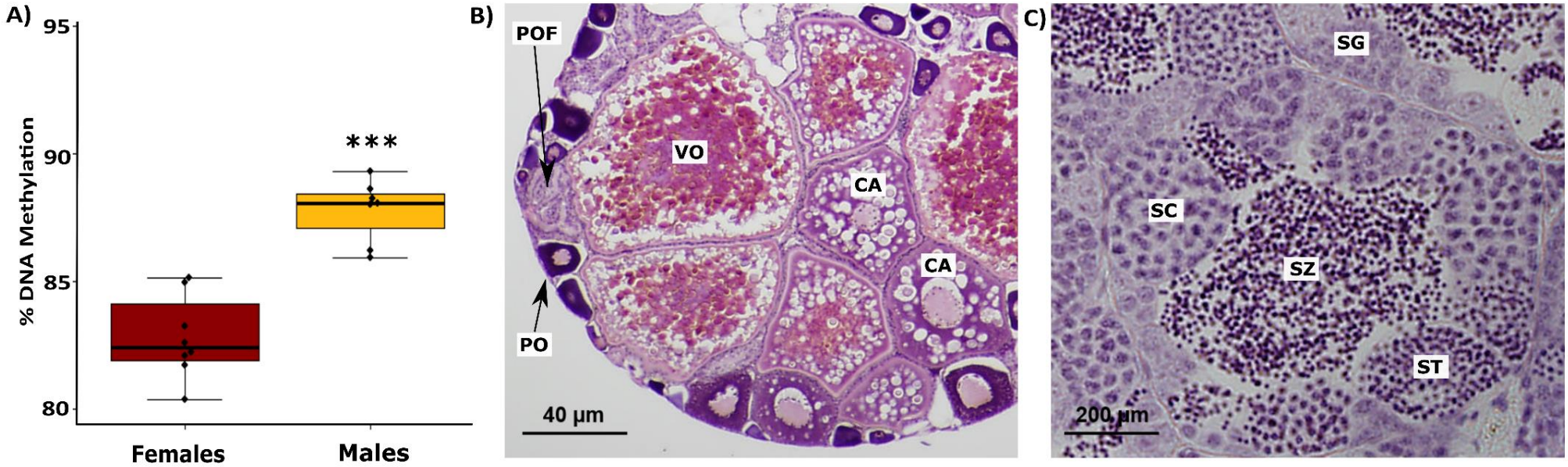
A)
95
90
85
80
% DNA Methylation
***
Females
Males
B)
POF
VO
CA
CA
PO
40 µm
C)
SG
SC
SZ
ST
200 µm

Figure 3. A) Comparison of the proportion of global DNA methylation (measured using the LUMA assay) between ovaries and testis in control adult zebrafish (n = 6-8 for each group). Asterisks indicate significant differences between males and females (Student's t-Test; ***P<0.001). Example of histological sections of zebrafish testicular (**B**) and ovarian tissue (**C**) of mature fish to illustrate the histological structure and proportion of the various cell types in ovaries and testes. The testes section is a normal testes showing different stages of spermatogenesis; SG spermatogonia, SC Spermatocytes, ST Spermatids and SZ spermatozoa. The ovarian section shows a normal ovary with oocytes at different stages of development; POF post ovulatory follicle, CA, Cortical alveoli, VO vitellogenic oocyte and PO primary oocytes. Histological images were obtained from samples analyzed as part of a different experiment but kept under similar husbandry conditions.

Table 1A. Correlation analysis between transcript expression and global methylation for *dnmt1* and *dnmt3*.				
Tissue	**Gene**	**-**	**Correlation coefficient**	P value
Testis	*dnmt1*	-	-0.919	**0.010**
Ovary	*dnmt1*	-	-0.225	0.668
Testis	*dnmt3*	-	-0.927	**0.003**
Ovary	*dnmt3*	-	0.052	0.934
Table 1B. Correlation analysis between transcript expression and specific CpG loci methylation.				
Tissue	**Gene**	**CpG Position**	**Correlation coefficient**	P value
Ovary	*esr1*	1	-0.765	**0.045**
		2	-0.470	**0.029**
	amh	1	-0.271	0.659
		2	-0.957	**0.017**
		3	-0.547	0.341
	dnmt1	1	-0.584	0.224
		2	-0.573	0.234
		3	-0.580	0.227
		4	-0.540	0.269
		5	-0.582	0.225
		6	-0.552	0.256
		7	-0.603	0.206
		8	-0.625	0.160
		9	-0.701	0.121
		10	-0.625	0.185
		11	-0.461	0.358
		Mean	-0.148	0.779
Testis	*esr1*	1	-0.875	**0.004**
		2	-0.697	0.055
	amh	1	-0.072	0.878
		2	-0.818	**0.025**
		3	-0.920	**0.003**
	dnmt1	1	0.526	0.284
		2	0.522	0.288
		3	0.525	0.285
		4	0.509	0.302
		5	0.503	0.309
		6	0.447	0.374
		7	0.461	0.358
		8	0.449	0.371
		9	0.532	0.277

		10	0.599	0.401
		11	0.484	0.516
		Mean	0.071	0.893
Liver Females	*esr1*	1	-0.971	**0.006**
		2	-0.906	**0.034**
	dnmt1	1	-0.761	0.079
		2	-0.686	0.132
		3	-0.751	0.085
		4	-0.268	0.663
		5	-0.912	**0.011**
		6	-0.736	0.993
		7	-0.813	**0.049**
		8	-0.689	0.199
		9	-0.717	0.173
		10	-0.836	**0.038**
		11	-0.758	0.080
		Mean	-0.843	**0.035**
Liver Males	*esr1*	1	-0.065	0.878
		2	-0.268	0.522
	dnmt1	1	-0.736	0.095
		2	-0.818	**0.047**
		3	-0.901	**0.014**
		4	-0.534	0.275
		5	0.431	0.394
		6	-0.843	0.352
		7	-0.683	0.135
		8	-0.669	0.146
		9	-0.826	**0.043**
		10	-0.683	0.135
		11	-0.847	**0.034**
		Mean	-0.872	**0.024**

Table 1. Relationships between transcription and global methylation for *dnmt1* and *dnmt3* (**A**) and gene transcription and promoter CpG loci methylation for specific target genes (**B**). Red shading indicates significant correlations ($P < 0.05$).

Sex-Specific Transcription and DNA Methylation Levels in the Liver

In the liver, at the transcriptional level, sex-specific differences were identified for a number of transcripts involved in reproductive function including the transcripts encoding vitellogenin (*vtg1*; 1322.96 fold; $P \leq 0.001$; Figure 4A), *esr1* (8 fold; $P \leq 0.001$; Figure 4B), and *esr2a* (2.8 fold; $P = 0.012$; Figure 4B), but not for *esr2b* (Figure 4A). *vtg1* (Figure 4A) and *esr1* (Figure 4B) were found to be predominantly expressed in ovarian tissue compared to the testis, while *esr2a* was found to be predominantly expressed in the testis (Figure 4B). For transcripts involved in epigenetic regulation, differences in transcription between males and females were identified only for *hdac1* ($P \leq 0.001$; over-expressed in males; Figure 4A).

Analysis of DNA methylation in the promoter region for *esr1* revealed consistently higher average DNA methylation at 2 CpG sites in males compared to females (male = 13.17%, female = 9.03%, $P = 0.013$, Figure 4C), but no significant differences in methylation were detected in the promoter region of *dnmt1*.

The transcription of *esr1* was significantly inversely correlated with its promoter DNA methylation in the liver of female fish (CpG positions 1 and 2; Table 1). In addition, for the *dnmt1* gene, transcription was significantly inversely correlated with promotor DNA methylation both in the livers of females (CpG positions 5, 7 and 10; Table 1) and males (CpG positions 2, 3, 9 and 11; Table 1).

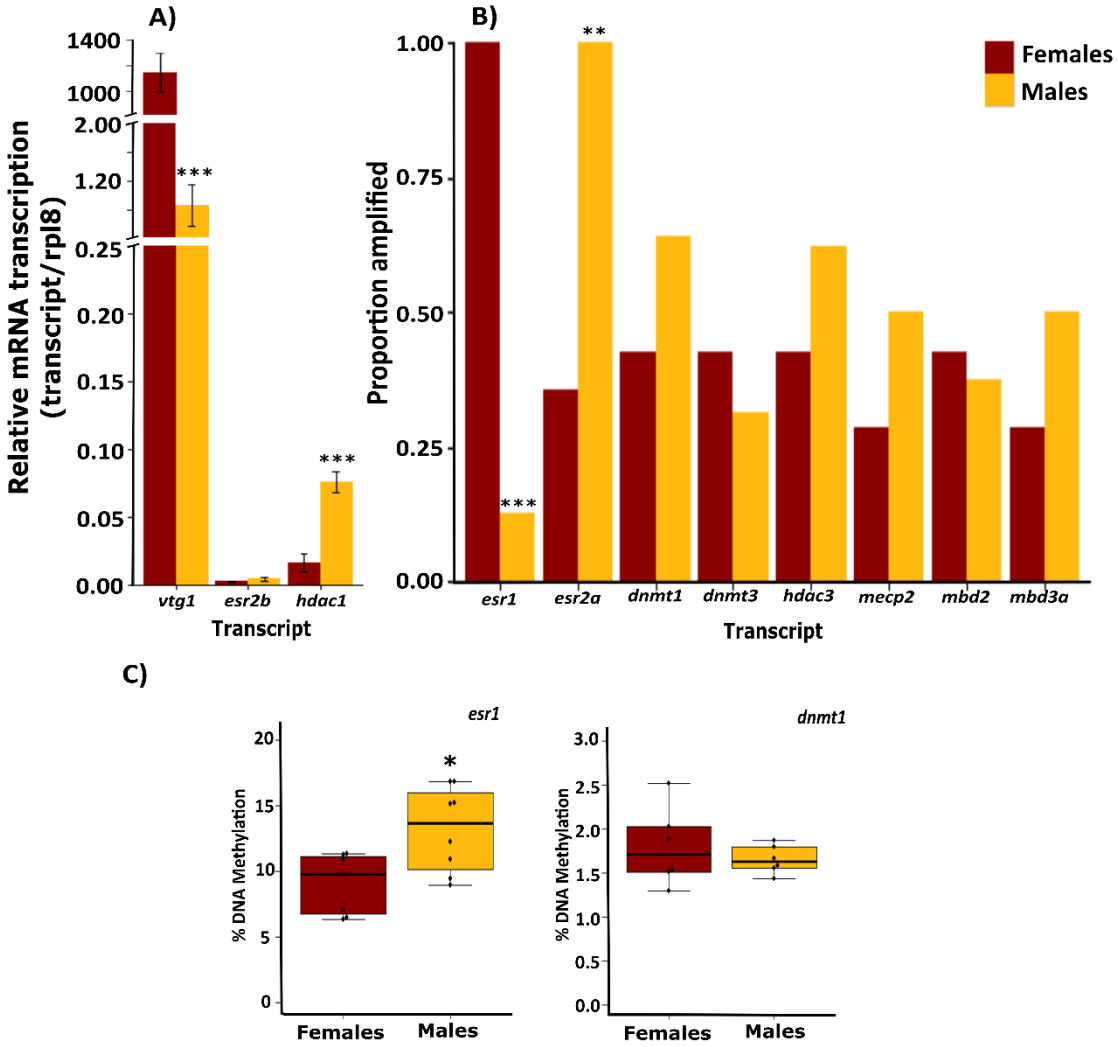

Figure 4. A and **B**) Comparison of the relative transcript profiles (target gene transcription/*rpl8* transcription) between females and males in adult zebrafish livers (n= 6-8 for each group). For each gene of interest, where amplification was detected in more than 70% individuals in both sexes, data are represented as relative expression compared to *rpl8*. Where amplification was detected in less than 70% of the individuals of one or both sexes, data are presented as the proportion of individuals for which the target genes of interest were detected**. C**) Comparison of gene specific average promoter methylation between females and males in adult zebrafish gonads livers. Data for individual CpG sites are presented in supplementary figure 3. Asterisks indicate significant differences between males and females (Student's t-Test; *P<0.05 **P<0.01 ***P<0.001)

Discussion:

A wealth of information exists about zebrafish reproductive biology at the physiological and molecular levels, but little is known about the role of epigenetic regulation on reproductive function in adult tissues. Here we report for the first time sexually dimorphic DNA methylation profiles, and associations between transcription and DNA methylation in the promoters of *esr1*, *amh* and *dnmt1*, providing a valuable contribution to our understanding of the epigenetic regulation of reproduction in this important model organism.

Sex-Specific Transcription and DNA Methylation in the Gonads

Comparisons of global DNA methylation between males and females revealed that the DNA in the testes was significantly hyper-methylated compared to that in the ovaries. The relative proportion of germ cells to somatic cells in oviparous animals differs in the male and female gonads, with testis containing a far greater proportion of maturing germ cells, including sperm, compared to ovaries, which contain large oocytes surrounded by many somatic cells [38–40]. The differences in global DNA methylation between testes and ovaries could be due to the differences in cell type composition between these two organs and in particular to the high proportion of maturing gametes, including mature sperm which are known to be hyper-methylated across vertebrates [41,42].

In the gonads, *amh* was over-expressed in the testis compared to the ovary, as previously reported in fish [3,43] and other vertebrates [44] where *amh* is known to play an important role in testis development. Studies in zebrafish have associated the upregulation of *amh* with the gonadal transcriptional profile in heat-induced masculinization of female fish [3]. In the ovary,

amh is expressed in granulosa cells and is thought to have multiple functions including the regulation of germ cell proliferation and follicular development [45], while in males *amh* is expressed by Sertoli cells, and inhibits the development of the Müllerian ducts during development and maintains the differentiation of the gonads in adult fish [43]. Comparisons between the mean gonadal DNA methylation levels in the 5' flanking region of *amh* revealed that the transcription of this gene and the level of DNA methylation measured in its promotor region were significantly inversely correlated both in testicular and ovarian tissues. DNA methylation of CpG sites located in gene promotors has previously been associated with the regulation of transcript expression in a number of organisms, suggesting that for *amh,* transcription may be influenced by DNA methylation in this region of the promotor [46].

In addition, the *amh* promotor region analysed was significantly hyper-methylated by 24.26% in testes compared to ovaries, despite the fact that transcription of *amh* is higher in males. We hypothesise that this may be due to the low proportion of cells expressing *amh* (Sertoli cells), compared to germ cells in the testis, which would have masked the DNA methylation profiles present in Sertoli cells. This result highlights the issues of conducting epigenetic studies in tissues with multiple cell types, each with unique DNA methylation patterns, where changes in DNA methylation on a specific cell type may not reflect the dynamics of the methylome in other cell types.

Comparisons between the relative expression levels for the three oestrogen receptor subtypes show that the predominantly expressed receptor in both males and females was *esr2a*, but no differences were observed between the sexes. In contrast, for *esr1*, there was a sex specific pattern of transcription and DNA methylation; we found hyper-methylation of

the 5' flanking region of the *esr1* gene in the testis (93.13%) compared to the ovary (18.95%). We also observed that *esr1 was* predominantly expressed in the ovaries, similar to previous reports in adult *Oryzias latipes* [47] and *Pimephales promelas* [23], associated with the important role of *esr1* in oestrogen signalling in females. In addition, for both ovarian and testicular tissues, *esr1* expression was found to be significantly inversely correlated with promotor DNA methylation, suggesting relative hypo-methylation of CpG sites in the promoter may be associated with elevated transcription for this gene.

It is important to note that the very prominent hyper-methylation of the targeted *esr1* promotor region reported in testicular tissue is potentially associated with the proportion of germ cells, including mature sperm, within the testis in addition to specific regulation within cell types where *esr1* is expressed [41,42].

In contrast to that observed for *esr1*, *esr2b* was overexpressed in the testis, similarly to previous reports in the Korean rockfish (*Sebastes schlegeli)* [48]. Our findings support the hypothesis that *esr2b* may play a role in mediating the effects of oestrogen on testicular function and spermatogenesis in fish, as previously proposed for another cyprinid species [23].

In ovaries, we found significant overexpression of *cyp19a1a*, as previously reported in a number of species including in *Perca flavescens* and *Pimephales promelas* [49,50]. Aromatase is responsible for the irreversible conversion of androgens into oestrogens and it is fundamental for female sex differentiation and development, with its inhibition resulting in masculinization of the population [28,51], accounting for its overexpression in ovaries (granulosa cells) compared to testes. For example, a study in zebrafish associated the downregulation of *cyp19a1a* with the heat-induced masculinization of female fish [3].

For the transcripts involved in epigenetic pathways, in the gonads, sex-specific levels of transcription were observed for six of the seven transcripts measured, and all were significantly overexpressed in ovaries compared to testes. These findings suggest an important role for these transcripts during oogenesis and/or during embryonic development. It is important to note that sperm cells contain very little cytoplasm, and within this, few transcripts are stored and delivered to the embryo during fertilization [52,53]. In contrast, oocytes contain large reserves of maternal transcripts that support embryo development prior to the zygotic genome activation (ZGA), with many maternal transcripts playing important roles well beyond this point [54,55]. In zebrafish, ZGA occurs at approximately the 1,000 cell blastula stage, much later in development than in mammalian species including mice (~2 cell) or humans (~4–8 cell) [41,56–58]. Therefore, in comparison to mammalian models, zebrafish are likely to be significantly more dependent on maternal transcripts to support the critical early stages of embryo development. We hypothesize that, in addition to their role within somatic and germ cells in the gonads, many of these transcripts involved in epigenetic regulation which we have shown to be overexpressed in ovaries, may be related to their role as maternal transcripts during embryo development, and are potentially involved in the dynamics of demethylation and re-methylation occurring prior to ZGA, as well as histone remodelling, occurring during this period [41,42]. This hypothesis is supported by the fact that several of these transcripts were reported to be strongly expressed during early embryo development prior to ZGA which begins during the maternal-to-zygotic transition as the embryo enters the mid blastula transition (reviewed in [55]).

In the gonads, there were no significant differences in the DNA methylation patterns for *dnmt1*, despite the fact that this gene was significantly overexpressed in ovaries at the transcript level. However, *the* expression of *dnmt1* and *dnmt3 was* significantly inversely correlated with global DNA methylation in the testis, supporting the idea that these enzymes may be important in regulating global DNA methylation levels [59]. For example; the expression of *dnmt1* has been associated with changes in global DNA methylation, and inactivation of *dnmt1* has been shown to cause global demethylation of the genome [59]. In addition, it has been demonstrated that *dnmt3* is important in the maintenance of DNA methylation patterns during *de novo* methylation processes [41,60]. The mean promoter DNA methylation of the 11 CpG sites measured for *dnmt1* was very low, 1.52% and 2.62% for males and females, respectively. It is important to note, that at this level of methylation the detection accuracy of the Pyrosequencer is compromised. Previous studies have reported little correlation between transcription and DNA hypomethylation, suggesting that hypo-methylated promoters create a transcriptionally permissive state [55], with no predictive value on gene activation [61]. The fact that the promoter of *dnmt1* was found to be hypo-methylated indicates that it was likely available to be regulated by other mechanisms including transcription factors, histone recruitment and modifications (reviewed in [55]). In addition to repression of transcription through DNA hyper-methylation, genes may be blocked indirectly through the recruitment of methyl-binding proteins or methyl-CpG-binding domain proteins, and these in turn may recruit co-repressors such as histone deacetylases [62]. Interestingly, in this study *hdac1* was the most highly expressed gene of those we studied in the gonads and was significantly over-expressed in ovarian tissue. *Mecp2, mbd2* and *mdb3a* were also significantly over-expressed in ovarian tissue compared to the testis; therefore, it is possible that the transcription of *dnmt1* in the ovaries may be

regulated indirectly through histone modifications or the recruitment of methyl-CpG-binding domain proteins.

Sex-Specific Transcriptional and DNA Methylation in the Liver

We found significant over-expression of the transcripts encoding *esr1* and *vtg1* in female livers, likely associated with the role of these genes in vitellogenesis [23]. In parallel, the 5' flanking region of the *esr1* gene was hyper-methylated in male compared to female livers. The transcription for *esr1* was found to be significantly inversely correlated with *esr1* promotor DNA methylation in the livers of female fish, suggesting that the level of promoter DNA methylation contributes to regulation of transcription for this gene. In contrast, the transcript encoding *esr2a* was over-expressed in male livers compared to females. This oestrogen receptor subtype is thought to be responsible for maintaining basal *esr1* levels and to act as an adjustment mechanism for oestrogen function [63,64]. The *esr2a* transcript profile levels are therefore variable and have been reported to be higher either in females [49] or in males [23,65].

For transcripts involved in epigenetic regulation, one gene (*hdac1*) was differentially expressed between males and females in the liver and was over-expressed in males. The significance of this finding is unknown, and to our knowledge this is the first time that a sex specific transcription pattern for this gene is reported for hepatic tissue. For *dnmt1*, significant inverse correlations were found between transcription and promotor DNA methylation in both male and female livers, suggesting that the DNA methylation present on the promotor of this gene may play a role in the regulation of its transcription in this tissue,

even though the promoter region analysed was strongly hypo-methylated, and therefore likely regulated by other mechanisms of transcriptional regulation. This demonstrates the importance of DNA methylation of the promoter region analysed for the functional regulation of this gene.

Given the functional role of the hepatic tissue in sexually mature females, including the production of vitellogenins and chorion proteins that are incorporated into developing oocytes, the physiology of the liver in females differs from that of males. This is clearly reflected in the higher hepatosomatic index of females compared to males and it is possible that the proportion and volume of hepatocytes compared to other cell types within the liver vary between males and females. If this is the case, this could also contribute to the differences in transcription and promoter DNA methylation observed between the livers of males and females.

There are some limitations to the methodologies used in this study: the locus- specific DNA methylation measurements were conducted only on a small number of CpG sites (2-11), within the regulatory regions of select target genes, hypothesized to be play important roles in the regulation of reproduction. CpG sites were chosen based on their proximity to the TSSs of the genes of interest and putative binding sites for transcription factors known to be key regulators of reproduction, including oestrogen-responsive elements (EREs). This analysis therefore does not provide a comprehensive view of the changes of methylation potentially occurring at other CpG sites, including those within gene bodies and those which may be important for the regulation of splicing [66].

In the future, we advocate that studies should perform measurements of transcription and methylation at the genome wide level at the various stages of sex development, and ideally

on isolated populations of cells to avoid the cellular heterogeneity of complex tissues, to better understand the potential for DNA methylation to play a role in transcriptional regulation and contribute to the establishment and maintenance of sexual dimorphism in zebrafish.

Conclusions

We provide evidence for sexual dimorphism in transcription and DNA methylation profiles in the livers and gonads of an important model organism. We report novel evidence for associations between DNA promotor methylation and transcription for *esr1, amh* and *dnmt1,* and between *dnmt1* and *dnmt3* transcription and global DNA methylation in the gonads. In addition, we document for the first time a significant overexpression of a group of genes involved in DNA and histone modifications in ovaries compared to testis, likely associated with their role as maternal transcripts to support embryo development. In addition, our data highlight critical considerations for investigating epigenetics in multicellular tissues, where each cell population is characterized by its own unique epigenetic signature.

Materials and Methods:

Fish husbandry

Adult wild-type WIK strain zebrafish (originating from a stock population at the University of Exeter) were maintained according to conditions reported by Paull and colleagues [67,68]. Mains tap water was filtered by reverse osmosis and reconstituted with Analar-grade mineral salts. Water was then heated to 28°C in a reservoir and aerated before it was supplied to each aquarium via a flow-through system. Preceding the start of the experiment, fish were allocated randomly into breeding groups (4 males and 4 females). These groups were then kept in individual 15 L tanks and allowed to breed naturally. Tanks were supplied with a flow rate of 48 L/day with constant aeration and maintained at 28 ± 0.5°C and pH 7-7.5. Fish were kept under a 12h light:dark cycle, including dawn and dusk transition periods of 30 minutes and were fed live *Artemia nauplii* once daily (ZM Premium Grade Artemia; ZM Ltd.) and TetraMin tropical flake food (Tetra; Melle, Germany) twice daily, to satiation [27]. All experiments were approved by the University of Exeter Ethics committee and conducted under approved protocols according to the UK Home Office regulations for use of animals in scientific procedures.

Initially, reproduction was monitored in several colonies and after a 10-day acclimation period, breeding groups that failed to spawn consistently were removed from the experiment. Reproduction (number of eggs produced per female, % of fertilization) was monitored for a total of 25 days on the two breeding groups selected for this study. At the end of this period fish were sacrificed humanely according to UK Home Office regulations, the fork length and weight were measured; the gonads and livers were dissected, weighted, immediately frozen in liquid nitrogen and stored at -80°C for molecular analysis. The

gonadosomatic index (GSI) = gonad weight (mg)/[total weight (mg)- gonad weight (mg)] x 100, hepatosomatic index (HSI) = liver weight (mg)/[total weight (mg)- liver weight (mg)] x 100 and the condition factor (k) = [weight (g) x 100]/[fork length (cm)]^3 were calculated for each fish.

DNA and RNA isolation

RNA and DNA were extracted from the livers and gonads of 8 male and 8 female fish using the AllPrep DNA/RNA Micro Qiagen Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extraction of both RNA and DNA was performed from the same tissue sample to allow for comparisons of the DNA methylation and transcription for the same gene within the same individual. In order to assess RNA and DNA purity and concentration, samples were analysed using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Transcript profiling

Transcript profiling of genes encoding epigenetic regulatory proteins (DNA (cytosine-5)-methyltransferase 1 (*dnmt1*), DNA (cytosine-5)-methyltransferase 3 (*dnmt3*), histone deacetylase 1 (*hdac1*), histone deacetylase 3 (*hdac3*), methyl-CpG-binding protein 2 (*mecp2*), methyl-CpG-binding domain protein 2 (*mbd2*) and methyl-CpG-binding domain protein 3a (*mbd3a*)) and genes involved in reproductive processes (aromatase (*cyp19a1a*), oestrogen receptor 1 (*esr1*), oestrogen receptor 2a (*esr2a*), oestrogen receptor 2b (*esr2b*), androgen receptor (*ar*), anti-Mullerian hormone (*amh*), and vitellogenin (*vtg*)) was

conducted using real-time quantitative PCR (RT-QPCR) as previously described [27]. Primers for each target gene were designed using Beacon Designer 3.0 software (Premier Biosoft International, Paulo Alto, CA) and using zebrafish NCBI RefSeq sequences. Primers were purchased from MWG-Biotech (Ebersburg, Germany). Assays were optimized for each transcript and standard curves were generated as previously described [69]. Primer specificity was confirmed by the observation of a single amplification product of the expected melting temperature throughout the range of detection of the assays. The linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was > 0.99 in each case, and efficiencies were between 1.86 - 2.24. The primer sequences, annealing temperatures, PCR product sizes and PCR efficiencies for each primer pair were previously described in Laing et al., 2016 and are shown in Supplementary Information Table S1.

RNA was treated with DNase I (Qiagen) to remove any potential DNA contamination. 2 µg of total RNA was then converted to cDNA using random hexamers (MWG-Biotech, Ebersberg, Germany) and M-MLV reverse transcriptase (Promega, Madison, USA), according to manufacturer's instructions. cDNA was then diluted 1:2 and RT-QPCR was performed in duplicate using an iCycler iQ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA) and SYBR Green chemistry as previously described [27]. A template-minus negative control was run in duplicate to verify the absence of contamination on each plate. Efficiency-corrected relative expression levels were determined using the ΔΔ Ct method [70]. Data were normalized to the control gene, ribosomal protein l8 (*rpl8*), shown to have stable expression in the livers and gonads in another cyprinid fish species [23,71].

Bisulfite-PCR-Pyrosequencing

The sequences of promoter regions of *esr1*, *amh* and *dnmt1* were obtained from Ensembl (release 83; Cunningham et al 2015) [72] using the Biomart portal [73]. Zebrafish *esr1* (ENSDARG00000004111) has three known transcripts (*esr1*-001 (3449 bp), *esr1*-201 (3502 bp) and *esr1*-202 (212 bp)) and two TSSs. The *dnmt1* gene (ENSDARG00000030756) also has two TSSs and three transcripts (*dnmt1*-001 (4896 bp), *dnmt1*-201 (4893 bp) and *dnmt1*-202 (5031 bp)). *amh* (ENSDARG00000014357) has one transcript (*amh*-001, 3243 bp) and one TSS (Figure 1). The promoter regions were screened for the presence of putative binding sites for transcription factors known to be involved in reproduction, including oestrogen-responsive elements (EREs), Dmrt3 and Sox9 using JASPAR [74], and the matrix models ESR1 (MA0112), ESR2 (MA0258), DMRT3 (MA0610) and SOX9 (MA0077). PCR and Pyrosequencing assays were designed using the PyroMark Assay design software (Qiagen, Hilden, Germany). Pyrosequencing primers and their corresponding target sequences were previously described in Laing et al., 2016 [27] and are shown in Supplementary Information Table S2.

Genomic DNA (500 ng) was treated with sodium bisulfite using the EZ-96 DNA Methylation-Gold Kit (Zymo Research, CA, USA) according to the manufacturers' standard protocol. Template preparation and pyrosequencing in a Qiagen Pyromark Q24 pyrosequencer was carried out as described by Tost and Gut (2007) [75] on bisulfite-treated DNA from the gonads and liver of 8 individual fish per treatment group. In this technique, the degree of methylation at each CpG position in a sequence is determined from the ratio of T and C and the analytical sensitivity is approximately 5%–10% for individual CpG dinucleotides [76]. To verify the absence of DNA contamination, negative controls were run in duplicate. Bisulfite-PCR amplification was performed in duplicate using the primers and assay conditions

provided in Supplementary Information Table S2. In order to confirm primer specificity for bisulfite-modified DNA, unmodified DNA samples were included during primer optimization. For figures 1-3, data are presented as an average of CpG sites within each amplicon.

Luminometric-Based Assay (LUMA) for Global DNA Methylation

The LUMA assay was performed using DNA extracted from gonad samples from 8 individual fish per sex, as described by Karimi and colleagues [77]. Analyses of global DNA methylation were conducted only for gonad samples since sufficient quantities of DNA were not available to perform the LUMA assay in liver samples. 250ng of each DNA sample were digested in duplicate with both HpaII and MspI, and data were normalized to the EcoRI peak to account for any technical differences between samples [78]. HpaII and MspI are restriction endonucleases which are sensitive and insensitive to CpG methylation in the sequence CCGG respectively while EcoRI is included in all reactions as a normalization reference. Global DNA methylation values were calculated according to the formula (HpaII(G)/EcoRI(T))/(MspI(G)/EcoRI(T)), where G and T refer to the peak heights for HpaII or MspI (DNA methylation) and EcoRI (input DNA), respectively.

Statistical analysis

Statistical analyses were carried out using R (version 3.0.2) [79]. Data were tested for equal variance and for normality using the Shapiro–Wilk test prior to analysis. Comparisons between male and female groups were performed using the Student's t-Test. P values of ≤0.05 were considered to be significant. All data are presented as mean ± SEM.

For transcript profiles, data points classified as outliers (using Chauvenet's criterion) and data points for which the expression was below the assay detection limit were excluded from analysis. Chauvenet's criterion is used to calculate a probability band based on the mean of a normally distributed data set. Any data points that lie outside this calculated probability band are considered to be outliers, and are subsequently removed from the data set. A new mean and standard deviation based on the remaining values and new sample size can then be calculated. Where for both of the sexes amplification was detected in more than 70% of individuals, data were represented as relative expression. Where amplification was detected in less than 70% of the individuals from one or more of the sexes, data were represented as the proportion of individuals for which the target genes were detected.

In order to determine if there were associations between the DNA methylation levels for specific loci in the promoter regions of genes of interest and their transcription, correlation analysis was conducted. Pearson correlation was used when data was normally distributed, and where data did not meet the assumptions of parametric testing, Spearman correlation analysis was performed. Correlation analyses were also conducted to determine the relationship between global DNA methylation and transcription for *dnmt1* and *dnmt3*, as above.

All graphs were created using the R packages ggplot2 [80], gplots [81], beeswarm [82] and ggbiplot [83].

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Author Contributions:

LVL and EMS conceived and designed the experiments. LVL, JV, ED, TMUW, JM, RVA and EMS performed the experiments. LVL, JM & EMS analysed data generated from these experiments. EMS, JV, EM, TMUW, RVA, and JM provided training and supervision throughout the project. LVL wrote the first version of the manuscript. The manuscript was written through contributions of all authors and all authors have given approval to the final version of the manuscript.

Additional Information

The authors declare no competing financial interest.

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Supporting Information

Sex-specific transcription and DNA methylation profiles of reproductive and epigenetic associated genes in the livers and gonads of breeding zebrafish.

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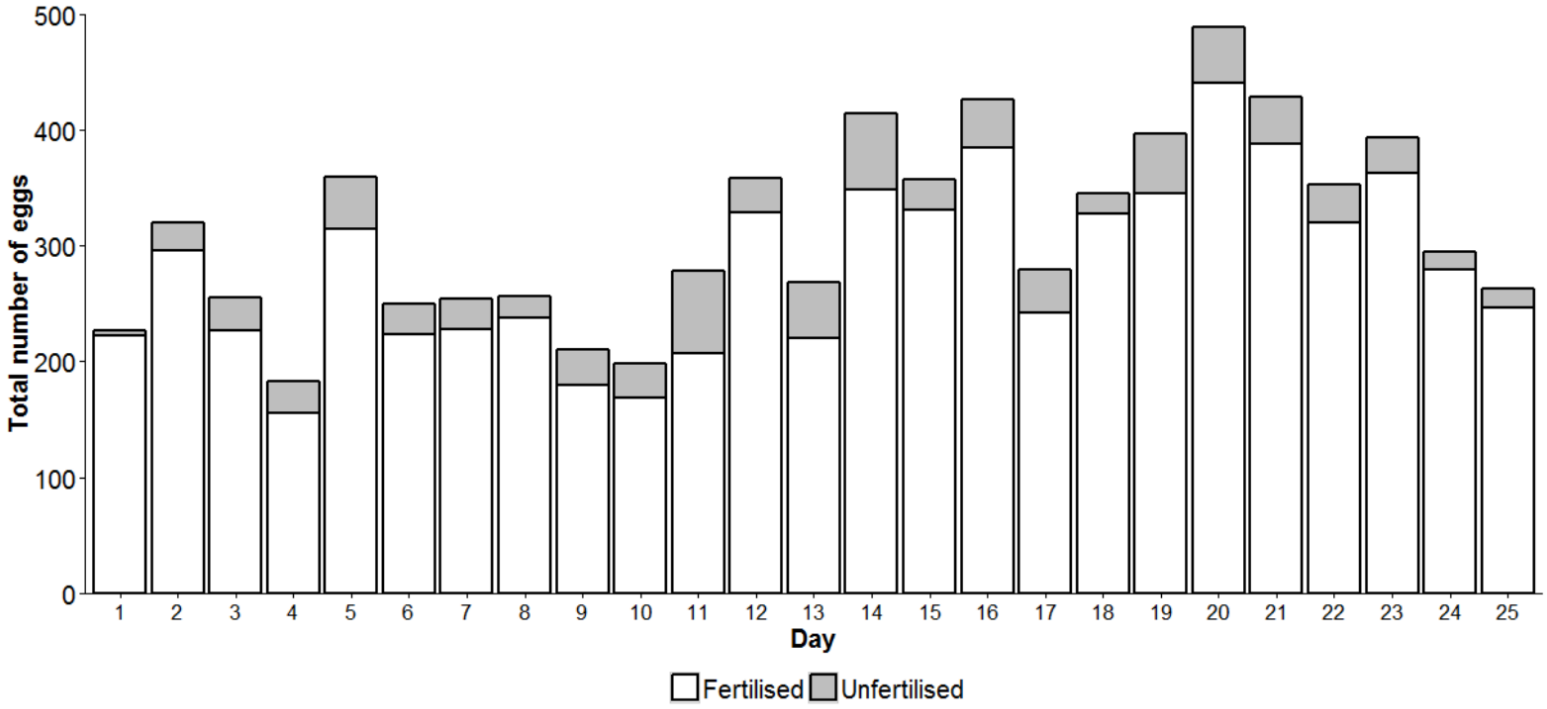
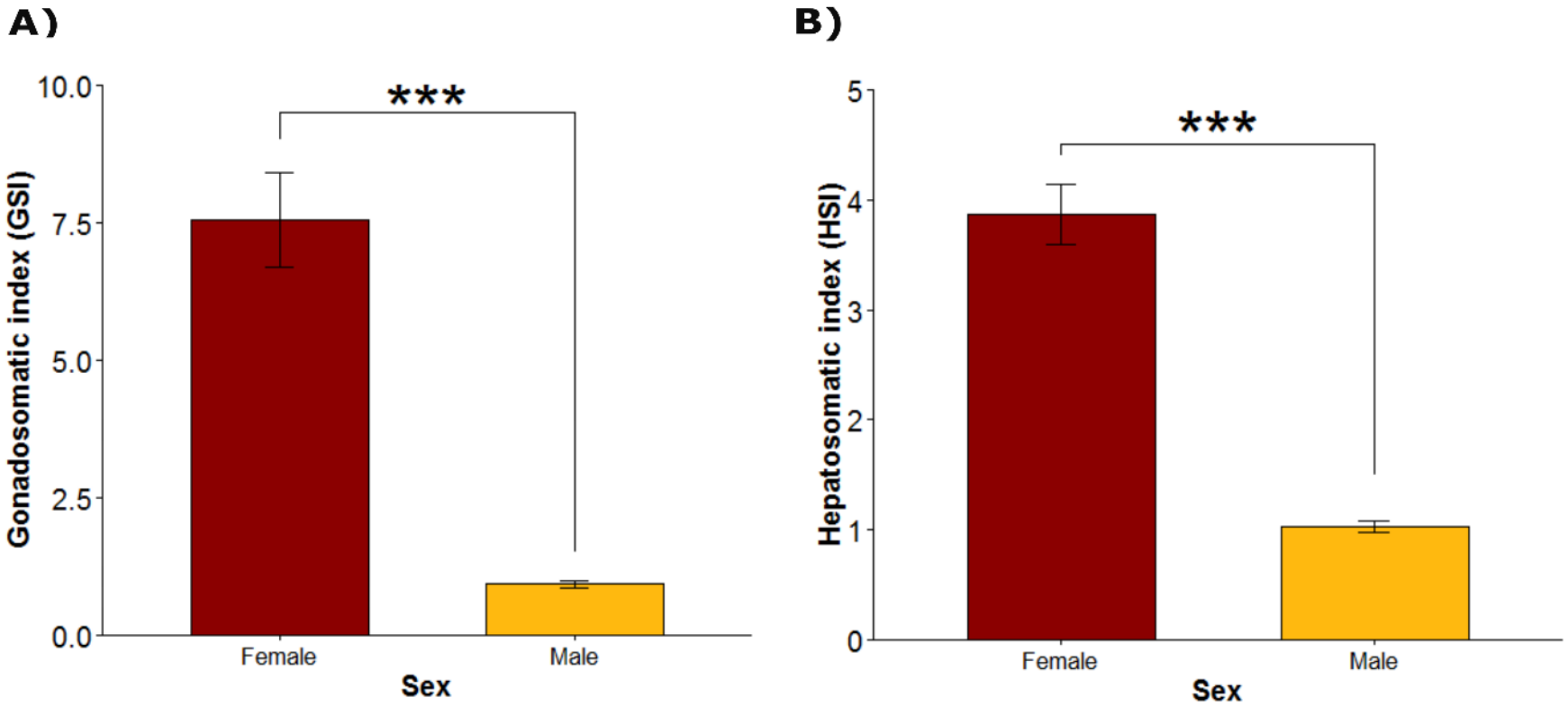
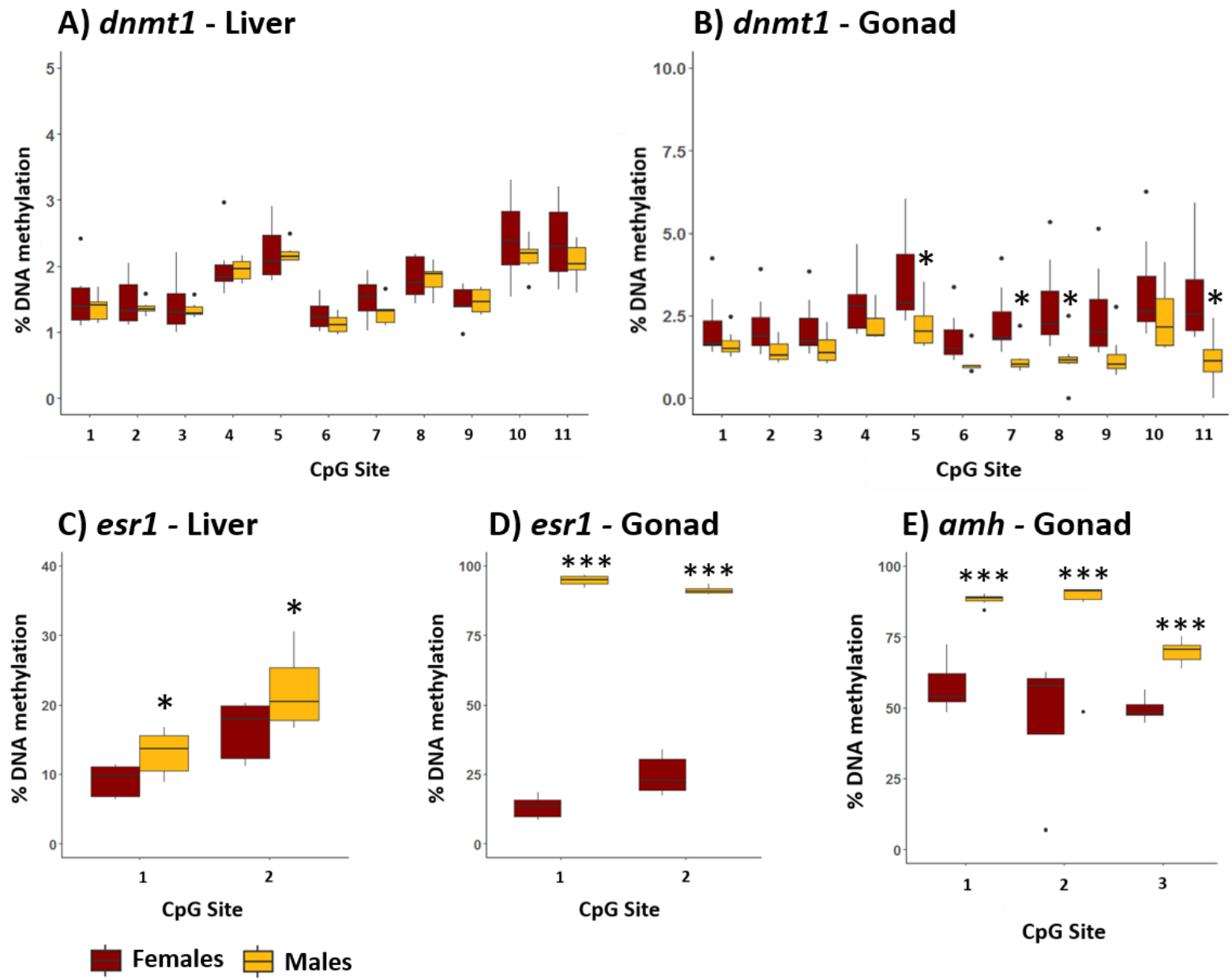


Supplementary Information Figure S1. Total number of eggs spawned for all females (n=8) during the 25 day period prior to sampling.

Proportion of eggs unfertilized and fertilized eggs are shown in grey and white, respectively.



Supplementary Information Figure S2. Morphometric parameters for female (red) and male (yellow) fish (n=8 individuals per group). Individual plots represent the gonadosomatic index (**A**) and hepatosomatic index (**B**)**.** Asterisks indicate significant differences between males and females (Student's t-Test, ***$p < 0.001$).


A) dnmt1 - Liver
% DNA methylation
CpG Site
B) dnmt1 - Gonad
% DNA methylation
CpG Site
C) esr1 - Liver
% DNA methylation
CpG Site
Females
Males
D) esr1 - Gonad
% DNA methylation
CpG Site
E) amh - Gonad
% DNA methylation
CpG Site

Supplementary Information Figure S3. Gene specific DNA methylation profiles for the individual CpG sites (shown in Figure 1 of the main manuscript) in the promoter region of *dnmt1* **(A– liver, B - gonad)** *esr1* **(C – liver, D - gonad),** and *amh* **(E**) in adult zebrafish. Data are presented as boxplots (n = 6-8 for each group). Asterisks indicate significant differences between males and females (*P<0.05 **P<0.01 ***P<0.001).

Supplementary Information Table 1. Target genes, primer sequences and assay details for the RT-QPCR analysis.

Name	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
Aromatase	*cyp19a1a*	AGCCGTCCAGCCTCAG	ATCCAAAAGCAGAAGCAGTAG	101	61.5	2.06
Oestrogen receptor 1	*esr1*	TATGACCTGTTGCTGGAGATG	CGCCGTTGGACTGAATGG	130	59.5	2.14
Oestrogen receptor 2a	*esr2a*	AGGAGAAAACCAAGTAAACCAATC	AGGCTGCTAACAAGGCTAATG	173	59.0	1.86
Oestrogen receptor 2b	*esr2b*	ATCTGCTAATGCTGCTCTCAC	CGCTCTGTTGTCTGTCTTCC	131	57.8	2.18
Androgen receptor	*ar*	ACGAGGGTGTTAGATGAGAC	AAGTATGAGGAAAGCGAGTAAAG	129	58.0	1.97
Anti-Mullerian hormone	*amh*	TGTCTCAACCATCGTCTTCAG	CAGTCAATCCATCCATCCAAAC	124	61.0	2.24
Vitellogenin	*vtg1*	AGCAGCAGCAGTCGTAAC	CAATGATGGTGGCAGTCTTAG	148	57.5	1.84
DNA (cytosine-5)-methyltransferase 1	*dnmt1*	CGCTGTCGTGTTGAGTATGC	TCCCTTGCCCTTTCCTTTCC	180	58.5	2.06
DNA (cytosine-5)-methyltransferase 3	*dnmt3*	TGATGCCGTGAAAGTGAGTC	TTGCCGTGTAGTGATAGTGC	172	58.5	2.19
Histone deacetylase 1	*hdac1*	TGACAAACGCATCTCCATTCG	CTCTTCTCCATCCTTCTCTTCTTC	157	58.0	2.04
Histone deacetylase 3	*hdac3*	GAATGTGTGGAGTTTGTGAAGG	CTGGATGAAGTGTGAAGTCTGG	190	57.0	1.98
Methyl CpG binding protein 2	*mecp2*	GAGGCAGAAACAGGACAG	TGGTGGTGATGATGATGG	176	58.0	2.13
Methyl-CpG-binding domain protein 2	*mbd2*	AACAGCCTCCATCTTCAAG	CGTCCTCAGCACTTCTTC	166	59.0	2.19
Methyl-CpG-binding domain protein 3a	*mbd3a*	ACTCTTCTTTCGGCTCTG	TCTTCCTGCTTCCTGATG	164	57.0	1.99

Supplementary Information Table 2. Bisulfite-pyrosequencing primers and assay details for the gene promoters analyzed.

Name	Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Sequence Primer (5'-3')	Sequence analysed (5'-3')	Ta (°C)
Oestrogen receptor 1	*esr1*	AGAGGAGGTAAATAAATTAAAGATAGTTAG	Biotin-TACTCCTTTAACATATAATTTCCCATAACA	GGTAAATAAATTAAAGATAGTTAGG	TYGATATTGAYGGTTATTTTTTAGAGTAGGTTATGGTAATTAG	58.0
Anti-Mullerian hormone	*amh*	GTTTTTTATTTTTATGGGATGGTAGTTAGG	Biotin-AAACACAACTTAAAAACTTCCACTTATAT	TTGTTTTGAAGTATATTTGGAT	TATAYGTAATGGGGAATGTTTTAGTTTAAGGAAYGGTATTTGGTATTATTAAYGGGTTATTTATAAAATAATGTTTTTA	58.0
DNA (cytosine-5)-methyltransferase 1	*dnmt1*	GGGTATTAATATGTGATAGTGTTAATTGTAG	Biotin - TAAACCCAAATACACTCACAACAC	TTATGAATTGTAGTTAGTAGTTGA	GAAATAYGYGYGGGTYGTTTTTTYGYGYGGAAAYGYGGGTGAGTYGGAYGTTATT	58.0

Chapter 3

Bisphenol A causes reproductive toxicity, decreases *dnmt1* transcription and reduces global DNA methylation in breeding zebrafish (*Danio rerio*).

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Bisphenol A causes reproductive toxicity, decreases *dnmt1* transcription, and reduces global DNA methylation in breeding zebrafish *(Danio rerio)*

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ABSTRACT

Bisphenol A (BPA) is a commercially important high production chemical widely used in epoxy resins and polycarbonate plastics, and is ubiquitous in the environment. Previous studies demonstrated that BPA activates estrogenic signaling pathways associated with adverse effects on reproduction in vertebrates and that exposure can induce epigenetic changes. We aimed to investigate the reproductive effects of BPA in a fish model and to document its mechanisms of toxicity. We exposed breeding groups of zebrafish (*Danio rerio*) to 0.01, 0.1, and 1 mg/L BPA for 15 d. We observed a significant increase in egg production, together with a reduced rate of fertilization in fish exposed to 1 mg/L BPA, associated with significant alterations in the transcription of genes involved in reproductive function and epigenetic processes in both liver and gonad tissue at concentrations representing hotspots of environmental contamination (0.1 mg/L) and above. Of note, we observed reduced expression of DNA methyltransferase 1 (*dnmt1*) at environmentally relevant concentrations of BPA, along with a significant reduction in global DNA methylation, in testes and ovaries following exposure to 1 mg/L BPA. Our findings demonstrate that BPA disrupts reproductive processes in zebrafish, likely via estrogenic mechanisms, and that environmentally relevant concentrations of BPA are associated with altered transcription of key enzymes involved in DNA methylation maintenance. These findings provide evidence of the mechanisms of action of BPA in a model vertebrate and advocate for its reduction in the environment.

Abbreviations: E2, 17β-estradiol; 5Fc, 5-formylcytosine; 5hmC, 5-hydroxymethylcytosine; *ar*, androgen receptor; *amh*, anti-Müllerian hormone; *cyp19a1a*, aromatase; BPA, bisphenol A; *dnmt3*, DNA (cytosine-5)-methyltransferase 3; *dnmt1*, DNA methyltransferase 1; *esr1*, estrogen receptor 1; *esr2a*, estrogen receptor 2a; *esr2b*, estrogen receptor 2b; ER, estrogen receptor; GSI, gonadosomatic index; HSI, hepatosomatic index; *hdac1*, histone deacetylase 1; *hdac3*, histone deacetylase 3; *mecp2*, methyl CpG binding protein 2; *mbd2*, methyl-CpG-binding domain protein 2; *mbd3a*, methyl-CpG-binding domain protein 3a; 5mC, methylcytosine; PCA, principal component analysis; *rpl8*, ribosomal protein L8; *vtg1*, vitellogenin 1

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Introduction

Bisphenol A (BPA) is a commercially important high production chemical widely used in the production of epoxy resins, utilized in food and beverage packaging, dental sealants, and as a monomer component of polycarbonate plastics.[1,2] With over three million tons produced globally per annum, environmental exposure is common,[3] and in the USA BPA was measurable in 75% of food products tested.[4] Human exposure occurs predominantly via ingestion of contaminated food, caused by leaching of BPA from linings of canned goods and polycarbonate packaging. BPA has also been detected in drinking water at concentrations up to 15 ng/L.[5] In addition, inhalation is thought to be a plausible secondary route of exposure,[3] with BPA present in 86% of domestic dust samples at concentrations ranging from 0.2 to 17.6 μg/g.[6] BPA has been detected in the urine of ~95% of adults in the USA and Asia.[7,8] It has also been measured in the serum of adult men and women [9] and in breast milk, fetal plasma, and placental tissue, raising concerns about human exposures during critical periods of development.[1,10]

BPA is moderately water soluble, entering the environment via direct discharge from BPA production and processing industries, wastewater treatment plants and leachate from landfill sites.[11] Its presence is ubiquitous in the aquatic environment and surface water concentrations have been detected up to the low μg/L range, with peak concentrations reaching up to 21 μg/L.[12] Concentrations in landfill leachate have been reported to reach up to 17,200 μg/L.[1] Due to its ubiquitous

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nature, the potential for environmental exposure in wildlife populations, including fish, is very significant. Levels of BPA reported in fish vary; values of 1-11 ng BPA/g dry weight in muscle and 2-75 ng BPA/g dry weight in liver have been reported.[13]

BPA has been shown to act as an estrogen receptor (ER) agonist,[14,15] able to bind to ERs, resulting in feminizing effects.[16,17] A study using the human cell line HepG2, found that BPA strongly activated estrogen receptor 1 (ESR1; previously known as ERα) mediated responses, but did not activate ESR2 (previously known as ERβ), while in the cell line HeLa, BPA was found to activate both ESR1 and ESR2.[14] In fish, BPA induced *esr1* expression in the livers of male fathead minnows *(Pimephales promelas)* exposed for 4 d to 10 μg BPA/L, consistent with an estrogenic mode-of-action.[18] BPA has also been shown to alter the transcriptional profile of steroidogenic enzyme genes in a time-dependent manner, including aromatase (*cyp19a1a*), which is responsible for the irreversible conversion of androgens into estrogens and is a key regulator of estrogen synthesis in the gonads. This enzyme was significantly upregulated in both the ovary and testis of *Gobiocypris rarus* exposed to 15 μg/L BPA for 7d, followed by suppression after 35 d exposure.[19]

Adverse impacts on reproduction have been observed in several fish models. A multi-generational study in fathead minnow showed that BPA reduced gonadal growth in males and females, reduced hatching in F1 offspring of fish exposed to 640 μg/L and induced the estrogen regulated egg yolk protein, vitellogenin, a well established biomarker of xenoestrogen exposure, in the liver of male fish exposed to 640 and 1280 μg/L BPA.[20] Further multigenerational studies have demonstrated the potential adverse effects associated with exposure to BPA.[21,22] Exposure to BPA in guppies has been associated with reduced sperm quality,[23] and the presence of necrotic cells in the seminiferous tubules of *Xiphophorus helleri* was also reported.[24] Together, these studies demonstrate the potential reproductive consequences following exposure to relatively high concentrations of BPA in fish.

Evidence also exists supporting the involvement of BPA in the etiology of a range of human disease phenotypes including cardiovascular disease,[25] altered behavior in children,[26] prostate cancer[27] and recurrent miscarriage.[28] In addition to the well-established estrogenic mode-of-action, additional mechanisms have been proposed, including potential anti-androgenic activity.[29] Low dose effects and non-monotonic dose response curves have been reported.[30,31] More recently, increasing evidence suggests that BPA may alter the epigenetic regulation of gene expression; for example, altered DNA methylation patterns have been observed both globally (i.e., changes to the total genomic content of DNA methylation) and at the regulatory regions of specific genes (i.e., locus-specific) in mammals.[32-36] In humans, exposure to BPA in the workplace has been associated with hypomethylation of LINE-1 in spermatozoa, a marker of global DNA methylation levels in the genome.[37] Understanding the effects of BPA exposure on epigenetic processes, and how these alterations perturb expression of genes that are related to development and reproduction, are important to the evaluation of adverse effects associated with BPA exposure, both in humans and wildlife, particularly for exposures at environmentally relevant concentrations.

To date, few studies have investigated the potential for BPA to induce epigenetic and transcriptional changes in fish. A study in *Gobiocypris rarus* found BPA exposure to be associated with altered DNA methylation in the 5′ flanking region of *cyp19a1a* (aromatase), and the effects to be time-dependent.[19] In addition, a significant decrease in the expression of DNA methyltransferase 1 (*dnmt1*) in ovarian tissue has been reported, with a significant decrease in global DNA methylation.[19]

Given the extensive use and ubiquity of BPA, it is important to understand the mechanisms mediating its toxic effects and the impacts these can have on both wild populations and human health. The present study aims to investigate the effects of BPA on reproduction in the zebrafish model and identify epigenetic and transcriptional changes associated with BPA exposure. We exposed breeding groups of zebrafish to BPA for 15 d to determine if reproduction was affected by the exposure. The concentrations tested included environmentally relevant concentrations found world-wide (0.01 mg/L) and at point sources (0.1 mg/L).[12,38] The highest concentration tested (1 mg/L) has only been reported in landfill leachate and is unlikely to occur in surface waters, but it was included to enable a mechanistic analysis of BPA toxicity. We quantified the transcription of genes involved in epigenetic signaling and reproductive function, together with global and locus-specific DNA methylation in exposed fish.

Results

Water chemistry

The mean measured concentrations of BPA in the tank water were between 100 and 139% of the nominal concentrations for all treatments, and are presented in Supporting Information Table S1.

Effects of BPA on morphometric parameters

The mean mass and length of male and female fish were 460.0 $\pm$ 0.008 mg and 36.5 $\pm$ 0.02 mm, and 480.6 $\pm$ 0.01 mg and 35.7 $\pm$ 0.03 mm, respectively. There were no significant differences in size or condition factor (mean 0.95 and 1.05 for males and females, respectively) between treatment groups.

No alterations in general feeding and swimming behavior were observed in any spawning group, with the exception of the mortality of one female in the 0.1 mg/L BPA treatment. The egg output calculations for that group were adjusted accordingly. Hepatosomatic index (HSI; the ratio of liver weight to body weight) in males was significantly increased in fish exposed to 1 mg/L BPA, but no effects of BPA were observed in females (Supporting Information Fig. S1). There were no significant differences in the gonadosomatic index (GSI; the ratio of gonad weight to body weight) of males or females as a result of the BPA exposure.

Effects of bisphenol A on reproduction

During the 10 d pre-exposure period there were no differences in cumulative egg production between treatment groups ($P = 0.098$). During the exposure, groups treated with 1 mg/L

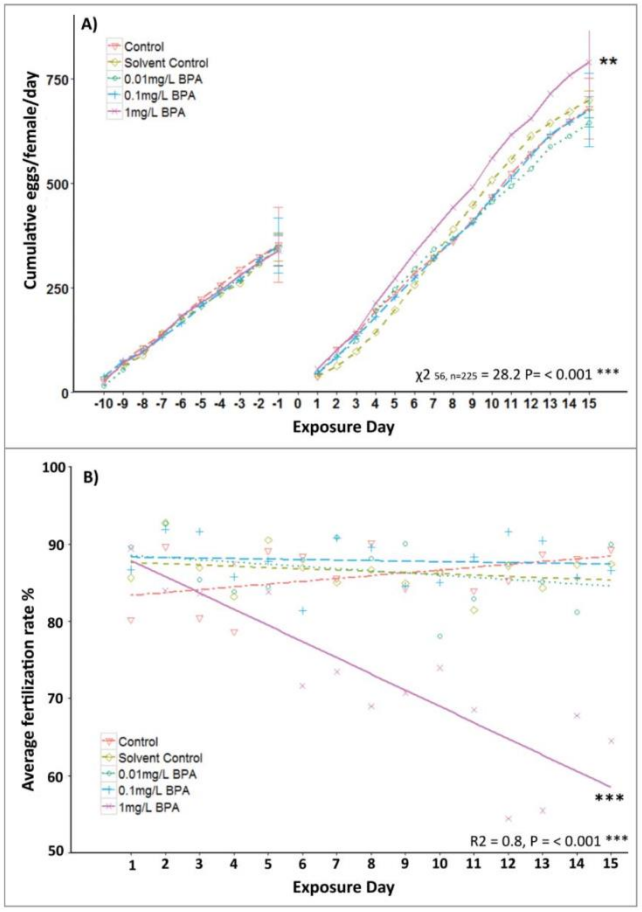

Figure 1. A) Cumulative number of eggs per female per day in breeding groups exposed to 0.01, 0.1, and 1 mg/L BPA. Data is presented for a 10 d pre-exposure followed by a 15 d chemical exposure periods (n = 3 replicate groups per treatment). Statistical comparisons were conducted in R (version 3.0.2), and the lme4 package was used to fit mixed effects linear models, followed by repeated measures ANOVA and Chi-squared Wald test to determine the effects of the exposure to BPA compared to the solvent control. B) Mean fertilization success (%) during the 15 d chemical exposure period (n = 3 replicate groups per treatment). Statistical analyses were conducted using R (version 3.0.2); the Regression coefficient (R^2) was calculated using linear modeling. Asterisks indicate significant differences between treatment groups ($^{**}P < 0.01$; $^{***}P < 0.001$).

BPA spawned a significantly greater number of eggs per female when compared to all other treatment groups ($P \leq 0.01$); this increased egg production intensified throughout the exposure period (Fig. 1A). During the pre-exposure, fertilization success remained consistently high with no significant differences between groups and an overall mean fertilization rate of 85.6%. During the 15 d exposure, fertilization success in colonies exposed to 1 mg/L BPA significantly declined ($P = 0.001$; Fig. 1B). Additionally, for this treatment group, there was a significant negative correlation between the length of the exposure (number of days) and the average percentage of fertilization ($R^2 = 0.80$; $P \leq 0.001$), indicating that the effects of BPA on fertilization became progressively more pronounced over the exposure period.

Effects of bisphenol A on gene transcription

Analysis of genes involved in reproductive processes in the liver revealed that *vtg1* and *esr2b* were significantly upregulated in males following exposure to 1 mg/L BPA when compared to the solvent control group (fold-change = 172.90, P=0.009 and fold-change = 5.40, P=0.014, respectively). In females, *esr2b* was significantly upregulated following exposure to 0.01 mg/L BPA (P = 0.044). For genes involved in epigenetic regulation, the most pronounced changes observed were for *dnmt1*, which was significantly downregulated in the livers of females exposed to 0.01 mg/L BPA ($P = 0.040$) and in both males and females exposed to 0.1 (males: $P = 0.020$; females: $P = 0.005$) and 1 mg/L BPA (males: P = 0.020; females: P = 0.005). In addition, changes were also observed for histone deacetylase 3 (*hdac3*), methyl-CpG-binding domain protein 2 (*mbd2*) and methyl CpG binding protein 2 (*mecp2*) in males, and for *mbd2* in females (Fig. 2A and B; Supporting Information Figs. S2 and 3).

In the gonads, BPA exposure was also associated with significant changes in transcription for genes involved in reproductive function and on epigenetic pathways (Figs. 2, 3). Principal component analysis (PCA) for the testis indicated clear separations between the transcription profiles of fish exposed to the solvent control and fish exposed 1 mg/L BPA, based on the data for all genes quantified (Fig. 3). For ovaries, changes were more pronounced and PCA revealed a separation between fish exposed to 0.1 and 1 mg/L BPA and the solvent control (Fig. 3).

In the testis, the transcript encoding *esr2a* and *cyp19a1a* were significantly downregulated in response to 1 mg/L BPA ($P = 0.002$ and 0.018, respectively; Fig. 2; Supporting Information Fig. S4). There was also a significant association between the concentration of BPA and the level of transcription for *cyp19a1a* (P = 0.025; Supporting Information Table S4), which decreased with increasing concentrations of BPA. In addition, for anti-Müllerian hormone (*amh*), BPA affected gene transcription ($P \leq$ 0.05) and a decreasing trend across all concentrations was observed, but this was not statistically significant ($P = 0.094$; Supporting Information Fig. S4). Similarly to the testis, in the ovaries of exposed females, the transcript encoding *esr2a* was significantly downregulated following exposure to 1 mg/L BPA ($P \leq 0.001$). In addition, there were similar (but non-significant) trends for other genes involved in reproductive function including *esr1* and *ar*, which appeared to decrease with increasing exposure concentrations (Fig. 2; Supporting Information Fig. S5).

As in the liver, *dnmt1* was significantly downregulated in ovaries following exposure to all three BPA concentrations tested (P = 0.032, 0.032, 0.032, respectively). Although no significant group-wise changes in *dnmt1* transcription were observed in the testis (Fig. 2; Supporting Information Fig. S4), the expression of *dnmt1* in the testis was associated with BPA exposure concentration ($R^2 = 0.110$; $P = 0.046$; Supporting Information Table S4). In addition, changes in *mbd2* transcription were observed in the testis, with a significant increase in transcription measured in males exposed to 0.01 mg/L BPA ($P = 0.020$), but reduced expression in males exposed to 1 mg/L BPA ($P = 0.030$; Fig. 2; Supporting Information Fig. S4).

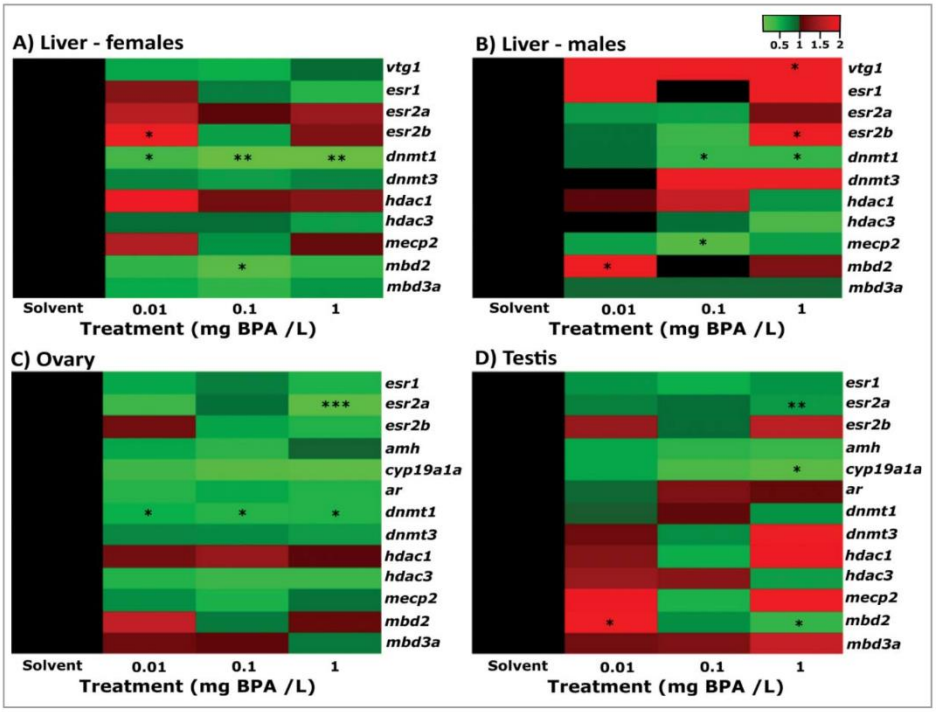

Figure 2. Transcript profiles for target genes in the livers of females (A) and males (B), and in the ovary (C) and testis (D) following exposure to 0.01, 0.1, and 1 mg/L BPA for 15 d. Data were collected for 6-8 fish per treatment, and data points classified as outliers (using the Chauvenet's criterion) and for which the expression was below the detection limit of the assay were excluded from analysis. Where amplification was detected in more than 70% of individuals, data are represented as fold-change relative to the expression in the solvent control group. Where amplification was detected in less than 70% of individuals, data are presented as the proportion of individuals for which the target genes were amplified. Asterisks represent significant differences between treatment groups compared to the solvent control group ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Effects of bisphenol A on global DNA methylation

Analysis of global DNA methylation in the gonads revealed significant decreases in the proportion of global methylation following exposure to 1 mg/L BPA in both males (by 3.2%; $P = 0.029$; Fig. 4A) and females (by 4.9%; $P = 0.041$; Fig. 4B).

Effects of bisphenol A on gene-specific DNA methylation

Targeted DNA methylation profiling in the promoter region of *amh* revealed that exposure to 1 mg/L BPA caused a small but significant increase in methylation compared to the solvent control for the first of the three CpG sites assessed in the testes

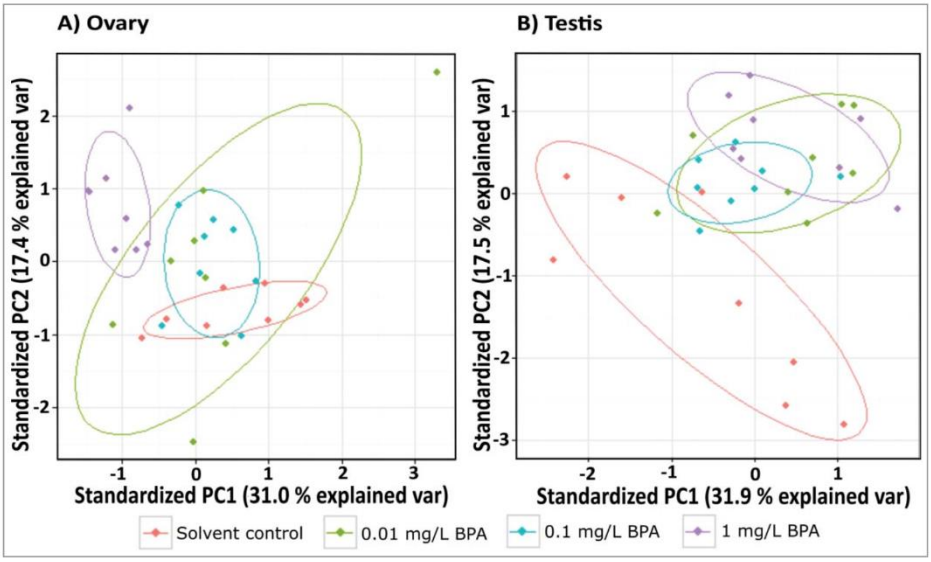

Figure 3. Principal components (PC) score plots showing the relative similarity of gonadal transcription profiles for zebrafish exposed to solvent, 0.01, 0.1, and 1 mg/L BPA for 15 d. A) Ovary. B) Testis. Points represent PC scores for individual fish along PCs 1 and 2. Circles represent a general characterization of the PC space occupied by each treatment group and were calculated using the prcomp package in R (version 3.0.2).

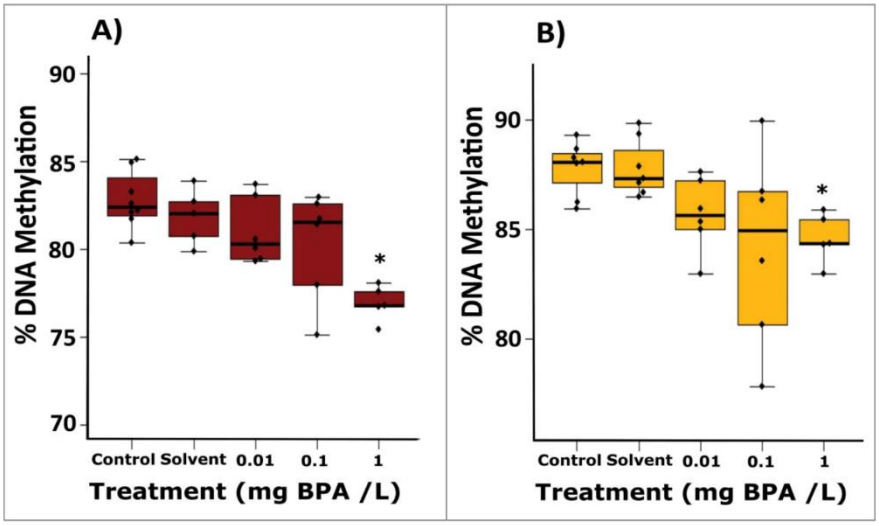

Figure 4. Global DNA methylation profiles in the gonads of adult zebrafish following exposure to 0.01, 0.1, and 1 mg/L BPA. Graphs present the percentage of global DNA methylation in ovaries (A) and testis (B). Data are presented as boxplots (n = 6-8 for each group). Asterisks indicate significant differences compared to the solvent control ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

($P = 0.032$; Fig. 5, see Supporting Information Fig. S6 for the position of this CpG site), with DNA methylation at this site being significantly correlated with BPA exposure concentration ($R^2 = 0.1625$; $P = 0.013$). No differences in DNA methylation were seen for this region in ovaries from exposed female fish (Fig. 5). BPA was also not associated with altered DNA methylation at two CpG sites in the 5′ flanking region of the *esr1* gene in either the liver or gonads (Supporting Information Fig. S7). The analysis of 11 CpG sites across the promoter of *dnmt1* identified significant increases in DNA methylation for a number of sites in the liver (in both males and females) and the testes (males). Although group-wise comparisons of this region revealed no significant differences in the female ovaries (Figs. 6 and 7), *dnmt1* promoter methylation was significantly correlated with BPA exposure at various sites (positions 4, 5, 6 and 8; Supporting Information Table S4).

Discussion

Exposure to BPA resulted in a consistent downregulation of *dnmt1* transcription in the ovary and in the liver of both males and females following exposure to BPA, including at

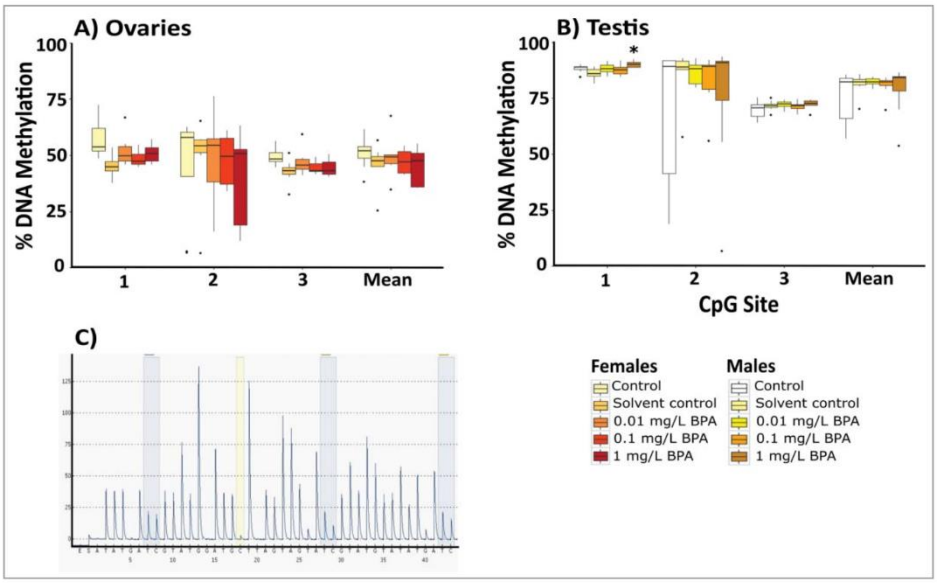

Figure 5. Gene specific DNA methylation profiles for three CpG sites in the promoter region of anti-Müllerian hormone (*amh*) in the ovaries (A) and testes (B) of adult zebrafish following exposure to 0.01, 0.1, and 1 mg/L BPA. C) Example pyrogram of three CpG sites in the 5′ flanking regions of the *amh* gene. Data are presented as boxplots (n = 6-8 for each group). Asterisks indicate significant differences compared to the solvent control ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

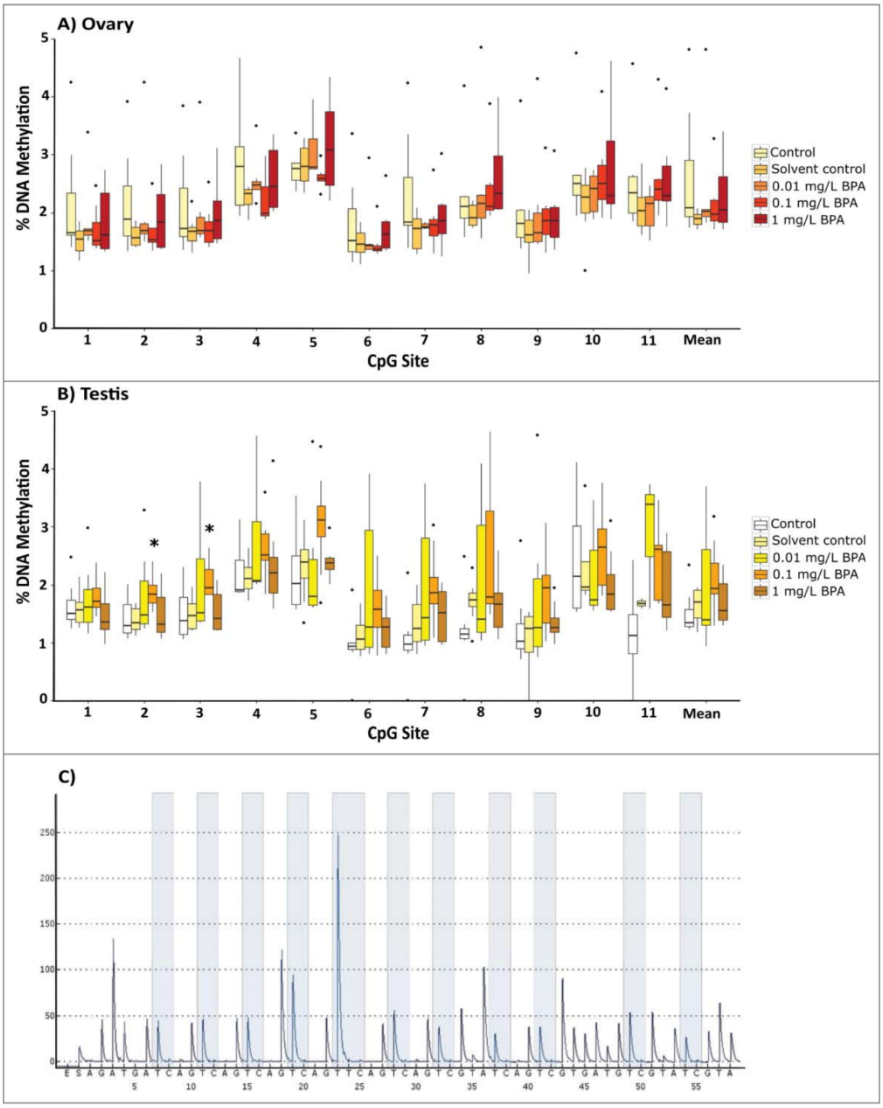

Figure 6. Gene-specific DNA methylation profiles for 11 CpG sites in the promoter region of DNA (cytosine-5)-methyltransferase 1 (*dnmt1*) in the ovaries (A) and testis (B) of adult zebrafish following exposure to 0.01, 0.1, and 1 mg/L BPA. C) Example pyrogram of 11 CpG sites in the 5′ flanking regions of the *dnmt1* gene. Data are presented as boxplots (n = 6-8 for each group). Asterisks indicate significant differences compared to the solvent control ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

environmentally relevant concentrations in females. In association with this, we found a reduction in global DNA methylation, probably due to the decrease in *dnmt1* expression. At the highest concentration tested, BPA caused reduced fertilization, potentially via estrogenic mechanisms. Together, our data provide evidence of the molecular mechanisms of action of BPA and the potential for it to cause adverse health impacts in vertebrates.

Reproductive effects of BPA on adult zebrafish

We provide evidence that BPA exposure results in an impairment of reproductive function in breeding zebrafish. These effects included an increase in the number of eggs spawned and a decrease in fertilization success in groups exposed to 1 mg/L BPA. A number of mechanisms may contribute to the observed effect of BPA on reproduction, including stimulation of estrogen responsive processes via the interaction of BPA or its metabolites with estrogen signaling pathways, as previously reported for a range of organisms.[39–41] We have investigated the effects of BPA on the expression of transcripts involved in reproductive function and known to be directly or indirectly regulated by estrogens.

We found no evidence for significant alterations in the transcription of *esr1* or DNA methylation across the *esr1* promoter in the gonads and livers of both sexes, but a significant association between BPA concentration and decreased transcription was found for the livers of females, and a trend for reduced expression was also observed in the ovaries and testis, similar to that described previously.[31] Disruption of ESR1 has been associated with alterations of spermatogenesis and subsequently infertility in mice,[42,43] therefore suggesting that the apparent decrease in *esr1* transcript in the testis may contribute toward the observed decline in fertilization success at this concentration.

BPA was found to downregulate *esr2a* in both ovaries and testes, but not in the liver. Similarly, a decrease in *esr2a*

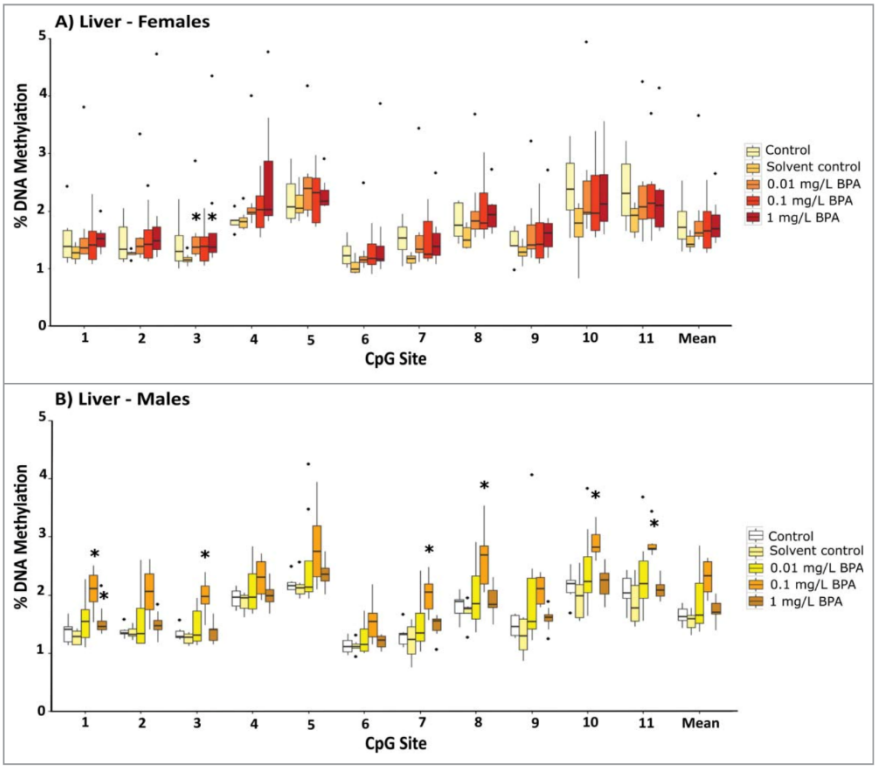

Figure 7. Gene specific DNA methylation profiles for 11 CpG sites in the promoter region of DNA (cytosine-5)-methyltransferase 1 (*dnmt1*) in the livers of female (A) and male (B) adult zebrafish following exposure to 0.01, 0.1, and 1 mg/L BPA. Data are presented as boxplots (n = 6-8 for each group). Asterisks indicate significant differences compared to the solvent control ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

transcription was reported in ovaries of *Gobiocypris rarus* exposed to 0.05 mg/L BPA for 35, d and was associated with disruption of oogenesis and the occurrence of atretic follicles.[31] These findings concur with previous studies reporting that *esr2a* is more sensitive compared to *esr1*, to the natural estrogen, 17β-estradiol (E2).[41] In contrast, BPA caused increased transcription of *esr2b* in the livers of males and females but not in the gonads, and, importantly, for females this effect was observed at the environmentally relevant concentration of 0.01 mg/L BPA. In parallel, BPA induced a significant increase in the transcription of the egg yolk protein, *vtg1*, and an increase in HSI in males, likely as a result of increased vitellogenin production in hepatocytes, indicating an association between the induction of *esr2b* in males and the induction of *vtg1*, as previously reported for fathead minnows.[44] Together, these findings suggest that the effects of BPA on reproduction involve disruption of estrogen receptor signaling principally via *esr1* and *esr2b* in the liver, and *esr2a* in the gonads.

In addition to the disruption in estrogen receptor signaling, changes in sex steroid biosynthesis may have contributed to the observed disruption of reproduction in colonies exposed to 1 mg/L BPA. We found a significant decrease in *cyp19a1a* transcript in the testis of males exposed to 1 mg/L BPA, and a significant association between transcription and BPA exposure concentration. In ovaries, a decreasing trend was also observed. These findings suggest potential feedback mechanisms were activated to counteract the estrogen/androgen ratio imbalance caused by BPA, through reducing the irreversible conversion of testosterone into estrogens. Similar findings have recently been reported for the Chinese rare minnow (*Gobiocypris rarus*) following a long term exposure to BPA,[19] and studies using the aromatase knockout (ArKO) mouse found ArKO males to have reduced fertility,[45] demonstrating the critical role of aromatase in gametogenesis in males.

In the testis, a decrease in *amh* transcription was associated with increased BPA exposure concentrations. Similarly, in mammals, downregulation of AMH has been reported following exposure to BPA.[46,47] Exposure to 1 mg/L BPA also caused significant DNA hypermethylation in the *amh* promoter in the testis (CpG 1), demonstrating that exposure to BPA caused epigenetic alterations at this specific gene locus. There was also a significant correlation between the level of methylation in CpG 1 and *amh* transcription, and with BPA exposure concentration. This suggests that epigenetic mechanisms may be playing a role in the observed decline in *amh* transcript in testis tissue, which in turn could have consequences for the functioning of the testis, resulting in de-masculinization.

Fertilization success decreased over time with the mean fertilization rate dropping from 89% on day 1 to 69% by day 15. These findings are consistent with those of Haubruge et al., who reported declines in sperm count of 40-75% in guppies exposed to 0.274 or 0.549 mg/L BPA.[23] BPA exposure has been linked to male sexual dysfunction in humans, and urinary concentrations of BPA have been associated with declines in sperm concentration, motility, and morphology in men.[48] The mechanism by which disruption of normal spermatogenesis takes place is hypothesized to be via disruption of the Sertoli cells, which are directly sensitive to xenobiotic chemicals, and whose functions are essential during spermatogenesis.[23] Our data are in agreement with these findings and further document the

importance of Sertoli cells as targets for BPA toxicity, by demonstrating its effects on *amh* and *cyp19a1a*, both expressed in these cells in the testis.

Changes in fertilization success may have occurred not only due to effects of BPA on spermatogenesis but also due to BPA-induced alterations in egg quality. Females exposed to 1 mg/L BPA produced an increased number of eggs, but these eggs may have lacked the quality required for fertilization success and embryo survival. Many factors contribute to egg quality, of which the hormonal environment during oogenesis is a critical one.[49] The observed changes in the expression of estrogen receptors and the trends observed for *cyp19a1a* in females indicate a disruption of the estrogen/androgen balance within ovaries and consequent alterations in sex steroid signaling pathways, putatively leading to alterations in oogenesis and oocyte quality. This hypothesis is supported by previous studies in which BPA was shown to affect oogenesis.[50] In addition, a study in pregnant mice exposed to BPA found gross abnormalities in the meiotic prophase of oogenesis, including synaptic defects, which were suggested to occur via Esr2 (ERβ) signaling.[51] Interestingly, in the present study, changes were also observed in the expression of an ERβ subtype (*esr2a*) in the gonads of both sexes, suggesting similar mechanisms could be occurring.

Effects of BPA on epigenetic regulation

There is now strong evidence demonstrating that BPA has the potential to induce changes in DNA methylation at both gene-specific and genome-wide levels in exposed organisms[32,33]; however, this has rarely been studied in fish.

In our study, we found a significant decrease in the expression of the DNA methylation maintenance enzyme, *dnmt1*, for all three BPA concentrations tested in ovaries of females—including at environmentally relevant concentrations—and the DNA methylation pattern in the promoter region of the *dnmt1* gene was found to be significantly associated with BPA exposure concentrations for four CpG sites. The expression of *dnmt1* is known to be associated with changes in global DNA methylation, and inactivation of *dnmt1* has been shown to cause global demethylation of the genome.[52] In this regard, it was interesting that global DNA methylation levels were significantly decreased in ovarian tissue of fish exposed to 1 mg/L BPA, potentially as a consequence of the suppression in *dnmt1* transcription. In contrast, previous studies in *Gobiocypris rarus*, have reported global DNA hypermethylation in ovaries exposed to 0.015 mg/L BPA for 35, d[19] suggesting these epigenetic effects may be concentration- and time-dependent, and potentially vary across vertebrate species. Importantly, *dnmt1* is reported to be an important maternal transcript involved in the regulation of DNA methylation during the first stages of embryo development, particularly prior to the zygote genome activation.[53,54] Therefore, the significant decrease in the expression of *dnmt1* observed in ovaries of females exposed to all three concentrations of BPA could have potential consequences for the appropriate development of offspring, in addition to influencing the level of DNA methylation in the ovary of exposed females.

For males, *dnmt1* transcription was also negatively associated with BPA exposure concentrations and a significant hypermethylation of two CpG sites in the promoter region of the *dnmt1* gene in fish exposed to 0.1 mg/L BPA was observed. In addition, we measured a significant decrease in global DNA methylation in the testis of fish exposed to 1 mg/L BPA, suggesting that the BPA-induced reduction in global methylation is likely to be functionally linked to the decrease in *dnmt1* transcription. These data align with the reported hypomethylation of sperm associated with the presence of BPA in urine, in a study of male factory workers in China.[37] There is evidence to suggest that DNA demethylation and methylation establishment events during early development are guided by the paternal DNA methylation program instructed by the sperm chromosomes.[55,56] Therefore, it is plausible that changes to the global DNA methylation pattern in testes such as those reported for fish exposed to 1 mg/L BPA may have the potential to impact on the epigenetic reprogramming of embryos, with potential consequences for their subsequent development.

In the liver, we observed a significant decrease in *dnmt1* transcription in males and females, including at environmentally relevant concentrations, demonstrating the very significant impact of BPA on the expression of this key DNA methylation maintenance enzyme. In addition, we report significant hypermethylation of the promoter region of the *dnmt1* gene in both male and female livers. Based on the positive association between the expression of this gene and global DNA methylation, it is plausible that the suppression of *dnmt1* may impact on global methylation as seen in other tissues. However, this could not be measured in the liver due to technical limitations related to the amount of DNA obtained from this tissue. The fact that changes in the transcript and methylation profile for *dnmt1* occur at environmentally relevant concentrations highlights the potential for BPA to cause epigenetic effects in exposed organisms within current exposure scenarios.

It is important to note that global DNA methylation in this study, measured using the LUMA assay, provides only an estimate of the total DNA methylation across all areas of the genome and all cell types in a given tissue. Decreased *dnmt1* transcription may be causing demethylation of specific areas of the genome or within specific cell types, but this may not be detectable by a global measurement of DNA methylation, including all cell types simultaneously. This may explain why *dnmt1* transcription appears to be more sensitive to BPA exposure compared to global methylation measurements.

The transcript profile for *mbd2* was significantly altered following exposure to BPA in both male testis and female livers. *mbd2* belongs to a family of nuclear proteins capable of binding specifically to methylated DNA, and may also function to repress transcription from methylated gene promoters.[57] We found also a significant decrease in *mecp2* transcription in male livers, a gene involved in transcriptional repression by associating with methylated CpG dinucleotides where it silences transcription by recruiting histone deacetylases, resulting in chromatin remodeling.[58] In addition, in male livers a significant decrease in *hdac3* transcription was also observed. These findings suggest that BPA is not only interacting with the processes linked to DNA methylation, but also has the potential to disrupt processes linked to chromatin structure and potentially impact on gene function via these mechanisms.

Despite the advances in our understanding of the epigenetic and transcriptional consequences of BPA in a model vertebrate, there are some limitations to the methodologies used: the locus-specific DNA methylation measurements conducted were

based on the sodium bisulphite treatment of genomic DNA and, therefore, cannot distinguish between DNA modifications such as 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and methylcytosine (5mC), which have unknown functional significance.[59] In addition, we explored the methylation status of specific CpG positions, within the regulatory regions of select target genes, hypothesized to be targets of BPA toxicity. This hypothesis-driven approach was successful in identifying some important mechanisms of BPA toxicity but may have missed other interesting effects outside these targeted regions, as suggested by the effects of BPA on global methylation levels. In addition, the global and locus-specific methylation measurements reported in this study are single measurements of DNA methylation across multiple cellular populations and cell types within each tissue. Both the gonad and liver are comprised of a mixture of cell types, whose genomic methylation and transcriptional activity is unique to the function of each cell type. In the testis for example, a large percentage of the cellular composition is made up of sperm cells containing very little cytoplasm and limited transcriptional activity, and the genomic DNA of sperm cells is also known to be hypermethylated. In contrast, the ovary contains oocytes characterized by very large cytoplasm where transcripts are stored to support the initial stages of embryogenesis before embryonic genome activation. Therefore, the datasets collected for these tissues are strongly dependent on the cellular composition of the tissue. In future studies, a genome-wide approach to measure methylation and also histone modifications, as well as analysis of single cells or pure populations of cells, may help to further characterize the effects of BPA on epigenetic signaling pathways.

Conclusions

Overall, we have found evidence that BPA caused significant disruption to reproduction in breeding zebrafish exposed to 1 mg/L BPA, likely via estrogenic mechanisms. The potential for BPA to cause disruption of reproduction shown here raises concerns for its toxicity when organisms are exposed to BPA in environments affected by other stressors, including other environmental endocrine disruptors with similar mechanistic pathways that may act additively to cause reproductive disruption. Importantly, BPA also caused significant alterations in the transcription of a number of genes involved in epigenetic regulation in both liver and gonad tissue, most notably on *dnmt1*, which occurred in conjunction with decreases in global DNA methylation. Of note, some changes were observed after exposure to environmentally relevant concentrations of BPA (0.01 mg/L), corresponding to current exposure scenarios for both humans and wildlife. These findings provide evidence of the adverse effects of BPA in a model vertebrate and advocate for BPA's replacement within consumer products and its reduction in the environment.

Materials and methods

Chemicals

All chemicals were obtained from Sigma-Aldrich, UK, unless stated otherwise.

Fish husbandry

Wild type WIK strain adult zebrafish (originating from a stock population at the University of Exeter) were maintained according to the conditions reported in Paull et al.[60] Prior to the start of the experiment, fish were randomly allocated into 18 breeding groups of 4 males and 4 females, kept in individual 15-L flow-through tanks and were allowed to breed naturally during an acclimation period of 7 d. After this period, colonies that failed to spawn consistently were removed prior to the start of the experiment. 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 heated to 28°C in a reservoir, before it was supplied to each aquarium using a flow-through system. Tanks were aerated and supplied with a flow rate of 48 L/day of water.[60] Tank water was maintained at 28 ± 0.5°C and pH 7-7.5 and fish were maintained under a 12 h light:dark cycle, including dawn and dusk transition periods of 30 min. Fish were fed live *Artemia nauplii* once daily (ZM Premium Grade Artemia; ZM Ltd.) and TetraMin tropical flake food (Tetra; Melle, Germany) twice daily, to satiation.

Exposures of breeding zebrafish to bisphenol A

The selected 15 groups that showed consistent breeding and behavioral patterns during the initial acclimation period were subjected to a 10 d pre-exposure period, followed by a 15 d exposure period. Reproductive data for the 10 d pre-exposure period were collected to ensure that all breeding groups were reproducing consistently and there were no differences between reproductive measurements for any of the breeding groups prior to the chemical exposure period. Three independent replicate breeding groups were assigned at random to each treatment. A flow-through system was used to dose the tanks for 15 d with three concentrations of BPA (0.01, 0.1, and 1 mg/L) using ethanol (0.0005%) as a solvent. An absolute control receiving water alone and a solvent control receiving the same concentration of ethanol as the chemical exposures were also included.

On day one of the exposure period, tanks were spiked with the appropriate amount of BPA to achieve the required exposure concentrations. Flow rates were monitored daily to ensure the chemical concentrations remained consistent and dosing stocks were replaced every day. Water samples from each tank were collected on days 5, 10, and 15 of the exposure, and were stored at −20°C until chemical analysis.

The effects of BPA on reproduction were determined by measuring the egg production and fertilization success of individual groups. Eggs were collected each morning approximately one hour post-fertilization (hpf), washed and transferred to petri dishes for analysis. The numbers of fertilized and unfertilized eggs were determined by visual inspection for each treatment using a dissection microscope (Motic DM143, Hong Kong).

On day 15 of the exposure period, all fish were sacrificed humanely using a lethal dose of benzocaine followed by

destruction of the brain, in accordance with UK Home Office regulations. The wet weight and fork length were recorded, and the condition factor for each fish was calculated (k) = [weight (g) × 100]/[fork length (cm)]^3. The gonads and livers were dissected and weighed, and the gonadosomatic index (GSI) = gonad weight (mg)/[total weight (mg)- gonad weight (mg)] × 100 and hepatosomatic index (HSI) = liver weight (mg)/[total weight (mg)- liver weight (mg)] × 100 were calculated. Gonads and livers were collected, snap frozen in liquid nitrogen and stored at −80°C until analysis for transcript profiling and DNA methylation.

Transcript profiling

Transcript profiling of genes encoding epigenetic regulatory proteins and genes involved in reproductive function was conducted using real-time quantitative PCR (RT-QPCR) as previously described.[61] Beacon Designer 3.0 software (Premier Biosoft International, Paulo Alto, CA) was used for designing primers for each target gene using zebrafish NCBI RefSeq sequences, and primers were purchased from MWG-Biotech (Ebersburg, Germany). Assays for each transcript were optimized and standard curves were generated as previously described.[61] Primer specificity was confirmed by observation of a single amplification product of the expected melting temperature throughout the range of detection. The linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was > 0.99 in each case, and efficiencies were between 1.86-2.24. The primer sequences, annealing temperatures, PCR product sizes and PCR efficiencies for each primer pair are shown in Supporting Information Table S2.

RNA and DNA were extracted together from the livers and gonads of 8 male and 8 female fish from each treatment group using the AllPrep DNA/RNA Micro Qiagen Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, which allows for extraction of both RNA and DNA from the same tissue sample. NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA) was used to assess RNA and DNA purity and concentration. RNA was treated with DNase I (Qiagen) to remove any potential DNA contamination. cDNA was synthesized from 2 μg of total RNA using random hexamers (MWG-Biotech, Ebersberg, Germany) and M-MLV reverse transcriptase (Promega, Madison, USA), according to manufacturer's instructions. cDNA was then diluted 1:2 and RT-QPCR was performed in duplicate using an iCycler iQ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA) and SYBR Green chemistry, as previously described.[61] On each plate, a template-minus negative control was run in duplicate to verify the absence of cDNA contamination. Efficiency-corrected relative expression levels were determined after normalization to a control gene, ribosomal protein l8 (*rpl8*), which has been shown to have stable expression in the livers and gonads following exposures to estrogens in another cyprinid fish species.[44,62]

Bisulfite pyrosequencing

DNA sequence data for the promoter regions of *esr1, amh*, and *dnmt1* were obtained from Ensembl (release 83; Cunningham et al. 2015) [63] using the Biomart portal.[64] Zebrafish *esr1* (ENSDARG00000004111) has 3 known transcripts [*esr1*-001 (3449 bp), *esr1*-201 (3502 bp) and *esr1*-202 (212 bp)] and 2 transcription start sites (TSSs). The *dnmt1* gene (ENSDARG00000030756) also has 2 TSSs and 3 transcripts [*dnmt1*-001 (4896 bp), *dnmt1*-201 (4893 bp) and *dnmt1*-202 (5031 bp)]. *amh* (ENSDARG00000014357) has one transcript (*amh*-001, 3243 bp) and one TSS (Supporting Information Fig. S6). Target sites within the promoter sequences were chosen based on their proximity to the TSSs and estrogen-responsive elements (EREs), identified using JASPAR,[65] and the matrix models ESR1 (MA0112) and ESR2 (MA0258). PCR and bisulfite pyrosequencing assays were designed using the PyroMark Assay design software (Qiagen, Hilden, Germany). Pyrosequencing primers and their corresponding target sequences are shown in Supporting Information Table S3.

Template preparation and pyrosequencing was carried out as described by Tost and Gut (2007) [66] on bisulfite-treated DNA from 8 individual fish (gonads and livers) per treatment group. Briefly, genomic DNA (500ng) was treated with sodium bisulfite using the EZ-96 DNA Methylation-Gold Kit (Zymo Research, CA, USA) according to the manufacturers' standard protocol. Water negative controls were run in duplicate to verify the absence of DNA contamination. Bisulfite-PCR amplification was performed in duplicate using the primers and assay conditions provided in Supporting Information Table S3. Unmodified DNA samples were included during primer optimization to confirm primer specificity for bisulfite-modified DNA.

Luminometric-based assay (LUMA) for global DNA methylation

The LUMA assay was performed as described by Karimi et al. (2006) using DNA extracted from gonad samples from 8 individual fish per treatment.[67] Sufficient quantities of DNA were not available to perform the LUMA assay in liver samples; therefore, analysis of global DNA methylation were conducted only for gonad samples. Each DNA sample (250 ng) was digested in duplicate with HpaII and MspI, and data were normalized to the EcoRI peak to account for any technical differences between samples.[68] Global DNA methylation values were calculated according to the formula [HpaII(G)/EcoRI(T)]/[MspI(G)/EcoRI(T)], where G and T refer to the peak heights for HpaII or MspI (methylation) and EcoRI (input DNA), respectively.

Water chemistry

For analysis of the concentrations of BPA in the exposure water, methanol, acetonitrile and water, both HPLC and LC-MS grade, HiPerSolv CHROMANORM®, were purchased from VWR Int. One mL of each water sample was added to a glass vial and mixed with 1 mL of HPLC-grade acetonitrile. Before LC-MS/MS analysis, aliquots were vortexed and diluted in a mixture of acetonitrile and water (1:3 v/v). Analyses were performed using a Surveyor MS Pump Plus HPLC pump with an HTC PAL autosampler coupled to a TSQ Vantage triple quadrupole mass spectrometer equipped with heated electrospray

(HESI II) source (ThermoFisher Scientific, Hemel Hempstead, UK). Chromatographic separation was achieved using a 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 water and methanol. The initial conditions for the gradient consisted of 10% methanol, which was increased to 100% in 4.5 min and maintained for 1 min before returning to the initial 10% methanol. The flow rate was 500 μL/min. The temperature of the autosampler was set at 8°C, and the column was kept at a room temperature. The HESI probe was operating in the negative mode and an ion-spray voltage of −4.0 kV was applied. The heated capillary temperature was set at 275°C and the vaporizer temperature was 60°C. Nitrogen was employed as sheath and auxiliary gas at a pressure of 30 and 5 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 BPA was performed using two characteristic multiple reaction monitoring (MRM) transitions of precursor ion 227.1→ 212.1 (CE: 20 V) and 227.1 → 133.1 (CE: 28 V).

Statistical analysis

Statistical analyses were carried out using R (version 3.0.2).[69] Prior to analysis, data were tested for equal variance and for normality using the Shapiro–Wilk test. Proportional data and variables with non-Gaussian distributions or non-homogeneous variances were subjected to variance-stabilizing arcsine transformations or log transformations. Non-parametric statistics were used when transformations did not result in distributions meeting the assumptions for parametric tests. All graphs were plotted using untransformed data for ease of interpretation. For the mean fertilization rates, comparisons between treatments were performed using Kruskal-Wallis tests followed by the Wilcoxon signed rank test. The Regression coefficient (R^2) was calculated using linear modeling for fertilization rates. Linear mixed effects models were generated using the lme4 package [70] in order to explore the effect of BPA concentration and length of exposure on egg numbers. Non-significant terms were removed from models; models were compared based on likelihood ratio testing to give the appropriate minimum adequate model. Model results were inspected to ensure residuals were normally distributed.

In order to determine the effects of BPA on the reproductive and molecular endpoints measured, statistical comparisons were performed between the solvent control and the groups exposed to BPA, and comparisons between the water control and the solvent control were also conducted to confirm that no significant differences occurred as a result of the presence of the solvent. Comparisons between treatments were performed using one-way analysis of variance (ANOVA) and Kruskal-Wallis tests. Where ANOVA analysis found a $P \leq 0.05$, post-hoc testing was carried out using the pairwise multiple comparisons of means method with false discovery rate P value adjustment. Where the Kruskal-Wallis test was used, post-hoc testing was carried out using the Wilcoxon signed rank test accounting for repeated measures within the data sets. P values of ≤ 0.05 were considered to be significant. All data are presented as mean ± SEM.

For transcript profiles, data points classified as outliers (using Chauvenet's criterion) and data points for which the expression was below the assay detection limit were excluded from analysis. Where amplification was detected in more than 70% of individuals, data were represented as fold-change relative to the expression in the water control group and groups were then compared using one-way ANOVA and Kruskal-Wallis tests with post-hoc tests as described previously. Where amplification was detected in less than 70% of individuals, data were represented as the proportion of individuals for which the target genes were detected, and analysis was conducted using a binomial generalized linear model. In the gonadal data sets, PCA was also performed using the prcomp function to identify the main trends in gene expression.

In order to determine if there were associations between the methylation levels for specific loci in the promoter regions of genes of interest and their transcription, correlation analysis was conducted. Where data was normally distributed Pearson correlation was used, and where data did not meet the assumptions of parametric testing, Spearman correlation analysis was performed. Correlation analyses were also conducted to determine the relationship between global methylation and *dnmt1* transcription, as above. The relationship between BPA concentration and transcript expression or methylation was also determined using regression analysis, calculated using linear modeling.

All graphs were plotted using untransformed data for ease of interpretation, and were created using the R packages ggplot2,[71] gplots,[72] beeswarm,[73] and ggbiplot.[74]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Supporting Information

Bisphenol A causes reproductive toxicity, decreases *dnmt1* transcription and reduces global DNA methylation in breeding zebrafish (*Danio rerio*).

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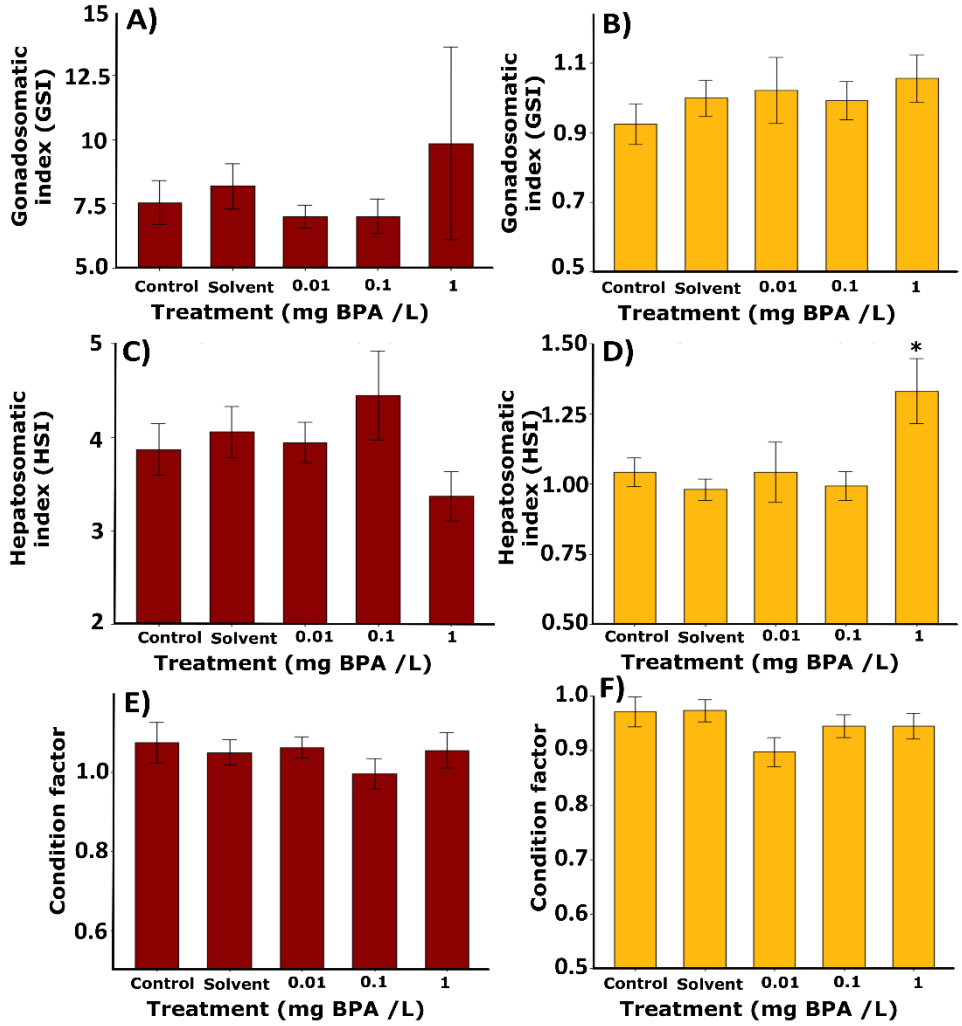
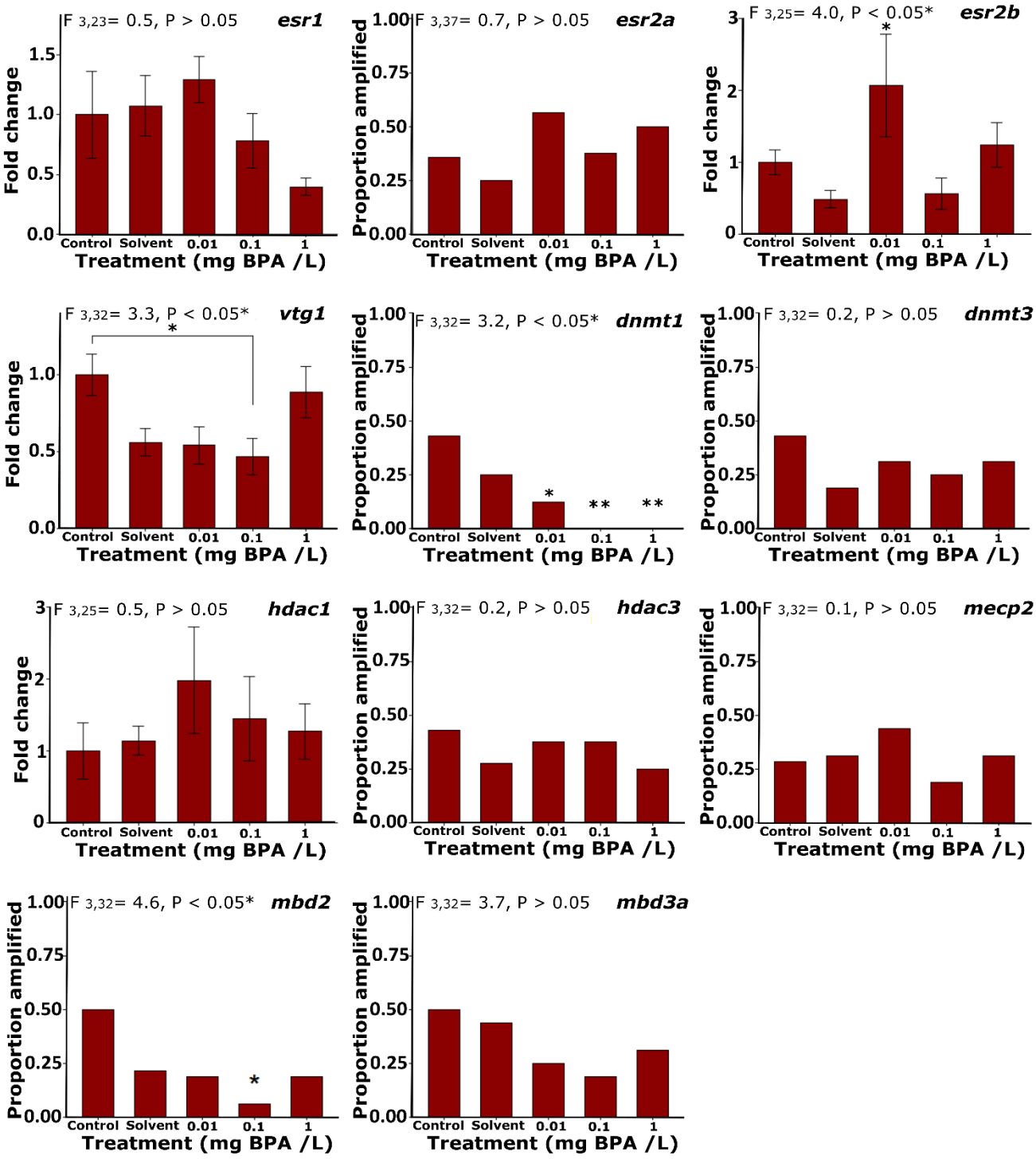
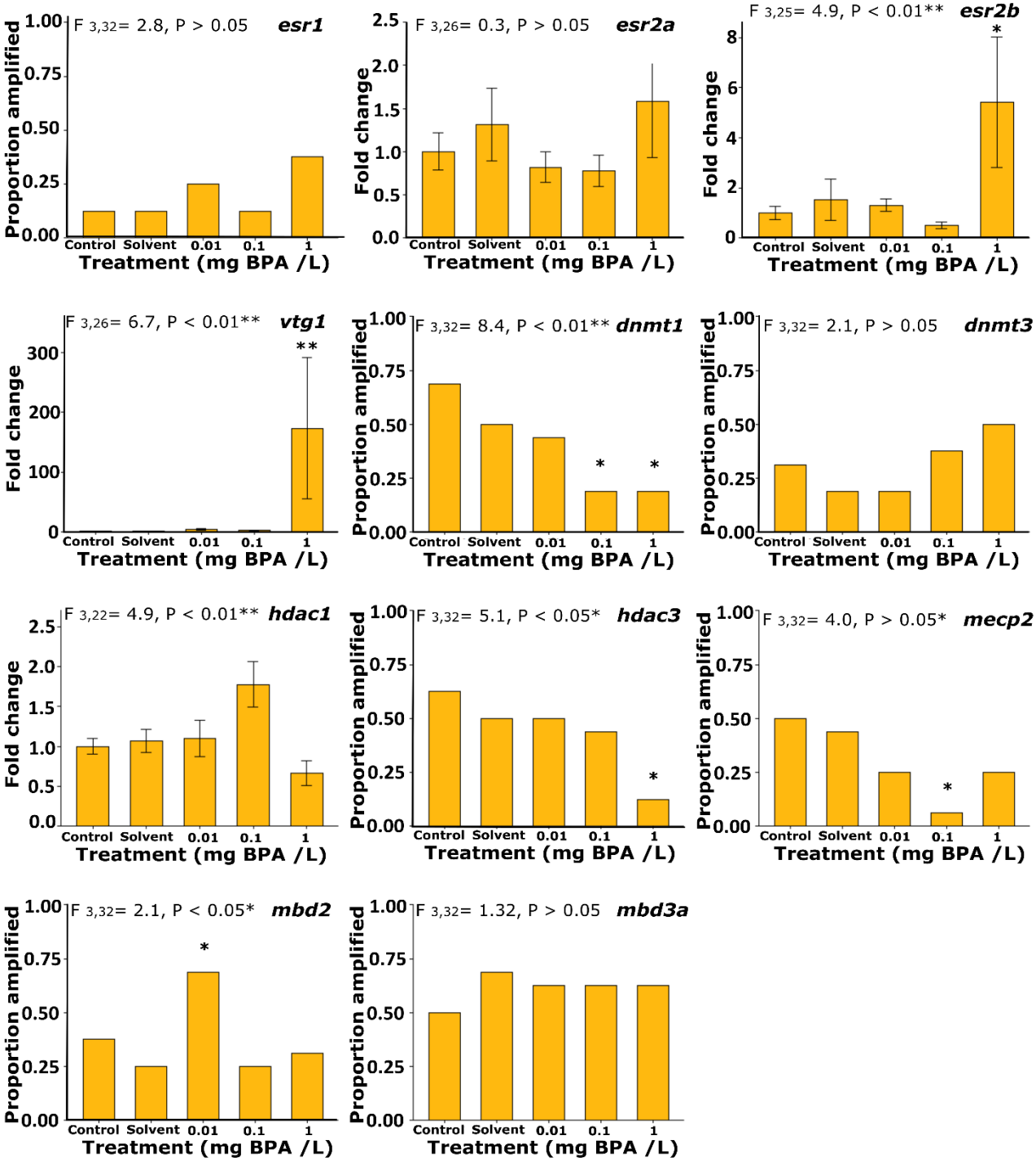


Supporting Information Figure S1. Morphometric parameters for males and females exposed to 0.01, 0.1 and 1 mg/L BPA (n=12 individuals per treatment). Individual plots represent the gonadosomatic index for females **(A)** and males **(B),** hepatosomatic index for females **(C)** and males **(D),** and the mean condition factor for females **(D)** and males **(E).** Statistical comparisons were conducted using Kruskal-Wallis one-way ANOVA on ranks followed by the pairwise Wilcox test, in R (version 3.0.2). All data are presented as mean ±SEM. Asterisks indicate significant differences compared to the solvent treatment (*$p<0.05$).


F 3,23= 0.5, P > 0.05
esr1
Fold change
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)
F 3,37= 0.7, P > 0.05
esr2a
Proportion amplified
F 3,25= 4.0, P < 0.05*
esr2b
F 3,32= 3.3, P < 0.05*
vtg1
F 3,32= 3.2, P < 0.05*
dnmt1
F 3,32= 0.2, P > 0.05
dnmt3
F 3,25= 0.5, P > 0.05
hdac1
F 3,32= 0.2, P > 0.05
hdac3
F 3,32= 0.1, P > 0.05
mecp2
F 3,32= 4.6, P < 0.05*
mbd2
F 3,32= 3.7, P > 0.05
mbd3a

Supporting Information Figure S2. Transcript profiling of target genes in female livers following exposure to 0.01, 0.1 and 1mg/L BPA for 15 days. Data were collected for 6-8 fish per treatment, and data points classified as outliers (using Chauvenet's criterion) and for which the expression was below the detection limit of the assay were excluded from analysis. Where amplification was detected in more than 70% individuals, data are represented as fold- change relative to the expression in the control group. Where amplification was detected in less than 70% individuals data are presented as the proportion of individuals for which the target genes were detected. Asterisks represent significant differences between treatment groups compared to the solvent control group (*P<0.05 **P<0.01 ***P<0.001).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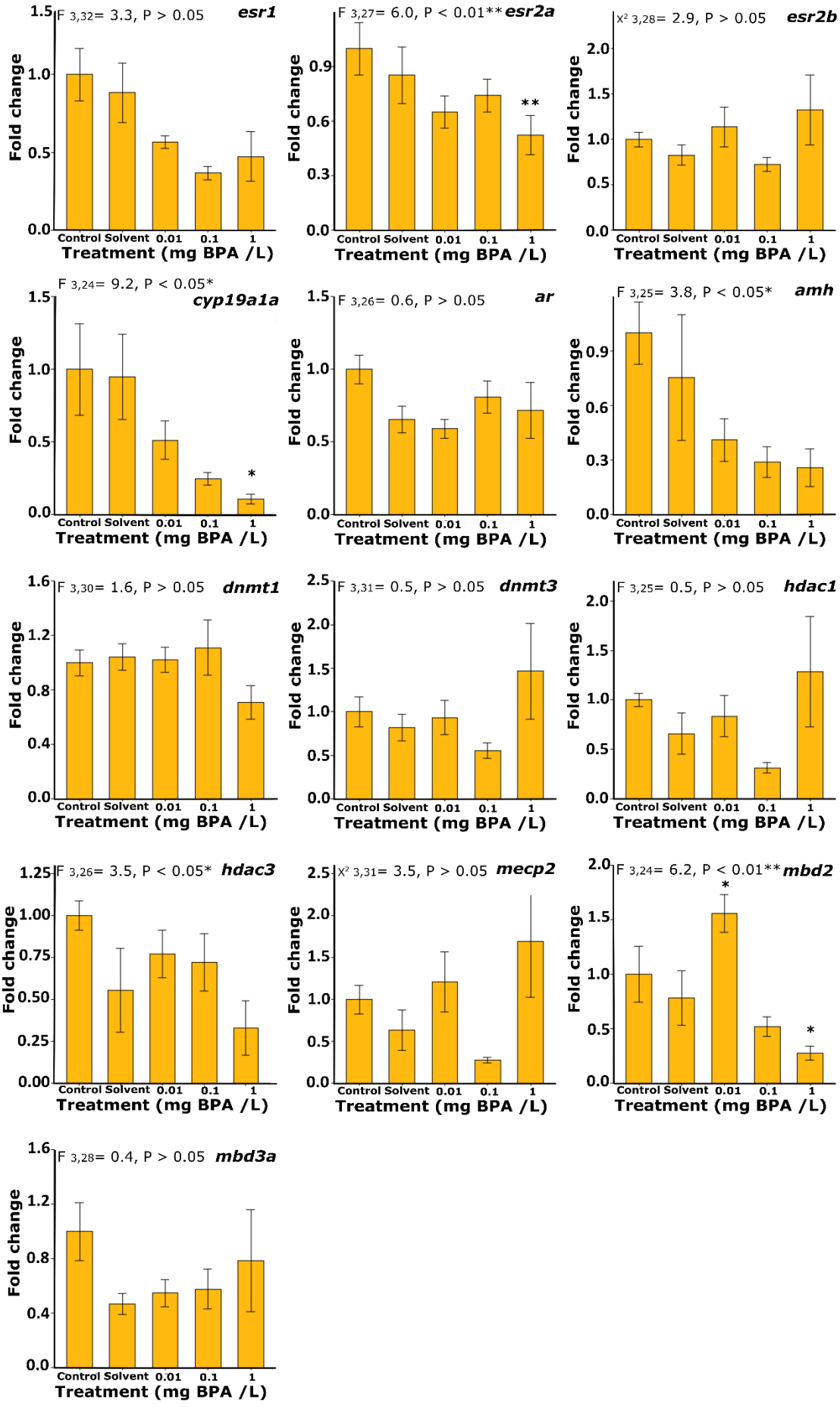
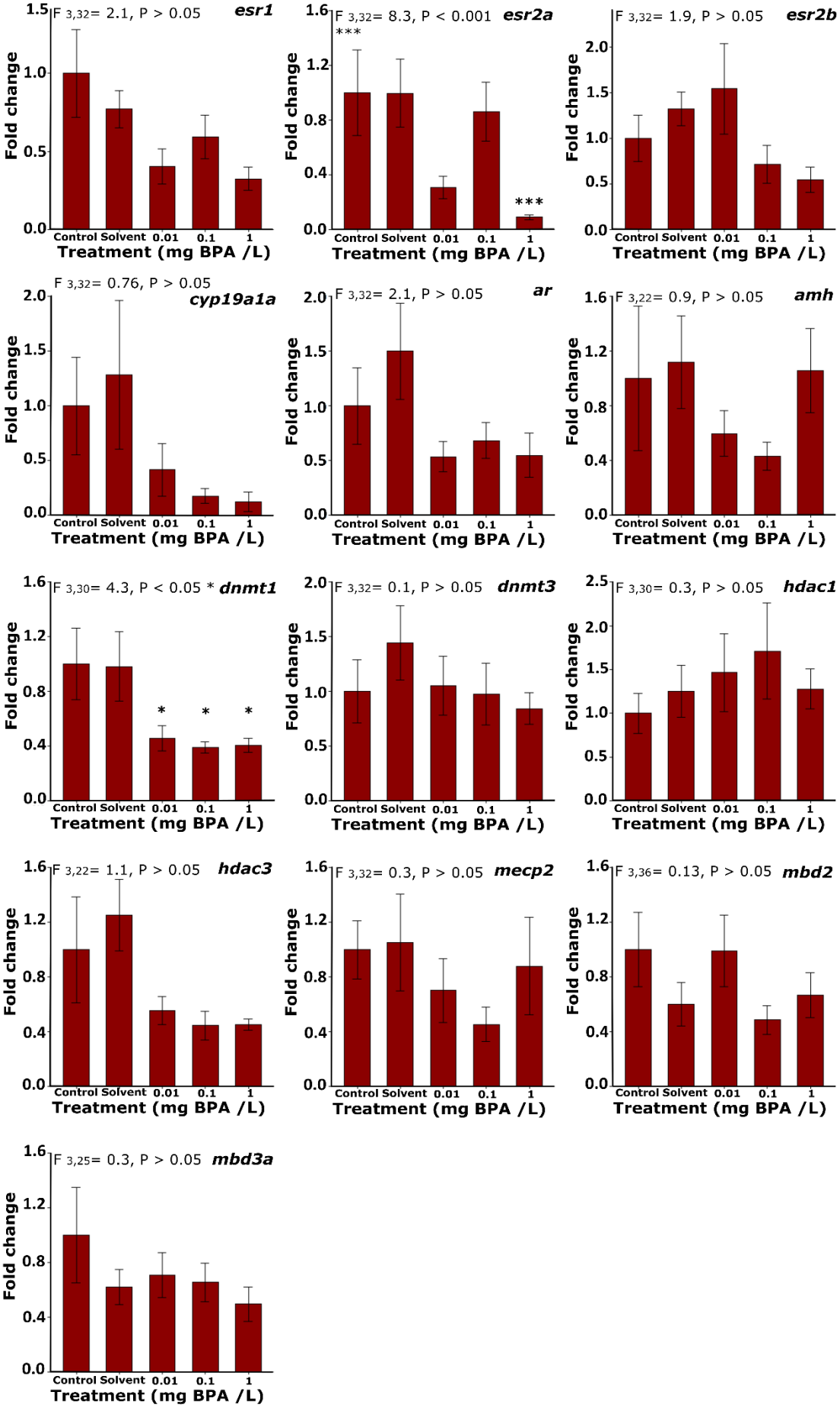
esr1
Proportion amplified
1.00
0.75
0.50
0.25
0.00
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)
F 3,26= 0.3, P > 0.05
esr2a
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)
F 3,25= 4.9, P < 0.01**
esr2b
*
Fold change
8
6
4
2
0
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)
F 3,26= 6.7, P < 0.01**
vtg1
**
Fold change
300
200
100
0
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)
F 3,32= 8.4, P < 0.01**
dnmt1
* *
Proportion amplified
1.00
0.75
0.50
0.25
0.00
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)
F 3,32= 2.1, P > 0.05
dnmt3
Proportion amplified
1.00
0.75
0.50
0.25
0.00
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)
F 3,22= 4.9, P < 0.01**
hdac1
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)
F 3,32= 5.1, P < 0.05*
hdac3
*
Proportion amplified
1.00
0.75
0.50
0.25
0.00
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)
F 3,32= 4.0, P > 0.05*
mecp2
*
Proportion amplified
1.00
0.75
0.50
0.25
0.00
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)
F 3,32= 2.1, P < 0.05*
mbd2
*
Proportion amplified
1.00
0.75
0.50
0.25
0.00
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)
F 3,32= 1.32, P > 0.05
mbd3a
Proportion amplified
1.00
0.75
0.50
0.25
0.00
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)

Supporting Information Figure S3. Transcript profiling of target genes in male livers following exposure to 0.01, 0.1 and 1mg/L BPA for 15 days. Data were collected for 6-8 fish per treatment, and data points classified as outliers (using Chauvenet's criterion) and for which the expression was below the detection limit of the assay were excluded from analysis. Where amplification was detected in more than 70% individuals, data are represented as fold-change relative to the expression in the control group. Where amplification was detected in less than 70% individuals data are presented as the proportion of individuals for which the target genes were detected. Asterisks represent significant differences between treatment groups compared to the solvent control group (*P<0.05 **P<0.01 ***P<0.001).

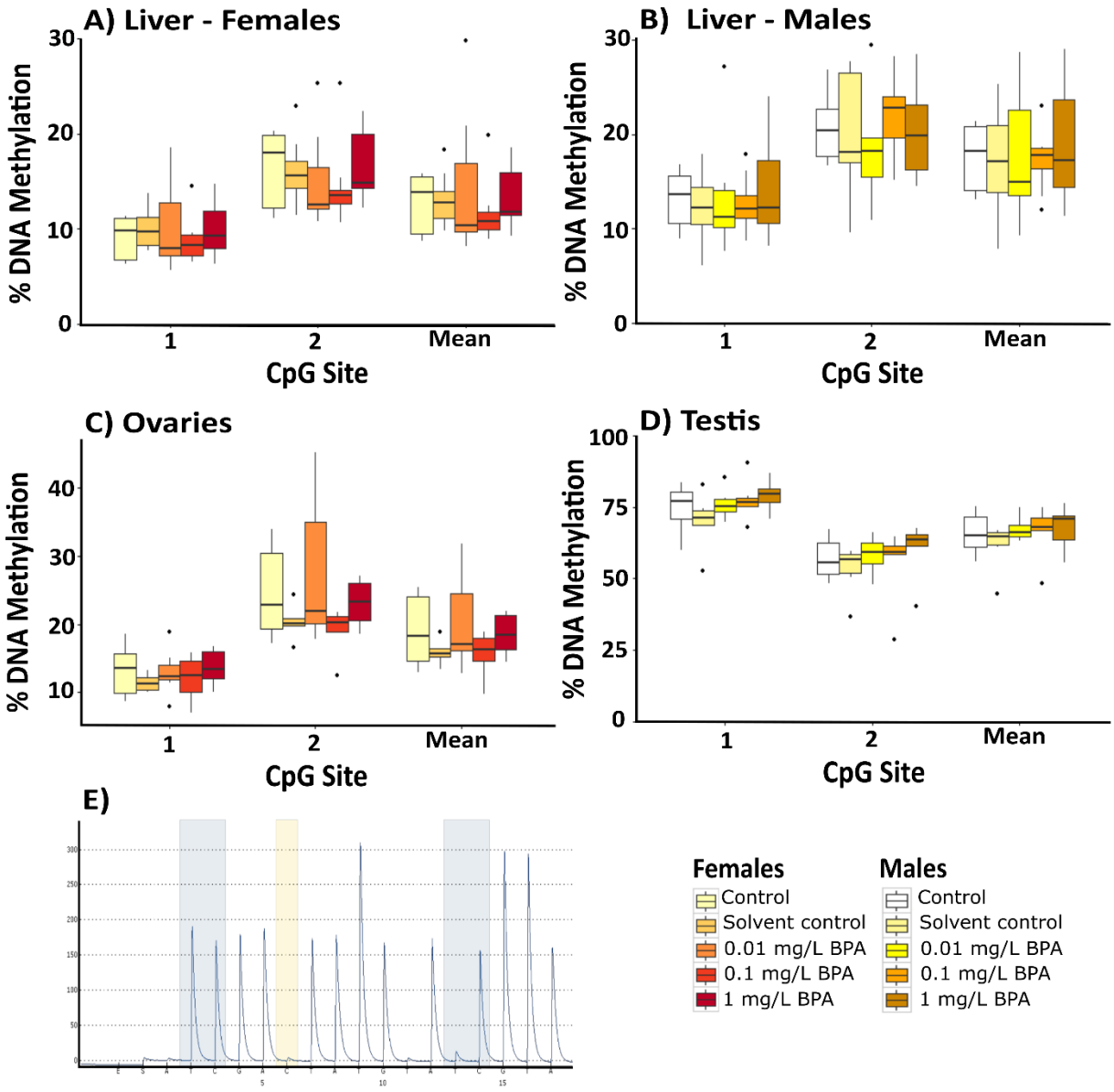

F 3,32= 3.3, P > 0.05
esr1
F 3,27= 6.0, P < 0.01**
esr2a
X² 3,28= 2.9, P > 0.05
esr2b
F 3,24= 9.2, P < 0.05*
cyp19a1a
F 3,26= 0.6, P > 0.05
ar
F 3,25= 3.8, P < 0.05*
amh
F 3,30= 1.6, P > 0.05
dnmt1
F 3,31= 0.5, P > 0.05
dnmt3
F 3,25= 0.5, P > 0.05
hdac1
F 3,26= 3.5, P < 0.05*
hdac3
X² 3,31= 3.5, P > 0.05
mecp2
F 3,24= 6.2, P < 0.01**
mbd2
F 3,28= 0.4, P > 0.05
mbd3a
Fold change
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)

Supporting Information Figure S4. Transcript profiling of target genes in the testis following exposure to 0.01, 0.1 and 1mg/L BPA for 15 days. Data are presented as fold-change relative to the control group. Relative expression was calculated as a ratio of the efficiency corrected expression data for the target gene / efficiency corrected expression data for *rpl8*. For each treatment, data were obtained for 6–8 individual fish. Individual data points classified as outliers, and for which the expression was below the detection limit of the assay were excluded from the analysis using the Chauvenet's criteria. Asterisks represent significant differences between treatment groups compared to the solvent control group (*P<0.05 **P<0.01 ***P<0.001).


F 3,32= 2.1, P > 0.05 esr1
F 3,32= 8.3, P < 0.001 esr2a
***
***
F 3,32= 1.9, P > 0.05 esr2b
F 3,32= 0.76, P > 0.05 cyp19a1a
F 3,32= 2.1, P > 0.05 ar
F 3,22= 0.9, P > 0.05 amh
F 3,30= 4.3, P < 0.05 * dnmt1
*
*
*
F 3,32= 0.1, P > 0.05 dnmt3
F 3,30= 0.3, P > 0.05 hdac1
F 3,22= 1.1, P > 0.05 hdac3
F 3,32= 0.3, P > 0.05 mecp2
F 3,36= 0.13, P > 0.05 mbd2
F 3,25= 0.3, P > 0.05 mbd3a
Fold change
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)

Supporting Information Figure S5. Transcript profiles of target genes in ovaries following exposure to 0.01, 0.1 and 1mg/L BPA for 15 days. Data are presented as fold-change relative to the control group. Relative expression was calculated as a ratio of the efficiency corrected expression data for the target gene / efficiency corrected expression data for *rpl8*. For each treatment, data were obtained for 6–8 individual fish. Individual data points classified as outliers, and for which the expression was below the detection limit of the assay were excluded from the analysis using the Chauvenet's criteria. Asterisks represent significant differences between treatment groups compared to the solvent control group (*P<0.05 **P<0.01 ***P<0.001).

Supporting Information Figure S6. Promoter regions of *amh*, *dnmt1* and *esr1*, showing the location of the CpG sites (indicated in bold), the target sequences used for pyrosequencing (underlined) and putative EREs (highlighted in blue) in relation to the transcription start sites (TSSs; highlighted in red). The sequences shown were derived from Ensembl Zv9 (release 83; assembly GRCz10) and correspond to the following genomic positions: chr22:20,736,779-20,737,279 (*amh*), chr3:54,352,519-54,352,819 (*dnmt1*) and chr20:26,483,369-26,484,513 (*esr1*).



Supporting Information Figure S7. Gene specific DNA methylation profiles for a series of two CpG sites in the promoter region of estrogen receptor 1 (*esr1*) in the liver of female **(A)** and male **(B)** adult zebrafish, and in the ovaries **(C)** and testes **(D)** of adult zebrafish following exposure to 0.01, 0.1 and 1 mg/L BPA. **E)** Example pyrogram of two CpG sites in the 5' flanking regions of the *esr1* gene. Data are presented as boxplots (n = 6-8 for each group). Asterisks indicate significant differences compared to the solvent control (*P<0.05 **P<0.01 ***P<0.001).

Supporting Information Table 1. Measured concentrations of BPA in the exposure water, using HPLC-MS. Concentrations were measured for the three replicate treatment tanks on days 5, 10 and 15 of the exposure and are presented as mean values ± SEM.

Nominal concentration	**Control**	**Solvent control**	**0.01 mg/L BPA**	**0.1 mg/L BPA**	**1 mg/L BPA**
Day 5	< 0.001	< 0.001	0.02 ± 0.00	0.14 ± 0.01	1.28 ± 0.05
Day 10	< 0.001	< 0.001	0.01 ± 0.00	0.14 ± 0.01	1.20 ± 0.14
Day 15	< 0.001	< 0.001	0.01 ± 0.00	0.09 ± 0.03	1.43 ± 0.06
Mean	**< 0.001**	**< 0.001**	**0.01**	**0.12**	**1.30**

Supporting Information Table 2. Target genes, primer sequences and assay details for the RT-QPCR analysis.

Name	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
Aromatase	*cyp19a1a*	AGCCGTCCAGCCTCAG	ATCCAAAAGCAGAAGCAGTAG	101	61.5	2.06
Estrogen receptor 1	*esr1*	TATGACCTGTTGCTGGAGATG	CGCCGTTGGACTGAATGG	130	59.5	2.14
Estrogen receptor 2a	*esr2a*	AGGAGAAAACCAAGTAAACCAATC	AGGCTGCTAACAAGGCTAATG	173	59.0	1.86
Estrogen receptor 2b	*esr2b*	ATCTGCTAATGCTGCTCTCAC	CGCTCTGTTGTCTGTCTTCC	131	57.8	2.18
Androgen receptor	*ar*	ACGAGGGTGTTAGATGAGAC	AAGTATGAGGAAAGCGAGTAAAG	129	58.0	1.97
Anti-Mullerian hormone	*amh*	TGTCTCAACCATCGTCTTCAG	CAGTCAATCCATCCATCCAAAC	124	61.0	2.24
Vitellogenin	*vtg1*	AGCAGCAGCAGTCGTAAC	CAATGATGGTGGCAGTCTTAG	148	57.5	1.84
DNA (cytosine-5)-methyltransferase 1	*dnmt1*	CGCTGTCGTGTTGAGTATGC	TCCCTTGCCCTTTCCTTTCC	180	58.5	2.06
DNA (cytosine-5)-methyltransferase 3	*dnmt3*	TGATGCCGTGAAAGTGAGTC	TTGCCGTGTAGTGATAGTGC	172	58.5	2.19
Histone deacetylase 1	*hdac1*	TGACAAACGCATCTCCATTCG	CTCTTCTCCATCCTTCTCTTCTTC	157	58.0	2.04
Histone deacetylase 3	*hdac3*	GAATGTGTGGAGTTTGTGAAGG	CTGGATGAAGTGTGAAGTCTGG	190	57.0	1.98
Methyl CpG binding protein 2	*mecp2*	GAGGCAGAAACAGGACAG	TGGTGGTGATGATGATGG	176	58.0	2.13
Methyl-CpG-binding domain protein 2	*mbd2*	AACAGCCTCCATCTTCAAG	CGTCCTCAGCACTTCTTC	166	59.0	2.19
Methyl-CpG-binding domain protein 3a	*mbd3a*	ACTCTTCTTTCGGCTCTG	TCTTCCTGCTTCCTGATG	164	57.0	1.99

Supporting Information Table 3. Bisulfite-pyrosequencing primers and assay details for the gene promoters analyzed.

Name	**Symbol**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Sequence Primer (5'-3')**	**Sequence analysed (5'-3')**	**Ta (°C)**
Estrogen receptor 1	*esr1*	AGAGGAGGTAAATAAATTAAAGATAGTTAG	Biotin-TACTCCTTTAACATATAATTTCCCATAACA	GGTAAATAAATTAAAGATAGTTAGG	TYGATATTGAYGGTTATTTTTTAGAGTAGGTTATGGTAATTAG	58.0
Anti-Mullerian hormone	*amh*	GTTTTTTATTTTTATGGGATGGTAGTTAGG	Biotin-AAACACAACTTAAAAACTTCCACTTATAT	TTGTTTTGAAGTATATTTGGAT	TATAYGTAATGGGGAATGTTTTAGTTTAAGGAAYGGTATTTGGTATTATTAAYGGGTTATTTATAAAATAATGTTTTTA	58.0
DNA (cytosine-5)-methyltransferase 1	*dnmt1*	GGGTATTAATATGTGATAGTGTTAATTGTAG	Biotin - TAAACCCAAATACACTCACAACAC	TTATGAATTGTAGTTAGTAGTTGA	GAAATAYGYGYGGGTYGTTTTTTYGYGYGGAAAYGYGGGTGAGTYGGAYGTTATT	58.0

Supporting Information Table 4. Statistical associations between **a)** BPA concentration and transcription; **b)** BPA concentration and global methylation; **c)** *dnmt1* transcription and global methylation; **d)** BPA concentration and specific CpG loci methylation; **e)** transcript expression and specific CpG loci methylation.

Table 4a. Regression analysis between BPA concentration and transcription .				
Tissue	**Gene**	**-**	**Adjusted R2**	**P value**
Liver Female	*vtg1*	-	0.155	**0.018**
	esr1	-	0.142	**0.049**
	esr2b	-	-0.036	0.896
	hdac1	-	-0.045	0.718
Liver Male	*vtg1*	-	0.181	**0.012**
	esr2a	-	0.021	0.246
	esr2b	-	0.117	0.057
	hdac1	-	0.141	**0.033**
Ovary	*esr1*	-	0.046	0.161
	esr2a	-	0.238	**0.017**
	esr2b	-	0.081	0.086
	amh	-	0.021	0.248
	cyp19a1a	-	0.031	0.220
	ar	-	0.020	0.245
	dnmt1	-	-0.021	0.449
	dnmt3	-	0.036	0.186
	hdac1	-	-0.035	0.674
	hdac3	-	0.048	0.166
	mecp2	-	-0.043	0.751
	mbd2	-	-0.039	0.722
	mbd3a	-	0.005	0.303
Testis	*esr1*	-	-0.031	0.619
	esr2a	-	0.053	0.121
	esr2b	-	0.049	0.148
	amh	-	0.075	0.094
	cyp19a1a	-	0.189	**0.025**
	ar	-	-0.032	0.754
	dnmt1	-	0.111	**0.046**
	dnmt3	-	0.132	**0.033**
	hdac1	-	0.059	0.117

	hdac3	-	0.080	0.092
	mecp2	-	0.083	0.072
	mbd2	-	0.135	**0.048**
	mbd3a	-	0.022	0.226

Table 4b. Regression analysis between BPA concentration and global methylation.

Tissue	Gene	-	Adjusted R2	P value
Testis	-	-	0.033	0.949
Ovary	-	-	0.051	0.121

Table 4c. Correlation analysis between *dnmt1* trancript expression and global methylation.

Tissue	Gene	-	Correlation coefficient	P value
Testis	*dnmt1*	-	0.110	0.576
Ovary	*dnmt1*	-	0.293	0.198

Table 4d. Regression analysis between BPA concentration and specific CpG loci methylation.

Tissue	Gene	CpG Position	Adjusted R2	P value
Liver Female	*esr1*	1	-0.031	0.075
		2	-0.033	0.834
	dnmt1	1	0.054	0.109
		2	0.029	0.179
		3	0.046	0.128
		4	0.076	0.073
		5	0.024	0.197
		6	0.040	0.144
		7	0.065	0.089
		8	0.085	0.069
		9	0.079	0.069
		10	0.070	0.081
		11	0.051	0.133
		Mean	0.063	0.093
Liver Male	*esr1*	1	-0.025	0.649
		2	-0.030	0.779
	dnmt1	1	-0.036	0.905
		2	-0.026	0.597
		3	-0.003	0.348
		4	-0.030	0.681
		5	-0.031	0.700
		6	-0.024	0.565
		7	-0.035	0.820
		8	-0.025	0.581

		9	-0.233	0.552
		10	-0.023	0.551
		11	-0.017	0.465
		Mean	-0.023	0.541
Ovary	*esr1*	1	0.052	0.115
		2	-0.024	0.583
	amh	1	0.005	0.295
		2	0.144	0.246
		3	-0.034	0.836
	dnmt1	1	0.082	0.068
		2	0.087	0.063
		3	0.092	0.057
		4	0.105	**0.045**
		5	0.114	**0.038**
		6	0.100	**0.049**
		7	0.044	0.137
		8	0.115	**0.038**
		9	0.091	0.058
		10	0.098	0.051
		11	0.061	0.100
		Mean	0.094	0.055
Testis	*esr1*	1	-0.016	0.465
		2	-0.009	0.397
	amh	1	0.163	**0.013**
		2	0.036	0.152
		3	-0.017	0.497
	dnmt1	1	-0.380	**0.047**
		2	0.003	0.304
		3	0.011	0.255
		4	-0.016	0.480
		5	0.000	0.318
		6	0.000	0.325
		7	-0.016	0.471
		8	-0.001	0.334
		9	0.006	0.290
		10	0.038	0.182
		11	0.005	0.313
		Mean	-0.001	0.335

Table 4e. Correlation analysis between trancript expression and specific CpG loci methylation.

Tissue	Gene	CpG Position	Correlation coefficient	P value
Ovary	*esr1*	1	-0.229	0.281
		2	-0.225	0.289

	amh	1	-0.323	0.164
		2	-0.286	0.235
		3	-0.286	0.221
	dnmt1	1	-0.050	0.830
		2	-0.026	0.912
		3	-0.003	0.991
		4	-0.142	0.540
		5	0.097	0.674
		6	0.082	0.724
		7	0.192	0.404
		8	0.065	0.780
		9	-0.033	0.887
		10	-0.055	0.814
		11	-0.068	0.771
		Mean	0.023	0.921
Testis	*esr1*	1	0.095	0.653
		2	0.386	**0.035**
	amh	1	-0.452	**0.014**
		2	-0.047	0.815
		3	-0.214	0.285
	dnmt1	1	-0.024	0.903
		2	-0.180	0.359
		3	-0.204	0.306
		4	-0.157	0.425
		5	-0.523	**0.004**
		6	-0.514	**0.006**
		7	-0.475	**0.014**
		8	-0.435	**0.023**
		9	-0.382	0.066
		10	-0.035	0.886
		11	-0.039	0.889
		Mean	-0.380	**0.047**

Chapter 4

Pre-exposure to copper caused a reduced transcriptional response and increased copper accumulation upon re-exposure in adult male three-spined stickleback (*Gasterosteus aculeatus*).

In Preparation

Pre-exposure to copper caused a reduced transcriptional response and increased copper accumulation upon re-exposure in adult male three-spined stickleback (*Gasterosteus aculeatus*).

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Abstract:

Metal contamination is widespread in the aquatic environment, and its sources include mining, agricultural runoff and industrial activity. Copper is particularly interesting due to its role as an essential element for life. To date, many studies addressing chemical toxicity in the lab focus on acute exposures. However, populations of fish inhabiting most aquatic environments are exposed to fluctuating exposure concentrations over time, and their response to a chemical will likely be influenced by the environmental conditions they have experienced over time. The present study aimed to investigate if prior exposure to environmentally relevant concentrations of copper result in differential responses upon re-exposure in adult three-spined stickleback, and to identify the molecular mechanisms which may be driving these differential responses. We exposed adult male three-spined stickleback to 0 or 20 μg/L copper for four days, followed by a depuration period of 30 days in clean water, and then re-exposed them to the same exposure concentrations for a further four days. We measured the copper content in the gill, liver and muscle, and performed transcriptomic analysis of gills and livers using the Illumina sequencing platform. We report that fish that had received prior exposure to copper had significantly more copper in the gill and elicited a reduced transcriptional response upon re-exposure, when compared to fish exposed for the first time. This suggests that pre-exposure to copper results in an altered transcriptional and physiological response and indicates a process of acclimation. Importantly, fish exposed to copper for four days, followed by depuration did not show any differences in copper content and transcriptional profile compared to control fish at the end of the depuration period, suggesting that differential responses observed in pre-exposed fish may occur via epigenetic mechanisms. Together, these findings support the hypothesis

that pre-exposure to copper has the potential to alter responses to copper in future exposure scenarios, highlighting the potential for significant variation in susceptibility to pollutants in wild populations as a result of their exposure history and suggesting that these responses can be induced even after short exposure windows in adult fish.

Keywords: teleost, acclimation, copper, multiple exposures, toxic metal, transcriptomics

Introduction:

Metal contamination is widespread in the aquatic environment, driven by sources such as mining, agricultural runoff and industrial activity. Chronic metal exposure can cause a number of toxic effects in wild populations of fish, including impaired growth, immune response and behaviour. Extreme metal exposure has also been shown to impact on genetic diversity [1–4].

Copper occurs naturally in the aquatic environment and is particularly interesting due to its role as an essential element for life, acting as a co-factor in a number of enzymatic reactions, including energy production and cellular metabolism [5]. However, when found in excess, copper is highly toxic, and aquatic organisms are particularly sensitive due to their continual exposure to contaminated waters via the gills and skin, as well as via the diet [6]. Copper has been shown to disrupt a number of biological processes including branchial ion-regulation and gas exchange, enzymatic activity, immune function, and to cause oxidative stress [7–9]. In the environment, copper concentrations vary widely reaching up to 0.2-30 µg/L in many freshwater environments, and in heavily contaminated systems concentrations can reach levels known to be toxic to fish [10–12]. This is particularly relevant for water bodies associated with industrial chemical discharge and mining activity where concentrations up to 100-200,000 µg/L have been measured [12]. In a recent analysis copper has been identified as the most significant metal pollutant in UK waters [13].

For metals, extreme cases exist of fish populations that are able to survive in highly contaminated waters, where concentrations of metals far exceed the LC50 for those species [4,14,15]. Although rare, well studied examples include the lakes in North America, which are highly contaminated with metals (particularly copper, cadmium and nickel) as a result of

local industrial and mining activity, but sustain viable populations of yellow perch (*Perca flavescens*) [1–3,16–19]. A further example of potential adaptation to chronic metal exposure has been reported in brown trout (*Salmo trutta*), inhabiting the River Hayle in Cornwall (Southwest England), where fish are chronically exposed to extreme metal concentrations (particularly zinc, iron and copper), likely entering the river via run off from the surrounding historic mines after peaks in rainfall [15,20]. For these populations, evidence suggests that metal tolerance is likely to be driven by genetic selection for a metal tolerant genotypes as a result of long term exposure to relatively high concentrations over many generations [1,4]. Furthermore, even for populations of fish inhabiting relatively un-impacted waters, their toxicological responses to metals can vary significantly. For example, the physiological responses to copper were compared across five populations of nine-spined stickleback (*Pungitius pungitius*) residing in similar latitudes and showed that there was significant physiological differences in metabolic rate and stress response following exposure among those populations [21].

To date, many studies addressing chemical toxicity in the laboratory focused on acute short term exposures [22]. However, repeated and continuous exposures of equivalent dose may not elicit the same toxicity [23]. Populations of fish inhabiting most aquatic environments are exposed to time-varying or repeated pulses of exposure, driven by runoff events or spills, or due to their mobility between polluted and clean waters [24,25]. Organisms must respond to these fluctuations occurring within the lifespan of an individual fish. Hydrologic dilution and dispersion may also contribute to variations in exposure concentration [26], and during periods of depuration from the chemical of interest, detoxification, elimination and recovery may occur within the exposed organism [26,27].

The sustainability of fish populations is critically dependent on their ability to adapt to frequent changes in the local environment [28]. Despite this, legislation to protect the environment from chemical contamination are often based on toxicological measurements conducted under optimal laboratory conditions, and that do not take into account the exposure history or the variation in susceptibility of wild populations.

Few examples have been reported where the role of sub-lethal exposure concentrations in modifying the responses of organisms during subsequent exposure scenarios were investigated. Often these studies focus on lethal concentrations which are not commonly found in the environment. A study exploring the effects of pulsed copper exposure versus continuous short term exposure in early life-stage fathead minnows (*Pimephales promelas*), reported that continuous exposures resulted in a greater mortality rate when compared to pulsed exposures of the same concentration [27]. In addition, exposure of a naïve population of the amphipod *Hyalella azteca* to lethal concentrations of copper sulfate ($CuSO_4$), followed by a depuration period in clean water and a subsequent second exposure resulted in the cumulative mortality curve to be lower than that of *Hyalella* that were never exposed, indicating some induced tolerance [29].

A study exploring the occurrence of copper acclimation in the least killifish (*Heterandria formosa*) found that fish pre-exposed to 15 µg/L copper for seven days, followed by exposure to a lethal concentration (150 µg/L copper) had a significantly longer time to death compared to the control fish, however these fish were not subject to a period of depuration or recovery [30]. In addition, Grosell *et al*., report that copper acclimated rainbow trout (*Oncorhynchus mykiss*) clear a single bolus of injected copper from their plasma more effectively than non-acclimated fish, reporting a four-fold increase in hepatobiliary copper

excretion in copper acclimated fish [31]. These data suggest that pre-exposure to an environmentally relevant sub-lethal concentration of copper can induce an acclimation response in fish, but they do not elucidate the molecular mechanisms responsible for these effects.

Given that exposure to pollutants such as copper in the wild are intermittent, it is important to understand the role of prior exposure to sub-lethal and environmentally realistic concentrations of copper in subsequent exposure scenarios. In addition to long term selection events, fish must respond to fluctuations in the concentration of, and type of pollutants or stressors within their local environment, within their lifespan. The present study aims to investigate if prior exposure to environmentally relevant concentrations of copper, result in differential responses upon re-exposure in adult three-spined stickleback, and to identify the molecular mechanisms which may be driving differential responses. We have controlled for genetic background, by using individuals form the same laboratory population. We exposed adult male three-spined stickleback to 20 µg/L copper for four days, followed by a depuration period of 30 days in clean water. Fish were then re-exposed to 20 µg/L copper for four days, and samples were collected for analysis of tissue metal concentration and transcriptomics using the Illumina sequencing platform to perform RNA-Seq.

Materials and Methods:

Fish source and maintenance

A freshwater population of three-spined stickleback (originating from the River Erme, Devon, United Kingdom, provided by the University of Plymouth) was maintained at the University of Exeter in the Aquatic Resource Centre. Fish were maintained in mixed sex stock tanks (112 L) and supplied with aerated synthetic freshwater [32]. Holding tanks were supplied with mains tap water which 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 photoperiod of 18:6 light/dark (with 30 minute dawn/dusk transitional periods) and a temperature of 15 ± 1 °C. Throughout the copper exposure periods (two periods of four days), fish were not fed to ensure that the conditions during the copper exposure were maintained as constant as possible and copper uptake occurred predominantly via the gill. During the 30 day depuration period, and prior to the exposure, fish were fed to satiation twice daily with blood worms (*Chironomus sp.*; Tropical Marine Centre, Chorleywood, UK) and once daily with live *Artemia nauplii* once daily (ZM Premium Grade Artemia; ZM Ltd.).

Exposure to copper and tissue sampling

All experiments were conducted under approved protocols according to the UK Home Office regulations for use of animals in scientific procedures.

Adult stickleback males were exposed to 0 or 20 µg/L of copper (added as copper sulphate, Sigma Aldrich) for four days for an initial pre-exposure treatment (Exposure 1; Figure 1), via a flow through system employing a flow rate of 144 L/day for each 40 L exposure tank (3.6x tank volumes per day). At the end of the fourth day, fish were then transferred to 122 L holding tanks and maintained at a flowrate of 250 L/day in clean water for a depuration period of 30 days (Depuration; Figure 1). On day 34 of the study, at end of the 30 day depuration phase, fish were re-exposed to 0 or 20 µg/L of copper for a four day second exposure treatment ending on day 38 (Exposure 2; Figure 1), using the same system described above. Appropriate controls were maintained in parallel, as described in figure 1. Treatment groups were classified as the control_control (absolute control), cu_control (fish exposed to copper for four days followed by 34 days of depuration), control_cu (fish maintained as controls until exposure 2, then exposed to copper for four days with no period of depuration), and cu_cu (fish pre-exposed to copper for four days followed by 30 days depuration and a second four day copper exposure; Figure 1).

For exposure 1, each treatment group comprised of four replicate exposure tanks (40 L) containing 12 fish per tank (n = 48 fish per treatment). During the 30 day depuration period the remaining fish from each replicate tank were mixed into two 122 L holding tanks per treatment group to ensure that fish were kept in large groups, avoiding excess aggressive behaviours from dominant males. For exposure 2, each treatment group comprised of two replicate exposure tanks (40 L) containing 7 fish per tank (n = 14 fish per treatment; Figure

1). The concentration of oxygen in each tank was measured twice daily throughout the 38 day experiment to ensure they were kept close to 100% air saturation. Daily measurements of conductivity and pH where also conducted.

On day four of exposure 1 (Figure 1), 9 fish from each treatment group (control and copper exposed) were sacrificed by lethal dose of benzocaine followed by destruction of the brain, in accordance with the UK Home Office regulations. The wet weight and fork length were recorded, and the condition factor for each fish was calculated (k= (weight (g) x 100)/ (fork length (cm)) ^{3}). The livers were dissected and weighed, and the hepatosomatic index (HSI = (liver weight (mg)/ (total weight (mg) - liver weight (mg)) x 100)) was calculated. Gill and liver samples were collected, snap frozen in liquid nitrogen and stored at -80 °C until RNA-Seq analysis. Gill, liver and muscle samples were also collected for metal analysis (placed in an acid washed tubes, weighed to determine wet mass of the tissue, then stored at -20 °C). On day 18 and 34 (after 14 and 30 days of depuration respectively), a further 9 fish from each treatment group (control and exposed to copper during the initial exposure) were sampled as described above for metal analysis. On day 38 of the experiment (at the end of exposure 2), a further 9 fish from each treatment group (control_control - absolute control, cu_control - exposed to copper followed by depuration, control_cu - exposed to copper with no period of depuration, and cu_cu - fish pre-exposed to copper followed by a second exposure treatment; Figure 1) were sampled as described above for transcriptional and metal analysis.

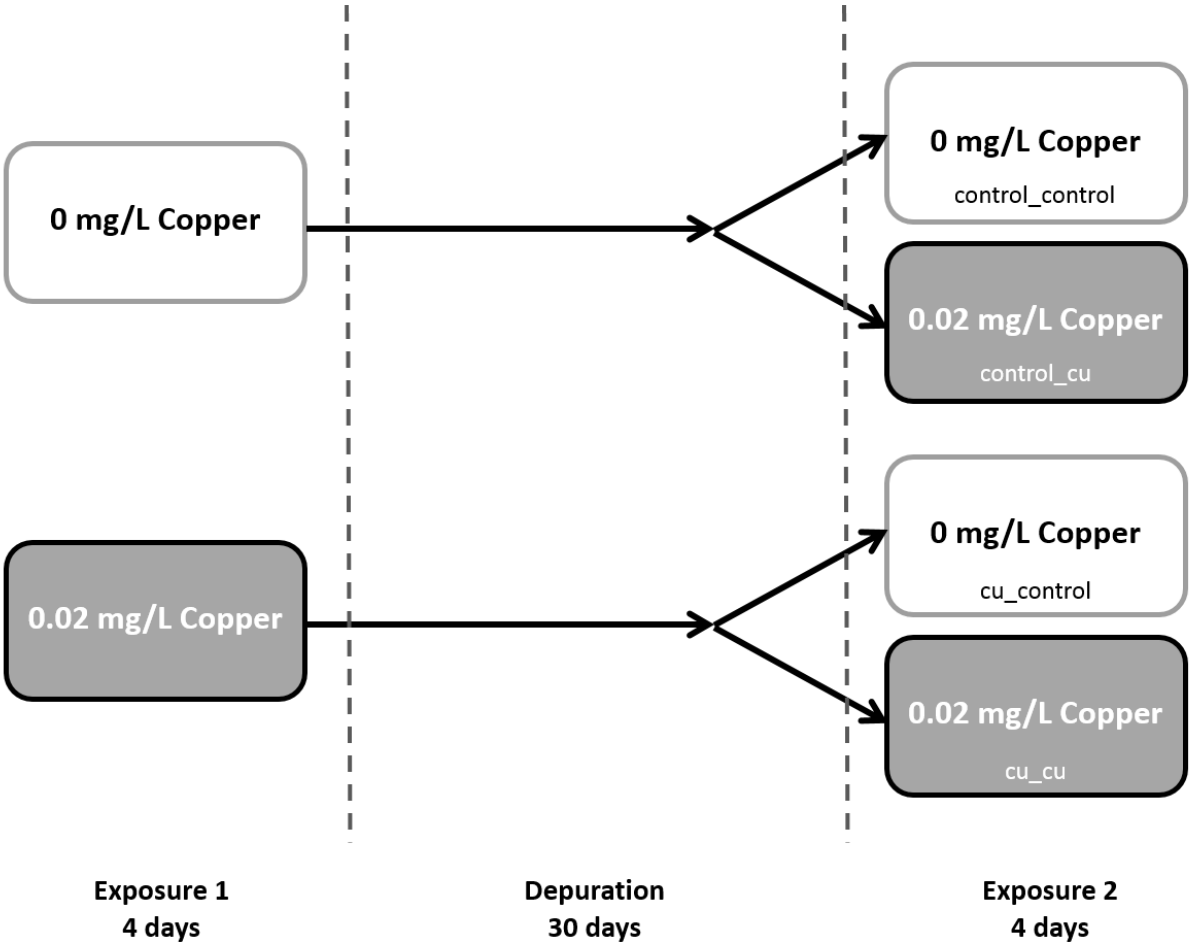


Figure 1. The experimental design for this study. Fish were exposed to copper or kept in control conditions for 4 days followed by a 30 day depuration period. After this period, fish from both the control and the copper exposed group were either re-exposed to copper for 4 days or maintained in control conditions. Sampling (n=9 per treatment group) occurred at the end of the first exposure, at days 14 and 30 of the depuration period and at the end of the second exposure.

Metal analysis in water and tissue samples

Water samples were collected from each tank on days one and three of each four day exposure (experimental days 1, 3, 36 and 38) and on the final day of the 30 day depuration period (experimental day 34). Samples were then 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 re-distillation, ≥ 99.999% trace metals basis, Sigma Aldrich) to a final concentration of 0.01% in each falcon tube. Tissue samples were freeze dried and the dry mass determined before acid digestion. Tissue digestion was conducted by adding 500 µl of nitric acid to each tube and incubated at room temperature for 48 hours with frequent vortexing until all samples had completely digested. 0.1% hydrogen peroxide (Fisher; Hydrogen Peroxide, 100 volume > 30%w/v) was added in order to facilitate the breakdown and removal of the fatty material. All samples were covered with Parafilm to prevent evaporation. The resulting digested solution was then diluted 1:10 with ultrapure water to a total volume of 10 ml. 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.

RNA extraction, library preparation and sequencing

Transcript profiling was conducted in the gills and livers of 6 fish from each treatment group at the end of exposure 2. RNA was extracted using the AllPrep DNA/RNA MiniQiagen Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Prior to RNA-Seq library preparation, RNA was treated to remove genomic DNA contamination on the AllPrep

column using the RNase-Free DNase Set (Qiagen, Hilden, Germany). Total RNA concentration, purity and integrity were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA) and an RNA Screen Tape on the 2200 TapeStation Instrument (Agilent Technologies, Inc., USA). All RNA input to library construction was of high quality with A260/A280 and A260/A230 ratios > 1.8. ERCC spike-in control mixes (Ambion) were added to all individual RNA samples, according to the manufacturer's instructions to be used for normalisation between sample libraries. cDNA libraries from all samples were then prepared using the Illumina TruSeq LT Stranded mRNA Sample Preparation kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions, multiplexed with 48 samples (24 livers and 24 gills) across four lanes. Libraries were sequenced using an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA), to generate 100 bp paired-end reads for the gill samples, and 100 bp single-end reads for liver samples.

Read mapping and differential expression analysis

All analyses were carried out on a local server running under the Linux environment. Prior to read mapping, sequences were filtered for quality using trimmomatic v0.35 [33] (default parameters and the TruSeq3-PE and TruSeq3-SE adapter sequences for the gill and liver sample libraries, respectively).

Reference stickleback genome sequences were retrieved from Ensembl (BROAD S1 Ensembl release 87, http://www.ensembl.org/Gasterosteus_aculeatus/Info/Index), and ERCC spike-in sequences from the Thermo Fisher Scientific website (Thermo Fisher Scientific, 2016). ERCC spike-in sequences and the nucleotide sequence for stickleback metallothionein (*mt*;

[11]) were appended to the reference genome. The reference library was prepared and reads aligned to the stickleback genome using bowtie [34] within RSEM version 1.2.29 [35].

The RUVSeq package was used to execute the RUVg function in order perform exploratory analysis to identify outliers, and to estimate factors of unwanted variation using ERCC spike-ins [36]. In the gill dataset, sample 84 was identified as an outlier, which could not be remediated using digital normalization, therefore this sample was excluded from the dataset. In addition, normalisation using the ERCC spike-ins was not found to be superior to the calcNormFactors() used as standard in the edgeR package and therefore the latter was used for the subsequent analysis.

Raw count data for each transcript were extracted and input into edgeR Bioconductor package [37] in R [38] in order to identify the expressed genes across all samples and differentially expressed genes between treatment groups. The calcNormFactors() function was used to perform digital normalisation of the RNA-Seq data for differences in library size between samples. Transcripts were considered differentially expressed with a FDR < 0.05 (Benjamini-Hochberg correction). Hierarchical clustering was performed on all differentially expressed transcripts using a Euclidean distance metric, in the cluster() package for R. Full annotations for the differentially expressed genes were downloaded from BioMart [39] using stickleback Ensembl gene IDs.

Biomart was also used to identify zebrafish (*Danio rerio*) orthologues for these genes and corresponding zebrafish Ensembl gene IDs were downloaded and subsequently used for functional annotation analysis. Functional annotation analysis was then performed for differentially expressed genes identified from each treatment when compared to the absolute control (control_control) using the Database for Annotation, Visualisation and Integrated Discovery (DAVID v6.8) [40]. Ensembl gene IDs for all expressed transcripts in this

study were used as background lists. KEGG pathways and Gene Ontology (GO) terms for Biological Process, Cellular Component and Molecular Function were considered significantly over-represented when the adjusted P value was < 0.05 (Benjamini-Hochberg correction). Significant KEGG pathways were annotated with fold change information using the pathview() package [41].

Further statistical analysis

For samples collected on the final day of exposure 1 (day four), the metal uptake data, morphometric parameters data from exposure 1 and depuration time points (days 4, 18 and 34), comparisons between control and copper exposed groups were performed using the Student's t-Test due to the fact comparisons were only made for one independent variable.

For samples collected on the final day of exposure 2 (day 38); metal uptake data and morphometric parameters were analysed using an analysis of variance model in R in order to test for the effect of multiple independent variables (including the exposure concentration for exposure 1 and for exposure 2) [38]. A separate model was used for each dataset, to test for effects of exposure 1, exposure 2 and the interaction between these variables on the endpoints measured at the end of exposure 2 (day 38). Minimum adequate models (MAM) were derived by model simplification using F tests based on analysis of deviance. Test results reported refer to the significance of each term within the MAM. When a significant effect of an interaction was identified, pairwise comparisons to determine which groups differed were conducted using Tukey's HSD post hoc test.

P values of ≤0.05 were considered to be significant. All data are presented as mean ± SEM. Data that did not meet the normality (Sharpo-Wilko test) and equal variance (Bartlett) test were log transformed prior to analysis.

Results:

Water chemistry and morphometric parameters

For both exposure 1 and the exposure 2 the mean measured concentration of copper in each tank water was 0.0231 ± 0.0007 mg/L for copper treatments, 0.0056 ± 0.0004 mg/L for the control treatment and 0.0064 ± 0.0002 mg/L during the depuration period for both control and copper pre-exposed fish, a detailed breakdown is presented in Supporting Information Table 1. The average conductivity (266.05 µs ± 0.50) and pH level (7.85 ± 0.05) measured in the tanks remained stable throughout the exposure, and were not affected by the treatment.

Throughout the study, no mortalities were recorded and fish remained in good condition. The mean fork length, condition factor and hepatosomatic index of the fish where 5.51 ± 0.08 cm, 1.04 ± 0.03 g/cm^3 and 2.92 ± 0.25, respectively, and there were no significant differences between treatment groups (Supporting Information Table 2, 3 and 4). On day four of the experiment (at the end of exposure 1), there was a significant difference in weight between fish exposed to control conditions and copper during the pre-exposure treatment (1.60 ± 0.08 g and 1.93 ± 0.11 g respectively, P = 0.042; Supporting Information Table 2), however for the remainder of the 38 day study the mean fish weight was 1.75 ± 0.08 g, and there were no significant differences between treatment groups (Supporting Information Table 3 and 4).

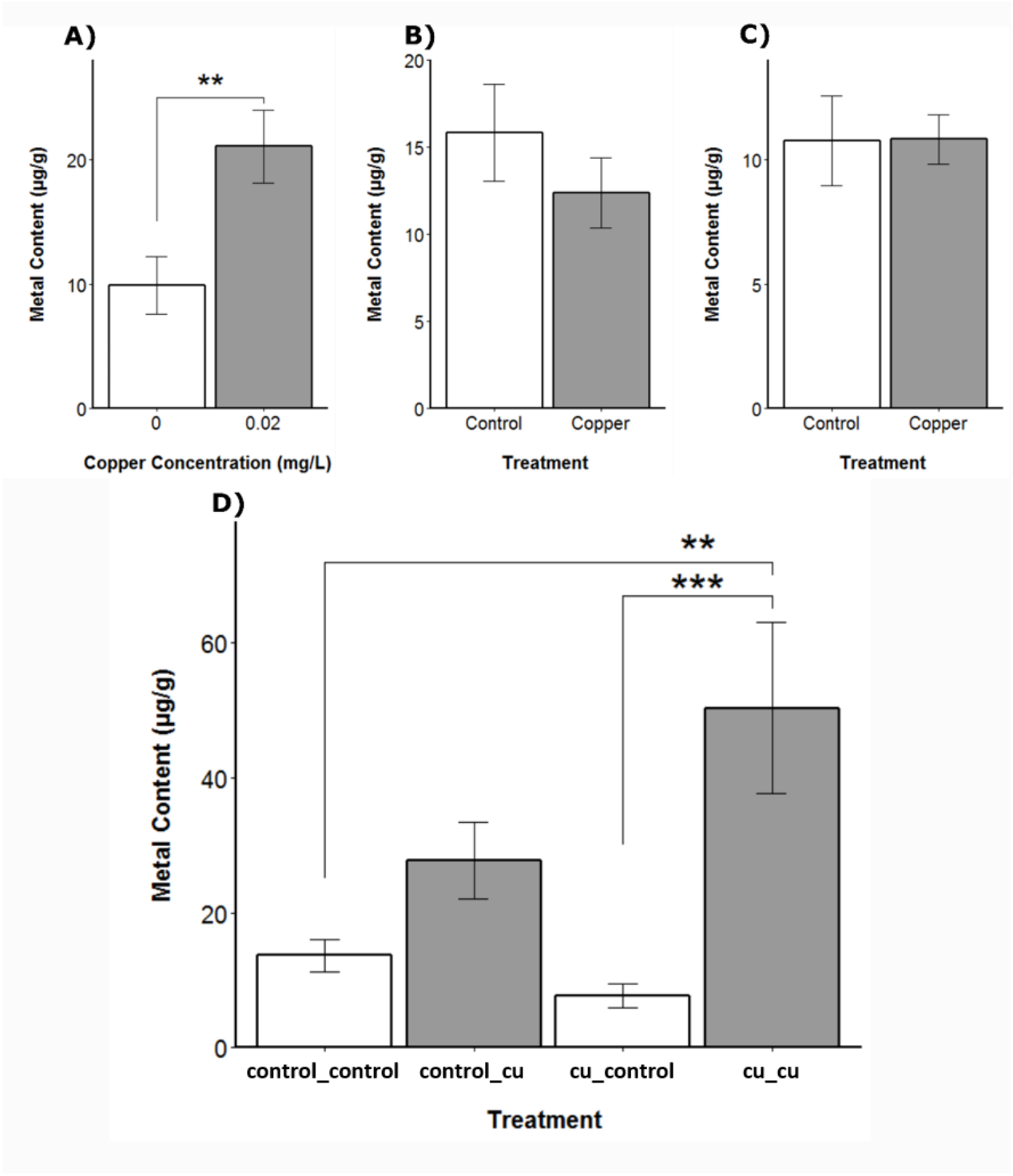

A)
**
Metal Content (µg/g)
20
10
0
0
0.02
Copper Concentration (mg/L)
B)
Metal Content (µg/g)
20
15
10
5
0
Control
Copper
Treatment
C)
Metal Content (µg/g)
10
5
0
Control
Copper
Treatment
D)
**
***
Metal Content (µg/g)
60
40
20
0
control_control
control_cu
cu_control
cu_cu
Treatment

Figure 2. Measured copper concentrations in the gill of male stickleback sampled **A)** on day 4 of the study, (after exposure 1) **B)** on day 18 (after 14 days depuration) and **C)** day 34 of the study (after 30 days depuration) and **D)** on day 38 of the study (after exposure 2). Treatment groups were classified as the control_control (absolute control), cu_control (fish exposed to copper for 4 days followed by 34 days of depuration), control_cu (fish maintained as controls until exposure 2, then exposed to copper for 4 days with no period of depuration), and cu_cu (fish pre-exposed to copper for 4 days followed by 30 days depuration and a second 4 day copper exposure; Figure 1). Tissue metal content was measured 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 and mean values reported in Supporting Information Tables 5-7. On day 38 of the experiment (at the end of exposure 2), general linear modelling revealed a significant interaction between exposure 1 and exposure 2 ($P = 0.0463$).

Tissue metal Concentration

In the gill, on day four of the experiment (at the end of exposure 1), there was a 2.13 fold greater tissue copper concentration in fish exposed to copper when compared to the control (P = 0.0097; Figure 2A; Supporting Information Table 5). However, during the depuration period, we report no significant difference in tissue copper concentration in the gills of fish from both treatments sampled on day 18 (P = 0.3255; Figure 2B; Supporting Information Table 5) or day 34 (P = 0.9745; Figure 2C; Supporting Information Table 5) of the study. However, at the end of exposure 2, on day 38 of the experiment, general linear modelling revealed a significant interaction between exposure 1 and exposure 2 (P = 0.0463; Figure 2D; Supporting Information Table 6 and 7). For fish exposed to copper once, with no period of depuration (control_cu treatment group) we report 2.03 fold greater tissue copper concentration in the gills compared to the absolute control (control_control). However, for fish exposed to copper twice (cu_cu), we found 3.69 fold greater tissue copper concentration in their gills compared to the absolute control (control_control; P=0.003; Figure 2D; Supporting Information Table 6 and 7).

In the liver, on day four of the experiment (at the end of exposure 1), there was no significant effect of copper exposure on the tissue copper concentration (P = 0.1252; Supporting Information Figure 1A; Supporting Information Table 5). Throughout the depuration period, there remained no significant effect of prior copper exposure on tissue copper concentration in the livers of fish sampled on day 18 (P = 0.9070; Supporting Information Figure 1B and 1C; Supporting Information Table 5) or day 34 (P = 0.5272; Supporting Information Figure 1C; Supporting Information Table 5) of the experiment. However, on day 38 of the experiment (at the end of exposure 2), there was no significant

effect of exposure 1 on tissue copper concentration, however exposure 2 was a significant explanatory variable of the data (P = 8.69e-05; Supporting Information Figure 1D; Supporting Information Table 6 and 7).

In the muscle, there was no significant effect of copper exposure on the tissue copper concentration at day four (P = 0.6181; Supporting Information Figure 2A; Supporting Information Table 5), 18 (P = 0.3272; Supporting Information Figure 2B; Supporting Information Table 5) or 34 (P = 0.3217; Supporting Information Figure 2C; Supporting Information Table 5). However, on day 38 of the experiment (at the end of exposure 2), general linear modelling revealed that exposure 1 was a significant explanatory variable (P = 0.0068; Supporting Information Figure 2; Supporting Information Table 6 and 7).

Transcript Profiling and Functional Analysis in the Gill

A summary of the raw sequencing statistics for the gill is available in Supporting Information Table 8. Expression analysis using edgeR identified a total of 574 transcripts which were differentially expressed in one of more treatment group compared to the control group (control_control), 343 of which increased and 231 decreased in expression (FDR < 0.05; Figure 3; Supporting Information Table 10). For fish exposed to control_cu conditions, a total of 565 transcripts were differentially expressed compared to control_control fish, while in the cu_cu treatment group a total of 51 genes were found to be differentially expressed compared to the control_control (Figure 3A and 3B). A total of 42 differentially expressed genes were common between these two treatment groups (control_cu and cu_cu), and just nine differentially expressed genes were unique to the cu_cu group.

Differential expression analysis using edgeR identified zero differentially expressed genes for fish exposed to cu_control compared to control_control fish (Figure 3A and 3B). A full list of differentially expressed transcripts and associated annotations, Log2-transformed fold-changes and FDR values are presented in Supporting Information Table 10.

Clustering of all samples, based on gene expression levels for all differentially expressed genes, is presented in Figure 3C. Cluster analysis showed that the expression profiles of all individual fish exposed to control_cu conditions were distinct from those of the other three treatment groups, corresponding to the highest number of differentially expressed genes when compared to the control_control (565; Figure 3C). However, samples from the cu_cu group did not differentiate in a separate cluster from the other treatment groups, reflecting the fact that this treatment group only had 51 differentially expressed genes compared to the control_control group (Figure 3C).

The lists of over-represented GO terms and KEGG pathways in fish exposed to control_cu and cu_cu are shown in Supporting Information Tables 11 and 12 respectively. For fish exposed to control_cu the most significantly enriched terms included *immune response*, *cell cycle, ion transport* and *cell division* (Supporting Information Table 11). The most significantly over-represented KEGG pathways included *cell cycle*, *DNA replication* and *glutathione metabolism* (Supporting Information Table 11; Figure 4, 5 and 6). For fish exposed to cu_cu conditions the only significant enriched GO term was *integral component of plasma membrane* (Supporting Information Table 12).

Transcript expression of metallothionein (*mt*) was significantly up-regulated in fish exposed to control_cu conditions, (2.83 fold increase compared to the control_control; FDR = 0.00008; Figure 7). For fish exposed to cu_control, there was no significant difference in

expression when compared to the control_control, while for fish exposed to cu_cu metallothionein (*mt*) appeared to be up-regulated by 1.67 fold, but this was not significant compared to the control_control group (Figure 7).

Transcript Expression and Functional Analysis – Liver

A summary of the raw sequencing statistics for the liver is available in Supporting Information Table 9. Differential expression analysis using edgeR identified a total of only 38 transcripts which were differentially expressed in one or more treatment group compared to the control group (control_control), 11 of which increased and 27 decreased in expression (FDR < 0.05). For fish exposed to control_cu conditions, a total of 7 transcripts were differentially expressed compared to control_control fish, while in the cu_cu treatment group a total of 32 genes were found to be differentially expressed compared to the control_control (Supporting Information Figure 3A and 3B). Expression analysis using edgeR identified two differentially expressed genes for fish exposed to cu_control compared to control_control fish (Supporting Information Figure 3A and 3B). A full list of differentially expressed transcripts and associated annotations, Log2-transformed fold-change and FDR values are presented in Supporting Information Table 13.

Clustering of all samples, based on the gene expression levels of the differentially expressed genes, is presented in Supporting Information Figure 3C. Clustering analysis did not show consistent differences between treatment groups except for the cu_control group, which appeared to cluster together (Supporting Information Figure 3C).

The lists of over-represented GO terms and KEGG pathways in fish exposed to control_cu and cu_cu are shown in Supporting Information Tables 14 and 15 respectively. For fish exposed to control_cu no significantly over-represented terms were identified (Supporting Information Table 14). For fish exposed to cu_cu conditions, significant enriched GO terms were associated with immune response (Supporting Information Table 15).

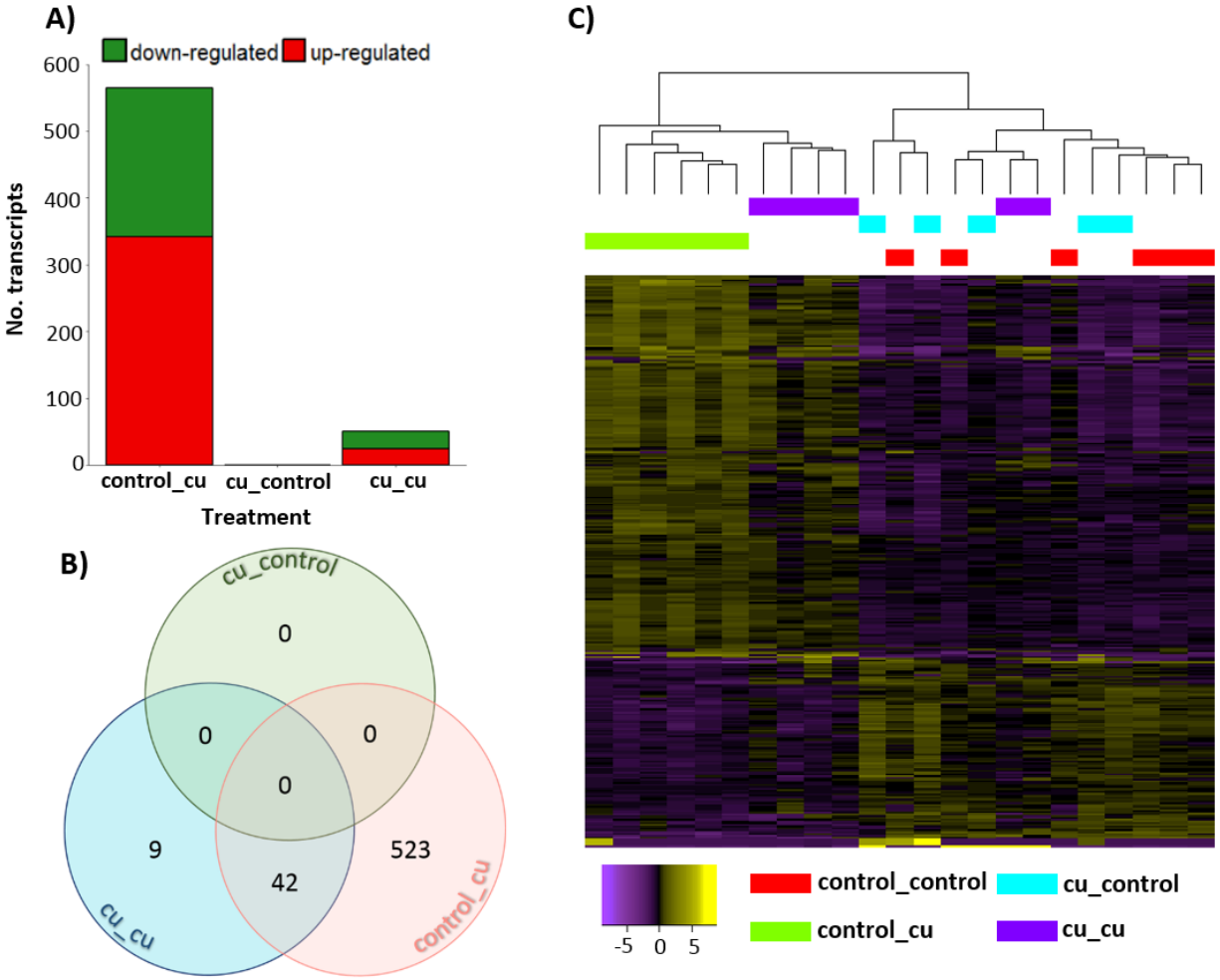
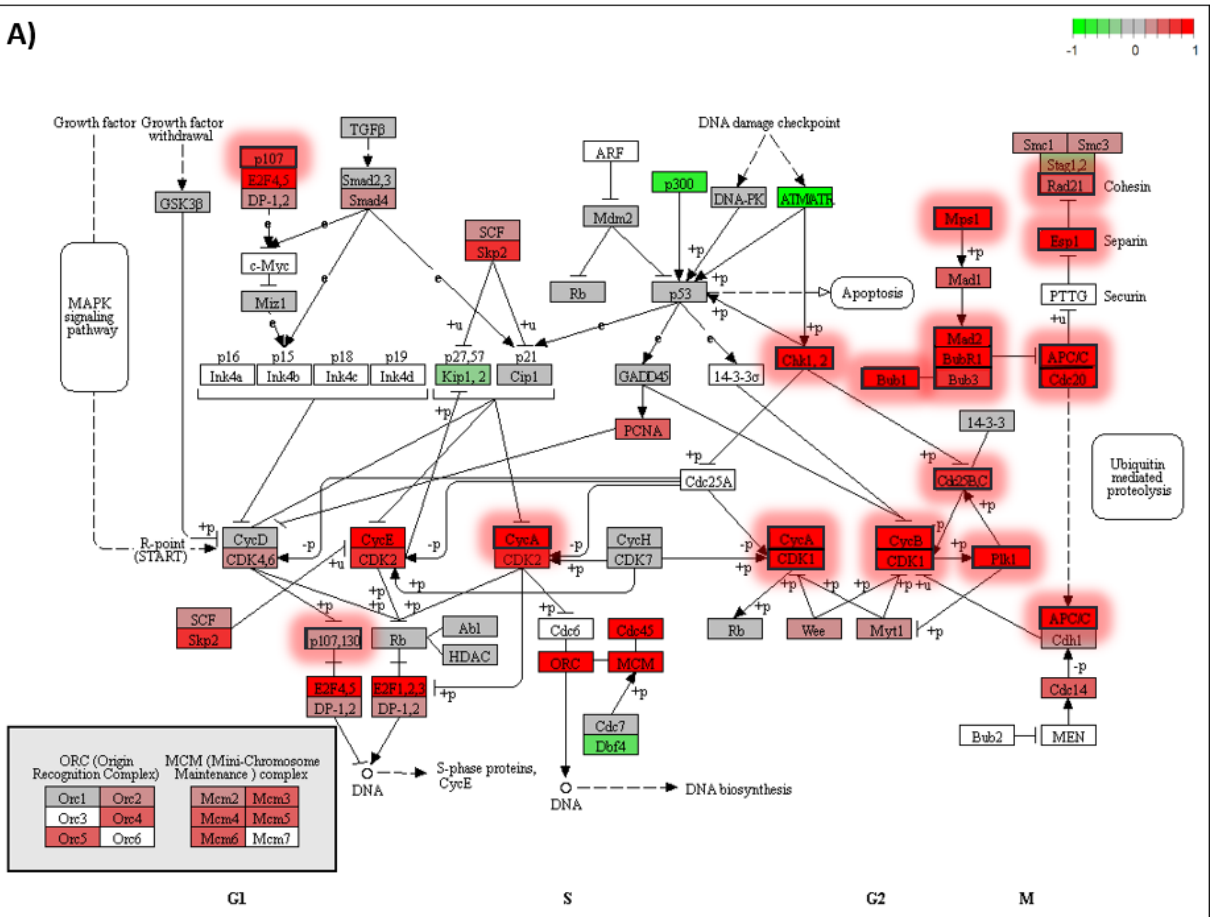


Figure 3. A) Number of differentially expressed genes in the gill within each treatment group when compared to fish maintained in control conditions throughout the experiment, obtained using edgeR with a FDR <0.05. **B)** Venn diagram displaying the number of differentially expressed genes in each treatment group compared to fish maintained in control conditions, and the overlay between these gene lists across treatments. **C)** Heatmap based on the expression level of all differentially regulated transcripts in all individual samples. The hierarchical clustering to generate gene and condition trees was conducted with an Euclidean distance metric, using the cluster() package in R. Treatment groups were classified as the control_control (absolute control), cu_control (fish exposed to copper for 4 days followed by 34 days of depuration), control_cu (fish maintained as controls until exposure 2, then exposed to copper for 4 days with no period of depuration), and cu_cu (fish pre-exposed to copper for 4 days followed by 30 days depuration and a second 4 day copper exposure; Figure 1).

A)


-1
0
1
Growth factor
Growth factor withdrawal
TGFβ
p107
E2F4,5
DP-1,2
Smad2,3
Smad4
GSK3β
MAPK signaling pathway
c-Myc
Miz1
SCF
Skp2
ARF
Mdm2
p300
DNA-PK
ATM/ATR
DNA damage checkpoint
Rb
p53
Apoptosis
Mps1
Mad1
Mad2
BubR1
Bub3
Bub1
Smc1
Smc3
Stag1,2
Rad21
Cohesin
Esp1
Separin
PTTG
Securin
APC/C
Cdc20
p16
Ink4a
p15
Ink4b
p18
Ink4c
p19
Ink4d
p27,57
Kip1, 2
p21
Cip1
GADD45
14-3-3σ
Chk1, 2
PCNA
14-3-3
Cdc25A
Cdc25BC
Ubiquitin mediated proteolysis
R-point
(START)
CycD
CDK4,6
CycE
CDK2
CycA
CDK2
CycH
CDK7
CycA
CDK1
CycB
CDK1
Plk1
Abl
HDAC
p107,130
Rb
Cdc6
Cdc45
ORC
MCM
Wee
Myt1
APC/C
Cdh1
Cdc14
Bub2
MEN
E2F4,5
DP-1,2
E2F1,2,3
DP-1,2
Cdc7
Dbf4
DNA
S-phase proteins, CycE
DNA
DNA biosynthesis
ORC (Origin Recognition Complex)
Orc1
Orc2
Orc3
Orc4
Orc5
Orc6
MCM (Mini-Chromosome Maintenance) complex
Mcm2
Mcm3
Mcm4
Mcm5
Mcm6
Mcm7
G1
S
G2
M

B)

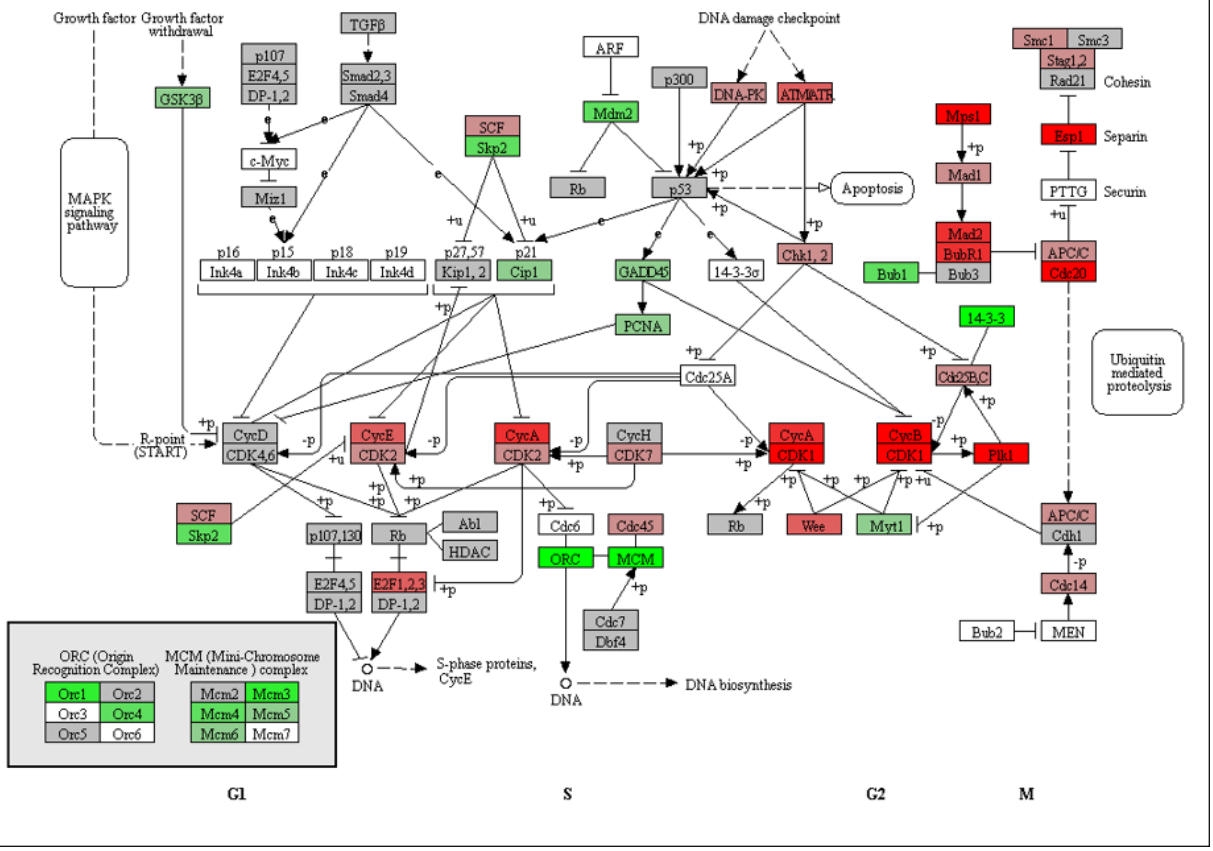

Growth factor
Growth factor withdrawal
TGFβ
p107
E2F4,5
DP-1,2
Smad2,3
Smad4
GSK3β
MAPK signaling pathway
c-Myc
Miz1
SCF
Skp2
ARF
Mdm2
p300
DNA-PK
ATM/ATR
DNA damage checkpoint
Rb
p53
Apoptosis
Mps1
Mad1
Mad2
BubR1
Bub3
Bub1
Smc1
Smc3
Stag1,2
Rad21
Cohesin
Esp1
Separin
PTTG
Securin
APC/C
Cdc20
p16
Ink4a
p15
Ink4b
p18
Ink4c
p19
Ink4d
p27,57
Kip1, 2
p21
Cip1
GADD45
14-3-3σ
Chk1, 2
PCNA
14-3-3
Cdc25A
Cdc25BC
Ubiquitin mediated proteolysis
R-point
(START)
CycD
CDK4,6
CycE
CDK2
CycA
CDK2
CycH
CDK7
CycA
CDK1
CycB
CDK1
Plk1
Abl
HDAC
p107,130
Rb
Cdc6
Cdc45
ORC
MCM
Wee
Myt1
APC/C
Cdh1
Cdc14
Bub2
MEN
E2F4,5
DP-1,2
E2F1,2,3
DP-1,2
Cdc7
Dbf4
DNA
S-phase proteins, CycE
DNA
DNA biosynthesis
ORC (Origin Recognition Complex)
Orc1
Orc2
Orc3
Orc4
Orc5
Orc6
MCM (Mini-Chromosome Maintenance) complex
Mcm2
Mcm3
Mcm4
Mcm5
Mcm6
Mcm7
G1
S
G2
M

Figure 4. Overview of the *cell cycle* signalling pathway (taken from the KEGG database; http://www.genome.jp/kegg/) for naïve fish exposed to copper for 4 days **(A)**, and for fish exposure to copper following a pre-exposure **(B)**. Proteins within this pathway are depicted by boxes, and arrows show signalling routes. Pathway components found to be up regulated are shown in red, while down regulated components are shown in green. Components highlighted by a bold black box and shading were found to be statistically significant in our differential expression analysis (compared to fish kept in control conditions throughout the experiment).

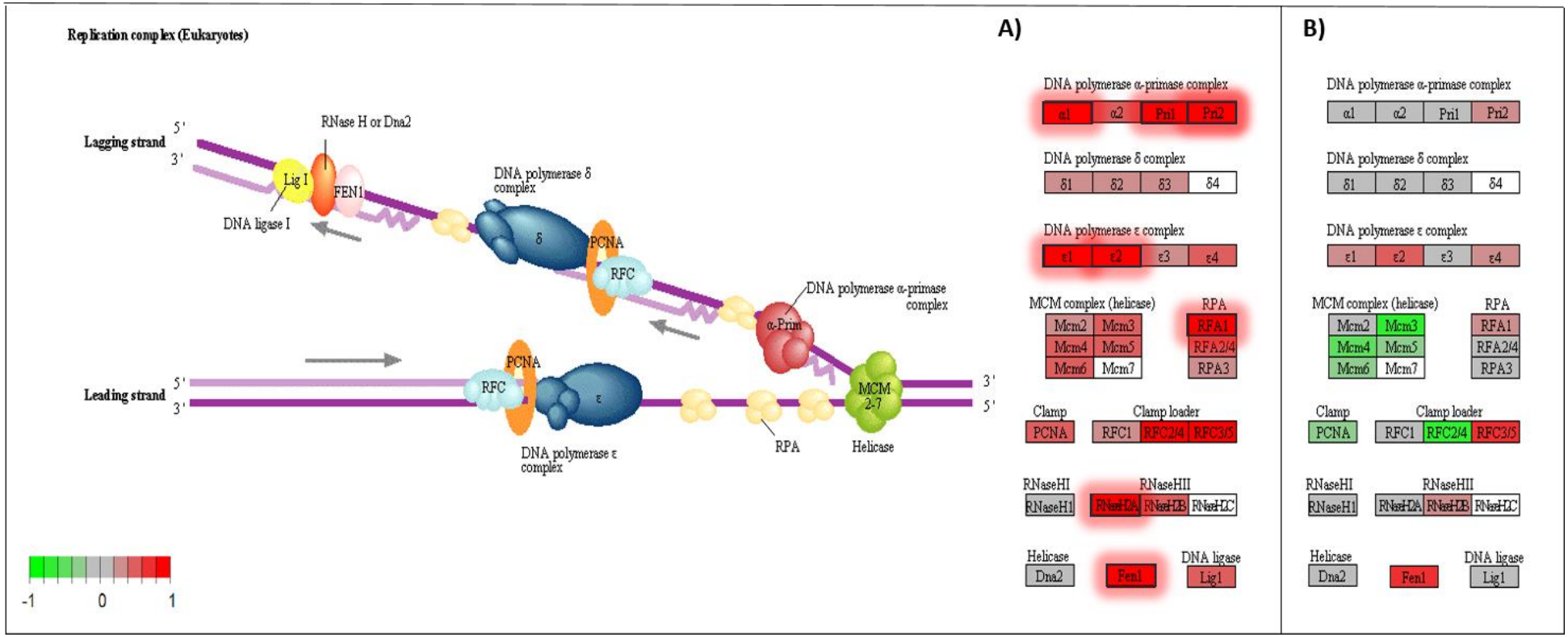

Replication complex (Eukaryotes)
Lagging strand
5'
3'
RNase H or Dna2
Lig I
FEN1
DNA ligase I
DNA polymerase δ complex
δ
PCNA
RFC
DNA polymerase α-primase complex
α-Prim
Leading strand
5'
3'
PCNA
RFC
ε
DNA polymerase ε complex
RPA
MCM 2-7
3'
5'
-1
0
1
A)
DNA polymerase α-primase complex
α1
α2
Pri1
Pri2
DNA polymerase δ complex
δ1
δ2
δ3
δ4
DNA polymerase ε complex
ε1
ε2
ε3
ε4
MCM complex (helicase)
Mcm2
Mcm3
Mcm4
Mcm5
Mcm6
Mcm7
RPA
RFA1
RFA2/4
RPA3
Clamp
PCNA
Clamp loader
RFC1
RFC2/4
RFC3/5
RNaseHI
RNaseH1
RNaseHII
RNaseH2A
RNaseH2B
RNaseH2C
Helicase
Dna2
Fen1
DNA ligase
Lig1
B)
DNA polymerase α-primase complex
α1
α2
Pri1
Pri2
DNA polymerase δ complex
δ1
δ2
δ3
δ4
DNA polymerase ε complex
ε1
ε2
ε3
ε4
MCM complex (helicase)
Mcm2
Mcm3
Mcm4
Mcm5
Mcm6
Mcm7
RPA
RFA1
RFA2/4
RPA3
Clamp
PCNA
Clamp loader
RFC1
RFC2/4
RFC3/5
RNaseHI
RNaseH1
RNaseHII
RNaseH2A
RNaseH2B
RNaseH2C
Helicase
Dna2
Fen1
DNA ligase
Lig1

Figure 5. Overview of the *DNA replication* signalling pathway (taken from the KEGG database; http://www.genome.jp/kegg/) for naïve fish exposed to copper for 4 days **(A)**, and for fish exposure to copper following a pre-exposure **(B)**. Proteins within this pathway are depicted by boxes, and arrows show signalling routes. Pathway components found to be up regulated are shown in red, while down regulated components are shown in green. Components highlighted by a bold black box and shading were found to be statistically significant in our differential expression analysis (compared to fish kept in control conditions throughout the experiment).

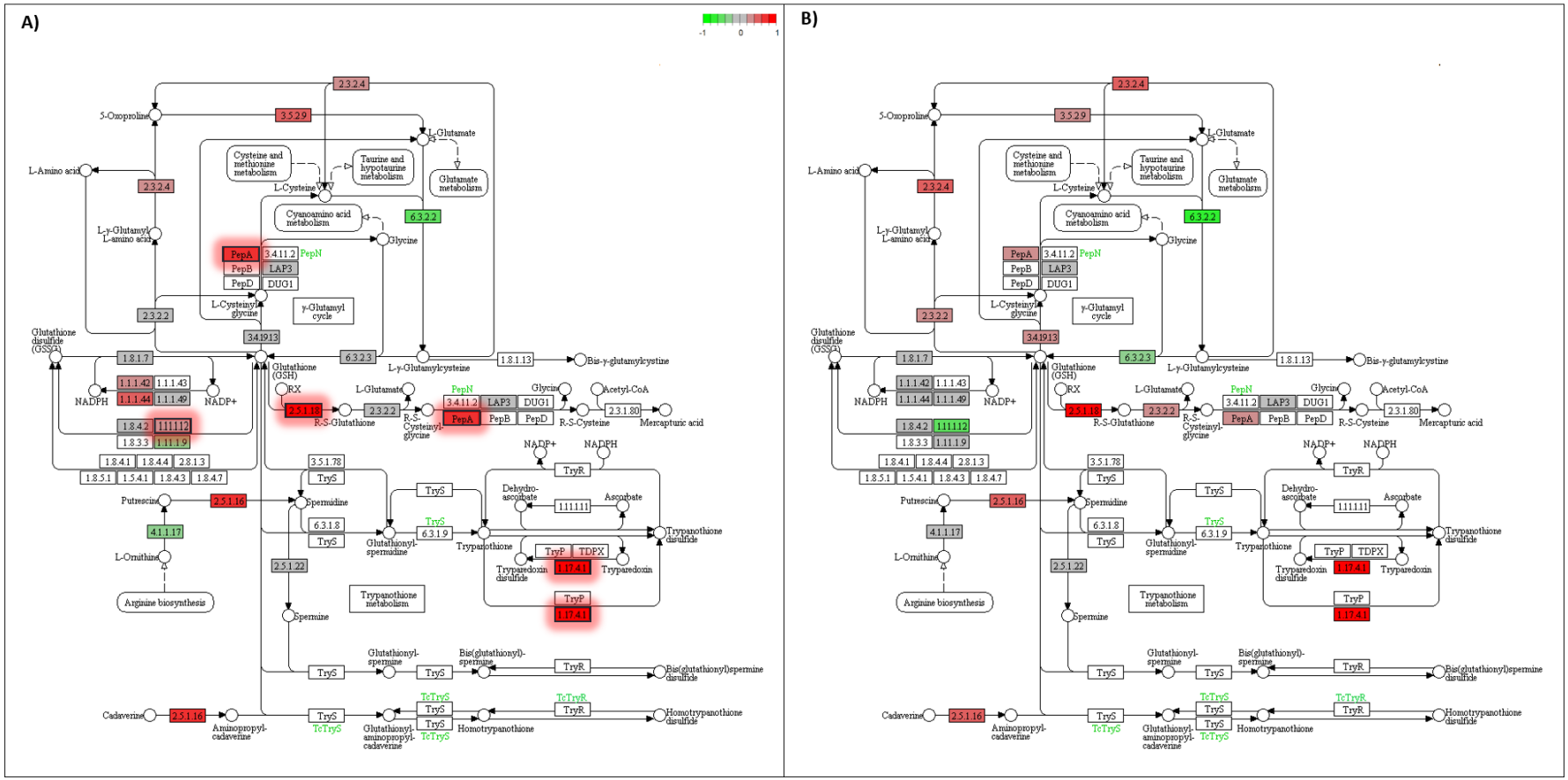

A)
B)
-1
0
1
5-Oxoproline
L-Glutamate
L-Amino acid
Cysteine and methionine metabolism
Taurine and hypotaurine metabolism
Glutamate metabolism
L-Cysteine
Cyanoamino acid metabolism
Glycine
L-γ-Glutamyl L-amino acid
PepA
PepB
PepD
LAP3
DUG1
PepN
L-Cysteinyl glycine
γ-Glutamyl cycle
Glutathione disulfide (GSSG)
Glutathione (GSH)
L-γ-Glutamylcysteine
Bis-γ-glutamylcystine
NADPH
NADP+
RX
R-S-Glutathione
L-Glutamate
Glycine
Acetyl-CoA
R-S-Cysteinyl-glycine
R-S-Cysteine
Mercapturic acid
Putrescine
Spermidine
Glutathionyl-spermidine
Trypanothione
Trypanothione disulfide
Dehydro-ascorbate
Ascorbate
Tryparedoxin disulfide
Tryparedoxin
TryR
TryS
TryP
TDPX
L-Ornithine
Arginine biosynthesis
Trypanothione metabolism
Spermine
Glutathionyl-spermine
Bis(glutathionyl)-spermine
Bis(glutathionyl)spermine disulfide
Cadaverine
Aminopropyl-cadaverine
Glutathionyl-aminopropyl-cadaverine
Homotrypanothione
Homotrypanothione disulfide
TcTryS
TcTryR

Figure 6. Overview of the *glutathione metabolism* signalling pathway (taken from the KEGG database; http://www.genome.jp/kegg/) for naïve fish exposed to copper for 4 days **(A)**, and for fish exposure to copper following a pre-exposure **(B)**. Proteins within this pathway are depicted by boxes, and arrows show signalling routes. Pathway components found to be up regulated are shown in red, while down regulated components are shown in green. Components highlighted by a bold black box and shading were found to be statistically significant in our differential expression analysis (compared to fish kept in control conditions throughout the experiment).

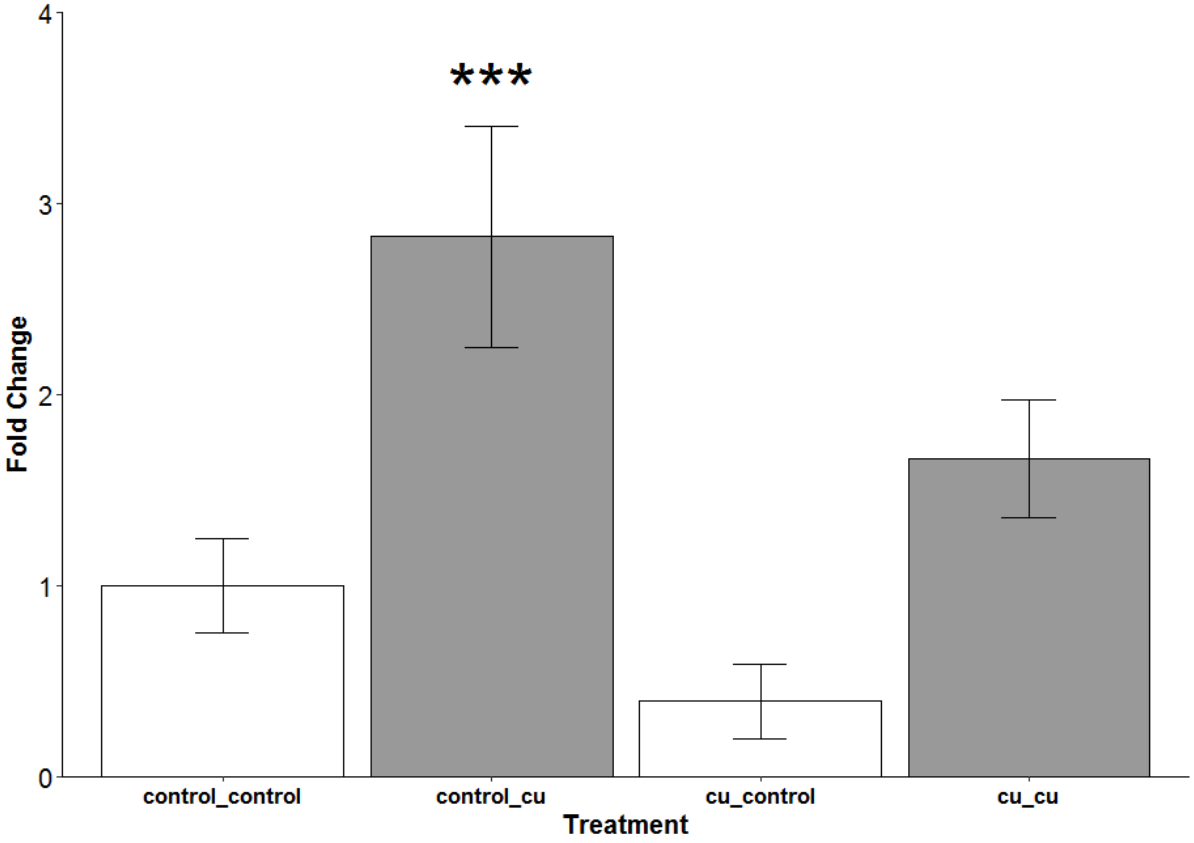


Figure 7. Transcript profile for metallothionein in the gill of exposed fish on day 38 of the study (day 4 of the second exposure; n = 5-6). Comparisons were made for each treatment group compared to fish maintained in control conditions throughout the experiment, obtained using edgeR with a FDR <0.05.

Discussion:

Tissue copper uptake

Copper measurements in the gill revealed a 2.13 fold increase in copper concentration following the first period of exposure but this effect was not present in other tissues, suggesting that the gills are the principal route of metal uptake in these fish when exposed to waterborne copper [42]. Although copper uptake via the gill usually makes up a small proportion of the overall copper intake in fish, there is evidence from aquatic toxicity studies that the gill can contribute considerably to copper uptake when fish are under significant elevated copper exposure via the water or dietary deficiency [6,43]. This is explained by the large gill surface area which is in continual and direct contact with the water and the abundance of metal specific carriers in addition to other ion/metal transporters, including putative epithelial sodium channels (ENaC) and copper transporters (CTR1) [44].

Interestingly, for fish exposed to copper during the initial exposure and then maintained in clean water for 30 days during the depuration period, gill copper concentrations were similar to that of control fish from as early as day 18 (after only 14 days in clean water), suggesting that copper accumulated during the initial four day exposure was then either excreted directly from the gill, or transported to other tissues for storage or excretion during the depuration period. In parallel, it may be that during the depuration period these fish reduce the amount of copper being taken up from the water via the gill. This has been reported previously in the rainbow trout (*Oncorhynchus mykiss*), where pre-exposure to 22 μg/L copper for 28 days, followed by a depuration period of between 10 and 30 days [45], resulted in declines in copper uptake in the gills of fish pre-exposed to copper during the depuration period [45].

In addition, in the gill there is a significant interaction between the pre-exposure and the second exposure, and 3.69 fold more copper was found in the gills of fish which have been repeatedly exposed to copper when compared to the control. These data suggest that fish that have been pre-exposed to copper are not only able to depurate the copper accumulated during the initial exposure after just 14 days of depuration, upon re-exposure these fish can uptake more copper compared to naïve fish. The effect of prior exposure to a stressor on the responses of organisms upon re-exposure in the absence of continuous exposure is thought to be modulated, at least in part, via epigenetic mechanisms, often referred to as the epigenetic memory (discussed in detail later in this discussion) [46]. However, we cannot exclude the possibly that increased metal concentrations in the gills of fish that have been exposed to copper twice could be explained by elevated metal binding proteins and metal transporters at the protein level.

Studies have also highlighted the possibility that pre-exposure to one metal, may modulate the uptake of alternative metals. For example copper acclimation in the gills of rainbow trout has been associated in a higher capacity to accumulate cadmium upon re-exposure [47]. It was hypothesised that this increase in cadmium uptake as a result of copper acclimation may be explained by the increased gill ion regulatory activity associated with copper exposure, for example, the increase in Na^+/K^+ ATPase activity to counteract copper induced Na^+ loss [47]. These data are concordant with our findings, suggesting that copper acclimation changes the way in which metals are handled, potentially via epigenetic mechanisms, but also suggests that exposure to metals such as copper may also alter the way in which other metals which utilise similar uptake routes are handled in the tissues of acclimated fish.

In control fish, the liver is the tissue where the most copper was detected, with 128 fold more copper than the gill, where the least copper load was identified. This is likely explained by the liver's key role in metal homeostasis and storage [45,48]. However, in the liver there was no effect of pre-exposure on copper uptake but the second exposure was found to cause a significant increase in tissue copper load. This may be explained by the fact that a four day exposure is likely to be too short for waterborne copper taken up via the gill to reach the liver. In addition to the liver, the kidney represents a key tissue which serves as scavenging and clearance organ, usually accumulating high concentrations of metals and shown in previous studies to play a key role in metal tolerance [15,44]. Although the transcriptional responses in the kidney were not measured in the present study, this would be an interesting tissue to study in the future.

In our study, although we observed no significant effects of copper exposure on muscle copper concentrations after the initial exposure (day four), by day 38 the initial exposure was found to be statistically significant in explaining the variation in muscle copper concentrations between treatment groups, suggesting that fish pre-exposed to copper (during exposure 1), are storing significantly more copper in their muscles, however the processes mediating this are not detectable after just four days of exposure. Toxic metals are transported via the blood to the liver, bound to metal binding proteins and may then be transported to muscle for long term storage or excreted via the bile [42]. A study by Grosell *et al*., demonstrated that in copper naïve fish exposed to a single bolus of injection of radiolabelled ^{64}Cu, concentrations of newly accumulated and total muscle Cu increased significantly over time [31]. In this study significant increases in muscle copper concentrations were observed after just 72 hours, however the speed of this copper movement will depend

on the route of copper uptake, the concentration to which fish are exposed and the test species.

In addition, in our study the concentration of copper present in the muscle of fish following repeated exposure was higher than that in naïve fish exposed only once. Similar findings have previously been reported for the least killifish (*Heterandria Formosa*), in which pre-exposure to copper was associated with increased whole body copper load when re-exposed to a lethal dose of copper [30]. This study however, did not allow fish to recover with a depuration period.

Transcriptome profiling

The gene expression patterns of the four treatment groups at the end of the second exposure were examined to identify potential mechanisms of toxicity and differential responses. In the gill the greatest number of differentially expressed genes following copper exposure were recorded for naïve fish which had been not been pre-exposed to copper, while for pre-exposed fish just 51 differentially expressed genes were identified upon re-exposure. This is particularly interesting due to the fact that fish were pre-exposed to copper were found to be able to uptake more copper upon re-exposure than naïve fish which were only exposed to copper once. In addition, fish which were exposed to copper followed by a period of depuration showed no differential gene expression compared to the control, suggesting that these fish were able to recover in terms of both their transcriptomic responses and copper accumulation as a result of copper exposure. To our knowledge this is the first time that these finding have been reported in the literature.

In the liver, across all treatment groups a total of only 38 differentially expressed genes were detected, and there was no functional over-representation of pathways. These data, together with the copper concentrations measured in the liver suggest that the main tissue affected by waterborne copper exposure is the gill under our experimental conditions. Therefore this discussion will focus principally on transcriptional responses in the gill.

Metal homeostasis: Exposure of naïve fish to copper resulted in a significant induction in mt expression, but not in pre-exposed fish.

Homeostatic regulation of copper is fundamental to ensuring an adequate supply of copper for essential metabolic processes, and controlling its cellular concentrations in order to prevent toxicity when found in excess. This process involves the regulation of copper uptake from the environment, circulation of copper bound to metal transporters [44], delivery to target organs, supply to metabolic pathways, storage and excretion [6]. Therefore, the significant metal accumulation observed in the gills of fish exposed in this study would be expected to be associated with changes in key components of this homeostatic system.

A number of metal binding proteins have been identified to play important roles in metal homeostasis, and are frequently used as classic biomarkers of metal exposure, of which Metallothionein (*mt*) is the best documented. *mt* is a metal binding protein with a high affinity for metal ions, involved in the detoxification, buffering and storage of toxic metals through sequestration and therefore reduction of the amount of the free metal ions [49]. Distributed in the liver, gill, kidney and intestine, it is widely acknowledged that *mt*s account for a major proportion of cellular storage of a number of metals, including copper [49–52].

Exposure to sub-lethal concentrations of a number of essential and non-essential metals including copper, mercury, zinc and cadmium have been shown to stimulate metallothionein transcription in both short term laboratory exposures and in chronically exposed wild fish [53–55]. Copper ions bind to metal response elements (MREs) in the promotor region of *mt*, stimulating transcription [49,56]. We found that *mt* was significantly upregulated in the gills of naïve fish exposed to copper (2.83 fold), representing an important response to the toxicity of excess copper in the gills cells, and corresponding to a significant increase in gill copper concentration. In fish exposed to copper followed by a period of depuration, *mt* expression was found to return to basal levels and in these fish, we also found the gill copper concentrations to have recovered to levels similar to the control. However, for fish exposed to copper following a pre-exposure period, *mt* transcription was increased to a lesser degree, and this was not significantly different compared to the control. This is in contrast to the levels of copper detected in the gills of these fish, which were found to be greater than that of naïve fish exposed to copper only once with no period of depuration. Interestingly, in a previous study in the least killifish, fish were pre-exposed to 15 µg/L copper for seven days followed by exposure to a lethal copper level (150 µg/L copper), *mt* induction was reported to be higher for the acclimated fish compared to the controls [30]. However, in this study, fish were not allowed to depurate, therefore preventing the possibility of recovery. In addition, upon re-exposure a lethal dose was used, which is likely to cause significant toxicity and stimulate a differing response to that of our study, where an environmentally realistic concentration was used.

Exposure to copper in naïve fish resulted in a significant increase in the transcription of genes associated with cell cycle

Functional analysis of the lists of differentially expressed transcripts revealed that some of the most significant changes in gene expression following exposure to copper in naïve fish were related to processes associated with cell division. *Cell cycle* and *DNA replication* were both significantly over represented GO terms and KEGG pathways in naïve copper exposed fish associated with significant increases in the expression of a large number of genes in these pathways. However, these GO terms and KEGG pathways were not found to be significantly over represented in fish pre-exposed to copper after the second exposure.

In the present study we report upregulation of a number of genes involved in cell cycle, including MAD2 mitotic arrest deficient-like 1 (*mad2l1*), RAD21 cohesin complex component a (*rad21a*), SHC SH2-domain binding protein 1 (*shcbp1*) and a number of centromere proteins including *cenpe*, *cenpf*, *cenpi*, *cenpk* and *cenpn.* These findings are consistent with studies in mice exhibiting Wilson's disease, a severe metabolic disorder associated with elevated hepatic copper, which report similar changes in these genes in the liver [57]. These data are also concordant with previous studies in fish, where copper exposure was associated with the upregulation of genes involved in cell cycle regulation and gene encoding components of cellular structure in liver cells [11]. Studies in fish have associated exposure to toxic levels of copper with increased cellular proliferation in the gill epithelium basal layer, particularly increasing the cellular proliferation of pavement and chloride cells [58]. Cellular proliferation is thought to play an important role in the restoration of ion homeostasis by increasing the capacity for ion uptake, and the significant over represented of the *cell cycle* and *DNA replication* GO terms and KEGG pathways in naïve fish exposed to

copper suggests that this process was taking place in these fish, but not to the same extent in pre-exposed fish. This suggests that fish that were pre-exposed to copper may have already undergone cellular proliferation during the initial pre-exposure as part of the acclimation process. If this is the case, the increase in cellular mass in the gills may also explain the higher levels of copper in the gills observed for these fish compared to fish sampled after the first period of exposure. It would be interesting to conduct histological analysis of the gills to confirm the cellular proliferation indicated by the changes in gene expression and to document the long term fate of those cells following a period of depuration.

Exposure to copper disrupted the expression of genes involved in immune responses

Gene expression analysis revealed that a predominant downregulation of a number of genes associated with immune responses in the gills of naïve fish exposed to copper once. This is particularly evident from the GO term analysis where *immune response* is one of the most significantly over-represented terms in this treatment group. In addition, GO terms related to immune responses were significantly over-represented in the liver of fish repeatedly exposed to copper. This suggests impairment of the immune system, and is concordant with findings from Uren Webster *et al*., Pierron *et al*., Santos *et al*., and Reynders *et al*., regarding alterations in immune response genes as a result of exposure to metals [11,15,16,59]. It has been hypothesised that these changes in the immune response can be explained by the direct inhibition of the immune system, in addition to disruptions to the energy budget, and commonly results in increased susceptibility to infection [15,60–62].

For fish repeatedly exposed to copper, only one of these immune response genes was found to change in the gill. Matrix metallopeptidase 13a (*mmp13a*) has been shown to play a role in tissue remodelling and the degradation of extracellular matrix proteins, and in this study was significantly downregulated in naïve fish exposed to copper only once and in fish pre-exposed to copper prior to the final exposure [63]. The impact of copper on fish gills has been has been associated with structural and functional damage to the gills [64,65]. This damage is thought to be brought about through the covalent binding of copper ions to the SH groups of the Na^+/K^+ ATPase, therefore interfering with the conformation changes of this protein [64]. Gill structural damage caused by exposure to elevated copper concentrations can be restored, despite this being a slow process [65]. The downregulation of this gene in both naïve fish exposed once to copper and the gills of fish exposed to copper following a period of pre-exposure could be associated with gill damage as a result of copper exposure, and may relate to the slow speed at which gill restoration has been reported to occur [65].

Copper exposure disrupted osmoregulation in naïve fish

In the present study, Na^+/K^+ ATPase *atp11a* was found to be upregulated by 1.40 fold and *atp8b3* downregulated by 2.25 fold in naïve fish exposed to copper, however the expression of these two transcripts were not found to be altered in fish that has been repeatedly exposed. To date, studies exploring the effects of acute metal exposure have reported alterations in the expression of a number of genes involved in membrane components and ion homeostasis, particularly in the gills. Copper has been shown to reduce Na^+ uptake competitively and by interfering with Na^+/K^+ ATPase [6]. When in excess, copper has been shown to enter the gill via Na^+/K^+ ATPase through 'ionic mimicry' and thereby competing

with nutritive ions for uptake [6]. Ion uptake via Na^+/K^+ ATPase is critical for life in freshwater fish, occurring continuously to offset the passive loss of Na^+ as part of the osmoregulation process. Copper competes with Na^+ for entry through a putative apical sodium channel and/or a Na^+/H^+ exchanger, eventually inhibiting Na^+/K^+ ATPase.

Additionally, copper has also been shown to inhibit carbonic anhydrase, reducing the supply of H^+ and $HCO3^-$ ions for Na^+ and Cl^- uptake exchange. Carbonic anhydrase has been reported to be important catalysing the reversible hydration/dehydration of carbon dioxide playing an important role on CO_2 excretion [66]. Lowered NaCl levels have been shown to lead to increased blood viscosity, with the potential to cause circulatory collapse [6]. In the present study there was a significant downregulation of carbonic anhydrase (*ca10a*) in the gills of naïve fish exposed to copper, but not in any other treatment group, suggesting respiratory and osmotic stress may be occurring in this group.

In naïve fish exposed to copper, the GO terms *potassium ion transport* and *ion transports* were significantly over represented. We also report alterations in the expression of a number of potassium voltage-gated channel proteins including *kcnip3*, *kcnj1a.6*, *kcnk5b* and *kctd12b*, all found to be downregulated only in naïve fish exposed to copper. However, *kcnj16* was found to be downregulated in both naïve fish exposed to copper and fish which had been repeatedly exposed to copper while *kcnj15* was found to be only downregulated in repeatedly exposed fish. As part of the Na^+/K^+ ATPase function, K^+ is a vital component, therefore the downregulation of potassium voltage-gated channel proteins could have considerable consequences for cellular processes. Disruption of Na^+/K^+ ATPase can lead to osmotic stress [30,67,68], potentially providing an explanation for the upregulation of aquaporin 3a (*aqp3a*) in both naïve fish under copper exposure and repeatedly exposed fish.

Aquaporin 3a is a small integral membrane protein which functions as a water channel; its upregulation could be explained by a compensatory response in order to balance osmotic stress as a result of copper exposure.

Several other genes responsible for membrane components and ion transport were downregulated in naïve fish under copper exposure, including the magnesium transporter NIPA-like domain containing 4 (*nipal4*), a number of voltage-gated proton channels that mediate H^+ currents (*nox1*, *noxo1a* and *noxo1b*) and 12 genes from the solute carrier family, only 1 of which was also found to be downregulated in the gills of fish which had been pre-exposed to copper. These data suggest that copper exposure in naïve fish, may result in a number of transcripts encoding membrane components to be regulated in order to counter their inhibition by copper exposure, however in fish which were pre-exposed to copper the lesser degree of response of these pathways suggests a reduced effect of the exposure on osmoregulation, indicating the existence of compensatory mechanisms.

Previous studies have reported acclimation of fish to copper following chronic exposure, in general these studies report acclimation to be driven by the restoration of plasma ionic balance and morphological changes in the gill structure [69,70]. Increased cellular proliferation in the gill membrane in response to copper exposure has been shown to result in increased distance of the water-blood diffusion distance for gaseous exchange, in turn resulting in impairment of normal osmoregulatory processes via the water [58]. Interestingly, in the present study the only significantly over represented GO term identified among the differentially expressed genes in pre-exposed fish upon re-exposure was *integral components of plasma membranes*, suggesting that in these fish may have acclimated through alterations of integral membrane components in turn resulting in increased

tolerance, thereby explaining why the effects observed on genes involved in oxidative stress, cell division and immune responses are also less pronounced than in fish which have been exposed to copper only once.

Exposure to copper in naïve fish stimulated a significantly greater oxidative stress response than in pre-exposed fish

Exposure to sub-lethal concentrations of copper have been shown to cause increased levels of reactive oxygen species (ROS) and subsequently oxidative stress owing to copper's high reactivity with H_2O_2 and ability to undergo redox reactions, forming ROS, a process known as the Fenton reaction [12,71]. ROS production can lead to lipid peroxidation, DNA damage and protein carbonyl production [12,72–74]. Oxidative stress can also be induced by copper through the inhibition of antioxidant enzymatic activity and alterations in the mitochondrial electron-transfer chain[6,74,75]. Oxidative stress can lead to DNA and protein damage or lipid peroxidation, with associated adverse health effects at the cellular level.

In the present study, *glutathione metabolism* was found to be a significantly over represented KEGG pathway in naïve fish exposed to copper only once, but not for fish exposed to copper following a pre-exposure . In addition, this is consistent with the expression of glutathione peroxidase 4a (*gpx4a*), found to be significantly increased by 2.15 fold in naïve fish exposed to copper after just four days of copper exposure. In response to this increase in ROS, the upregulation of the cellular antioxidant defence system is stimulated in order to limit oxidative damage. Gene expression studies provide evidence that copper exposure may stimulate the expression of a suite of enzymes in the antioxidant

defence system. For example, both glutathione reductase and glutathione peroxidase expression have been shown to correlate with copper exposure in the gills and livers of brown trout [76]. Glutathione is a metal binding peptide identified to play an important role in metal homeostasis, frequently used as classic biomarkers of metal exposure or oxidative stress, and is an integral component in the cellular antioxidant defence system. Generally found to be present at high metal concentrations, glutathione acts as a first line of cellular defence through the scavenging of oxyradicals and protecting cells against oxidative stress [6]. Glutathione peroxidase determines the ratio of reduced (GSH) versus oxidized glutathione (GSSH), and in this way regulates the homeostatic redox balance within the cell [6]. Therefore, the observed increase in glutathione peroxidase 4a (*gpx4a*) expression and the over representation of the *glutathione metabolism* KEGG pathway in the gills of naïve fish exposed to copper suggests that these fish are eliciting a significant oxidative stress response. However, no changes in gene expression were observed in fish exposed to copper followed by depuration or in fish exposed to copper following a pre-exposure period, suggesting that in both of these treatment groups the oxidative stress caused by copper present in their gill cell may be more modest or has stimulated a different cellular antioxidant response.

It is possible that the copper present in gill cells of fish exposed to copper twice is still stimulating the cellular antioxidant defence system, even if the extent of this effect is modest. DNA-damage-inducible transcript 4 (*ddit4*), shown to respond to cellular stress and DNA damage [77,78], was significantly upregulated in both naïve fish exposed to copper and in fish exposed to copper following a pre-exposure period. *ddit4* is important in the regulation of the cells own production of ROS in mitochondria, and is an important component of the

cellular antioxidant defence system. In addition, 70 kD heatshock protein (*hsp70.3*), which temporarily binds and stabilizes damaged proteins, and heat shock protein 90 alpha (cytosolic) class A member 1 (*hsp90aa1.2*), which aids protein folding and quality control were also found to be significantly up-regulated in the gills of both the naïve fish exposed to copper and in the fish exposed to copper following a pre-exposure period. Interestingly, in a study exploring the role of zinc acclimation, pre-exposure of large yellow croaker (*Pseudosciaena croceato*) to 2 mg Zn L^{-1} was found to significantly reduce the oxidative stress caused by a subsequent and immediate exposure to 10 mg Zn L^{-1} [79]. These data suggest that early acclimation to mild metal stress may prime individuals to withstand episodes of high metal stress experienced later in life thereby altering the transcriptomic response and in parallel reducing the extent to which oxidative stress is induced. However, an oxidative stress response may still be induced, albeit significantly reduced.

In addition, it cannot be ruled out, that in fish that have been pre-exposed to copper a greater proportion of antioxidant molecules may still be present during the second exposure, induced by the pre-exposure, and therefore allowing fish to respond more efficiently to oxidative stress independently of transcription. Alternatively, fish which have been repeatedly exposed to copper may be more efficient at binding free copper ions, meaning that less free copper ions would be present in the cells, potentially providing an explanation for the detection of fewer changes in transcripts associated with oxidative stress responses.

Epigenetics

Data from this study demonstrates that fish exposed to copper can recover during a depuration period both in terms of copper load in the gills, and in terms of transcriptional activity. Despite this, we observed that in fish repeatedly exposed to copper, less differentially expressed transcripts were detected upon re-exposure compared to naïve fish. These data suggest that after an initial exposure to copper, following the elimination of any additional copper in the gill taken up during the initial exposure, epigenetic mechanisms may be at play in order to establish a molecular memory of previous copper exposure. This prior exposure may modulate the differential response observed in re-exposed individuals via a number of potentially contributing molecular mechanisms.

In mammalian models, a number of examples exist where copper exposure has been associated with changes in DNA methylation. In the Jackson toxic milk mouse model of Wilson disease, changes in hepatic copper concentration has been associated with alterations in global DNA methylation [80]. In addition, studies using the Cohen diabetic sensitive rats, also associated changes in global DNA methylation with copper exposure, specifically hyper global DNA methylation in placental tissue, in response to copper supplementation [81].

In the present study we observed significant upregulation of histone acetyltransferase 1 (*hat1*), involved in the modification of histones, integral components of chromatin structure, in naïve fish exposed to copper, but not in fish that had been repeatedly exposed [82]. In addition, significant upregulation of H2A histone family, member Vb (*h2afvb*) and H3 histone, family 3A (*h3f3a*) were also observed only in naïve fish exposed to copper. Copper exposure has previously been associated with changes to epigenetic marks within

chromatin. Exposure of HL-60 human leukemia cells to copper has been shown to cause a concentration dependant decrease in histone acetylation, attributed to Cu-induced oxidative stress [83,84]. These studies are concordant with previous findings from Kang *et al*., who found copper exposure of Hep3B cells to cause inhibition of histone acetylation both at a toxic as well as at a non-toxic concentrations [85,86]. In the present study both structural maintenance of chromosomes 2 (*smc2*) and structural maintenance of chromosomes 4 (*smc4*) are significantly upregulated in naïve fish exposed to copper, but not for fish exposed repeatedly to copper. These data are concordant with a study by Huster *et al*., exploring the gene expression profile in the liver of *Atp7b-/-* Wilson's disease mice [57]. Huster *et al*., observed the significant upregulation of a number of genes known to be involved in chromatic structure; including *smc2* and *smc4*, similarly to that observed for naïve fish in our study [57]. It has been reported that changes in chromatin structure, as a consequence of modifications such as histone acetylation is associated with the regulation of gene expression [87], and modifications at the chromatin level can contribute to what is often referred to as 'epigenetic memory'. In this way, the observed cellular responses to copper re-exposure may be modulated by previous exposures.

In addition to changes to the histone code, it is hypothesised that some of the differential responses observed in genes expression may be driven by changes at the protein level stimulated by the initial exposure. The initial exposure to copper was reported to stimulate the differential expression of 565 transcripts in the gill of naïve fish; beyond transcription there are further layers of regulation, including the rate of translation of mRNA molecules into proteins and protein degradation rates. However, studies in human cell lines suggest that although protein degradation rates can vary from minutes to days [88,89], it is unlikely that

this many proteins would evade degradation over a 30 day period [90,91]. The use of high-resolution mass spectrometry to quantify protein dynamics in non-dividing HeLa cells, revealed that the median calculated half-live of the HeLa cell proteome is 35.52 hours [91]. Turnover rates were found to be significantly correlated with protein orthologues in mouse cell lines, suggesting high conservation between mammalian species. To our knowledge, this has not been studied in fish cells [91]. It is important to note, that the ambient body temperature in fish, such as the stickleback, is considerably lower than that of mammalian models, suggesting that protein synthesis and degradation processes may also be slower in fish. However, providing similar protein degradation rates are conserved in fish cells, these data, coupled with the relatively short half-life of mRNA [92], suggest that in our study the significant reduction in the number of differentially expressed transcripts observed in pre-exposed fish upon re-exposure, compared to in naïve fish exposed to copper, is unlikely to be explained by memory of pre-exposure at the protein level alone. Therefore, we hypothesize that epigenetic mechanisms may be playing a key role in the increased tolerance to copper observed in these fish.

Conclusions

The data generated in this study provide evidence that the exposure history of fish populations can significantly affect transcriptional responses and copper accumulation in future exposure scenarios, even at environmentally relevant concentrations and when exposures occur in adult fish. These data suggest that each wild population is likely to be uniquely acclimated as a result of prior exposure, highlighting the need for regulatory and management strategies to take a tailored approach to manage local populations. The

findings also raise a number of important biological questions regarding the extent to which epigenetic process may be involved in these acclimation processes, which developmental windows may be more likely to result in epigenetic changes and the potential for epigenetic marks to be inherited to future generations (see chapter 5).

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Supporting Information

Pre-exposure to copper caused a reduced transcriptional response and increased copper accumulation upon re-exposure in adult male three-spined stickleback (*Gasterosteus aculeatus*).

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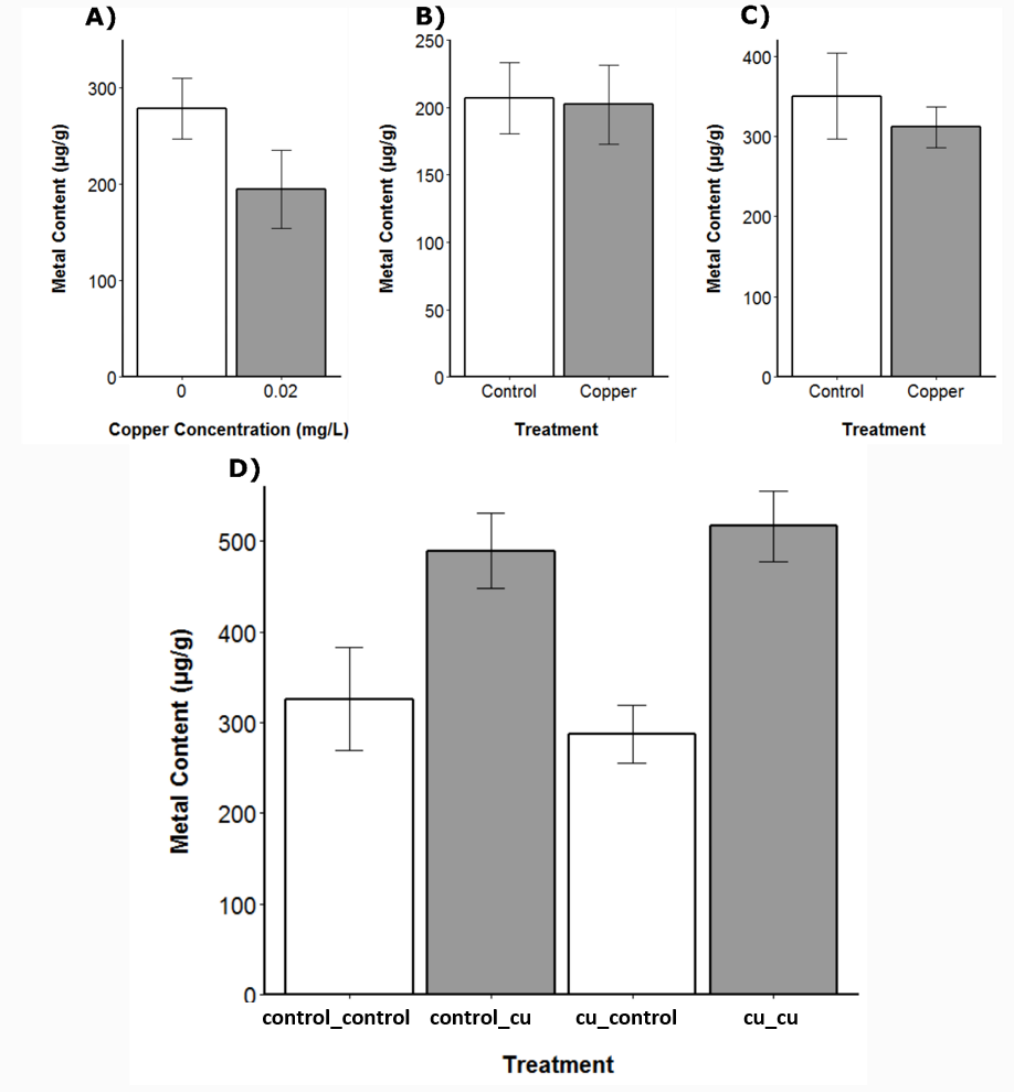
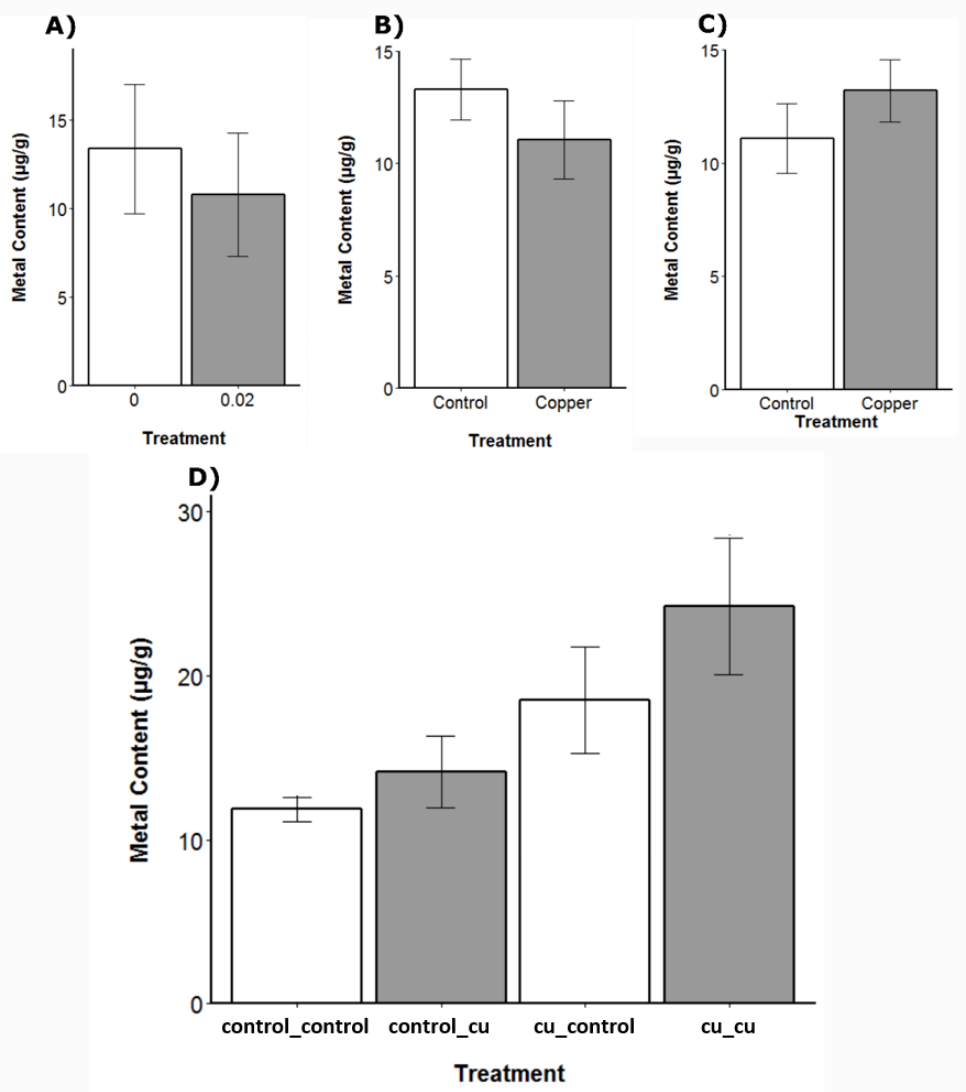
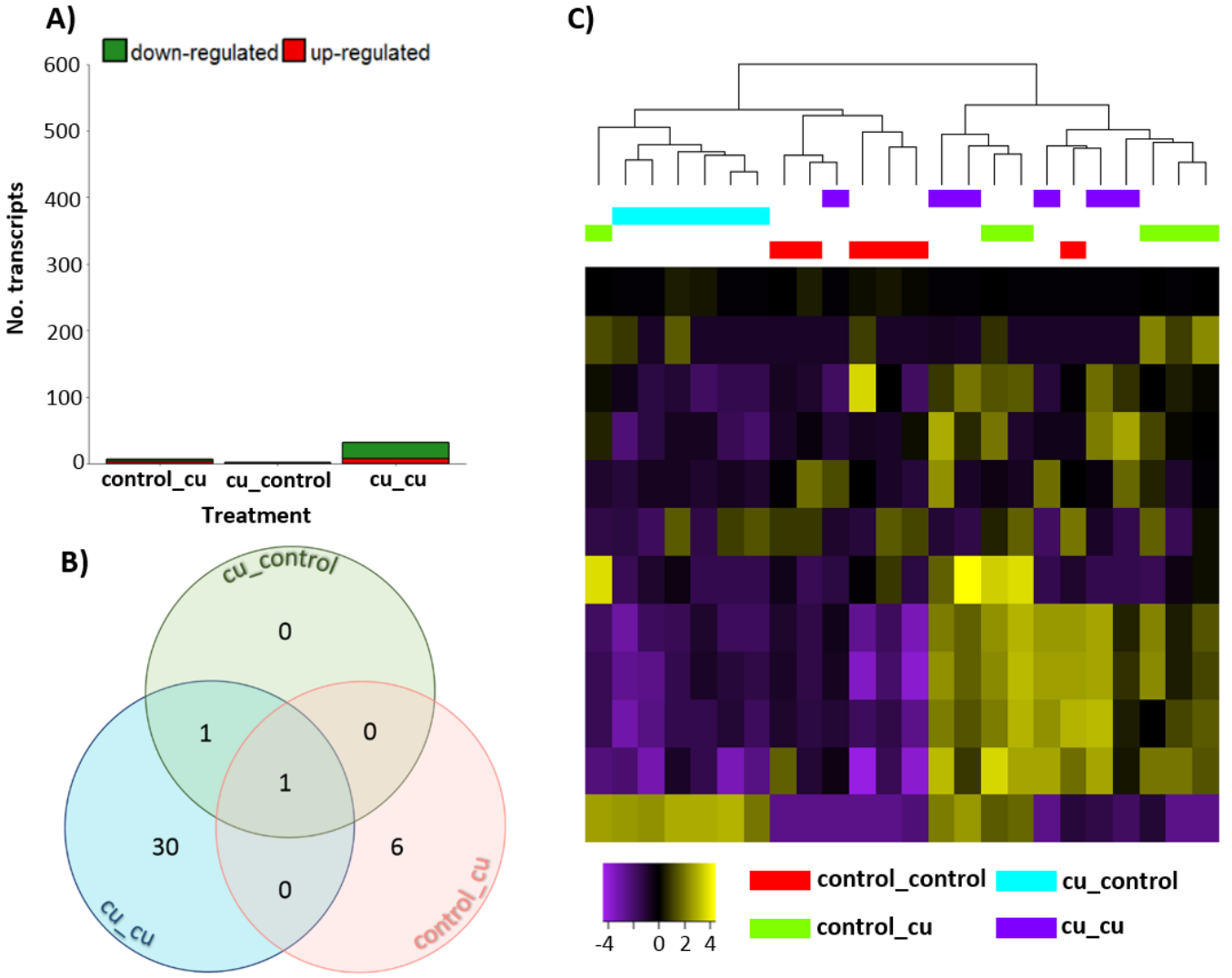


Supporting Information Figure 1. Measured copper concentrations in the liver of male stickleback sampled **A)** on day 4 of the study, (after exposure 1), **B)** on day 18 (after 14 days depuration), **C)** 34 of the study (after 30 days depuration) and **D)** on day 38 of the study (after exposure 2). Tissue metal content was measured by ICPMS (n = 7/8 fish for each treatment group). Data is presented as mean µg Cu/g ± standard error mean. Statistics were conducted using accepted minimum adequate models (analysis of variance model, R; $P<0.05$) with model details and mean values reported in Supporting Information Tables 5-7. On day 38 of the experiment (at the end of exposure 2), exposure 2 was a significant explanatory variable ($P = 8.69e-05$).



Supporting Information Figure 2. Measured copper concentrations in the muscle of male stickleback sampled **A)** on day 4 of the study (after exposure 1), **B)** on day 18 (after 14 days depuration), **C)** 34 of the study (after 30 days depuration) and **D)** on day 38 of the study (after exposure 2). Tissue metal content was measured by ICPMS (n = 7/8 fish for each treatment group). Data is presented as mean µg Cu/g ± standard error mean. Statistics were conducted using accepted minimum adequate models (analysis of variance model, R; $P<0.05$) with model details and mean values reported in Supporting Information Tables 5-7. On day 38 of the experiment (at the end of exposure 2), exposure 1 was a significant explanatory variable ($P = 0.0068$).


A)
down-regulated
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No. transcripts
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Supporting Information Figure 3. A) Number of differentially expressed genes within each treatment group when compared to fish maintained in control conditions throughout the experiment (control_control) in the liver, obtained using edgeR with a FDR <0.05. **B)** Venn diagram displaying the number of differentially expressed genes in each treatment group compared to the control_control, and the overlay between these gene lists across treatments. **C)** Heatmap illustrating the expression level of all differentially regulated transcripts in all individual samples. The hierarchical clustering to generate gene and condition trees was conducted with an Euclidean distance metric, using the cluster() package in R.

Supporting Information Table 1. Measured concentrations of copper in the exposure water. Concentrations were measured for four replicate treatment tanks on day three, and two replicate tanks for days 34 and 37, using ICP-MS, and are presented as mean values ± SEM.

Exposure scenario	Day	Copper treatment mg/L	Mean measured [Cu] mg/L	SEM	% of the nominal concentration
1	3	0	0.006	0.000312	100
1	3	0.02	0.023	7.55E-05	114.66
Depuration	34	0	0.006	0.000175	100
Depuration	34	0	0.006	5.27E-05	100
2	37	0	0.005	6.56E-05	100
2	37	0.02	0.023	0.000626	113.448

Supporting Information Table 2. Morphometric parameters for fish sampled after exposure 1 and depuration days 18 and 34 (n = 9 fish per treatment group). Statistical comparisons were conducted using the Student's t-Test in R (version 3.2.4). All data are presented as mean ± SEM. Asterisks indicate significant differences compared to the control treatment (*p<0.05).

Exposure scenario	Day	Exposure 1	Exposure 2	Morphometric parameter	Mean	SEM	t statistic	P value
1	4		control	Weight	1.60	0.08	-2.24	**0.04 ***
			copper		1.93	0.12		
Depuration	18	control			1.83	0.15	-0.01	0.99
		copper			1.84	0.08		
	34	control			1.62	0.10	-0.80	0.44
		copper			1.72	0.09		
1	4		control	Fork length	5.56	0.09	-0.87	0.40
			copper		5.67	0.09		
Depuration	18	control			5.43	0.14	-0.43	0.68
		copper			5.50	0.06		
	34	control			5.45	0.09	0.17	0.87
		copper			5.43	0.08		
1	4		control	Condition factor (g/cm^3)	0.93	0.02	-1.95	0.08
			copper		1.06	0.06		
Depuration	18	control			1.12	0.02	0.47	0.65
		copper			1.10	0.04		
	34	control			1.00	0.04	-1.73	0.11
		copper			1.07	0.02		
1	4		control	HSI	2.55	0.21	-2.08	0.06
			copper		3.58	0.45		
Depuration	18	control			3.83	0.40	0.30	0.77
		copper			3.68	0.31		
	34	control			2.86	0.25	0.84	0.42
		copper			3.06	0.09		

Supporting Information Table 3. Morphometric parameters for fish sampled after exposure 2 (n = 9 fish per treatment group). All data are presented as mean ± SEM. Statistical analyses are presented in Supporting Information Table 4.

Exposure scenario	Day	Exposure 1	Exposure 2	Morphometric parameter	Mean	SEM
2	38	control	control	Weight	1.79	0.06
		control	copper		1.74	0.06
		copper	control		1.77	0.06
		copper	copper		1.68	0.072
2	38	control	control	Fork length	5.49	0.06
		control	copper		5.49	0.05
		copper	control		5.65	0.14
		copper	copper		5.46	0.06
2	38	control	control	Condition factor (g/cm^3)	1.07	0.02
		control	copper		1.05	0.02
		copper	control		1.01	0.04
		copper	copper		1.02	0.02
2	38	control	control	HSI	2.37	0.20
		control	copper		2.31	0.12
		copper	control		2.53	0.23
		copper	copper		2.46	0.29

Supporting Information Table 4. Statistical analysis for the morphometric parameters for fish sampled after exposure 2 (n = 9 fish per treatment group). Analysis of variance models for the relationships between exposure 1, exposure 2 and an exposure 1/ exposure 2 interaction are presented. Analyses were conducted in R (version 3.2.4).

Exposure scenario	Day	Test	Morphometric parameter	Exposure 1		Exposure 2		Exposure 1 / Exposure 2 interaction	
				F value	P value	F value	P value	F value	P value
2	38	General Linear Model	Weight	NS	NS	NS	NS	NS	NS
			Fork length	NS	NS	NS	NS	NS	NS
			Condition factor	NS	NS	NS	NS	NS	NS
			HIS	NS	NS	NS	NS	NS	NS

Supporting Information Table 5. Measured concentrations of copper in the tissues of fish sampled after exposure 1 and depuration days 18 and 34 (n = 9 fish per treatment group). Statistical comparisons were conducted using the Student's t-Test in R (version 3.2.4). All data are presented as mean ± SEM. Asterisks indicate significant differences compared to the control treatment (*p<0.05).

Exposure scenario	Day	Exposure 1	Exposure 2	Tissue	Metal uptake (µg/g)			
					Mean	SEM	t statistic	P value
1	4		control	Liver	278.92	31.51	1.63	0.13
			copper		194.95	40.99		
Depuration	18	control			206.73	26.31	0.12	0.91
		copper			202.07	29.31		
	34	control			350.16	53.22	0.66	0.53
		copper			311.40	25.03		
1	4		control	Gill	9.88	2.31	-3.00	**0.01 ***
			copper		21.06	2.91		
Depuration	18	control			15.84	2.78	1.02	0.33
		copper			12.36	2.00		
	34	control			10.75	1.81	0.03	0.97
		copper			10.82	0.99		
1	4		control	Muscle	13.36	3.65	0.51	0.62
			copper		10.79	3.49		
Depuration	18	control			13.28	1.35	1.01	0.33
		copper			11.06	1.73		
	34	control			11.09	1.53	1.02	0.32
		copper			13.19	1.37		

Supporting Information Table 6. Measured concentrations of copper in the tissues of fish sampled after exposure 2 (n = 9 fish per treatment group). All data are presented as mean ± SEM. Statistical analyses are presented in Supporting Information Table 7.

Exposure scenario	Day	Exposure 1	Exposure 2	Metal uptake (µg/g)		
				Tissue	Mean	SEM
2	38	control	control	Liver	325.82	56.39
		control	copper		489.12	41.28
		copper	control		287.30	31.70
		copper	copper		516.15	38.59
2	38	control	control	Gill	13.64	2.45
		control	copper		27.79	5.69
		copper	control		7.68	1.73
		copper	copper		50.39	12.77
2	38	control	control	Muscle	11.84	0.72
		control	copper		14.13	2.20
		copper	control		18.51	3.25
		copper	copper		24.20	4.17

Supporting Information Table 7. Statistical comparisons for the measured concentrations of copper in the tissues of fish sampled after exposure 2 (n = 9 fish per treatment group). Analysis of variance models for the relationships between exposure 1, exposure 2 and an exposure 1/ exposure 2 interaction were conducted in R (version 3.2.4). Test results reported refer to the significance of each terms within the minimum adequate model (MAM).

Exposure scenario	**Day**	**Test**	**Tissue**	**Exposure 1**		**Exposure 2**		**Exposure 1 / Exposure 2 interaction**	
				F value	P value	F value	P value	F value	P value
2	38	General Linear Model	Liver	NS	NS	21.84	**8.69e-05 *****	NS	NS
			Gill	1.05	0.3147	18.15	**0.0001968 *****	4.33	**0.0463 ***
			Muscle	8.51	**0.006762 ****	1.07	0.30991	NS	NS

Supporting Information Table 8. Summary statistics of raw sequencing reads for the gill dataset, numbers of reads retained after adaptor removal and quality filtering and retained for alignment to reference genome.

Sample_no	Treatment	Tissue	Read characterization	No. Raw reads	No. paired trimmed reads	No. forward only surviving reads	No. reverse only surviving reads	% reads dropped
68	control_control	Gill	100 bp Paired end	16,764,641	15,713,435	991,327	30,184	0.18%
70	control_control			17,389,478	16,397,695	950,183	33,104	0.05%
97	control_control			13,863,995	12,874,542	948,775	25,972	0.11%
101	control_control			22,896,388	21,611,765	1,206,974	39,596	0.17%
125	control_control			18,426,206	17,020,129	1,336,078	33,745	0.20%
126	control_control			31,524,348	28,825,279	2,629,603	51,110	0.06%
89	control_cu			15,764,945	14,689,694	1,034,605	30,729	0.06%
90	control_cu			15,971,492	14,577,186	1,350,793	27,182	0.10%
103	control_cu			14,951,836	14,099,160	814,106	26,113	0.08%
104	control_cu			15,207,681	14,050,328	1,117,108	26,902	0.09%
131	control_cu			16,900,006	15,784,740	1058170	30,016	0.16%
133	control_cu			16,820,700	15,601,969	1,182,094	29,377	0.04%
84	cu_control			15,069,568	12,991,243	2,033,657	31,767	0.09%
85	cu_control			24,266,773	23,072,001	1,135,341	41,281	0.07%
112	cu_control			19,161,042	18,125,530	991,269	35,746	0.04%
113	cu_control			15,794,026	14,666,570	1,091,417	25,771	0.07%
140	cu_control			14,455,332	13,887,330	529,650	26,664	0.08%
141	cu_control			17,941,353	16,925,834	968,480	32,466	0.08%
76	cu_cu			17,702,374	16,052,465	1,600,719	28,918	0.11%
77	cu_cu			17,135,365	16,255,166	834,637	29,519	0.09%
118	cu_cu			16,605,105	15,615,379	944,720	28,610	0.10%
79	cu_cu			15,216,575	14,101,938	1,077,556	27,862	0.06%
145	cu_cu			18,094,856	17,094,010	951,076	31,596	0.10%
146	cu_cu			16,910,164	15,894,900	971,959	29,678	0.08%

Supporting Information Table 9. Summary statistics of raw sequencing reads for the liver dataset, numbers of reads retained after adaptor removal and quality filtering and retained for alignment to reference genome.

Sample_no	Treatment	Tissue	Read characterization	No. Raw single reads	No. trimmed single reads	% reads dropped
101	control_control	Liver	100 bp Single end	10,038,330	10,014,388	0.24
124	control_control			19,669,106	19,520,721	0.75
125	control_control			9,345,121	9,278,439	0.71
68	control_control			17,569,750	17,529,548	0.23
69	control_control			18,906,204	18,774,676	0.7
97	control_control			16,517,712	16,402,428	0.7
103	control_cu			17,157,607	17,117,190	0.24
104	control _cu			14,032,124	13,927,785	0.74
131	control_cu			20,883,907	20,740,445	0.69
132	control_cu			7,975,883	7,955,697	0.25
89	control_cu			8,616,006	8,594,511	0.25
90	control_cu			13,045,514	13,013,875	0.24
112	cu_control			13,262,649	13,172,474	0.68
113	cu_control			7,301,460	7,249,337	0.71
140	cu_control			8,571,755	8,511,355	0.7
141	cu_control			9,362,867	9,338,894	0.26
84	cu_control			9,152,910	9,088,915	0.7
85	cu_control			10,774,707	10,735,666	0.36
118	cu_cu			6,990,739	6,974,234	0.24
119	cu_cu			13,148,573	13,050,011	0.75
145	cu_cu			6,157,212	6,142,571	0.24
146	cu_cu			18,821,574	18,690,113	0.7
75	cu_cu			12,303,953	12,275,722	0.23
77	cu_cu			7,601,993	7,584,216	0.23

Supporting Information Table 10. List of all differentially expressed transcripts in gill samples. Values presented are Log2 transformed fold changes and FDR values for each treatment group compared to fish maintained under control conditions throughout the experiment (control_control group) in the gill. Red shading indicates significant up-regulation (FDR < 0.05) and green shading represents significant down-regulation (FDR < 0.05).

Gene ID	Name	Annotation	Database	control_cu		cu_control		cu_cu	
				LogFC	FDR	LogFC	FDR	LogFC	FDR
ENSGACG00000003128	AAAS	aladin WD repeat nucleoporin	Ensembl	0.633	0.008	-0.19	0.898	0.355	0.944
ENSGACG00000016707	abca4b	ATP-binding cassette, sub-family A (ABC1), member 4b	Ensembl	-0.9	0.014	0.091	0.968	-0.38	1
ENSGACG00000014650	ACOT11 (1 of many)	acyl-CoA thioesterase 11	Ensembl	0.829	0.001	-0.08	0.963	0.509	0.381
ENSGACG00000018279	acrc	acidic repeat containing	Ensembl	1.744	1E-07	0.411	0.885	1.069	0.122
ENSGACG00000004121	acsf2 (1 of many)	acyl-CoA synthetase family member 2	Ensembl	0.939	0.005	-0.49	0.885	0.451	1
ENSGACG00000005819	ACVR1C	activin A receptor type 1C	Ensembl	-1.17	0.024	0.525	0.885	-1.46	0.003
ENSGACG00000009317	adamts9	ADAM metallopeptidase with thrombospondin type 1 motif, 9	Ensembl	-1.22	0.016	0.77	0.885	-0.4	1
ENSGACG00000008575	ADCY6 (1 of many)	adenylate cyclase 6	Ensembl	-0.65	0.007	0.106	0.937	-0.42	0.64
ENSGACG00000009438	add3b	adducin 3 (gamma) b	Ensembl	-0.85	0.011	0.119	0.948	-0.26	1
ENSGACG00000010492	adss	adenylosuccinate synthase	Ensembl	-0.56	0.043	-0.15	0.918	-0.3	1
ENSGACG00000003641	ahcy	Adenosylhomocysteinase	Ensembl	1.877	4E-17	-0.21	0.919	1.006	0.003
ENSGACG00000007894	ahcyl1 (1 of many)	adenosylhomocysteinase-like 1	Ensembl	-0.8	0.006	0.032	0.995	-0.39	1
ENSGACG00000001035	ahcyl1 (1 of many)	adenosylhomocysteinase-like 1	Ensembl	-0.62	0.03	0.169	0.899	-0.26	1
ENSGACG00000007812	amigo1	adhesion molecule with Ig-like domain 1	Ensembl	-1.76	0.002	0.736	0.885	-1.77	0.031
ENSGACG00000004467	ampd1	adenosine monophosphate deaminase 1	Ensembl	-0.81	0.039	-0.29	0.898	-0.64	0.63
ENSGACG00000015694	ampd3a	adenosine monophosphate deaminase 3a	Ensembl	-0.55	0.041	0.411	0.885	-0.24	1

ENSGACG00000014142	anapc4	anaphase promoting complex subunit 4	Ensembl	0.677	0.035	-0.08	0.973	0.432	0.919
ENSGACG00000015330	ankrd10a	ankyrin repeat domain 10a	Ensembl	1.255	8E-04	-0.08	0.983	0.148	1
ENSGACG00000008118	anln	anillin, actin binding protein	Ensembl	2.26	1E-18	0.179	0.944	1.241	0.048
ENSGACG00000003178	anp32e (1 of many)	acidic (leucine-rich) nuclear phosphoprotein 32 family, member E	Ensembl	0.714	0.01	-0.28	0.898	0.241	1
ENSGACG00000017044	anxa5b	annexin A5b	Ensembl	0.598	0.02	-0.16	0.905	0.454	0.691
ENSGACG00000008510	apnl	actinoporin-like protein	ZFIN	1.161	0.002	-0.44	0.893	0.348	1
ENSGACG00000010315	aqp3a	aquaporin 3a	Ensembl	1.152	1E-04	0.443	0.885	1.424	2E-05
ENSGACG00000000258	arhgap19	Rho GTPase activating protein 19	Ensembl	2.098	1E-09	-0.25	0.942	0.894	0.872
ENSGACG00000010684	arhgap32a	Rho GTPase activating protein 32a	ZFIN	-1.02	0.006	-0.08	0.979	-0.46	1
ENSGACG00000017541	arhgef38	Rho guanine nucleotide exchange factor (GEF) 38	ZFIN	-1.36	7E-08	0.231	0.897	-1.08	0.007
ENSGACG00000017545	arhgef38	Rho guanine nucleotide exchange factor (GEF) 38	Ensembl	-1.34	0.001	0.244	0.898	-0.71	0.436
ENSGACG00000019259	arhgef39	Rho guanine nucleotide exchange factor (GEF) 39	Ensembl	1.573	0.002	-0.08	0.993	0.631	1
ENSGACG00000011079	armc1l	armadillo repeat containing 1	Ensembl	1.684	0.004	-0.22	0.96	0.752	1
ENSGACG00000007983	arrdc2	arrestin domain containing 2	Ensembl	0.849	0.005	-0.24	0.915	0.89	0.496
ENSGACG00000008955	asap2a	ArfGAP with SH3 domain, ankyrin repeat and PH domain 2a	Ensembl	-0.55	0.03	0.136	0.921	-0.32	1
ENSGACG00000005911	aspm	abnormal spindle microtubule assembly	ZFIN	1.939	2E-13	-0.33	0.893	1.084	0.084
ENSGACG00000014851	atp11a (1 of many)	ATPase, class VI, type 11A	Ensembl	0.487	0.049	0.034	0.991	0.21	1
ENSGACG00000012658	atp8b3	ATPase, aminophospholipid transporter, class I, type 8B, member 3	Ensembl	-1.17	4E-06	-0.14	0.925	-0.76	0.181
ENSGACG00000006354	aurka	aurora kinase A	Ensembl	2.634	3E-18	0.305	0.919	1.85	0.002
ENSGACG00000016381	aurkb	aurora kinase B	Ensembl	2.119	3E-09	0.129	0.971	1.125	0.243

ENSGACG00000019282	B2M (1 of many)	beta-2-microglobulin	Ensembl	-0.55	0.027	-0.17	0.898	-0.45	0.593
ENSGACG00000003839	bcl6a	B-cell CLL/lymphoma 6a (zinc finger protein 51)	Ensembl	-1.57	3E-04	-0.58	0.885	-1.52	0.001
ENSGACG00000015981	bcl6ab	B-cell CLL/lymphoma 6a, genome duplicate b	Ensembl	-1.07	0.015	-0.69	0.885	-0.89	0.199
ENSGACG00000009685	birc5a	baculoviral IAP repeat containing 5a	Ensembl	1.69	7E-08	0.037	1	0.965	0.618
ENSGACG00000009876	bora	bora, aurora kinase A activator	Ensembl	1.917	5E-04	0.04	1	0.356	1
ENSGACG00000006394	bub1bb	BUB1 mitotic checkpoint serine/threonine kinase Bb	ZFIN	1.16	0.027	0.203	0.944	0.714	0.853
ENSGACG00000002522	bub3	BUB3 mitotic checkpoint protein	Ensembl	0.636	0.034	0.116	0.948	0.127	1
ENSGACG00000018802	BX957278.1	Chromosome 7: 19,560,805-19,573,975	ZFIN	0.685	0.02	0.17	0.927	1.296	0.005
ENSGACG00000015243	ca10a	carbonic anhydrase Xa	Ensembl	-1.57	0.02	-0.73	0.885	-0.68	1
ENSGACG00000004999	ca2	carbonic anhydrase II	Ensembl	-1.17	2E-06	-0.27	0.913	-0.97	0.079
ENSGACG00000007449	CABZ01046997.1	Chromosome 3: 4,845,352-4,850,386	ZFIN	-2.91	0.036	-2.55	0.885	-2.53	0.185
ENSGACG00000007447	CABZ01046997.1	CABZ01046997.1	ZFIN	-3.27	0.017	-2.93	0.746	-2.48	0.317
ENSGACG00000008073	CABZ01055534.1	Chromosome 24: 37,086,314-37,091,016	ZFIN	-2.66	6E-05	-0.66	0.885	-2.47	0.003
ENSGACG00000018966	CABZ01067151.1	Chromosome 1: 53,480,127-53,502,501	ZFIN	-1.64	0.046	-0.75	0.885	-1.77	0.132
ENSGACG00000011358	camk1gb	calcium/calmodulin-dependent protein kinase IGb	Ensembl	-0.71	0.031	-0.01	1	-0.64	0.644
ENSGACG00000020227	camkk1a	calcium/calmodulin-dependent protein kinase kinase 1, alpha a	Ensembl	-0.92	0.001	0.221	0.897	-0.3	1
ENSGACG00000006707	CARMIL1	capping protein regulator and myosin 1 linker 1	Ensembl	-0.76	0.001	0.494	0.885	-0.43	0.86
ENSGACG00000007552	casc5	cancer susceptibility candidate 5	ZFIN	1.625	8E-06	0.239	0.922	0.938	0.293
ENSGACG00000018765	casp3b (1 of many)	caspase 3, apoptosis-related cysteine peptidase b	Ensembl	-3.72	4E-09	-0.67	0.885	-0.6	1
ENSGACG00000000185	casq1b	calsequestrin 1b	ZFIN	-0.74	0.045	-0.16	0.928	-0.57	0.497
ENSGACG00000010988	ccdc77	coiled-coil domain containing 77	Ensembl	1.71	0.002	0.198	0.962	1.148	0.497

ENSGACG00000016665	ccna2	cyclin A2	Ensembl	1.744	2E-06	-0.1	0.985	0.686	0.951
ENSGACG00000018014	ccnb1	cyclin B1	Ensembl	2.571	2E-15	-0.01	1	1.624	0.009
ENSGACG00000017487	ccnb2 (1 of many)	cyclin B2	Ensembl	2.566	5E-06	-0.49	0.918	1.086	0.874
ENSGACG00000011284	ccnf	cyclin F	Ensembl	2.284	1E-06	-0.16	0.979	0.849	1
ENSGACG00000019847	cct7	chaperonin containing TCP1, subunit 7 (eta)	Ensembl	0.5	0.034	-0.01	1	0.386	0.669
ENSGACG00000018016	cd74a	CD74 molecule, major histocompatibility complex, class II invariant chain a	ZFIN	-0.62	0.022	-0.28	0.885	-0.4	0.915
ENSGACG00000013626	cdc20	cell division cycle 20 homolog	Ensembl	2.191	1E-09	0.178	0.952	1.474	0.07
ENSGACG00000004851	cdc25b	cell division cycle 25B	Ensembl	0.703	0.014	-0.23	0.898	0.268	1
ENSGACG00000002675	cdca5	cell division cycle associated 5	Ensembl	1.806	0.022	-0.59	0.916	0.434	1
ENSGACG00000002694	cdca8	cell division cycle associated 8	Ensembl	1.698	3E-07	0.249	0.917	1.053	0.21
ENSGACG00000002518	cdk1	cyclin-dependent kinase 1	Ensembl	2.233	3E-13	0.019	1	1.227	0.064
ENSGACG00000003875	cdk5rap3	CDK5 regulatory subunit associated protein 3	Ensembl	0.548	0.043	-0.2	0.898	0.194	1
ENSGACG00000011791	cdkn3	cyclin-dependent kinase inhibitor 3	Ensembl	1.936	0.002	0.009	1	0.958	0.974
ENSGACG00000009412	ceacam1	carcinoembryonic antigen-related cell adhesion molecule 1	ZFIN	-1.18	4E-06	0.408	0.885	-0.84	0.192
ENSGACG00000018561	CENPA	centromere protein A	Ensembl	2.347	2E-08	-0.13	0.979	0.775	1
ENSGACG00000012261	cenpe	centromere protein E	ZFIN	2.173	4E-13	0.233	0.929	1.098	0.216
ENSGACG00000016190	cenpf	centromere protein F	ZFIN	1.984	1E-09	0.404	0.885	1.223	0.07
ENSGACG00000017312	cenpi	centromere protein I	Ensembl	1.802	8E-09	-0.32	0.902	0.837	0.381
ENSGACG00000018295	cenpk	centromere protein K	ZFIN	1.406	0.007	0.182	0.965	0.879	0.835
ENSGACG00000015976	cenpn	centromere protein N	Ensembl	1.408	6E-05	0.109	0.973	0.576	1

ENSGACG00000014325	cep135	centrosomal protein 135	Ensembl	1.007	0.011	0.084	0.979	0.586	0.886
ENSGACG00000006663	cep55l	centrosomal protein 55 like	Ensembl	2.157	2E-06	0.491	0.885	1.323	0.177
ENSGACG00000008882	cep57l1	centrosomal protein 57, like 1	Ensembl	0.808	0.026	0.101	0.963	0.295	1
ENSGACG00000015229	cep70	centrosomal protein 70	Ensembl	0.903	0.014	0.124	0.958	0.613	0.913
ENSGACG00000009039	cftr	cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	Ensembl	-1.13	0.002	-0.39	0.885	-1	0.115
ENSGACG00000012120	cgna	cingulin a	Ensembl	-0.62	0.047	0.477	0.885	-0.3	1
ENSGACG00000003690	chaf1b	chromatin assembly factor 1, subunit B	Ensembl	1.297	0.007	0.166	0.965	0.301	1
ENSGACG00000008196	chchd6b	coiled-coil-helix-coiled-coil-helix domain containing 6b	Ensembl	-0.6	0.013	-0.05	0.982	-0.25	1
ENSGACG00000020635	chek1	checkpoint kinase 1	Ensembl	1.4	0.026	0.128	0.983	0.224	1
ENSGACG00000012557	chia.4	chitinase, acidic.4	ZFIN	-2.86	0.009	-1.04	0.885	-0.65	1
ENSGACG00000009700	chst15	carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	Ensembl	0.874	0.005	-0.49	0.885	0.404	1
ENSGACG00000008628	CIT	citron rho-interacting serine/threonine kinase	Ensembl	1.903	7E-11	-0.05	0.993	1.065	0.081
ENSGACG00000010461	clcn2b	chloride channel, voltage-sensitive 2b	ZFIN	-1.5	5E-07	0.64	0.885	-0.71	0.504
ENSGACG00000004159	cldn10l2	claudin 10-like 2	Ensembl	-1.01	1E-07	0.464	0.885	-0.51	0.618
ENSGACG00000011753	cldn17	claudin 17	Ensembl	-0.93	0.003	0.042	0.991	-0.63	0.582
ENSGACG00000012739	cldna	claudin a	Ensembl	0.763	0.016	-0.36	0.885	0.334	1
ENSGACG00000013961	COL28A1 (1 of many)	collagen type XXVIII alpha 1 chain	Ensembl	1.237	0.017	0.306	0.921	0.948	0.827
ENSGACG00000013156	commd8	COMM domain containing 8	Ensembl	1.005	0.013	0.088	0.982	0.365	1
ENSGACG00000002233	COMTD1 (1 of many)	catechol-O-methyltransferase domain containing 1	Ensembl	0.59	0.016	-0.17	0.899	0.169	1
ENSGACG00000011303	coro7	coronin 7	Ensembl	0.688	0.015	-0.24	0.898	0.151	1

ENSGACG00000012306	cpt1b	carnitine palmitoyltransferase 1B	Ensembl	-0.78	0.032	0.176	0.919	-0.81	0.219
ENSGACG00000002769	cratb	carnitine O-acetyltransferase b	Ensembl	0.721	0.001	-0.3	0.885	0.309	1
ENSGACG00000002600	ctbp2a	C-terminal binding protein 2a	ZFIN	0.84	0.049	-0.38	0.895	0.453	1
ENSGACG00000009382	cthl	cystathionase (cystathionine gamma-lyase)	Ensembl	0.832	3E-04	0.102	0.948	0.461	0.854
ENSGACG00000016202	ctsba	cathepsin Ba	Ensembl	0.513	0.049	0.056	0.975	0.368	0.968
ENSGACG00000018739	cxcl12b	chemokine (C-X-C motif) ligand 12b (stromal cell-derived factor 1)	Ensembl	-2.84	0.023	-2.16	0.885	-0.76	1
ENSGACG00000013804	cyfip1	cytoplasmic FMR1 interacting protein 1	Ensembl	0.468	0.043	-0.09	0.945	0.115	1
ENSGACG00000011080	cyp26c1	cytochrome P450, family 26, subfamily C, polypeptide 1	Ensembl	-1.25	7E-04	-0.6	0.885	-0.78	0.915
ENSGACG00000014692	cyt1	type I cytokeratin, enveloping layer	Ensembl	1.354	0.049	0.149	0.984	0.514	1
ENSGACG00000009894	dachc	dachshund c	Ensembl	-0.91	0.015	-0.34	0.885	-0.64	0.551
ENSGACG00000015866	dck	deoxycytidine kinase	Ensembl	1.438	0.007	0.164	0.978	0.411	1
ENSGACG00000002379	ddit4	DNA-damage-inducible transcript 4	Ensembl	1.157	2E-04	-0.32	0.885	1.072	0.047
ENSGACG00000008597	degs2	delta(4)-desaturase, sphingolipid 2	Ensembl	-0.8	3E-04	0.038	0.992	-0.47	0.817
ENSGACG00000005920	depdc1a	DEP domain containing 1a	Ensembl	1.99	7E-06	0.004	1	1.176	0.192
ENSGACG00000005507	DEPDC1B	DEP domain containing 1B	Ensembl	2.313	2E-11	0.07	0.997	1.401	0.061
ENSGACG00000015574	desmb	desmin b	Ensembl	-2.17	0.05	-1.52	0.885	-0.31	1
ENSGACG00000003734	dhfr	dihydrofolate reductase	Ensembl	1.206	0.004	-0.35	0.899	0.329	1
ENSGACG00000012455	dhps (1 of many)	deoxyhypusine synthase	Ensembl	2.966	4E-04	0.155	0.973	1.877	0.191
ENSGACG00000015270	diaph3	diaphanous-related formin 3	Ensembl	1.99	1E-10	0.36	0.895	1.401	0.036
ENSGACG00000009474	dlgap5	discs, large (Drosophila) homolog-associated protein 5	Ensembl	1.758	2E-09	-0.17	0.952	0.868	0.615
ENSGACG00000015397	dnah9l	dynein, axonemal, heavy polypeptide 9 like	Ensembl	-2	0.023	-0.54	0.898	-1.77	0.215

ENSGACG00000006408	dnajb2	DnaJ (Hsp40) homolog, subfamily B, member 2	Ensembl	-0.66	0.008	0.032	0.995	-0.31	1
ENSGACG00000015279	DONSON	downstream neighbor of SON	Ensembl	1.276	2E-04	0.203	0.926	0.266	1
ENSGACG00000009383	DSCAML1 (1 of many)	DS cell adhesion molecule like 1	Ensembl	-1.01	2E-04	0.263	0.885	-0.45	0.935
ENSGACG00000004502	dtymk	deoxythymidylate kinase (thymidylate kinase)	Ensembl	1.08	0.013	-0.8	0.885	-0.28	1
ENSGACG00000014118	dyx1c1	dyslexia susceptibility 1 candidate 1	Ensembl	-1.61	0.001	0.316	0.902	-0.29	1
ENSGACG00000017086	e2f8	E2F transcription factor 8	Ensembl	1.312	0.032	0.271	0.947	0.079	1
ENSGACG00000003031	ecrg4a	esophageal cancer related gene 4a	Ensembl	1.129	0.002	-0.41	0.885	0.747	0.26
ENSGACG00000002063	ect2	epithelial cell transforming 2	Ensembl	2.174	3E-12	0.399	0.885	1.273	0.067
ENSGACG00000001421	eef1db	eukaryotic translation elongation factor 1 delta b (guanine nucleotide exchange protein)	Ensembl	0.574	0.035	-0.44	0.885	0.173	1
ENSGACG00000004748	eif2a	eukaryotic translation initiation factor 2A	Ensembl	0.776	1E-04	0.037	0.986	0.555	0.29
ENSGACG00000003191	eif3s10	eukaryotic translation initiation factor 3, subunit 10 (theta)	Ensembl	0.703	6E-04	0.185	0.89	0.521	0.217
ENSGACG00000006781	emp3b	epithelial membrane protein 3b	Ensembl	2.193	0.044	1.928	0.64	-0.24	1
ENSGACG00000005388	engase	endo-beta-N-acetylglucosaminidase	Ensembl	0.649	0.047	-0.24	0.895	0.161	1
ENSGACG00000006345	eomesb	eomesodermin homolog b	Ensembl	-1.48	0.036	-0.68	0.885	-0.64	1
ENSGACG00000001096	ercc6l	excision repair cross-complementation group 6-like	ZFIN	1.735	3E-04	-0.29	0.937	0.727	1
ENSGACG00000000685	espl1	extra spindle pole bodies like 1, separase	Ensembl	2.12	3E-14	0.139	0.955	1.341	0.008
ENSGACG00000008646	etv5a	ets variant 5a	Ensembl	-1	0.023	-0.18	0.949	-0.64	0.832
ENSGACG00000002181	exo1	exonuclease 1	Ensembl	1.559	1E-04	0.457	0.885	0.528	1
ENSGACG00000005912	eya2	EYA transcriptional coactivator and phosphatase 2	Ensembl	-0.8	0.046	0.336	0.885	-0.65	0.279
ENSGACG00000019474	fabp2	fatty acid binding protein 2	Ensembl	-1.5	1E-06	0.2	0.902	-0.86	0.199
ENSGACG00000018140	FABP6 (1 of many)	fatty acid binding protein 6	Ensembl	0.725	0.037	0.056	0.986	0.599	0.35

ENSGACG00000016129	fam120b	family with sequence similarity 120B	Ensembl	-0.77	0.041	-0.04	0.999	-0.13	1
ENSGACG00000000836	fam212ab (1 of many)	family with sequence similarity 212	Ensembl	0.884	0.004	-0.53	0.885	0.451	0.943
ENSGACG00000010183	fam83d	family with sequence similarity 83, member D	ZFIN	1.287	7E-04	-0.09	0.982	0.436	1
ENSGACG00000000884	fancd2	Fanconi anemia, complementation group D2	Ensembl	1.235	7E-06	0.32	0.885	0.568	0.84
ENSGACG00000009634	fancg	Fanconi anemia, complementation group G	ZFIN	1.462	0.02	0.557	0.898	0.443	1
ENSGACG00000011591	fanci	Fanconi anemia, complementation group I	Ensembl	1.818	6E-09	0.554	0.885	1.015	0.193
ENSGACG00000013422	fancl	Fanconi anemia, complementation group L	Ensembl	1.791	4E-04	0.015	1	0.601	1
ENSGACG00000004228	FARP1 (1 of many)	FERM, ARH/RhoGEF and pleckstrin domain protein 1	Ensembl	0.628	0.043	-0.05	0.984	0.247	1
ENSGACG00000002244	fen1	flap structure-specific endonuclease 1	Ensembl	0.86	0.002	0.205	0.914	0.37	1
ENSGACG00000013900	fgf12a	fibroblast growth factor 12a	Ensembl	-3.7	4E-14	-0.6	0.885	-1.6	0.031
ENSGACG00000005948	fnbp1l	formin binding protein 1-like	Ensembl	-0.58	0.011	-0.09	0.948	-0.42	0.551
ENSGACG00000018474	foxi3b	forkhead box I3b	Ensembl	-0.73	0.019	-0.14	0.927	-0.68	0.179
ENSGACG00000019929	foxm1	forkhead box M1	Ensembl	1.976	6E-08	-0.2	0.948	0.515	1
ENSGACG00000003402	foxp1a	forkhead box P1a	Ensembl	-0.55	0.028	-0.15	0.913	-0.46	0.51
ENSGACG00000015236	FZR1 (1 of many)	fizzy/cell division cycle 20 related 1	Ensembl	0.648	0.026	0.109	0.948	0.39	1
ENSGACG00000012915	g2e3	G2/M-phase specific E3 ubiquitin protein ligase	Ensembl	1.903	0.003	-0.86	0.885	1.202	0.618
ENSGACG00000019030	gal3st3	galactose-3-O-sulfotransferase 3	Ensembl	-5.81	0.002	0.15	1	-2.72	0.51
ENSGACG00000002489	galm	galactose mutarotase	Ensembl	0.598	0.007	-0.24	0.885	0.305	1
ENSGACG00000010729	gcnt3 (1 of many)	glucosaminyl (N-acetyl) transferase 3	Ensembl	-1.12	9E-04	-0.18	0.913	-0.56	0.794
ENSGACG00000013095	gdpd3a	glycerophosphodiester phosphodiesterase domain containing 3a	Ensembl	0.954	2E-05	0.146	0.925	0.578	0.974
ENSGACG00000006348	gdpd5b	glycerophosphodiester phosphodiesterase domain containing 5b	Ensembl	-0.83	0.006	0.16	0.918	-0.57	0.578
ENSGACG00000012166	gins1	GINS complex subunit 1 (Psf1 homolog)	Ensembl	1.008	0.032	0.202	0.947	0.065	1

ENSGACG00000013715	GLDC	glycine decarboxylase	Ensembl	0.799	1E-04	0.086	0.951	0.439	0.608
ENSGACG00000003096	gls2a	glutaminase 2a (liver, mitochondrial)	Ensembl	-1.15	0.011	-0.22	0.925	-0.87	0.293
ENSGACG00000017251	GNAZ	G protein subunit alpha z	Ensembl	-1.4	5E-07	0.168	0.918	-0.99	0.091
ENSGACG00000006942	gpnmb	glycoprotein (transmembrane) nmb	Ensembl	1.944	1E-08	-1.26	0.885	1.974	0.006
ENSGACG00000015136	gpsm2	G-protein signaling modulator 2	Ensembl	0.586	0.027	-0.01	1	0.21	1
ENSGACG00000013272	gpx4a	glutathione peroxidase 4a	Ensembl	1.104	8E-04	0.008	1	0.656	0.709
ENSGACG00000011755	grik1b	glutamate receptor, ionotropic, kainate 1b	Ensembl	-2.05	0.035	-0.19	0.988	-0.6	1
ENSGACG00000009427	grik5	glutamate receptor, ionotropic, kainate 5	Ensembl	-1.57	5E-04	-0.32	0.898	-1.32	0.064
ENSGACG00000007655	gstm.1	glutathione S-transferase mu, tandem duplicate 1	ZFIN	1.145	0.027	0.056	0.993	0.09	1
ENSGACG00000008981	gtse1	G-2 and S-phase expressed 1	Ensembl	1.368	0.002	-0.01	1	0.098	1
ENSGACG00000007516	gucy2c	guanylate cyclase 2C	Ensembl	-1.84	2E-11	0.433	0.885	-0.83	0.193
ENSGACG00000014618	h2afvb	H2A histone family, member Vb	Ensembl	0.854	3E-04	-0.27	0.885	0.189	1
ENSGACG00000008983	h3f3a	H3 histone, family 3A	Ensembl	0.593	0.042	0.049	0.982	0.315	1
ENSGACG00000004939	hat1	histone acetyltransferase 1	Ensembl	0.606	0.015	-0.01	1	0.313	0.979
ENSGACG00000010745	haus5	HAUS augmin-like complex, subunit 5	Ensembl	0.841	0.024	0.105	0.974	0.297	1
ENSGACG00000006322	hepacam2	HEPACAM family member 2	Ensembl	-1.06	3E-04	-0.06	0.98	-0.88	0.064
ENSGACG00000015887	hexb	hexosaminidase B (beta polypeptide)	Ensembl	0.656	0.002	-0.15	0.927	0.367	0.935
ENSGACG00000016482	hmgb2a	high mobility group box 2a	Ensembl	0.719	0.029	0.259	0.885	0.463	0.949
ENSGACG00000001173	hmgb2b	high mobility group box 2b	Ensembl	0.73	0.002	0.021	1	0.324	0.919
ENSGACG00000001258	hmgcra	3-hydroxy-3-methylglutaryl-CoA reductase a	Ensembl	0.801	0.008	-0.24	0.889	0.024	1
ENSGACG00000018490	hmmr	hyaluronan-mediated motility receptor (RHAMM)	Ensembl	2.138	3E-11	0.307	0.898	1.414	0.025
ENSGACG00000019469	HMOX1 (1 of many)	heme oxygenase 1	Ensembl	1.09	0.023	-0.21	0.919	0.916	0.31

ENSGACG00000006771	hmox1a	heme oxygenase 1a	Ensembl	1.125	0.003	-0.41	0.885	0.945	0.279
ENSGACG00000014680	hsd17b14	hydroxysteroid (17-beta) dehydrogenase 14	Ensembl	0.775	0.008	-0.24	0.913	0.357	1
ENSGACG00000013051	hsp70.3	heat shock cognate 70-kd protein, tandem duplicate 3	ZFIN	2.615	7E-06	-0.71	0.89	2.73	4E-05
ENSGACG00000012888	hsp90aa1.2	heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2	Ensembl	1.657	4E-09	0.23	0.897	1.426	1E-05
ENSGACG00000019293	hspa14	heat shock protein 14	Ensembl	0.642	0.003	-0.01	1	0.362	0.881
ENSGACG00000010827	IDH2 (1 of many)	isocitrate dehydrogenase (NADP(+)) 2	Ensembl	-0.54	0.02	-0.01	1	-0.31	1
ENSGACG00000005812	ifrd2	interferon-related developmental regulator 2	Ensembl	-0.68	0.02	0.123	0.937	-0.4	0.874
ENSGACG00000011125	igf2b	insulin-like growth factor 2b	Ensembl	-0.94	0.013	0.059	0.986	-0.46	1
ENSGACG00000014275	igfbp5a	insulin-like growth factor binding protein 5a	Ensembl	-1.05	0.087	-0.21	0.898	-1.49	5E-05
ENSGACG00000012767	ighd	immunoglobulin heavy constant delta	ZFIN	-1.06	0.006	-0.29	0.898	-0.26	1
ENSGACG00000009475	igic1s1	immunoglobulin light iota constant 1, s1	ZFIN	-1.98	0.41	-4.01	0.543	-4.18	0.048
ENSGACG00000009493	igic1s1	immunoglobulin light iota constant 1, s1	ZFIN	-1.98	0.41	-4.01	0.543	-4.18	0.048
ENSGACG00000003503	im:7150988	im:7150988	Ensembl	5.619	3E-05	-0.54	0.944	4.636	0.007
ENSGACG00000005590	INCENP	inner centromere protein	Ensembl	2.054	2E-11	0.489	0.885	1.23	0.109
ENSGACG00000020608	inpp5kb	inositol polyphosphate-5-phosphatase Kb	Ensembl	-1.23	0.111	-1.02	0.885	-1.92	0.03
ENSGACG00000003101	IQGAP3	IQ motif containing GTPase activating protein 3	Ensembl	1.972	3E-11	-0.12	0.967	0.825	0.474
ENSGACG00000010580	isoc2	isochorismatase domain containing 2	Ensembl	0.786	0.005	-0.02	1	0.264	1
ENSGACG00000018485	itk	IL2-inducible T-cell kinase	Ensembl	-0.82	0.001	0.04	0.991	-0.46	0.582
ENSGACG00000005703	KCNIP3 (1 of many)	potassium voltage-gated channel interacting protein 3	Ensembl	-1.33	0.024	-0.16	0.972	-0.75	0.885
ENSGACG00000020871	KCNJ15	potassium voltage-gated channel subfamily J member 15	Ensembl	-1.21	0.073	-0.83	0.885	-1.7	0.003
ENSGACG00000014456	kcnj16	potassium inwardly-rectifying channel, subfamily J, member 16	Ensembl	-0.92	0.019	-0.31	0.885	-0.97	0.01
ENSGACG00000006583	kcnj1a.6	potassium inwardly-rectifying channel, subfamily J, member 1a, tandem duplicate 6	Ensembl	-1.94	0.002	-0.24	0.913	-0.73	1

ENSGACG00000007871	kcnk5b	potassium channel, subfamily K, member 5b	Ensembl	-0.74	0.003	0.297	0.885	-0.43	0.827
ENSGACG00000018268	kctd12b	potassium channel tetramerisation domain containing 12b	ZFIN	-1.03	0.015	0.08	0.978	-0.6	0.888
ENSGACG00000008998	kidins220a	kinase D-interacting substrate 220a	Ensembl	-0.54	0.03	-0.06	0.975	-0.33	0.974
ENSGACG00000011110	kif11	kinesin family member 11	Ensembl	2.316	2E-11	0.117	0.977	1.342	0.031
ENSGACG00000008878	kif14	kinesin family member 14	ZFIN	1.893	7E-06	0.056	0.998	0.805	0.862
ENSGACG00000015507	kif18a	kinesin family member 18A	Ensembl	1.942	8E-07	0.233	0.924	1.221	0.356
ENSGACG00000013708	kif1ab	kinesin family member 1Ab	Ensembl	-1.69	7E-08	0.003	1	-0.76	0.449
ENSGACG00000020689	kif20a	kinesin family member 20A	Ensembl	1.302	5E-08	0.228	0.898	0.761	0.319
ENSGACG00000011003	kif20ba	kinesin family member 20Ba	Ensembl	2.052	5E-10	0.288	0.913	1.32	0.064
ENSGACG00000010271	kif20bb	kinesin family member 20Bb	ZFIN	2.707	3E-05	-0.03	1	1.381	0.306
ENSGACG00000007521	kif22	kinesin family member 22	Ensembl	1.576	1E-10	-0.02	1	0.84	0.109
ENSGACG00000005226	kif23	kinesin family member 23	Ensembl	2.25	3E-12	-0.2	0.946	1.241	0.125
ENSGACG00000016920	KIF23 (1 of many)	kinesin family member 23	Ensembl	1.874	1E-10	0.266	0.899	1.006	0.208
ENSGACG00000013814	kif2c	kinesin family member 2C	ZFIN	1.124	0.005	0.217	0.929	0.532	1
ENSGACG00000020289	kif4	kinesin family member 4	Ensembl	1.891	3E-08	0.087	0.979	1.034	0.23
ENSGACG00000001865	kifc1	kinesin family member C1	Ensembl	2.031	1E-12	-0.28	0.913	0.836	0.249
ENSGACG00000017582	klf6b	Kruppel-like factor 6b	Ensembl	0.731	0.011	0.164	0.927	0.756	0.081
ENSGACG00000008197	kntc1	kinetochore associated 1	Ensembl	1.706	2E-08	0.281	0.897	0.804	0.506
ENSGACG00000006925	kpna2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	Ensembl	2.313	2E-17	-0.02	1	1.26	0.01

ENSGACG00000019844	krcp	kelch repeat-containing protein	Ensembl	1.275	0.026	0.083	0.997	0.442	1
ENSGACG00000018310	krt15	keratin 15	Ensembl	0.942	0.007	-0.24	0.898	0.605	0.763
ENSGACG00000003787	krt5	keratin 5	Ensembl	0.944	4E-04	-0.08	0.976	0.469	0.848
ENSGACG00000014689	krtt1c19e	keratin type 1 c19e	Ensembl	1.201	0.019	-0.33	0.91	0.564	1
ENSGACG00000004093	lbr	lamin B receptor	Ensembl	0.745	0.015	-0.14	0.934	0.247	1
ENSGACG00000006705	ldlra	low density lipoprotein receptor a	Ensembl	0.605	0.015	-0.09	0.948	0.516	0.636
ENSGACG00000012167	lgmn	legumain	Ensembl	0.513	0.032	-0.17	0.898	0.342	0.933
ENSGACG00000016922	macrod1	MACRO domain containing 1	Ensembl	0.989	0.016	-0.49	0.885	0.323	1
ENSGACG00000001594	mad2l1	MAD2 mitotic arrest deficient-like 1	Ensembl	1.84	2E-06	0.164	0.959	0.953	0.48
ENSGACG00000005087	mastl	microtubule associated serine/threonine kinase-like	Ensembl	1.914	2E-08	0.235	0.934	0.954	0.286
ENSGACG00000019155	mcm10	minichromosome maintenance 10 replication initiation factor	Ensembl	0.753	0.036	-0.2	0.922	-0.02	1
ENSGACG00000005828	mcm9	minichromosome maintenance 9 homologous recombination repair factor	ZFIN	1.063	0.02	0.207	0.942	0.729	1
ENSGACG00000009823	MCOLN2	mucolipin 2	Ensembl	-2.53	0.035	-3.24	0.438	-2.58	0.119
ENSGACG00000006537	MCPH1	microcephalin 1	Ensembl	1.424	0.011	0.509	0.885	0.884	0.717
ENSGACG00000016110	melk	maternal embryonic leucine zipper kinase	Ensembl	1.825	1E-08	-0.25	0.921	1.087	0.064
ENSGACG00000014882	mfsd11	major facilitator superfamily domain containing 11	Ensembl	0.85	0.049	-0.45	0.885	0.405	1
ENSGACG00000000124	mhc1uka	major histocompatibility complex class I UKA	ZFIN	-1.15	0.035	-0.04	1	-0.69	0.826
ENSGACG00000001910	mhc1uka	major histocompatibility complex class I UKA	ZFIN	-1.04	0.022	0.188	0.947	0.01	1
ENSGACG00000001919	mhc1uka	major histocompatibility complex class I UKA	ZFIN	-0.87	0.018	-0.24	0.901	-0.3	1
ENSGACG00000007471	miip	migration and invasion inhibitory protein	Ensembl	1.362	0.035	0.031	1	0.461	1
ENSGACG00000020579	mis18a	MIS18 kinetochore protein A	Ensembl	2.284	2E-05	0.228	0.966	1.216	0.504
ENSGACG00000008539	mis18bp1	MIS18 binding protein 1	ZFIN	1.538	4E-07	0.06	0.991	0.698	0.822
ENSGACG00000020141	mmp13a (1 of many)	matrix metallopeptidase 13a	Ensembl	-1.66	0.044	-1.24	0.885	-2.03	0.009

ENSGACG00000003264	mpc2	mitochondrial pyruvate carrier 2	Ensembl	-0.65	0.01	-0.21	0.893	-0.55	0.568
ENSGACG00000017166	mpx	myeloid-specific peroxidase	Ensembl	-0.76	0.002	-0.04	1	0.112	1
Stickleback_Metallothionein	mt	metallothionein	Ensembl	1.711	8E-05	-1.15	0.885	0.995	0.197
ENSGACG00000010763	mybl2b	v-myb avian myeloblastosis viral oncogene homolog-like 2b	Ensembl	1.507	8E-05	-0.26	0.929	0.442	1
ENSGACG00000005023	mybphb	myosin binding protein Hb	Ensembl	-0.72	0.023	-0.5	0.885	-0.58	0.595
ENSGACG00000014959	myh11b	myosin, heavy chain 11b, smooth muscle	Ensembl	-2.11	0.048	-0.86	0.885	-2.23	0.063
ENSGACG00000010018	MYH13 (1 of many)	myosin heavy chain 13	Ensembl	-0.82	0.007	0.102	0.956	-0.24	1
ENSGACG00000016622	myo15ab	myosin XVAb	Ensembl	-2.28	2E-06	0.13	0.973	-1.36	0.149
ENSGACG00000020245	MYO19	myosin XIX	Ensembl	-0.6	0.024	-0.07	0.976	-0.51	0.409
ENSGACG00000016512	myo5aa	myosin Vaa	Ensembl	-0.91	0.008	0.397	0.885	-0.21	1
ENSGACG00000010907	MYOZ1 (1 of many)	myozenin 1	Ensembl	-1.18	0.004	-0.47	0.885	-0.68	0.845
ENSGACG00000008630	naa10	N(alpha)-acetyltransferase 10, NatA catalytic subunit	Ensembl	0.608	0.008	-0.29	0.885	0.197	1
ENSGACG00000013389	naga	N-acetylgalactosaminidase, alpha	Ensembl	0.957	0.001	-0.51	0.885	0.413	1
ENSGACG00000019459	nap1l1	nucleosome assembly protein 1-like 1	Ensembl	0.578	0.041	0.214	0.886	0.151	1
ENSGACG00000010292	ncapd2	non-SMC condensin I complex, subunit D2	Ensembl	1.065	4E-05	0.208	0.895	0.626	0.402
ENSGACG00000003922	ncapd3	non-SMC condensin II complex, subunit D3	Ensembl	1.996	4E-14	0.167	0.929	1.15	0.083
ENSGACG00000016204	ncapg	non-SMC condensin I complex, subunit G	Ensembl	2.312	3E-09	-0.31	0.925	0.809	0.652
ENSGACG00000003962	ncapg2	non-SMC condensin II complex, subunit G2	Ensembl	1.947	1E-07	-0.59	0.885	0.912	0.5
ENSGACG00000013606	ncaph	non-SMC condensin I complex, subunit H	Ensembl	1.888	3E-10	-0.04	1	0.829	0.364
ENSGACG00000000636	ncaph2	non-SMC condensin II complex, subunit H2	Ensembl	1.63	4E-04	0.178	0.969	0.795	0.955
ENSGACG00000007732	ndc80	NDC80 kinetochore complex component	Ensembl	2.063	1E-15	0.441	0.885	1.246	0.044
ENSGACG00000017687	nefla	neurofilament, light polypeptide a	Ensembl	-1.81	0.001	-0.69	0.885	-0.48	1

ENSGACG00000016544	neil3	nei-like DNA glycosylase 3	Ensembl	1.319	0.013	-0.53	0.89	-0.05	1
ENSGACG00000009916	nek2	NIMA-related kinase 2	Ensembl	2.341	9E-06	0.015	1	1.797	0.028
ENSGACG00000010747	nemp1	nuclear envelope integral membrane protein 1	Ensembl	1.272	7E-04	0.454	0.885	0.564	1
ENSGACG00000004380	NET1 (1 of many)	neuroepithelial cell transforming 1	Ensembl	1.621	9E-06	0.229	0.937	0.834	0.481
ENSGACG00000001755	nipal4	NIPA-like domain containing 4	Ensembl	-0.87	0.039	-0.23	0.899	-0.74	0.504
ENSGACG00000016311	nipsnap3a	nipsnap homolog 3A	Ensembl	0.919	0.032	0.124	0.973	0.303	1
ENSGACG00000019966	nitr9	novel immune-type receptor 9	ZFIN	-1.01	0.013	0.165	0.954	-0.31	1
ENSGACG00000019971	nitr9	novel immune-type receptor 9	ZFIN	-0.87	0.044	0.009	1	-0.15	1
ENSGACG00000009229	nkl.4	NK-lysin tandem duplicate 4	Ensembl	-1.97	0.002	-0.87	0.885	-1.14	0.683
ENSGACG00000018296	nlgn3a	neuroligin 3a (nlgn3a), mRNA	Ensembl	-1.98	0.04	0.167	0.982	-0.48	1
ENSGACG00000009105	nlrc3	NLR family, CARD domain containing 3	Ensembl	-0.56	0.01	-0.22	0.885	-0.16	1
ENSGACG00000017324	nox1	NADPH oxidase 1	Ensembl	-0.67	0.01	-0.02	1	-0.65	0.067
ENSGACG00000012005	noxo1a	NADPH oxidase organizer 1a	Ensembl	-1.14	0.001	-0.06	0.979	-1.04	0.023
ENSGACG00000009244	noxo1b (1 of many)	NADPH oxidase organizer 1b	Ensembl	-2.12	0.015	-0.65	0.893	-2.62	0.016
ENSGACG00000006863	npy	neuropeptide Y	Ensembl	-2.18	0.031	-1.49	0.885	-0.86	1
ENSGACG00000014782	NTRK3 (1 of many)	neurotrophic receptor tyrosine kinase 3	Ensembl	-2.36	0.02	0.626	0.885	-0.88	1
ENSGACG00000014129	nudt1	nudix (nucleoside diphosphate linked moiety X)-type motif 1	Ensembl	1.968	6E-06	0.288	0.948	0.654	1
ENSGACG00000011074	nuf2	NUF2, NDC80 kinetochore complex component	Ensembl	1.731	2E-06	-0.09	0.982	0.53	1
ENSGACG00000000064	nup210	nucleoporin 210	Ensembl	0.721	0.012	0.182	0.898	0.207	1
ENSGACG00000012186	nusap1	nucleolar and spindle associated protein 1	Ensembl	1.494	9E-05	0.098	0.978	0.619	1
ENSGACG00000017898	odam	odontogenic, ameloblast asssociated	Ensembl	-0.61	0.969	-7.71	0.389	-6.41	0.031
ENSGACG00000010083	ola1	Obg-like ATPase 1	Ensembl	0.634	0.046	0.31	0.885	0.703	0.314
ENSGACG00000002331	oscp1a	organic solute carrier partner 1a	Ensembl	1.546	0.044	-0.38	0.964	1.1	0.91
ENSGACG00000010948	otop2 (1 of many)	otopetrin 2	Ensembl	-1.5	0.018	-0.44	0.885	-0.87	0.822
ENSGACG00000010964	otop2 (1 of many)	otopetrin 2	Ensembl	-1.52	0.004	-0.33	0.898	-0.5	1
ENSGACG00000018794	ovol1	ovo-like zinc finger 1	Ensembl	0.961	0.024	-0.35	0.903	0.555	0.902

ENSGACG00000006876	oxct1b	3-oxoacid CoA transferase 1b	Ensembl	-1.06	5E-05	0.3	0.885	-0.58	0.472
ENSGACG00000013355	p2rx4b	purinergic receptor P2X, ligand-gated ion channel	Ensembl	-1.74	0.03	-0	1	-1.14	0.822
ENSGACG00000001239	paics	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	Ensembl	1.149	0.031	-0.73	0.885	-0.09	1
ENSGACG00000015999	pane1	proliferation associated nuclear element	Ensembl	1.84	6E-05	-0.5	0.893	0.257	1
ENSGACG00000016615	PAPPA	pappalysin 1	Ensembl	-1.11	0.009	0.587	0.885	-0.26	1
ENSGACG00000020045	parpbp	PARP1 binding protein	Ensembl	2.391	2E-09	0.418	0.899	1.394	0.154
ENSGACG00000015034	pask	PAS domain containing serine/threonine kinase	Ensembl	1.455	0.004	0.228	0.948	0.615	1
ENSGACG00000006932	pbk	PDZ binding kinase	Ensembl	1.883	1E-10	0.215	0.925	0.905	0.23
ENSGACG00000017913	pcdh2g17	protocadherin 2 gamma 17	Ensembl	-2.19	0.001	-0.8	0.885	-0.51	1
ENSGACG00000019032	pcyt2 (1 of many)	phosphate cytidylyltransferase 2, ethanolamine	Ensembl	-0.62	0.035	0.122	0.948	-0.07	1
ENSGACG00000018691	pdcd4b	programmed cell death 4b	Ensembl	-0.49	0.033	0.096	0.948	-0.18	1
ENSGACG00000013825	pde4a	phosphodiesterase 4A, cAMP-specific	Ensembl	-1.32	4E-06	0.348	0.885	-0.69	0.098
ENSGACG00000007263	pde4ba	phosphodiesterase 4B, cAMP-specific a	Ensembl	-0.59	0.041	-0.16	0.92	-0.24	1
ENSGACG00000015168	PDE4DIP	phosphodiesterase 4D interacting protein	Ensembl	-0.68	0.034	-0.12	0.948	-0.43	1
ENSGACG00000015037	PDE8A	phosphodiesterase 8A	Ensembl	-0.59	0.015	0.449	0.885	-0.22	1
ENSGACG00000016879	pdlim5b	PDZ and LIM domain 5b	Ensembl	0.715	0.026	-0.27	0.895	0.27	1
ENSGACG00000001786	pfkpa	phosphofructokinase, platelet a	Ensembl	-0.87	0.01	-0.34	0.885	-0.67	0.317
ENSGACG00000010695	phf19	PHD finger protein 19	Ensembl	1.5	2E-05	0.278	0.907	0.734	0.745
ENSGACG00000008901	phkg1a	phosphorylase kinase, gamma 1a	Ensembl	0.629	0.104	0.153	0.925	0.925	0.001
ENSGACG00000015607	pif1	PIF1 5'-to-3' DNA helicase homolog	Ensembl	2.469	1E-07	0.55	0.918	1.522	0.193
ENSGACG00000005180	pik3r3b (1 of many)	phosphoinositide-3-kinase, regulatory subunit 3b (gamma)	Ensembl	-0.75	0.008	0.065	0.977	-0.32	1

ENSGACG00000000601	pip4k2cb	phosphatidylinositol-5-phosphate 4-kinase, type II, gamma b	Ensembl	-1.7	0.035	0.358	0.922	0.014	1
ENSGACG00000013689	pipox	pipecolic acid oxidase	Ensembl	-2.19	8E-04	0.363	0.898	-1.08	0.826
ENSGACG00000016526	plk1	polo-like kinase 1	Ensembl	2.278	2E-13	0.062	0.998	1.122	0.145
ENSGACG00000008202	plk4	polo-like kinase 4	Ensembl	1.091	1E-04	0.115	0.958	0.469	1
ENSGACG00000015198	pnpla4	patatin-like phospholipase domain containing 4	Ensembl	1.232	0.038	0.273	0.942	0.626	1
ENSGACG00000017517	pnpla7a	patatin-like phospholipase domain containing 7a	Ensembl	-0.55	0.027	0.173	0.898	-0.13	1
ENSGACG00000020839	pof1b	premature ovarian failure, 1B	ZFIN	0.557	0.03	-0.42	0.885	0.066	1
ENSGACG00000002594	pola1	polymerase (DNA directed), alpha 1	Ensembl	0.977	0.002	0.086	0.969	0.189	1
ENSGACG00000009265	pole	polymerase (DNA directed), epsilon	Ensembl	0.937	0.009	0.38	0.885	0.26	1
ENSGACG00000010602	POLE2	DNA polymerase epsilon 2, accessory subunit	Ensembl	1.272	7E-04	0.352	0.898	0.478	1
ENSGACG00000012184	porcn	porcupine homolog (Drosophila)	Ensembl	-3.76	0.031	-1.61	0.885	-4.26	0.154
ENSGACG00000000860	ppiab	peptidylprolyl isomerase Ab (cyclophilin A)	Ensembl	0.995	1E-04	-0.49	0.885	0.139	1
ENSGACG00000012983	ppp1r13l	protein phosphatase 1, regulatory subunit 13 like	Ensembl	0.72	0.001	-0.21	0.898	0.12	1
ENSGACG00000005957	prc1a	protein regulator of cytokinesis 1a	Ensembl	1.754	1E-05	0.275	0.921	1.041	0.497
ENSGACG00000017394	prc1b	protein regulator of cytokinesis 1b	Ensembl	2.358	2E-16	0.271	0.937	1.106	0.061
ENSGACG00000012026	prcp	prolylcarboxypeptidase	Ensembl	0.527	0.025	0.022	0.999	0.414	0.64
ENSGACG00000018500	PRF1 (1 of many)	perforin 1	Ensembl	-1.65	0.005	-0.04	1	-0.77	1
ENSGACG00000018504	PRF1 (1 of many)	perforin 1	Ensembl	-1.47	0.013	0.328	0.9	-0.32	1
ENSGACG00000013573	prf1.9	perforin 1.9	Ensembl	-1.21	0.011	-0.65	0.885	-0.65	0.974
ENSGACG00000015249	prim1	primase, DNA, polypeptide 1 [S	Ensembl	0.959	0.029	0.209	0.927	0.128	1
ENSGACG00000006105	prim2	primase, DNA, polypeptide 2	Ensembl	0.896	0.005	0.186	0.918	0.29	1
ENSGACG00000017658	primpol	primase and polymerase (DNA-directed)	Ensembl	1.134	0.02	0.355	0.91	0.443	1

ENSGACG00000017842	prkacbb	protein kinase, cAMP-dependent, catalytic, beta b	Ensembl	-0.58	0.032	-0.01	1	-0.45	0.582
ENSGACG00000005629	prkag2a	protein kinase, AMP-activated, gamma 2 non-catalytic subunit a	Ensembl	-1.13	0.006	0.159	0.936	-0.46	1
ENSGACG00000016775	prkcba	protein kinase C, beta a	Ensembl	0.59	0.011	-0.06	0.975	0.473	0.414
ENSGACG00000013018	prkcz	protein kinase C, zeta	Ensembl	-1.03	0.017	-0.18	0.944	-0.18	1
ENSGACG00000015210	prkx	protein kinase, X-linked	Ensembl	0.902	2E-04	0.018	1	0.701	0.13
ENSGACG00000014224	psmc3ip	PSMC3 interacting protein	Ensembl	1.394	0.002	-0.25	0.929	0.255	1
ENSGACG00000002475	ptgdsb.1	prostaglandin D2 synthase b, tandem duplicate 1	Ensembl	0.737	0.019	-0.17	0.934	0.582	0.356
ENSGACG00000007384	ptgs2b	prostaglandin-endoperoxide synthase 2b	Ensembl	-0.72	0.012	-0.24	0.893	-0.39	1
ENSGACG00000020652	pygma	phosphorylase, glycogen, muscle A	Ensembl	-0.85	0.023	-0.27	0.898	-0.51	0.827
ENSGACG00000002887	qars	glutaminyl-tRNA synthetase	Ensembl	0.722	5E-04	-0.06	0.978	0.516	0.44
ENSGACG00000008215	racgap1	Rac GTPase activating protein 1	Ensembl	1.705	1E-04	-0.1	0.984	0.809	0.955
ENSGACG00000006276	rad21a	RAD21 cohesin complex component a	Ensembl	0.494	0.048	0.112	0.929	0.17	1
ENSGACG00000012241	rad51	RAD51 recombinase	Ensembl	1.553	3E-04	-0.02	1	0.194	1
ENSGACG00000019903	rad51ap1	RAD51 associated protein 1	Ensembl	1.524	4E-04	0.153	0.967	0.572	1
ENSGACG00000003111	rad54l	RAD54-like	Ensembl	0.946	0.002	0.181	0.925	0.348	1
ENSGACG00000013818	raver1	ribonucleoprotein, PTB-binding 1	Ensembl	1.506	0.034	0.013	1	0.703	1
ENSGACG00000002106	rbbp8	retinoblastoma binding protein 8	Ensembl	1.505	1E-04	0.131	0.969	0.776	0.815
ENSGACG00000000024	rbl1	retinoblastoma-like 1 (p107)	Ensembl	0.635	0.046	-0.16	0.922	0.135	1
ENSGACG00000015224	rbm38 (1 of many)	RNA binding motif protein 38	Ensembl	-1.24	0.025	-0.51	0.885	-1.07	0.51
ENSGACG00000006395	RGL3 (1 of many)	ral guanine nucleotide dissociation stimulator like 3	Ensembl	-0.7	0.008	0.309	0.885	-0.51	0.548
ENSGACG00000003256	rgs9b	regulator of G-protein signaling 9b	Ensembl	-0.72	0.043	-0.05	0.995	-0.43	1
ENSGACG00000013668	rhbdl2	rhomboid, veinlet-like 2	Ensembl	0.668	0.014	-0.17	0.906	0.445	0.835

ENSGACG00000015923	rhcg	ammonium transporter Rh type C	Ensembl	2.016	3E-07	-0.56	0.885	1.786	8E-04
ENSGACG00000011548	rhcgb	Rh family, C glycoprotein b	Ensembl	-1.32	7E-08	0.095	0.952	-0.86	0.073
ENSGACG00000009574	rhocb	ras homolog family member Cb	Ensembl	0.563	0.013	-0.08	0.956	0.455	0.512
ENSGACG00000016019	rnaseh2a	ribonuclease H2, subunit A	Ensembl	0.928	0.008	0.063	0.99	0.115	1
ENSGACG00000010856	rpa1	replication protein A1	Ensembl	0.973	0.023	0.314	0.893	0.29	1
ENSGACG00000010916	rps12	ribosomal protein S12	Ensembl	0.686	0.031	-0.56	0.885	0.157	1
ENSGACG00000011905	rps2	ribosomal protein S2	Ensembl	0.587	0.031	-0.24	0.885	0.344	1
ENSGACG00000003895	rps23	ribosomal protein S23	Ensembl	0.586	0.013	-0.29	0.885	0.322	1
ENSGACG00000007027	rps6kb1b	ribosomal protein S6 kinase b, polypeptide 1b	Ensembl	-0.62	0.009	0.081	0.961	-0.38	0.763
ENSGACG00000010761	rps9	ribosomal protein S9	Ensembl	0.575	0.03	-0.07	0.973	0.363	1
ENSGACG00000006018	rpsa	ribosomal protein SA	Ensembl	0.528	0.035	-0.04	0.988	0.381	0.992
ENSGACG00000020382	rrm1 (1 of many)	ribonucleotide reductase M1 polypeptide	Ensembl	1.704	1E-06	0.447	0.885	0.844	0.627
ENSGACG00000009741	rrm1 (1 of many)	ribonucleotide reductase M1 polypeptide	Ensembl	0.771	0.002	-0.36	0.885	-0.04	1
ENSGACG00000001494	RRM2 (1 of many)	ribonucleotide reductase regulatory subunit M2	Ensembl	2.096	0.014	-0.65	0.941	1.006	0.916
ENSGACG00000010606	rtkn2a	rhotekin 2a	Ensembl	2.379	2E-13	0.203	0.957	1.219	0.149
ENSGACG00000016739	SAPCD2	suppressor APC domain containing 2	Ensembl	1.301	0.044	0.388	0.924	0.368	1
ENSGACG00000002023	sass6	SAS-6 centriolar assembly protein	ZFIN	1.582	5E-04	0.457	0.885	0.447	1
ENSGACG00000008487	scdb	stearoyl-CoA desaturase b	Ensembl	-0.86	0.002	-0.01	1	-0.89	0.071
ENSGACG00000004130	scinla	scinderin like a	Ensembl	0.699	0.007	-0.05	0.98	0.366	0.985
ENSGACG00000014733	sdr16c5a	short chain dehydrogenase/reductase family 16C, member 5a	Ensembl	-0.83	0.002	-0.06	0.98	-0.74	0.177
ENSGACG00000012282	sephs1	selenophosphate synthetase 1	Ensembl	0.612	0.03	0.222	0.886	0.163	1
ENSGACG00000010973	sgk1	serum/glucocorticoid regulated kinase 1	Ensembl	0.764	8E-04	-0.07	0.973	0.726	0.063
ENSGACG00000006063	sgk2a	serum/glucocorticoid regulated kinase 2a	Ensembl	-1.25	3E-10	0.342	0.885	-0.71	0.064
ENSGACG00000004980	sgol1	shugoshin-like 1 (S. pombe)	Ensembl	1.779	0.002	-0.33	0.937	0.877	1

ENSGACG00000014127	shcbp1	SHC SH2-domain binding protein 1	Ensembl	1.715	2E-09	0.001	1	0.7	0.379
ENSGACG00000012290	si:ch1073-155h21.1 (1 of many)	si:ch1073-155h21.1	Ensembl	-3.38	2E-15	0.335	0.898	-0.82	1
ENSGACG00000012285	si:ch1073-155h21.1 (1 of many)	si:ch1073-155h21.1	Ensembl	-1.8	0.004	0.522	0.885	-0.6	1
ENSGACG00000004690	si:ch1073-406l10.2	si:ch1073-406l10.2	Ensembl	0.799	0.014	-0.38	0.885	0.242	1
ENSGACG00000013053	si:ch211-113e8.6 (1 of many)	si:ch211-113e8.6	Ensembl	0.968	0.004	0.58	0.885	0.659	0.595
ENSGACG00000020401	si:ch211-137i24.10	si:ch211-137i24.10	Ensembl	0.683	0.042	0.241	0.89	0.68	0.271
ENSGACG00000000489	si:ch211-152p11.4	si:ch211-152p11.4	ZFIN	0.679	0.03	-0.05	0.988	0.687	0.407
ENSGACG00000008347	si:ch211-156j22.4	si:ch211-156j22.4	ZFIN	2.367	4E-06	0.265	0.955	1.579	0.149
ENSGACG00000014043	si:ch211-165b10.3	si:ch211-165b10.3	Ensembl	1.725	4E-04	0.012	1	1.277	0.5
ENSGACG00000007370	si:ch211-165b19.9	si:ch211-165b19.9	Ensembl	-1.66	0.021	-0.55	0.885	-0.97	0.794
ENSGACG00000008464	si:ch211-173b16.3	si:ch211-173b16.3	Ensembl	-0.72	0.031	0.247	0.907	-0.34	1
ENSGACG00000013052	si:ch211-173d10.4	si:ch211-173d10.4	ZFIN	3.029	6E-09	-0.09	0.998	2.853	9E-08
ENSGACG00000000201	si:ch211-173d10.4	si:ch211-173d10.4	ZFIN	3.541	0.015	2.839	0.885	3.672	0.012
ENSGACG00000007890	si:ch211-173d10.4	si:ch211-173d10.4	ZFIN	-2.58	2E-04	-0.56	0.885	-2.36	0.059
ENSGACG00000005129	si:ch211-242b18.1	si:ch211-242b18.1	Ensembl	0.709	0.006	0.012	1	0.33	1
ENSGACG00000002583	si:ch211-244a23.1	si:ch211-244a23.1	Ensembl	1.408	5E-05	-0.04	0.999	1.299	0.067
ENSGACG00000020337	si:ch211-26b3.2	si:ch211-26b3.2	Ensembl	1.587	3E-04	0.516	0.885	0.906	0.532
ENSGACG00000013766	si:ch211-66e2.3	si:ch211-66e2.3	ZFIN	0.747	5E-04	0.035	0.988	0.538	0.325
ENSGACG00000005562	si:ch211-66i15.5	si:ch211-66i15.5	Ensembl	-1.32	6E-05	-0.03	1	-1.08	0.437
ENSGACG00000011633	si:ch211-93f2.1	si:ch211-93f2.1	Ensembl	1.173	4E-04	0.722	0.885	0.976	0.078

ENSGACG00000010953	si:dkey-166c18.1	si:dkey-166c18.1	Ensembl	-0.9	0.031	0.594	0.885	-0.71	0.497
ENSGACG00000011044	si:dkey-205h13.1	si:dkey-205h13.1	Ensembl	-0.81	2E-04	-0.13	0.918	-0.36	0.853
ENSGACG00000016599	si:dkey-222f8.3	si:dkey-222f8.3	Ensembl	0.766	2E-04	0.201	0.893	0.503	0.445
ENSGACG00000008748	si:dkey-228b2.5	si:dkey-228b2.5	Ensembl	-1.36	2E-05	0.401	0.885	-0.37	1
ENSGACG00000014596	si:dkey-31e10.1	si:dkey-31e10.1	ZFIN	1.711	5E-07	0.482	0.885	1.138	0.064
ENSGACG00000002020	ska1	spindle and kinetochore associated complex subunit 1	Ensembl	1.68	2E-04	-0.54	0.892	1.146	0.363
ENSGACG00000018955	slc12a10.2	solute carrier family 12 (sodium/potassium/chloride transporters), member 10, tandem duplicate 2	ZFIN	-1.16	3E-04	-0.17	0.913	-0.54	1
ENSGACG00000018343	slc12a2	solute carrier family 12 (sodium/potassium/chloride transporter), member 2	Ensembl	-0.99	6E-05	-0.22	0.898	-0.75	0.071
ENSGACG00000018992	slc25a23a	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23a	Ensembl	-0.88	0.012	-0.52	0.885	-0.04	1
ENSGACG00000018368	SLC27A3	solute carrier family 27 member 3	Ensembl	0.707	0.012	0.026	1	0.587	0.193
ENSGACG00000019384	SLC2A4	solute carrier family 2 member 4	Ensembl	-0.99	0.008	-0.14	0.934	-1.23	0.008
ENSGACG00000018031	slc44a1a	solute carrier family 44 (choline transporter), member 1a	Ensembl	0.585	0.016	-0.03	0.991	0.44	0.582
ENSGACG00000002956	SLC44A4	solute carrier family 44 member 4	Ensembl	-0.67	0.026	0.463	0.885	-0.13	1
ENSGACG00000007018	slc4a1b	solute carrier family 4 (anion exchanger), member 1b (Diego blood group)	Ensembl	-1.23	3E-06	0.336	0.885	-0.62	0.381
ENSGACG00000019748	slc6a1l	solute carrier family 6 (neurotransmitter transporter, GABA), member 1	Ensembl	0.766	0.013	-0.13	0.948	0.612	0.444
ENSGACG00000019764	slc8a4b	solute carrier family 8 (sodium/calcium exchanger), member 4b	Ensembl	-2.01	0.002	0.299	0.915	-1.61	0.149
ENSGACG00000002959	slc9a2 (1 of many)	solute carrier family 9, subfamily A (NHE2, cation proton antiporter 2), member 2	Ensembl	-0.81	7E-04	0.073	0.967	-0.68	0.124

ENSGACG00000002446	slc9a3.2	solute carrier family 9, subfamily A (NHE3, cation proton antiporter 3), member 3, tandem duplicate 2	Ensembl	-1.48	9E-10	0.4	0.885	-0.91	0.063
ENSGACG00000015927	smc2	structural maintenance of chromosomes 2 [	Ensembl	1.714	1E-08	0.009	1	0.644	0.595
ENSGACG00000010151	smc4	structural maintenance of chromosomes 4	Ensembl	1.273	5E-08	0.054	0.98	0.657	0.381
ENSGACG00000005655	snrnp40	small nuclear ribonucleoprotein 40 (U5)	Ensembl	0.563	0.015	-0.25	0.885	0.363	0.993
ENSGACG00000006733	spc24	SPC24, NDC80 kinetochore complex component	Ensembl	1.937	5E-06	0.14	0.973	1.18	0.356
ENSGACG00000005296	spc25	SPC25, NDC80 kinetochore complex component	Ensembl	1.622	5E-05	0.731	0.885	0.991	0.47
ENSGACG00000019214	spdl1	spindle apparatus coiled-coil protein 1	Ensembl	1.058	0.006	0.143	0.952	0.658	0.835
ENSGACG00000017934	SPOCK1	SPARC/osteonectin, cwcv and kazal like domains proteoglycan 1	Ensembl	-1.27	0.024	0.531	0.885	-0.76	0.888
ENSGACG00000017249	sppl3	signal peptide peptidase 3	Ensembl	0.579	0.047	0.421	0.885	0.414	0.872
ENSGACG00000012535	sstr5	somatostatin receptor 5	Ensembl	-1.27	9E-08	0.339	0.885	-0.75	0.079
ENSGACG00000011549	stard13b	StAR-related lipid transfer (START) domain containing 13b	Ensembl	-0.52	0.041	0.217	0.885	-0.2	1
ENSGACG00000017146	stard14	START domain containing 14	Ensembl	-0.59	0.031	-0.35	0.885	-0.3	1
ENSGACG00000005352	STK39	serine/threonine kinase 39	Ensembl	-1.64	3E-12	0.397	0.885	-0.96	0.012
ENSGACG00000007379	stmn1b	stathmin 1b	Ensembl	0.903	0.016	0.027	1	0.39	1
ENSGACG00000020598	stoml3b	stomatin (EPB72)-like 3b	Ensembl	-0.79	0.002	0.085	0.955	-0.63	0.154
ENSGACG00000006760	STX19	syntaxin 19	Ensembl	-0.87	0.001	0.293	0.885	-0.54	0.492
ENSGACG00000010251	SUCNR1	succinate receptor 1	Ensembl	-1.38	0.01	0.054	1	-1.35	0.061
ENSGACG00000000520	syne2b	spectrin repeat containing, nuclear envelope 2b	ZFIN	0.522	0.042	-0.13	0.921	0.133	1
ENSGACG00000008324	taar20i	trace amine associated receptor 20i	ZFIN	-5.75	0.004	0.26	0.98	0.635	1
ENSGACG00000008294	tacc3	transforming, acidic coiled-coil containing protein 3	Ensembl	1.556	8E-10	0.225	0.898	0.923	0.13
ENSGACG00000004880	TCP11X2	t-complex 11 family, X-linked 2	Ensembl	2.171	4E-04	-0.44	0.927	1.012	1

ENSGACG00000009277	tert	telomerase reverse transcriptase	Ensembl	1.381	5E-05	0.067	0.988	0.293	1
ENSGACG00000005944	tfap2c	transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)	Ensembl	0.687	0.005	-0.29	0.885	0.194	1
ENSGACG00000003196	thop1	thimet oligopeptidase 1	Ensembl	1.229	8E-07	-0.32	0.885	0.276	1
ENSGACG00000000545	timeless	timeless circadian clock	Ensembl	1.052	0.009	0.637	0.885	0.486	1
ENSGACG00000019062	tk1	thymidine kinase 1, soluble	Ensembl	1.997	1E-09	0.46	0.885	1.012	0.274
ENSGACG00000007178	tmem222a	transmembrane protein 222a	Ensembl	1.839	0.047	1.019	0.885	1.652	0.31
ENSGACG00000005272	TMEM87A	transmembrane protein 87A	Ensembl	-0.52	0.035	0.159	0.908	-0.22	1
ENSGACG00000009369	tmprss13b	transmembrane protease, serine 13b	Ensembl	-0.57	0.046	0.256	0.885	-0.31	1
ENSGACG00000008384	tnnt3a	troponin T type 3a (skeletal, fast)	Ensembl	-0.84	0.01	-0.38	0.885	-0.48	0.951
ENSGACG00000009209	tonsl	tonsoku-like, DNA repair protein	Ensembl	1.41	4E-06	0.084	0.983	0.405	1
ENSGACG00000001596	top1	topoisomerase (DNA) I	Ensembl	-1.79	2E-04	0.426	0.895	-0.6	1
ENSGACG00000008498	top2a	topoisomerase (DNA) II alpha	Ensembl	2.272	1E-10	0.181	0.941	1.195	0.113
ENSGACG00000002460	tpk1	thiamin pyrophosphokinase 1	Ensembl	2.041	0.001	0.687	0.885	1.487	0.356
ENSGACG00000018599	tpm2	tropomyosin 2 (beta)	Ensembl	-0.76	0.037	-0.2	0.899	-0.46	0.845
ENSGACG00000013242	tpx2	TPX2, microtubule-associated	ZFIN	1.836	0.035	-0.96	0.885	-0.07	1
ENSGACG00000010396	trim35-12	tripartite motif containing 35-12	Ensembl	-0.64	0.008	0.313	0.885	-0.31	1
ENSGACG00000008559	trip13	thyroid hormone receptor interactor 13	Ensembl	1.053	0.038	0.07	1	0.743	0.919
ENSGACG00000005050	trmt1l	tRNA methyltransferase 1-like	Ensembl	0.777	0.042	0.176	0.942	0.394	1
ENSGACG00000010470	trpv6	transient receptor potential cation channel, subfamily V, member 6	Ensembl	-1.75	1E-11	0.35	0.885	-0.69	0.5
ENSGACG00000005236	tspan2a	tetraspanin 2a	Ensembl	-1.05	8E-06	0.037	0.991	-0.87	0.061
ENSGACG00000005223	ttk	ttk protein kinase	Ensembl	1.8	1E-09	0.014	1	1.051	0.132
ENSGACG00000011524	tuba1c (1 of many)	tubulin, alpha	Ensembl	0.965	2E-06	-0.01	1	0.489	0.5

ENSGACG00000008411	tuba8l4	tubulin, alpha 8 like 4	Ensembl	0.786	0.002	-0.37	0.885	0.166	1
ENSGACG00000004468	tuft1b	tuftelin 1b	ZFIN	-0.81	0.036	-0.43	0.885	-0.93	0.048
ENSGACG00000004111	tyms	thymidylate synthetase	Ensembl	2.103	1E-07	-0.81	0.885	0.407	1
ENSGACG00000003290	ube2c	ubiquitin-conjugating enzyme E2C	Ensembl	1.978	1E-09	-0.18	0.948	0.967	0.54
ENSGACG00000002213	VDAC2 (1 of many)	voltage dependent anion channel 2	Ensembl	-0.52	0.027	0.063	0.973	-0.23	1
ENSGACG00000016386	vegfc	vascular endothelial growth factor c	Ensembl	-0.78	0.008	0.092	0.967	-0.44	0.924
ENSGACG00000013081	vrk1	vaccinia related kinase 1	Ensembl	0.805	1E-03	-0.29	0.885	0.219	1
ENSGACG00000017321	wdhd1	WD repeat and HMG-box DNA binding protein 1	Ensembl	1.687	0.014	-0.19	0.974	0.171	1
ENSGACG00000003470	WNK4 (1 of many)	WNK lysine deficient protein kinase 4	Ensembl	-1.08	0.011	-0.09	0.972	-0.9	0.316
ENSGACG00000007861	wu:fb44b02	wu:fb44b02	ZFIN	2.492	2E-15	0.439	0.885	1.649	0.004
ENSGACG00000006045	ZG16	zymogen granule protein 16	Ensembl	-1.08	0.037	-0.28	0.913	-0.49	1
ENSGACG00000018321	zgc:110712	zgc:110712	Ensembl	-2.27	1E-08	0.056	0.987	-0.59	1
ENSGACG00000003168	zgc:112285	zgc:112285	Ensembl	-0.82	0.496	-0.97	0.885	-2.02	0.03
ENSGACG00000011845	zgc:113054	zgc:113054	Ensembl	1.254	1E-03	-0.08	0.982	0.497	1
ENSGACG00000005295	zgc:113162	zgc:113162	Ensembl	-0.85	0.017	0.136	0.945	-0.89	0.437
ENSGACG00000005772	zgc:152830	zgc:152830	Ensembl	0.753	8E-04	-0.26	0.885	0.269	1
ENSGACG00000010507	zgc:154061	zgc:154061	Ensembl	0.799	0.03	0.197	0.927	0.394	1
ENSGACG00000011906	zgc:158222	zgc:158222	Ensembl	-0.77	7E-04	0.188	0.898	-0.24	1
ENSGACG00000010669	zgc:162595	zgc:162595	Ensembl	-1.8	0.001	-0.09	0.987	-1.4	0.14
ENSGACG00000004948	zgc:163107	zgc:163107	Ensembl	0.547	0.029	-0.24	0.885	0.159	1
ENSGACG00000012895	zgc:174160	zgc:174160	Ensembl	2.015	3E-06	0.237	0.954	1.212	0.244
ENSGACG00000018991	zgc:55262	zgc:55262	Ensembl	0.823	0.017	-0.29	0.889	0.401	0.921
ENSGACG00000005552	zgc:63587	zgc:63587	Ensembl	0.527	0.03	-0.19	0.898	0.099	1
ENSGACG00000006889	zgc:65895	zgc:65895	ZFIN	-0.85	0.049	-0.36	0.885	-0.81	0.31

ENSGACG00000013147	zgc:85932	zgc:85932	Ensembl	0.684	0.003	0.027	0.996	0.438	0.582
ENSGACG00000004610	zgc:86896	zgc:86896	Ensembl	0.501	0.048	-0.06	0.978	0.521	0.364
ENSGACG00000010374	zgc:92161	zgc:92161	Ensembl	0.909	0.004	-0.39	0.898	-0.01	1
ENSGACG00000006715	znf367	zinc finger protein 367	Ensembl	1.166	0.049	0.267	0.922	0.312	1
ENSGACG00000005043	zwilch	zwilch kinetochore protein	Ensembl	1.376	8E-05	0.445	0.885	0.763	0.582
ENSGACG00000019813	NA	NA	Ensembl	-2.47	0.258	-1.23	0.885	-4.75	0.003
ENSGACG00000012488	NA	NA	Ensembl	-1.49	7E-04	-0.37	0.885	-1.92	2E-07
ENSGACG00000012491	NA	NA	Ensembl	-1.43	0.003	-0.6	0.885	-1.63	0.014
ENSGACG00000014580	NA	NA	Ensembl	-1.39	0.003	-0.63	0.885	-1.58	0.022
ENSGACG00000019379	NA	NA	Ensembl	-1.33	3E-05	-0.12	0.939	-1.28	0.002
ENSGACG00000006398	NA	NA	Ensembl	-1.6	7E-05	0.289	0.898	-1.27	0.03
ENSGACG00000005345	NA	NA	Ensembl	-1.11	1E-03	0.305	0.898	-1.17	0.031
ENSGACG00000011229	NA	NA	Ensembl	3.658	0.005	3.607	0.693	4	0.001
ENSGACG00000006110	NA	NA	Ensembl	-1.32	5E-08	0.27	0.885	-0.81	0.064
ENSGACG00000006427	NA	NA	Ensembl	1.903	4E-15	-0.35	0.898	1.18	0.084
ENSGACG00000005908	NA	NA	Ensembl	2.369	3E-08	0.22	0.948	1.393	0.09
ENSGACG00000018416	NA	NA	Ensembl	1.086	3E-05	0.323	0.885	0.845	0.145
ENSGACG00000001077	NA	NA	Ensembl	-0.67	0.016	-0.25	0.885	-0.62	0.192
ENSGACG00000006985	NA	NA	Ensembl	1.575	5E-07	0.209	0.931	1.05	0.197
ENSGACG00000001369	NA	NA	Ensembl	-3.3	0.013	-0.76	0.907	-2.77	0.199
ENSGACG00000011404	NA	NA	Ensembl	-1.9	1E-05	0.28	0.914	-1.19	0.338
ENSGACG00000004582	NA	NA	Ensembl	0.662	0.048	0.224	0.898	0.668	0.43

ENSGACG00000009578	NA	NA	Ensembl	0.589	0.016	0.184	0.895	0.512	0.437
ENSGACG00000010154	NA	NA	Ensembl	1.594	4E-06	-0.52	0.885	0.919	0.474
ENSGACG00000005566	NA	NA	Ensembl	-1.16	9E-06	-0.11	0.965	-0.93	0.48
ENSGACG00000014598	NA	NA	Ensembl	1.414	7E-06	0.406	0.885	0.791	0.492
ENSGACG00000009904	NA	NA	Ensembl	2.603	7E-12	-0.17	0.973	0.999	0.546
ENSGACG00000012390	NA	NA	Ensembl	1.666	6E-04	1.163	0.801	1.005	0.549
ENSGACG00000007987	NA	NA	Ensembl	-0.79	0.02	-0.1	0.967	-0.61	0.554
ENSGACG00000008388	NA	NA	Ensembl	0.783	0.009	-0.07	0.979	0.51	0.651
ENSGACG00000017385	NA	NA	Ensembl	2.314	8E-04	0.614	0.89	1.449	0.677
ENSGACG00000017382	NA	NA	Ensembl	2.001	4E-04	-0.27	0.955	1.082	0.717
ENSGACG00000015247	NA	NA	Ensembl	0.604	0.016	-0.14	0.925	0.413	0.719
ENSGACG00000017229	NA	NA	Ensembl	1.086	0.008	0.138	0.961	0.714	0.789
ENSGACG00000009927	NA	NA	Ensembl	0.918	0.002	0.315	0.885	0.485	0.802
ENSGACG00000019051	NA	NA	Ensembl	-1.3	0.05	0.136	0.966	-0.86	0.827
ENSGACG00000001629	NA	NA	Ensembl	-0.93	0.002	-0.33	0.885	-0.56	0.848
ENSGACG00000008868	NA	NA	Ensembl	1.578	0.004	-0.04	1	1.024	0.85
ENSGACG00000010139	NA	NA	Ensembl	1.945	0.003	0.422	0.914	0.998	0.85
ENSGACG00000000377	NA	NA	Ensembl	1.797	0.005	0.324	0.944	0.928	0.874
ENSGACG00000015806	NA	NA	Ensembl	-0.71	0.019	0.013	1	-0.47	0.897
ENSGACG00000018761	NA	NA	Ensembl	2.434	3E-04	-0.65	0.92	1.325	0.902
ENSGACG00000007076	NA	NA	Ensembl	1.875	0.015	0.995	0.885	1.208	0.913
ENSGACG00000006466	NA	NA	Ensembl	-0.54	0.035	-0.18	0.904	-0.32	0.943
ENSGACG00000017942	NA	NA	Ensembl	-1.06	0.009	0.237	0.898	-0.49	0.954
ENSGACG00000019432	NA	NA	Ensembl	-1.01	0.008	-0.04	0.997	-0.62	0.955

ENSGACG00000009970	NA	NA	Ensembl	1.742	2E-05	-0.37	0.907	0.692	0.961
ENSGACG00000002256	NA	NA	Ensembl	2.173	8E-04	-1.35	0.885	1.135	0.974
ENSGACG00000010896	NA	NA	Ensembl	-3.08	0.02	0.137	0.993	-0.36	1
ENSGACG00000000935	NA	NA	Ensembl	-2.96	0.006	-1.94	0.885	0.509	1
ENSGACG00000001847	NA	NA	Ensembl	-2.05	0.019	0.226	0.968	-0.8	1
ENSGACG00000012210	NA	NA	Ensembl	-1.42	0.014	-0.18	0.957	-0.31	1
ENSGACG00000000336	NA	NA	Ensembl	-1.4	1E-08	-0.25	0.885	-0.31	1
ENSGACG00000000188	NA	NA	Ensembl	-1.33	0.007	0.282	0.903	0.307	1
ENSGACG00000018719	NA	NA	Ensembl	-1.27	0.033	0.003	1	-0.63	1
ENSGACG00000014302	NA	NA	Ensembl	-1.22	0.023	-0.05	1	0.146	1
ENSGACG00000020258	NA	NA	Ensembl	-1.19	0.021	-0.5	0.885	0.278	1
ENSGACG00000009921	NA	NA	Ensembl	-1.1	0.013	0.133	0.963	-0.04	1
ENSGACG00000000348	NA	NA	Ensembl	-1.04	6E-04	-0.15	0.95	-0.32	1
ENSGACG00000000350	NA	NA	Ensembl	-1.01	2E-04	-0.49	0.885	-0.4	1
ENSGACG00000006275	NA	NA	Ensembl	-0.98	0.005	0.096	0.973	0.054	1
ENSGACG00000019052	NA	NA	Ensembl	-0.95	8E-04	0.307	0.885	-0.26	1
ENSGACG00000000330	NA	NA	Ensembl	-0.93	4E-04	0.175	0.913	-0.3	1
ENSGACG00000001641	NA	NA	Ensembl	-0.91	0.027	-0.12	0.969	-0.08	1
ENSGACG00000004162	NA	NA	Ensembl	-0.89	0.002	0.437	0.885	-0.4	1
ENSGACG00000003210	NA	NA	Ensembl	-0.71	0.011	0.154	0.921	-0.35	1
ENSGACG00000004768	NA	NA	Ensembl	-0.69	0.015	0.289	0.885	-0.17	1
ENSGACG00000001651	NA	NA	Ensembl	-0.69	0.022	0.552	0.885	0.157	1
ENSGACG00000008445	NA	NA	Ensembl	-0.64	0.046	0.242	0.899	-0.16	1
ENSGACG00000001357	NA	NA	Ensembl	0.61	0.038	-0.15	0.929	0.15	1
ENSGACG00000020803	NA	NA	Ensembl	0.647	0.023	0.262	0.885	-0.01	1

ENSGACG00000000326	NA	NA	Ensembl	0.704	0.006	-0.27	0.893	0.096	1
ENSGACG00000004053	NA	NA	Ensembl	0.749	0.002	-0.25	0.885	0.236	1
ENSGACG00000008514	NA	NA	Ensembl	0.825	0.002	-0.37	0.885	0.174	1
ENSGACG00000007173	NA	NA	Ensembl	0.862	0.012	-0.15	0.949	0.278	1
ENSGACG00000012538	NA	NA	Ensembl	0.887	7E-04	-0.19	0.919	0.324	1
ENSGACG00000001108	NA	NA	Ensembl	0.895	2E-04	-0.32	0.885	0.217	1
ENSGACG00000007432	NA	NA	Ensembl	0.979	0.017	0.086	0.982	0.058	1
ENSGACG00000016770	NA	NA	Ensembl	1.141	7E-04	-0.5	0.885	0.302	1
ENSGACG00000011552	NA	NA	Ensembl	1.272	0.041	0.391	0.898	-0.17	1
ENSGACG00000016167	NA	NA	Ensembl	1.274	0.024	0.086	0.989	0.429	1
ENSGACG00000012387	NA	NA	Ensembl	1.344	0.02	0.101	0.987	0.444	1
ENSGACG00000018947	NA	NA	Ensembl	1.511	0.004	0.679	0.885	0.098	1
ENSGACG00000004694	NA	NA	Ensembl	1.665	2E-04	-0.62	0.885	0.761	1
ENSGACG00000002258	NA	NA	Ensembl	1.698	0.005	-0.23	0.973	0.319	1

Supporting Information Table 11. Gene Ontology Terms and KEGG Pathways over-represented in the lists of differentially expressed transcripts following exposure to control_cu in the gill. Values presented are the number and percentage of transcripts associated with each GO term, the P value, fold enrichment and adjusted P value (adjusted P value < 0.05; Benjamini-Hochberg) associated with over-representation. Pale blue shading indicates significant enrichment. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.8) using all transcripts expressed in this study as a background list.

GOTERM_BP_DIRECT	Count	%	P Value	Fold Enrichment	Benjamini
antigen processing and presentation	22	3.84	3.48E-21	14.76	2.27E-18
immune response	24	4.19	3.30E-12	6.01	1.07E-09
cell cycle	30	5.24	1.68E-11	4.42	3.64E-09
mitotic nuclear division	21	3.66	4.75E-11	6.24	7.73E-09
cell division	23	4.01	1.47E-10	5.31	1.91E-08
mitotic sister chromatid segregation	8	1.40	1.85E-08	18.48	2.01E-06
microtubule-based movement	14	2.44	4.17E-07	5.82	3.88E-05
mitotic chromosome condensation	6	1.05	1.43E-06	20.79	1.17E-04
chromosome segregation	10	1.75	2.69E-06	7.70	1.94E-04
DNA replication	14	2.44	1.41E-05	4.34	9.19E-04
potassium ion import	7	1.22	2.57E-04	7.28	1.51E-02
ion transport	23	4.01	7.15E-04	2.20	3.80E-02
mitotic cytokinesis	5	0.87	8.71E-04	10.40	4.27E-02
chromosome condensation	4	0.70	0.001021	16.63	4.64E-02
cytokinesis	5	0.87	0.001902	8.66	7.93E-02
ammonium transmembrane transport	4	0.70	0.003323	11.88	1.13E-01
ammonium transport	4	0.70	0.003323	11.88	1.13E-01
DNA repair	16	2.79	0.003284	2.34	1.18E-01
G-protein coupled receptor signaling pathway	22	3.84	0.003251	2.00	1.24E-01
mitotic cell cycle	6	1.05	0.005952	4.99	1.85E-01
S-adenosylmethionine cycle	3	0.52	0.006673	20.79	1.96E-01
chromosome organization	4	0.70	0.00742	9.24	2.06E-01
regulation of mitotic nuclear division	4	0.70	0.00742	9.24	2.06E-01
mitotic spindle organization	4	0.70	0.00742	9.24	2.06E-01
single organismal cell-cell adhesion	7	1.22	0.008816	3.83	2.31E-01
regulation of ion transmembrane transport	7	1.22	0.008816	3.83	2.31E-01

regulation of pH	4	0.70	0.010227	8.32	2.52E-01
mitotic spindle assembly checkpoint	4	0.70	0.010227	8.32	2.52E-01
deoxyribonucleotide biosynthetic process	3	0.52	0.012922	15.59	2.97E-01
mitotic metaphase plate congression	3	0.52	0.012922	15.59	2.97E-01
potassium ion transport	7	1.22	0.019648	3.23	3.80E-01
spermatogenesis	5	0.87	0.019227	4.73	3.85E-01
anaphase-promoting complex-dependent catabolic process	3	0.52	0.020857	12.48	3.87E-01
hypotonic salinity response	3	0.52	0.020857	12.48	3.87E-01
intracellular signal transduction	20	3.49	0.018658	1.76	3.88E-01
sodium ion transport	6	1.05	0.02747	3.47	4.65E-01
organic cation transport	3	0.52	0.030301	10.40	4.76E-01
nitrogen utilization	3	0.52	0.030301	10.40	4.76E-01
signal transduction	35	6.11	0.031721	1.42	4.81E-01
cellular response to DNA damage stimulus	10	1.75	0.030023	2.28	4.84E-01
response to oxidative stress	5	0.87	0.033824	4.00	4.93E-01
double-strand break repair via homologous recombination	5	0.87	0.038252	3.85	5.26E-01
centriole replication	3	0.52	0.041093	8.91	5.42E-01
microtubule cytoskeleton organization	6	1.05	0.049166	2.97	5.88E-01
protein phosphorylation	30	5.24	0.047883	1.42	5.88E-01
sodium ion import across plasma membrane	3	0.52	0.053081	7.80	6.07E-01
lipid metabolic process	12	2.09	0.062027	1.83	6.57E-01
lipoprotein metabolic process	3	0.52	0.066127	6.93	6.72E-01
phosphorylation	30	5.24	0.068116	1.37	6.74E-01
DNA metabolic process	3	0.52	0.080101	6.24	7.26E-01
regulation of intracellular pH	4	0.70	0.085988	3.78	7.36E-01
cation transport	5	0.87	0.084913	2.97	7.39E-01
peptidyl-serine phosphorylation	6	1.05	0.096312	2.45	7.54E-01
fin regeneration	6	1.05	0.096312	2.45	7.54E-01
cyclic nucleotide biosynthetic process	3	0.52	0.094887	5.67	7.56E-01
response to cadmium ion	3	0.52	0.094887	5.67	7.56E-01
regulation of mitotic spindle organization	2	0.35	0.093672	20.79	7.59E-01
negative regulation of meiotic nuclear division	2	0.35	0.093672	20.79	7.59E-01
de novo' L-methionine biosynthetic process	2	0.35	0.093672	20.79	7.59E-01
type 2 immune response	2	0.35	0.093672	20.79	7.59E-01
DNA replication, synthesis of RNA primer	2	0.35	0.093672	20.79	7.59E-01
regulation of mitotic cell cycle, embryonic	2	0.35	0.093672	20.79	7.59E-01
chromosome separation	2	0.35	0.093672	20.79	7.59E-01
centromere complex assembly	2	0.35	0.093672	20.79	7.59E-01
negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	2	0.35	0.093672	20.79	7.59E-01
chitin metabolic process	2	0.35	0.093672	20.79	7.59E-01
cleavage furrow formation	2	0.35	0.093672	20.79	7.59E-01

spindle checkpoint	2	0.35	0.093672	20.79	7.59E-01
transsulfuration	2	0.35	0.093672	20.79	7.59E-01
GOTERM_MF_ALL	**Count**	**%**	**P Value**	**Fold Enrichment**	**Benjamini**
adenyl nucleotide binding	84	14.66	2.58E-07	1.74	3.52E-05
adenyl ribonucleotide binding	84	14.66	2.49E-07	1.74	4.25E-05
motor activity	19	3.32	2E-07	4.41	4.54E-05
microtubule motor activity	14	2.44	1.68E-07	6.30	5.72E-05
ATP binding	84	14.66	1.52E-07	1.76	1.04E-04
ATP-activated inward rectifier potassium channel activity	6	1.05	9.68E-07	22.50	1.10E-04
nucleoside binding	99	17.28	1.41E-06	1.59	1.38E-04
carbohydrate derivative binding	102	17.80	2.64E-06	1.55	1.50E-04
purine ribonucleoside binding	98	17.10	2.27E-06	1.57	1.54E-04
purine nucleoside binding	98	17.10	2.27E-06	1.57	1.54E-04
ribonucleoside binding	98	17.10	2.5E-06	1.57	1.55E-04
microtubule binding	18	3.14	1.83E-06	4.01	1.56E-04
tubulin binding	19	3.32	3.49E-06	3.65	1.59E-04
purine ribonucleoside triphosphate binding	98	17.10	2.12E-06	1.58	1.61E-04
purine ribonucleotide binding	98	17.10	3.19E-06	1.56	1.67E-04
purine nucleotide binding	98	17.10	3.44E-06	1.56	1.67E-04
ribonucleotide binding	98	17.10	4.11E-06	1.55	1.75E-04
inward rectifier potassium channel activity	7	1.22	1.62E-05	11.25	6.51E-04
ligand-gated ion channel activity	10	1.75	0.00013	5.00	4.90E-03
ligand-gated channel activity	10	1.75	0.00013	5.00	4.90E-03
potassium ion transmembrane transporter activity	11	1.92	0.000271	4.12	8.76E-03
nucleoside phosphate binding	107	18.67	0.000301	1.37	8.90E-03
nucleotide binding	107	18.67	0.000301	1.37	8.90E-03
voltage-gated potassium channel activity	8	1.40	0.000268	6.00	9.11E-03
G-protein coupled receptor activity	24	4.19	0.000296	2.30	9.13E-03
small molecule binding	110	19.20	0.000267	1.36	9.56E-03
DNA polymerase activity	7	1.22	0.000388	6.85	1.10E-02
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	39	6.81	0.000528	1.79	1.38E-02
pyrophosphatase activity	39	6.81	0.000508	1.79	1.38E-02
nucleoside-triphosphatase activity	37	6.46	0.000683	1.80	1.65E-02
hydrolase activity, acting on acid anhydrides	39	6.81	0.000665	1.77	1.67E-02
transmembrane receptor activity	37	6.46	0.001043	1.76	2.42E-02
integrin binding	6	1.05	0.001161	7.10	2.61E-02
potassium channel activity	8	1.40	0.001436	4.61	3.11E-02
ion transmembrane transporter activity	29	5.06	0.002544	1.82	5.28E-02
protein serine/threonine kinase activity	25	4.36	0.002956	1.91	5.94E-02
receptor activity	43	7.50	0.003085	1.58	6.01E-02

molecular transducer activity	43	7.50	0.003085	1.58	6.01E-02
RNA-directed DNA polymerase activity	4	0.70	0.004102	11.25	7.70E-02
telomerase activity	4	0.70	0.004102	11.25	7.70E-02
hydrolase activity, acting on ether bonds	4	0.70	0.004102	11.25	7.70E-02
ammonium transmembrane transporter activity	4	0.70	0.004102	11.25	7.70E-02
voltage-gated cation channel activity	8	1.40	0.004333	3.83	7.90E-02
DNA-directed DNA polymerase activity	5	0.87	0.004532	7.03	8.03E-02
cell adhesion molecule binding	8	1.40	0.005486	3.67	9.40E-02
passive transmembrane transporter activity	16	2.79	0.005784	2.21	9.65E-02
channel activity	16	2.79	0.005784	2.21	9.65E-02
cation:chloride symporter activity	4	0.70	0.005952	10.00	9.68E-02
substrate-specific channel activity	15	2.62	0.006299	2.26	9.98E-02
inorganic anion transmembrane transporter activity	8	1.40	0.006855	3.53	1.03E-01
cation transmembrane transporter activity	23	4.01	0.006774	1.84	1.05E-01
gated channel activity	12	2.09	0.009394	2.45	1.33E-01
voltage-gated ion channel activity	9	1.57	0.009199	3.02	1.33E-01
cytoskeletal protein binding	26	4.54	0.009853	1.71	1.37E-01
ribonucleoside-diphosphate reductase activity	3	0.52	0.011089	16.87	1.47E-01
adenosylhomocysteinase activity	3	0.52	0.011089	16.87	1.47E-01
oxidoreductase activity, acting on CH or CH2 groups, disulfide as acceptor	3	0.52	0.011089	16.87	1.47E-01
ribonucleoside-diphosphate reductase activity, thioredoxin disulfide as acceptor	3	0.52	0.011089	16.87	1.47E-01
trialkylsulfonium hydrolase activity	3	0.52	0.011089	16.87	1.47E-01
hydrolase activity	91	15.88	0.010882	1.26	1.47E-01
ion channel activity	14	2.44	0.012095	2.17	1.50E-01
voltage-gated channel activity	9	1.57	0.011874	2.89	1.50E-01
transferase activity, transferring phosphorus-containing groups	48	8.38	0.011719	1.42	1.51E-01
chloride transmembrane transporter activity	6	1.05	0.012525	4.22	1.52E-01
ATPase activity	22	3.84	0.014822	1.74	1.75E-01
metal ion transmembrane transporter activity	15	2.62	0.016294	2.02	1.87E-01
ion antiporter activity	5	0.87	0.017266	4.89	1.94E-01
substrate-specific transmembrane transporter activity	30	5.24	0.019871	1.54	2.17E-01
substrate-specific transporter activity	35	6.11	0.021091	1.47	2.22E-01
protein kinase activity	32	5.58	0.020784	1.51	2.22E-01
cation:cation antiporter activity	4	0.70	0.026461	6.00	2.63E-01
anion:cation symporter activity	4	0.70	0.026461	6.00	2.63E-01
oxidoreductase activity, acting on CH or CH2 groups	3	0.52	0.02613	11.25	2.64E-01
protein complex binding	13	2.27	0.031411	1.98	2.96E-01

transmembrane signaling receptor activity	30	5.24	0.032153	1.48	2.98E-01
transporter activity	41	7.16	0.031398	1.38	3.00E-01
DNA/RNA helicase activity	3	0.52	0.035521	9.64	3.20E-01
CTP:2,3-di-O-geranylgeranyl-sn-glycero-1-phosphate cytidyltransferase activity	3	0.52	0.035521	9.64	3.20E-01
UDP-N-acetylgalactosamine diphosphorylase activity	3	0.52	0.035521	9.64	3.20E-01
phospholactate guanylyltransferase activity	3	0.52	0.035521	9.64	3.20E-01
DNA primase activity	3	0.52	0.035521	9.64	3.20E-01
ATP:coenzyme F420 adenylyltransferase activity	3	0.52	0.035521	9.64	3.20E-01
solute:cation antiporter activity	4	0.70	0.03705	5.29	3.27E-01
transmembrane transporter activity	32	5.58	0.04109	1.42	3.52E-01
cation channel activity	10	1.75	0.043048	2.14	3.61E-01
structural molecule activity	24	4.19	0.044902	1.52	3.65E-01
monovalent inorganic cation transmembrane transporter activity	13	2.27	0.044307	1.87	3.65E-01
potassium:proton antiporter activity	3	0.52	0.045995	8.44	3.68E-01
tRNA guanylyltransferase activity	3	0.52	0.045995	8.44	3.68E-01
monovalent cation:proton antiporter activity	3	0.52	0.045995	8.44	3.68E-01
sodium:proton antiporter activity	3	0.52	0.045995	8.44	3.68E-01
peroxidase activity	4	0.70	0.04948	4.74	3.86E-01
kinase activity	39	6.81	0.050911	1.34	3.86E-01
anion transmembrane transporter activity	8	1.40	0.050829	2.37	3.90E-01
secondary active transmembrane transporter activity	10	1.75	0.057652	2.03	4.17E-01
phosphotransferase activity, alcohol group as acceptor	34	5.93	0.058762	1.36	4.19E-01
mannose-phosphate guanylyltransferase activity	3	0.52	0.057435	7.50	4.20E-01
sulfate adenylyltransferase activity	3	0.52	0.057435	7.50	4.20E-01
RNA guanylyltransferase activity	3	0.52	0.057435	7.50	4.20E-01
endopeptidase activity	19	3.32	0.059863	1.57	4.21E-01
receptor binding	19	3.32	0.059863	1.57	4.21E-01
transferase activity, transferring alkyl or aryl (other than methyl) groups	6	1.05	0.064671	2.75	4.43E-01
potassium ion antiporter activity	3	0.52	0.069738	6.75	4.64E-01
solute:proton antiporter activity	3	0.52	0.082803	6.14	5.21E-01
guanylyltransferase activity	3	0.52	0.082803	6.14	5.21E-01
adenylate cyclase activity	3	0.52	0.082803	6.14	5.21E-01
cytidylyltransferase activity	3	0.52	0.082803	6.14	5.21E-01
DNA polymerase binding	2	0.35	0.086726	22.50	5.34E-01
trace-amine receptor activity	2	0.35	0.086726	22.50	5.34E-01
cystathionine gamma-synthase activity	2	0.35	0.086726	22.50	5.34E-01
cystathionine gamma-lyase activity	2	0.35	0.086726	22.50	5.34E-01
oxidoreductase activity, acting on	4	0.70	0.088009	3.75	5.35E-01

peroxide as acceptor					
single-stranded DNA binding	5	0.87	0.092742	2.88	5.51E-01
antiporter activity	5	0.87	0.092742	2.88	5.51E-01
uridylyltransferase activity	3	0.52	0.096541	5.62	5.61E-01
protein serine/threonine/tyrosine kinase activity	3	0.52	0.096541	5.62	5.61E-01
antigen binding	3	0.52	0.096541	5.62	5.61E-01

GOTERM_CC_ALL	Count	%	P Value	Fold Enrichment	Benjamini
MHC protein complex	18	3.14	2.44E-22	21.61	8.28E-20
MHC class II protein complex	18	3.14	2.44E-22	21.61	8.28E-20
chromosomal part	42	7.33	2.42E-16	4.52	3.76E-14
chromosome	45	7.85	1.22E-15	4.07	1.38E-13
non-membrane-bounded organelle	113	19.72	2.38E-15	2.10	1.98E-13
intracellular non-membrane-bounded organelle	113	19.72	2.38E-15	2.10	1.98E-13
chromosome, centromeric region	20	3.49	3.64E-14	9.40	2.46E-12
cytoskeletal part	58	10.12	5.96E-14	3.01	3.37E-12
condensed chromosome	19	3.32	4.28E-12	8.05	2.07E-10
chromosomal region	21	3.66	9.51E-12	6.77	4.03E-10
cytoskeleton	62	10.82	1.82E-10	2.38	6.87E-09
supramolecular fiber	28	4.89	1.14E-09	3.98	3.87E-08
polymeric cytoskeletal fiber	28	4.89	1.14E-09	3.98	3.87E-08
condensed chromosome, centromeric region	12	2.09	2.57E-09	10.81	7.93E-08
microtubule cytoskeleton	38	6.63	7.40E-09	2.90	2.09E-07
plasma membrane part	68	11.87	1.16E-08	2.05	3.03E-07
nuclear chromosome	22	3.84	1.35E-08	4.44	3.26E-07
nuclear chromosome part	21	3.66	1.79E-08	4.58	4.04E-07
plasma membrane protein complex	25	4.36	8.00E-08	3.60	1.70E-06
kinetochore	12	2.09	2.71E-07	7.41	5.41E-06
nuclear replication fork	9	1.57	3.24E-07	11.44	6.11E-06
plasma membrane	89	15.53	3.80E-07	1.69	6.44E-06
intermediate filament	12	2.09	3.74E-07	7.20	6.68E-06
intermediate filament cytoskeleton	12	2.09	5.11E-07	7.01	7.87E-06
cell periphery	91	15.88	5.08E-07	1.67	8.19E-06
replisome	7	1.22	6.96E-07	16.81	9.83E-06
nuclear replisome	7	1.22	6.96E-07	16.81	9.83E-06
microtubule associated complex	17	2.97	6.72E-07	4.54	9.91E-06
replication fork	10	1.75	1.94E-06	8.00	2.64E-05
condensed chromosome kinetochore	9	1.57	2.30E-06	9.26	3.01E-05
kinesin complex	12	2.09	6.85E-06	5.52	8.60E-05
protein-DNA complex	11	1.92	7.50E-06	6.10	9.08E-05
condensed nuclear chromosome	8	1.40	1.32E-05	9.10	1.54E-04
spindle	13	2.27	4.06E-05	4.26	4.59E-04
apical plasma membrane	8	1.40	5.46E-05	7.52	5.97E-04

microtubule	16	2.79	6.98E-05	3.36	7.39E-04
apical part of cell	8	1.40	7.41E-05	7.20	7.61E-04
condensed nuclear chromosome, centromeric region	5	0.87	1.40E-04	15.44	1.40E-03
condensin complex	4	0.70	3.77E-04	21.61	3.65E-03
pore complex	5	0.87	4.68E-04	12.01	4.40E-03
DNA packaging complex	7	1.22	6.17E-04	6.30	5.64E-03
protein complex	106	18.50	8.32E-04	1.33	7.40E-03
integral component of plasma membrane	38	6.63	0.003459	1.62	2.97E-02
macromolecular complex	121	21.12	0.003792	1.25	3.17E-02
spindle microtubule	5	0.87	0.004069	7.20	3.31E-02
membrane attack complex	4	0.70	0.004596	10.81	3.65E-02
spindle midzone	3	0.52	0.006182	21.61	4.77E-02
Ndc80 complex	3	0.52	0.006182	21.61	4.77E-02
chromosome passenger complex	3	0.52	0.006182	21.61	4.77E-02
intrinsic component of plasma membrane	38	6.63	0.006969	1.56	5.25E-02
alpha DNA polymerase:primase complex	3	0.52	0.011987	16.21	8.68E-02
ribonucleoside-diphosphate reductase complex	3	0.52	0.011987	16.21	8.68E-02
cell part	337	58.81	0.012353	1.06	8.75E-02
membrane protein complex	31	5.41	0.015734	1.55	1.08E-01
condensed nuclear chromosome kinetochore	3	0.52	0.019371	12.97	1.29E-01
cell	337	58.81	0.022021	1.05	1.43E-01
plasma membrane region	10	1.75	0.029061	2.30	1.81E-01
centrosome	10	1.75	0.036673	2.21	2.20E-01
spindle pole	5	0.87	0.038104	3.86	2.24E-01
cytosolic small ribosomal subunit	5	0.87	0.047484	3.60	2.67E-01
organelle part	123	21.47	0.075326	1.13	3.88E-01
intracellular organelle part	121	21.12	0.079878	1.13	4.01E-01
microtubule organizing center	11	1.92	0.090659	1.77	4.32E-01
epsilon DNA polymerase complex	2	0.35	0.090196	21.61	4.36E-01
DNA replication factor A complex	2	0.35	0.090196	21.61	4.36E-01
spindle pole centrosome	2	0.35	0.090196	21.61	4.36E-01
primosome complex	2	0.35	0.090196	21.61	4.36E-01

KEGG Pathway	Count	%	P Value	Fold Enrichment	Benjamini
Cell cycle	17	2.97	1.30E-05	3.64	1.34E-03
Progesterone-mediated oocyte maturation	14	2.44	7.80E-05	3.72	4.01E-03
DNA replication	8	1.40	2.28E-04	6.22	4.69E-03
Purine metabolism	18	3.14	1.96E-04	2.80	5.03E-03
Intestinal immune network for IgA production	8	1.40	3.18E-04	5.91	5.45E-03
Pyrimidine metabolism	13	2.27	1.87E-04	3.66	6.41E-03
Oocyte meiosis	14	2.44	5.60E-04	3.06	8.21E-03
Glutathione metabolism	8	1.40	0.002263	4.30	2.88E-02

p53 signaling pathway	8	1.40	0.010179	3.28	1.10E-01
Fanconi anemia pathway	6	1.05	0.029679	3.41	2.67E-01
Neuroactive ligand-receptor interaction	21	3.66	0.055708	1.52	4.15E-01
Cell adhesion molecules (CAMs)	9	1.57	0.087113	1.95	5.14E-01
Herpes simplex infection	11	1.92	0.08108	1.80	5.16E-01

Supporting Information Table 12. Gene Ontology Terms and KEGG Pathways over-represented in the lists of differentially expressed transcripts following exposure to cu_cu in the gill. Values presented are the number and percentage of transcripts associated with each GO term, the P value, fold enrichment and adjusted P value (adjusted P value < 0.05; Benjamini-Hochberg) associated with over-representation. Pale blue shading indicates significant enrichment. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.8) using all transcripts expressed in this study as a background list.

GOTERM_BP_DIRECT	Count	%	P Value	Fold Enrichment	Benjamini
response to heat	3	6.98	1.59E-03	48.45	5.78E-01
chromosome segregation	4	9.30	5.33E-03	10.89	6.19E-01
response to temperature stimulus	3	6.98	4.44E-03	29.07	7.00E-01
mitotic nuclear division	4	9.30	1.56E-02	7.34	7.04E-01
cation transmembrane transport	4	9.30	2.94E-02	5.77	7.39E-01
single-organism process	32	74.42	1.52E-02	1.26	7.48E-01
positive regulation of mitotic cell cycle	2	4.65	2.78E-02	69.22	7.50E-01
ammonium transmembrane transport	2	4.65	2.78E-02	69.22	7.50E-01
ammonium transport	2	4.65	2.78E-02	69.22	7.50E-01
reproduction	3	6.98	7.18E-02	6.61	7.51E-01
organelle fission	4	9.30	3.55E-02	5.35	7.53E-01
reproductive process	3	6.98	7.06E-02	6.67	7.57E-01
positive regulation of cell cycle process	2	4.65	5.10E-02	37.27	7.57E-01
mitotic cell cycle	5	11.63	1.33E-02	5.24	7.64E-01
superoxide metabolic process	2	4.65	5.48E-02	34.61	7.66E-01
regulation of sister chromatid segregation	2	4.65	6.99E-02	26.92	7.66E-01
potassium ion import	2	4.65	7.74E-02	24.23	7.66E-01
microtubule-based process	4	9.30	8.55E-02	3.73	7.69E-01
camera-type eye morphogenesis	3	6.98	5.04E-02	8.08	7.71E-01
ion transmembrane transport	4	9.30	5.82E-02	4.38	7.71E-01
regulation of chromosome segregation	2	4.65	8.11E-02	23.07	7.72E-01
mitotic sister chromatid separation	2	4.65	6.62E-02	28.50	7.73E-01
regulation of mitotic sister chromatid separation	2	4.65	6.62E-02	28.50	7.73E-01
regulation of mitotic sister chromatid segregation	2	4.65	6.62E-02	28.50	7.73E-01
regulation of cell cycle	4	9.30	3.50E-02	5.38	7.73E-01
regulation of cell cycle process	3	6.98	4.84E-02	8.26	7.75E-01

cell division	3	6.98	8.46E-02	6.01	7.76E-01
cell cycle	5	11.63	6.95E-02	3.11	7.77E-01
eye development	4	9.30	6.47E-02	4.20	7.79E-01
nuclear division	4	9.30	2.76E-02	5.91	7.80E-01
GOTERM_BP_DIRECT	**Count**	**%**	**P Value**	**Fold Enrichment**	**Benjamini**
mitotic spindle organization	2	4.65	4.72E-02	40.38	7.85E-01
sensory organ morphogenesis	4	9.30	2.55E-02	6.09	7.88E-01
single organism reproductive process	3	6.98	6.40E-02	7.06	7.89E-01
chromosome separation	2	4.65	9.58E-02	19.38	7.89E-01
mitotic cell cycle process	4	9.30	4.50E-02	4.87	7.89E-01
reactive oxygen species metabolic process	2	4.65	9.95E-02	18.64	7.93E-01
eye morphogenesis	4	9.30	1.16E-02	8.21	7.93E-01
nitrogen compound transport	3	6.98	9.43E-02	5.63	7.93E-01
response to cadmium ion	2	4.65	4.33E-02	44.05	7.98E-01
organic cation transport	2	4.65	2.39E-02	80.76	8.05E-01
nitrogen utilization	2	4.65	2.39E-02	80.76	8.05E-01
GOTERM_MF_ALL	**Count**	**%**	**P Value**	**Fold Enrichment**	**Benjamini**
adenyl nucleotide binding	10	23.26	1.34E-02	2.45	4.12E-01
ribonucleotide binding	10	23.26	6.31E-02	1.87	4.18E-01
substrate-specific transmembrane transporter activity	6	13.95	2.07E-02	3.62	4.24E-01
purine nucleotide binding	10	23.26	6.17E-02	1.88	4.28E-01
transmembrane transporter activity	6	13.95	3.57E-02	3.13	4.37E-01
superoxide-generating NADPH oxidase activator activity	2	4.65	1.81E-02	106.33	4.39E-01
purine ribonucleotide binding	10	23.26	6.12E-02	1.88	4.44E-01
nucleoside binding	10	23.26	5.92E-02	1.89	4.53E-01
carbohydrate derivative binding	11	25.58	3.39E-02	1.97	4.54E-01
ion transmembrane transporter activity	5	11.63	4.18E-02	3.66	4.59E-01
ribonucleoside binding	10	23.26	5.90E-02	1.90	4.73E-01
transporter activity	7	16.28	3.24E-02	2.77	4.78E-01
ammonium transmembrane transporter activity	2	4.65	2.89E-02	66.46	4.84E-01
inward rectifier potassium channel activity	2	4.65	5.00E-02	37.97	4.91E-01
purine nucleoside binding	10	23.26	5.83E-02	1.90	4.92E-01
purine ribonucleoside binding	10	23.26	5.83E-02	1.90	4.92E-01
cation transmembrane transporter activity	4	9.30	8.53E-02	3.72	5.06E-01
adenyl ribonucleotide binding	10	23.26	1.33E-02	2.45	5.06E-01
purine ribonucleoside triphosphate binding	10	23.26	5.78E-02	1.90	5.15E-01
ATP binding	10	23.26	1.24E-02	2.48	6.27E-01
substrate-specific transporter activity	7	16.28	1.18E-02	3.48	8.46E-01

GOTERM_CC_ALL	Count	%	P Value	Fold Enrichment	Benjamini
integral component of plasma membrane	8	18.60	1.26E-03	4.56	4.07E-02
NADPH oxidase complex	2	4.65	1.54E-02	125.60	2.26E-01
cytoplasm	12	27.91	3.45E-02	1.88	3.21E-01
KEGG Pathway	**Count**	**%**	**P Value**	**Fold Enrichment**	**Benjamini**
Protein processing in endoplasmic reticulum	4	9.30	8.12E-03	8.58	1.44E-01
Spliceosome	3	6.98	3.89E-02	8.76	2.22E-01
MAPK signaling pathway	4	9.30	3.60E-02	4.91	2.94E-01

Supporting Information Table 13. List of all differentially expressed transcripts in liver samples. Values presented are Log2 transformed fold changes and FDR values for each treatment group compared to fish maintained under control conditions throughout the experiment (control_control group) in the liver. Red shading indicates significant up-regulation (FDR < 0.05) and green shading represents significant down-regulation (FDR < 0.05).

Gene ID	Name	Annotation	Database	control_cu		cu_control		cu_cu	
				LogFC	FDR	LogFC	FDR	LogFC	FDR
ENSGACG00000006807	alas2	aminolevulinate, delta-, synthase 2	Ensembl	-0.507	0.938	0.0801	1	-1.287	0.0181
ENSGACG00000008912	comtd1	catechol-O-methyltransferase domain containing 1	Ensembl	-4.876	0.1576	-6.701	0.026	-6.135	0.0125
ENSGACG00000006275	CU984600.2	Chromosome 9: 561,485-582,765	Ensembl	-1.453	0.5675	-2.02	0.3871	-2.438	0.0059
ENSGACG00000002379	ddit4	DNA-damage-inducible transcript 4	Ensembl	0.9032	0.7316	0.3725	1	1.8938	0.0487
ENSGACG00000018137	egr1	early growth response 1	Ensembl	-1.332	0.9081	-1.726	0.4486	-2.42	0.0487
ENSGACG00000016488	elovl6	ELOVL fatty acid elongase 6	Ensembl	-1.992	0.0135	-0.318	1	-2.013	0.0897
ENSGACG00000011586	EML6 (1 of many)	echinoderm microtubule associated protein like 6	Ensembl	-1.92	0.3398	-0.622	1	-6.795	6E-05
ENSGACG00000002655	GRM7 (1 of many)	glutamate metabotropic receptor 7	Ensembl	-5.511	0.0536	-3.255	0.5361	-5.399	0.0401
ENSGACG00000005417	hamp	hepcidin antimicrobial peptide	ZFIN	3.0198	0.8558	-4.649	0.0623	-4.61	0.0194
ENSGACG00000005425	hamp	hepcidin antimicrobial peptide	ZFIN	4.0459	0.6585	-2.722	1	-4.777	0.0352
ENSGACG00000004848	igfbp1a	insulin-like growth factor binding protein 1a	Ensembl	1.439	0.3431	-1.364	0.907	2.5835	0.0145
ENSGACG00000013082	igfbp1b	insulin-like growth factor binding protein 1b	Ensembl	1.9328	0.0436	-0.055	1	1.4895	0.5474
ENSGACG00000020944	mt-nd3	NADH dehydrogenase 3, mitochondrial	Ensembl	0.4477	0.988	0.8554	0.6765	1.4423	0.0059
ENSGACG00000009200	mxa	myxovirus (influenza) resistance A	ZFIN	-1.656	0.8403	-2.151	1	-3.925	0.0155

ENSGACG00000009188	mxa	myxovirus (influenza) resistance A	ZFIN	-1.775	0.8558	-1.53	1	-4.228	0.0352
ENSGACG00000019437	phax	phosphorylated adaptor for RNA export	ZFIN	-1.524	0.9897	-0.043	1	-6.205	0.0352
ENSGACG00000013212	rin3	Ras and Rab interactor 3	ZFIN	-0.445	1	-0.604	1	-2.488	2E-06
ENSGACG00000009046	rsad2	radical S-adenosyl methionine domain containing 2	Ensembl	-0.521	0.988	-0.64	1	-1.842	0.0065
ENSGACG00000020775	sc5d (1 of many)	sterol-C5-desaturase	Ensembl	-2.879	0.0494	-0.767	1	-1.421	0.911
ENSGACG00000013749	sh3d21	SH3 domain containing 21	Ensembl	2.2172	0.1576	0.8975	1	2.3908	0.024
ENSGACG00000012642	si:ch211-170d8.5	si:ch211-170d8.5	ZFIN	5.1663	0.0283	6.8599	7E-43	4.8534	0.0487
ENSGACG00000006710	si:dkey-188i13.6	si:dkey-188i13.6	ZFIN	-2.291	0.3706	-1.748	1	-3.303	0.0059
ENSGACG00000006713	si:dkey-188i13.6	si:dkey-188i13.6	ZFIN	-1.223	0.6106	-0.48	1	-1.891	0.0298
ENSGACG00000012181	slc15a4	solute carrier family 15 (oligopeptide transporter), member 4	Ensembl	0.6822	0.646	0.2758	1	0.9289	0.0487
ENSGACG00000011100	sptb	spectrin, beta, erythrocytic	Ensembl	-0.405	0.9958	-0.244	1	-1.313	0.0493
ENSGACG00000014094	tesca	tescalcin a	Ensembl	2.1133	0.0587	1.5642	0.6298	2.26	0.0326
ENSGACG00000019954	ugt5g1 (1 of many)	UDP glucuronosyltransferase 5 family, polypeptide G1	Ensembl	0.8669	0.833	0.5549	1	1.4842	0.0059
ENSGACG00000019912	usp18	ubiquitin specific peptidase 18	Ensembl	-0.958	0.9081	-1.027	1	-2.265	0.0155
ENSGACG00000014781	WNK4 (1 of many)	WNK lysine deficient protein kinase 4	Ensembl	-0.756	0.8428	-0.14	1	-1.471	0.036
ENSGACG00000009076	NA	NA	Ensembl	-3.339	0.0963	-2.627	0.573	-4.333	0.0065
ENSGACG00000007228	NA	NA	Ensembl	-1.237	0.9662	-0.992	1	-5.502	0.0155
ENSGACG00000009770	NA	NA	Ensembl	-1.612	0.8886	-2.305	0.8171	-3.603	0.0269
ENSGACG00000019775	NA	NA	Ensembl	-0.127	1	-0.437	1	-1.566	0.0401
ENSGACG00000007879	NA	NA	Ensembl	-0.437	0.9896	-0.302	1	-1.346	0.0438
ENSGACG00000006711	NA	NA	Ensembl	-1.998	0.7316	-1.767	1	-3.586	0.0487
ENSGACG00000001537	NA	NA	Ensembl	-8.994	0.0343	2.2665	0.3297	-5.722	0.1299
ENSGACG00000000509	NA	NA	Ensembl	3.4098	0.0046	4.214	0.4158	2.4566	0.4815
ENSGACG00000000076	NA	NA	Ensembl	3.4098	0.0046	4.214	0.4158	2.4566	0.4815

Supporting Information Table 14. Gene Ontology Terms and KEGG Pathways over-represented in the lists of differentially expressed transcripts following exposure to control_cu in the liver. Values presented are the number and percentage of transcripts associated with each GO term, the P value, fold enrichment and adjusted P value (adjusted P value < 0.05; Benjamini-Hochberg) associated with over-representation. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.8) using all transcripts expressed in this study as a background list.

GOTERM_BP_DIRECT	Count	%	P Value	Fold Enrichment	Benjamini
cellular lipid metabolic process	3	60	5.15E-03	17.82	4.65E-01
lipid metabolic process	3	60	8.83E-03	13.56	4.15E-01
fatty acid biosynthetic process	2	40	1.52E-02	98.46	4.60E-01
monocarboxylic acid biosynthetic process	2	40	1.67E-02	89.08	4.00E-01
sphingolipid metabolic process	2	40	1.99E-02	74.83	3.85E-01
carboxylic acid biosynthetic process	2	40	3.17E-02	46.77	4.78E-01
organic acid biosynthetic process	2	40	3.29E-02	45.08	4.39E-01
membrane lipid metabolic process	2	40	3.33E-02	44.54	4.01E-01
fatty acid metabolic process	2	40	3.84E-02	38.57	4.09E-01
small molecule biosynthetic process	2	40	5.16E-02	28.56	4.73E-01
monocarboxylic acid metabolic process	2	40	6.13E-02	23.98	5.01E-01
lipid biosynthetic process	2	40	6.47E-02	22.68	4.91E-01
single-organism metabolic process	3	60	9.08E-02	4.03	5.88E-01

Supporting Information Table 15. Gene Ontology Terms and KEGG Pathways over-represented in the lists of differentially expressed transcripts following exposure to cu_cu in the liver. Values presented are the number and percentage of transcripts associated with each GO term, the P value, fold enrichment and adjusted P value (adjusted P value < 0.05; Benjamini-Hochberg) associated with over-representation. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.8) using all transcripts expressed in this study as a background list.

GOTERM_BP_DIRECT	Count	%	P Value	Fold Enrichment	Benjamini
defense response to other organism	4	14.29	3.26E-05	57.20	1.02E-02
response to virus	3	10.71	7.30E-04	68.25	4.48E-02
response to biotic stimulus	4	14.29	6.07E-04	21.53	4.65E-02
response to other organism	4	14.29	4.99E-04	23.01	5.09E-02
response to external biotic stimulus	4	14.29	4.99E-04	23.01	5.09E-02
defense response to virus	3	10.71	4.32E-04	88.32	6.56E-02
defense response	4	14.29	1.91E-03	14.51	7.22E-02
viral process	3	10.71	1.46E-03	48.43	7.34E-02
multi-organism cellular process	3	10.71	1.46E-03	48.43	7.34E-02
multi-organism process	4	14.29	2.20E-03	13.81	7.39E-02
symbiosis, encompassing mutualism through parasitism	3	10.71	1.75E-03	44.16	7.56E-02
interspecies interaction between organisms	3	10.71	1.75E-03	44.16	7.56E-02
immune effector process	3	10.71	3.05E-03	33.36	9.16E-02
immune system process	5	17.86	6.12E-03	5.97	1.48E-01
response to stress	6	21.43	6.01E-03	4.42	1.58E-01
innate immune response	3	10.71	7.87E-03	20.57	1.74E-01
response to external stimulus	4	14.29	1.58E-02	6.79	3.01E-01
immune response	3	10.71	3.59E-02	9.21	5.35E-01
erythrocyte development	2	7.14	5.10E-02	35.75	6.42E-01
response to stimulus	8	28.57	7.66E-02	1.85	6.96E-01
myeloid cell development	2	7.14	7.39E-02	24.41	7.00E-01
embryonic hemopoiesis	2	7.14	8.25E-02	21.76	7.07E-01
embryonic organ development	3	10.71	7.35E-02	6.18	7.17E-01
response to oxygen levels	2	7.14	7.21E-02	25.02	7.29E-01
response to decreased oxygen levels	2	7.14	7.21E-02	25.02	7.29E-01
response to hypoxia	2	7.14	7.04E-02	25.66	7.40E-01

GOTERM_MF_ALL	Count	%	PValue	Fold Enrichment	Bonferroni
cytoskeletal protein binding	3	10.71	6.51E-02	6.58	9.99E-01
GOTERM_CC_ALL	**Count**	**%**	**PValue**	**Fold Enrichment**	**Bonferroni**
integral component of membrane	11	39.29	9.81E-02	1.55	8.93E-01
membrane part	12	42.86	9.56E-02	1.49	9.62E-01
membrane	13	46.43	8.79E-02	1.46	9.97E-01

Chapter 5

Exposure to copper during embryogenesis caused a differential response to copper in later life and increased tolerance in subsequent generations, in a fish model.

In Preparation

Exposure to copper during embryogenesis caused a differential response to copper in later life and increased tolerance in subsequent generations, in a fish model.

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Abstract:

Aquatic environments worldwide are impacted by chemical pollution. The sustainability of fish populations within these polluted ecosystems is critically dependent on their ability to adapt to change via genetic and/or epigenetic mechanisms. We conducted a series of copper exposures in the stickleback to explore if prior exposure can result in altered susceptibility in later life or in subsequent generations. Stickleback embryos were exposed to 0.01 mg/L copper during early life (1-217 hpf), a concentration causing less than 1.5% mortality, ensuring that selection for a tolerant genotype did not occur. Fish were then kept under control conditions until sexual maturation, when they showed differential copper accumulation compared to a control population. Under control conditions, adult fish that had been pre-exposed to copper during embryogenesis had a higher copper concentration in the liver, gill and muscle tissue. In addition, fish pre-exposed to copper were able to accumulate more copper when exposed to 0.01 or 0.02 mg/L Cu in adult life, compared to the control population. Mortality curves on F1 embryos revealed that the embryos originating from parents who were exposed to copper during embryogenesis were significantly more tolerant to copper when compared to the control population kept in parallel, and this was found to be inherited in the F2 generation. Our data support the hypothesis that exposure to copper during early life has the potential to reduce the susceptibility of a vertebrate model across generations, and highlight the importance of the conditions fish experience during embryogenesis, including exposure to stressors present in their environment, for the future physiology of those individuals both within their life span and in subsequent generations.

Keywords: multigenerational, inheritance, teleost, vertebrate, teleost, susceptibility, toxic metals.

Introduction:

Most aquatic environments in the UK and worldwide are impacted by environmental stressors of anthropogenic origin. Such stressors vary from chemical pollutants to habitat fragmentation and to changes in abiotic parameters such as temperature and dissolved oxygen or carbon dioxide [1–3], and often environments are impacted by multiple stressors simultaneously. The sustainability of wild populations, therefore, is critically dependent on their ability to adapt to the complex changes in their local environment, and is likely to be significantly influenced by population genetic structure and exposure history [4].

Differential responses to chemical pollutants have been widely reported in the environment and variation in chemical tolerance has been observed in species commonly used in environmental risk assessment (ERA) [5,6]. Despite this, legislation to protect the environment from chemical contaminants are often based on toxicological measurements conducted under optimal laboratory conditions, that do not take into account exposure history or the variation in susceptibility of wild populations. If not addressed, this can result in the misinterpretation of experimental data regarding species tolerances to a given chemical [5].

Variations in chemical tolerance have been reported for a number of pollutants and across evolutionarily distant species. Examples of differential susceptibility as a result of historic exposure have been reported for antibiotics and toxic metals in several bacterial species [7]. In addition, numerous copepod species have displayed variation in their responses to tributyltin, an anti-fouling agent used in paints [5]. A population of *Daphnia longispina* sourced from a copper polluted location was found to have a greater copper tolerance than individuals collected from a local uncontaminated site [8], and historic metal exposure (copper and zinc) was shown to result in differential responses upon re-exposure in the

earthworm (*Aporrectodea tuberculate*) [9]. In the Mediterranean land snail, *Xeropicta derbentina*, populations historically exposed to elevated temperatures were shown to be more resistant to heat-shock upon re-exposure in the lab, and these snails were shown to recover at a quicker rate [10]. In fish, historical exposure to pollutants has been associated with rapid adaptive evolution in several species including a population of guppies (*Poecilia reticulata*) historically exposed to crude oil [11], a population of least killifish, (*Heterandria Formosa*) chronically exposed to cadmium [12] and populations of brown trout (*Salmo trutta*) [13,14], three-spined stickleback (*Gasterosteus aculeatus*) [15] and wild yellow perch (*Perca flavescens*) [16] historically exposed to metal mixtures.

Examples also exist where pre-exposure to a given chemical may cause a decrease in tolerance upon re-exposure. For example, studies in the nematode *Caenorhabditis elegans* reveal that exposure to pristine and sulfidized Ag ENPs and $AgNO_3$ over 10 generations increased sensitivity when compared to a control population [17]. Similarly, evidence from studies using three *Daphnia* species exposed to high concentrations of Ag ENPs over five generations, supports increased sensitivity, but in contrast, a greater tolerance was observed at lower test concentrations [18]. It is important to note that in these studies, organisms were exposed to relatively high concentrations over multiple generations. For example in Volker *et al.,* exposures caused between 20-30% mortality [18], while in Schultz *et al.,* although the authors do not report mortality rates, one of the five Ag_2S-exposed populations stopped reproducing entirely, suggesting that the concentrations used resulted in significant toxicity [17]. These data suggest that exposure to the high concentrations tested likely resulted in selection for tolerant genotypes, and the phenotypes observed could be explained, at least in part, by genetic selection.

Prior exposure to a given stressor can also influence a population's response to other stressors. For example, the exposure of the malaria mosquito (*Anopheles gambiae s.s.*) larvae for 72h to sub-lethal concentrations of an agrochemical mixture containing pesticides, herbicides and fungicides was shown to cause a significant increase in larval tolerance to detamethrin, DDT and bendiocarb, the insecticides currently used for vector control [19]. In addition, studies exploring the variation in tolerance to copper of marine copepod species, *Tigriopus japonicus* and *Tigriopus californicus*, hypothesized that the differences in copper tolerance observed may be a by-product of adaptation to other stressors such as high temperature, changes in local salinity, pH and dissolved oxygen [5].

A large proportion of the studies reporting differential responses to a pollutant between wild populations of the same species are in fact describing unique selection events, where exposure to a relatively high concentration of a chemical, often over multiple generations, has resulted in the selection for (genetic) tolerance within the population. Extreme concentrations of chemical pollutants in the field where successful selection events for tolerance have occurred are generally rare. Instead, in the wild, populations inhabiting most aquatic environments are exposed to variations in the concentration of chemical pollutants present in their habitats, driven by rainfall events, and by human activity, for example during spillage events [20,21]. Concentrations may also vary spatially, as organisms move between polluted and clean waters [22,23]. These exposures may result in differential responses in subsequent exposure scenarios [24,25].

Some examples exist where low dose exposure has resulted in changes to the physiological responses of organisms in subsequent exposure scenarios within one generation, without causing significant mortality. For example, pre-exposure of the earthworm species, *Eisenia*

fetida to non-lethal concentrations of mercury strongly increased survival in subsequent tolerance tests when compared to earthworms kept in control conditions during the pre-exposure [26]. Laboratory pre-exposure to non-lethal episodes of hypoxia has also been shown to increase hypoxia tolerance in both tolerant [27] and sensitive fish species [28,29]. In addition, pre-exposure to low levels of cadmium in the rainbow trout (*Oncorhynchus mykiss*) was shown to cause a greater tolerance upon re-exposure to high doses of cadmium [30,31].

The effect of prior exposure to a chemical on the responses of organisms upon re-exposure in the absence of selection for genetic changes, is thought to be modulated, at least in part, via epigenetic mechanisms, often referred to as the epigenetic memory [32]. Epigenetics can be defined as mitotically heritable changes in gene expression that cannot be explained by changes in DNA sequence itself [32–34]. Modifications include but are not limited to; DNA and histone modifications, including DNA methylation, histone acetylation, ubiquitination and phosphorylation, changes in chromatin structure and noncoding RNAs [35]. It is well established that various environmental stressors, including dietary fluctuations [36,37], changes to thermal regimes [38], and a wide range of chemical pollutants including commonly studied examples such as bisphenol A [39–41], phthalates [42], copper [43], vinclozolin [44–46], mono(2-ethylhexyl) phthalate [47], 5-azacytidine [47] and arsenic [48] can modulate the epigenome [32]. The majority of these examples have focussed on identifying changes in DNA methylation; and this choice of endpoint was likely driven by the ease of its analysis relative to other epigenetic modifications, such as that of histones.

It is likely that many of the examples of altered tolerance to environmental stressors attributed to genetic selection reported to date, also involve epigenetic mechanisms, either in addition to, or as a result of alterations in the genomic structure [49]. There is increased

interest in determining if epigenetic modifications associated with exposure to chemical contaminants, such as DNA methylation, can be inherited via the germline, in the absence of genetic change [32,50,51]. If this is the case, exposure to chemical contaminants could influence future generations in the absence of genetic selection. This topic has received significant attention in recent years, and studies to date have suggested that experimental design is extremely important in order to rule out changes in genetic sequence when determining if the observed 'inheritance' of a phenotype is explained by the inheritance of epigenetic modifications alone [44–46]. In addition, determining if phenotypic or epigenetic modifications can be maintained to future generations in the absence of continued exposure to the toxicant of interest is considered to be a question of critical importance in environmental epigenetics.

It has been suggested that certain critical windows of development are more susceptible to epigenetic changes, for example during epigenetic reprogramming events, shown in the zebrafish model to occur after fertilisation, prior to zygotic genome activation (ZGA) [32,52,53], and shown in mammals to also occur in developing germ cells [54]. During these reprogramming events moderate DNA de-methylation occur, followed by re-methylation to levels comparable to that of somatic cells [52,55]. It is thought that during re-programming, chemical pollutants may interfere with this process of re-methylation and sequences that escape reprogramming may be involved in epigenetic inheritance [32,56,57].

Fish represent a useful model for studying transgenerational inheritance, due to the fact that an initial exposure, only results in the exposure of the F0 adult and its gametes (F1), but not F2 gametes, as is the case for exposures of mammalian pregnant females. In addition, the critical stages of early embryonic development where the epigenome of an individual is

thought to be most sensitive to environmental stress do not occur within the body of the female, as it is the case for viviparous and ovoviviparous animals, with significant advantages for exposure experiments. Therefore, transgenerational inheritance of the phenotypic effects or molecular marks can be demonstrated in the F2 generation following the initial exposure of the F0 generation to the stressor of interest. In mammalian models however, inheritance can only be demonstrated if alterations are present in F3 offspring, significantly lengthening the time required to conduct multigenerational studies.

For our study, we selected copper as a model toxicant to investigate the consequences of exposure during early life within the lifespan of the exposed organisms and in their progeny. Copper occurs naturally in the aquatic environment, and is particularly interesting due to its role as an essential element for life, and acts as a co-factor in a number of enzymatic reactions, including energy production and cellular metabolism [58]. When found in excess copper can be highly toxic, and fish are particularly sensitive due to their continual exposure to contaminated waters via the skin and the gills, in addition to via the diet [59]. When in excess, copper is known to disrupt biological processes including branchial ion regulation, enzyme activities, immune suppression and to causes oxidative stress [60–62]. In the environment, copper concentrations reported to date fluctuate and can reach levels known to be toxic to fish [63], and in a recent analysis copper has been identified as the most significant metal pollutant in UK waters [64].

In the present study, we aimed to test if early life exposure to an environmentally relevant concentration of copper could alter the responses of a fish population in subsequent exposure scenarios both later in life and in subsequent generations. In order to do this, we set out an initial exposure experiment during embryogenesis and tested the responses to

copper of the exposed and control populations when adults and in their progeny (up to the F2 generation).

Materials and Methods:

Fish source, culture and husbandry

A freshwater population of three-spined stickleback (originated from the: River Erme, Devon, United Kingdom) were maintained in the Aquatic Resource Centre at the University of Exeter in mixed sex stock tanks (112 L). Fish were maintained at densities of 20 fish per tank, and were supplied with aerated synthetic freshwater [65], with background copper concentrations of 0.0056 ± 0.0004 mg/L (as described in chapter 4). Holding tanks were supplied with mains tap water which 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 CaCl22H2O, 9.4 mg/L NaHCO3, 50 mg/L MgSO47H2O, 2.5 mg/L KCl, 50 mg/L, Tropic Marin Sea Salt), aerated, and maintained at 15°C in a reservoir. All exposure experiments were conducted during the summer months between March and September. During these months, fish were maintained at a photoperiod of 18:6 light/dark (with 30 minute dawn/dusk transitional periods) and a temperature of 15 ± 1°C. During the winter months, when new generations were growing to sexual maturity in clean water holding tanks, fish were maintained at a photoperiod of 6:18 light/dark (with 30 minute dawn/dusk transitional periods) and a temperature of 10 ± 1°C. Transitions in day light hours and temperature between summer and winter seasons were conducted in a controlled manner over a 6 week period. In the summer months (March – September), adult fish were fed to satiation twice daily with blood worms (Chironomus sp.; Tropical Marine Centre, Chorleywood, UK) and once daily with live *Artemia nauplii* (ZM Premium Grade Artemia; ZM Ltd.). In the winter months, adults were fed to satiation twice daily with blood worms (*Chironomus sp*.; Tropical

Marine Centre, Chorleywood, UK). Larval fish were fed twice daily to ZM-000 or ZM-100 (10 dpf – 28 dpf or 28 dpf – until large enough to feed on blood worms (ZM Ltd.)) and once daily with live *Artemia nauplii* (ZM Premium Grade Artemia; ZM Ltd.).

The stickleback embryos generated in this study were obtained via *in vitro* fertilisation (IVF; method adapted from Barber and Arnott (2000) [66]). Eggs which were not successfully fertilised were identified and removed by visual observation as described by Swarup (1958) [67], using a dissection microscope (Motic DM143, Hong Kong). The remaining 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 1 - 217 hpf.

All experiments were approved by the University of Exeter Ethics committee and conducted under approved protocols according to the UK Home Office regulations for use of animals in scientific procedures.

F0 generation embryo exposure

Using IVF, a pool of embryos was produced from between 20-26 females and 3 - 5 males , and randomly assigned to 600 ml acid-washed glass tanks at 1 hpf (corresponding to the one cell stage (blastodermic cap)). Exposures were started from the 1 cell stage in order to ensure the organism was exposed during the critical process of epigenetic reprogramming [52]. Each exposure was run in quadruplicate, and was repeated three times using different parental fish (see Figure 1 for a scheme of the experimental design). Embryos were either exposed to water control (0 mg/L Cu) or 0.01 mg/L Cu copper (added as copper sulphate,

Sigma Aldrich) from 1- 217 hpf (Figure 1). The exposure concentration was chosen for its environmental relevance and based on data generated during a prior experiment using the same population of fish [2], showing that this concentration causes less than 5% mortality, in order to avoid selecting for a copper tolerant genotype. Under these experimental conditions, sticklebacks have yolk sacs up until 217 hpf, and as such are not considered to be free feeding. This has regulatory implications under UK legislation as fish embryos become protected under the Animals (Scientific Procedures) Act from the free feeding stage onwards.

Each glass tank contained 40 – 50 embryos in 500 ml of water. The artificial freshwater was changed daily in order to avoid local hypoxia, and air saturation (AS%) was monitored daily using an optical dissolved oxygen meter (Mettler SevenGo Pro OptiOx, U.S.) and was found to be maintained between 80 – 99.8% AS. 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. At 217 hpf of exposure, a subset of the larvae were collected for metal analysis (placed in an acid washed tube, weighed to determine wet mass of the tissue, then stored at -20°C). 200 larvae from the control and copper pre-exposed populations were transferred to holding tanks, and were maintained until sexual maturation as described previously (Figure 1). Water samples were collected from each tank on day 5 and 9 of the exposure for measurements of the copper concentrations in the exposure water.

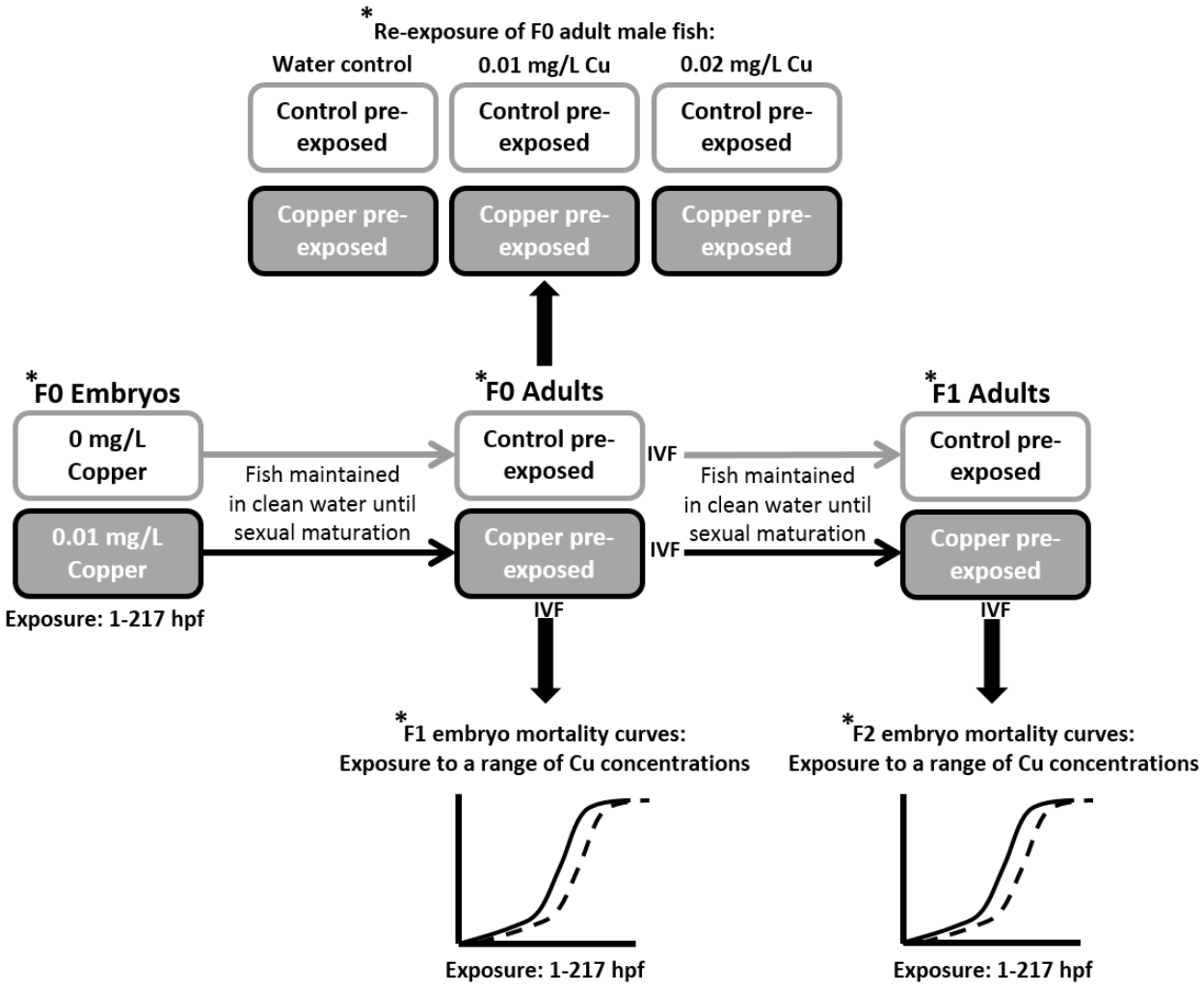
*Re-exposure of F0 adult male fish:
Water control
0.01 mg/L Cu
0.02 mg/L Cu
Control pre-exposed
Control pre-exposed
Control pre-exposed
Copper pre-exposed
Copper pre-exposed
Copper pre-exposed
*F0 Embryos
0 mg/L Copper
0.01 mg/L Copper
Exposure: 1-217 hpf
Fish maintained in clean water until sexual maturation
*F0 Adults
Control pre-exposed
Copper pre-exposed
IVF
IVF
Fish maintained in clean water until sexual maturation
*F1 Adults
Control pre-exposed
Copper pre-exposed
IVF
IVF
*F1 embryo mortality curves:
Exposure to a range of Cu concentrations
Exposure: 1-217 hpf
*F2 embryo mortality curves:
Exposure to a range of Cu concentrations
Exposure: 1-217 hpf

Figure 1. Schematic representation of the experimental design employed in these experiments**.** Exposure of naïve F0 embryos to control conditions or 0.01 mg/L copper during embryogenesis (1-217 hpf; n = 3 replicate exposures, each made up of 4 independent biological replicate dishes containing 20 embryos), followed by maintenance in clean water until sexual maturation. F0 adults were breed (IVF) to produce F1 and F2 embryos. Embryos were maintained in clean water holding tanks until sexual maturation for breeding and were not re-exposed to copper. For testing the tolerance of each generation, a subset of embryos were removed at 1 hpf and subjected to toxicity testing via exposure to a range of copper concentrations (0, 0.015, 0.025, 0.05, 0.0625, 0.075, 0.0875, 0.1, 0.125, 0.15 mg Cu/L), to generate mortality curves (n = 3-4). Exposure of naïve F0 embryos to control conditions or 0.01 mg/L copper during embryogenesis (1-217 hpf), followed by maintenance in clean water until sexual maturation. Adult male fish from both the control and copper populations were then re-exposed to 0, 0.01 or 0.02 mg/L Cu. Sampling points are represented by an asterisk (*).

F1 and F2 generation embryo generation

F1 generation embryos for both the control and copper pre-exposed populations were generated using the IVF technique described above (Figure 1). A selection of these fish were used to conduct copper toxicity testing as described below. In addition 200 larvae per population were reared in control conditions and were maintained until sexual maturation as described above.

F2 generation embryos for both the control and copper pre-exposed populations were generated from F1 generation adults maintained in clean water holding tanks, using the IVF technique (the F1 generation adults used for gamete collection were never re-exposed to copper after the initial exposure during the F0 generation). A selection of these fish were used to conduct copper toxicity testing as described below (Figure 1).

F0 copper exposures in adult fish

A subset of F0 adult male stickleback from both the control and copper pre-exposed populations were exposed to 0, 0.01 or 0.02 mg/L of copper in duplicate (9 fish per tank) for four days via a flow through system employing a flow rate of 144 L/day for each 40 L exposure tank (3.6x tank volumes per day; Figure 1). Throughout the 4 day copper exposure period, fish were not fed in order to minimise the amount of free organic matter in the tank to which copper can bind, therefore helping to maintain water copper concentrations close to the nominal exposure concentrations. In addition, copper exposure has been shown to affect appetite, therefore fasting the fish during the exposure was used to reduce the probability of nutritional changes acting as a confounding factor in our study [68].

At the end of the fourth day, 9 fish from each treatment group were sacrificed by lethal dose of benzocaine followed by destruction of the brain, in accordance with the UK Home Office regulations. The wet weight and fork length were recorded, and the condition factor (g/cm^3) for each fish was calculated (k= (weight (g) x 100)/ (fork length (cm))^3). The livers were dissected and weighed, and the hepatosomatic index (HSI = (liver weight (mg)/ (total weight (mg)- liver weight (mg)) x 100)) was calculated. Gill, liver and muscles were collected for metal analysis (placed in an acid washed tube, weighed to determine wet mass of the tissue, then stored at -20°C). Water samples were collected from each tank on day 1 and 3 of the exposure to determine the concentration of copper in the exposure water.

Assessment of copper tolerance in F1 and F2 embryos from both control fish and fish pre-exposed to copper during embryogenesis in the F0 generation

Copper tolerance in F1 and F2 embryos from both control fish and fish pre-exposed to copper during embryogenesis in the F0 generation was determined generating mortality curves for each population (Figure 1). Exposures were initiated at 1 hpf. Embryos from both the control and copper populations 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; Figure 1). The range of concentrations was selected based on previously published data, which demonstrated that these concentrations cause between 0% and 100% mortalities [2]. For each treatment, and for both populations IVF was performed as above to generate pools of fertilised embryos. Twenty embryos were randomly selected and allocated to each of 600 ml acid washed glass tanks, with water prepared as above, containing the appropriate

concentration of copper. After each 24 h exposure period, the proportion of mortalities and hatched embryos were recorded for each exposure tank. The water was then completely replaced with freshly made exposure water at the appropriate copper concentration, as described above to ensure that the water chemistry was maintained relatively constant throughout the experiment. All exposures were conducted in triplicate or quadruplicate using different parental fish. Water samples were collected from each tank on day 5 of the exposure to verify the copper concentration in the exposure water.

In addition to toxicity testing, a subset of F1 embryos were generated as described above and were either exposed to water control (0 mg/L Cu) or 0.0625 mg/L Cu for 9 days (217 hpf) in quadruplicate. Each glass tank contained 20 embryos. The artificial freshwater was changed as previously described. Individual embryos were observed daily 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. One embryo was removed daily from each tank, 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) fitted with a digital camera, to allow visualisation of any developmental effects in both the control and copper pre-exposed populations, resulting from re-exposure to either control conditions or 0.0625 mg/L Cu. A complete water change was carried out at each 24 hour period, as described above, and the experiment was maintained until 217 hpf.

Sampling of F1 adult fish for metal content analysis

F1 adult male and female stickleback (n =10) from both the pre-exposed and control populations which had been maintained in clean water holding tanks were sacrificed by lethal dose of benzocaine followed by destruction of the brain, in accordance with the UK Home Office regulations. The wet weight and fork length were recorded, and the condition factor, HSI and GSI for each fish was calculated. Gill, liver and muscles were collected for metal analysis as described above.

Metal analysis in water and tissue samples

Water samples were stored at -20°C prior to quantification of copper. Prior to analysis of total copper concentrations, water 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% in each falcon tube. Tissue samples were freeze dried and the dry mass was determined 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 with frequent vortexing until all samples had completely digested. 0.1% hydrogen peroxide (Fisher; Hydrogen Peroxide, 100 volume > 30%w/v) was added in order to facilitate the breakdown and removal of the fat materials. All samples were covered with in Parafilm to prevent evaporation. The resulting digested solution was then diluted 1:10 with ultrapure water to a total volume of 10 ml. 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.

Statistical analysis

Statistical analyses were conducted in R [69]. Prior to analysis, data were tested for equal variance and for normality using the Shapiro–Wilk test. The effects of copper exposure in naïve F0 embryos on whole organism metal content and mortality were analysed using the Student's t-Test. In addition, differences between the tissue metal content and morphometric parameters in F1 adult fish maintained in clean water holding tanks were also analysed using the Student's t-Test.

Effects of pre-exposure to copper on the survival and hatching success of F1 and F2 embryos exposed to a range of copper concentrations was analysed using a general linear model. A separate model was used for each time period after fertilisation (day 1-9), using a binomial error structure to test for effects of copper concentration on the proportion of mortality (as a continuous variable), pre-exposure (as a categorical variable) and the interaction between the two. In addition, the effects of re-exposure to copper on tissue metal content and morphometric parameters in F0 adults was assessed using generalised linear models as described for F1 and F2 exposed embryos. Minimum adequate models (MAM) were derived by model simplification using F tests based on analysis of deviance. F tests reported refer to the significance of each term within the MAM. All data was considered statistically significant when $p < 0.05$.

Results:

Effects of copper exposure in F0 embryos

For the initial exposure to copper of F0 embryos (exposed from 1 hpf; Figure 1), the mean measured concentration of copper in the tank water was 0.0114 ± 0.0003 mg/L for copper treatments and 0.0002 ± 4.1145e-05 mg/L for the control treatment (a detailed breakdown is presented in Supporting Information Table 1). Exposure to 0.01 mg/L Cu caused a significant increase in embryo mortality by 217 hpf (1.24%; P = 0.0384; Figure 2A), when compared to the background mortality in embryos exposed to control conditions (0.69%; Figure 2A). Fish exposed to 0.01 mg/L Cu had significantly greater whole organism copper concentrations when compared to the control (0.0031 ± 8.2687e-05 µg/Larvae and 0.0013 ± 1.7487e-04 µg/Larvae respectively; P = 1.2e-07; Figure 2B).

Effects of pre-exposure to copper during embryogenesis on copper tissue concentrations in later life (F0 Adults)

Fish that had been pre-exposed to copper (0.01 mg/L Cu; copper pre-exposed population) or control conditions (0.00 mg/L Cu; control population) during early life (1-217 hpf) were maintained in clean water until sexual maturation. A subset of male fish were then re-exposed to either 0, 0.01 or 0.02 mg/L Cu for 4 days (see Figure 1 for a scheme of the experimental design). The mean measured concentration of copper in each tank water was 0.0050 ± 4.668e-05 mg/L for the control treatment, 0.0135 ± 0.0003 mg/L for the 0.01 mg/L Cu treatment and 0.0218 ± 4.6680e-05 mg/L for the 0.02 mg/L Cu treatment (full details are presented in Supporting Information Table 2).

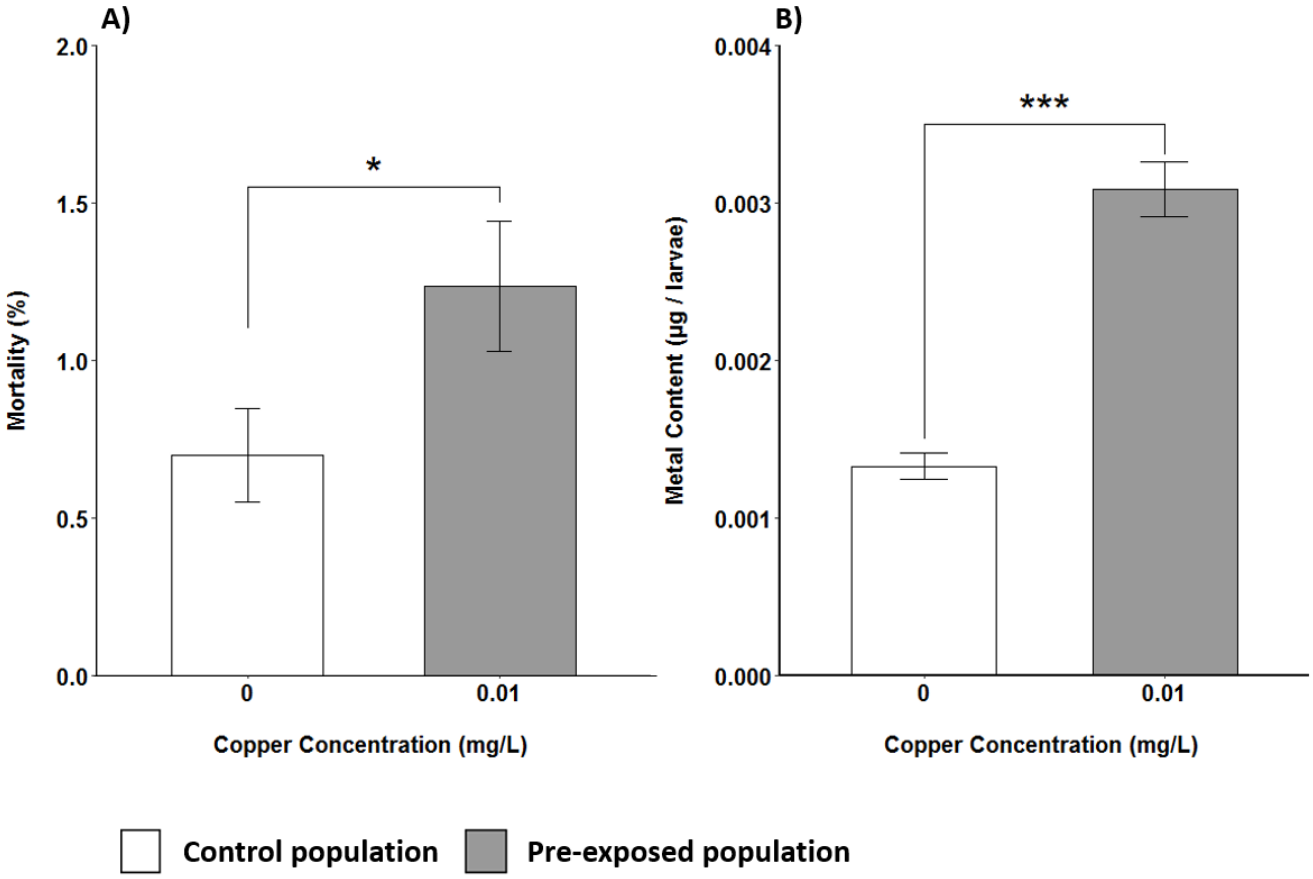

Figure 2. A) Average mortality and **B)** measured copper concentrations observed in F0 embryos exposed to 0.01 mg/L copper or kept in control conditions during embryogenesis (1-217 hpf; n = 3 replicate exposures, each made up of 4 replicate dishes containing 20 embryos). Statistical comparisons were conducted using the Student's t-Test in R (version 3.2.4). All data are presented as mean ± SEM. Asterisks indicate significant differences compared to the control population (*P<0.05, **P<0.01, ***P<0.001).

Throughout the exposure, no mortalities were recorded and fish remained in good condition. The mean fork length, weight, condition factor and hepatosomatic index were 5.6197 ± 0.0058 cm, 1.8455 ± 0.0056 g, 1.0365 ± 0.0023 g/cm^3 and 2.3296 ± 0.0223, respectively, and there were no significant differences between treatment groups (Supporting Information Table 3 and 4).

In the gill, there was 2.14 fold more copper in adult fish which had been pre-exposed to copper during early life and maintained under control conditions compared to the fish that were not pre-exposed to copper during early life. Exposure to 0.01 and 0.02 mg/L Cu caused a significant increase in copper concentration in the gills of both pre-exposed and control fish (P = 6.671e-07; Figure 3A; Supporting Information Tables 5 and 6). In addition, pre-exposure was found to be a significant explanatory variable determining the amount of copper detected in the gills of F0 adult fish (P = 0.0029; Figure 3A; Supporting Information Tables 5 and 6). Under exposure to 0.01 and 0.02 mg/L Cu, a 1.45 fold and 1.81 fold increase in copper concentration was measured in the gills of fish that had been pre-exposed to 0.01 mg/L Cu during early life (1-217 hpf) when compared to the control population, exposed in parallel.

In the both the liver and muscle, there was no significant effect of re-exposure on tissue copper concentration. However, pre-exposure was found to be a significant explanatory variable determining copper content, with on average 1.42 and 2.54 fold more copper in the liver and muscle of pre-exposed fish (P = 0.0143 and P = 0.0303 respectively; Figure 3B and 3C; Supporting Information Tables 5 and 6).

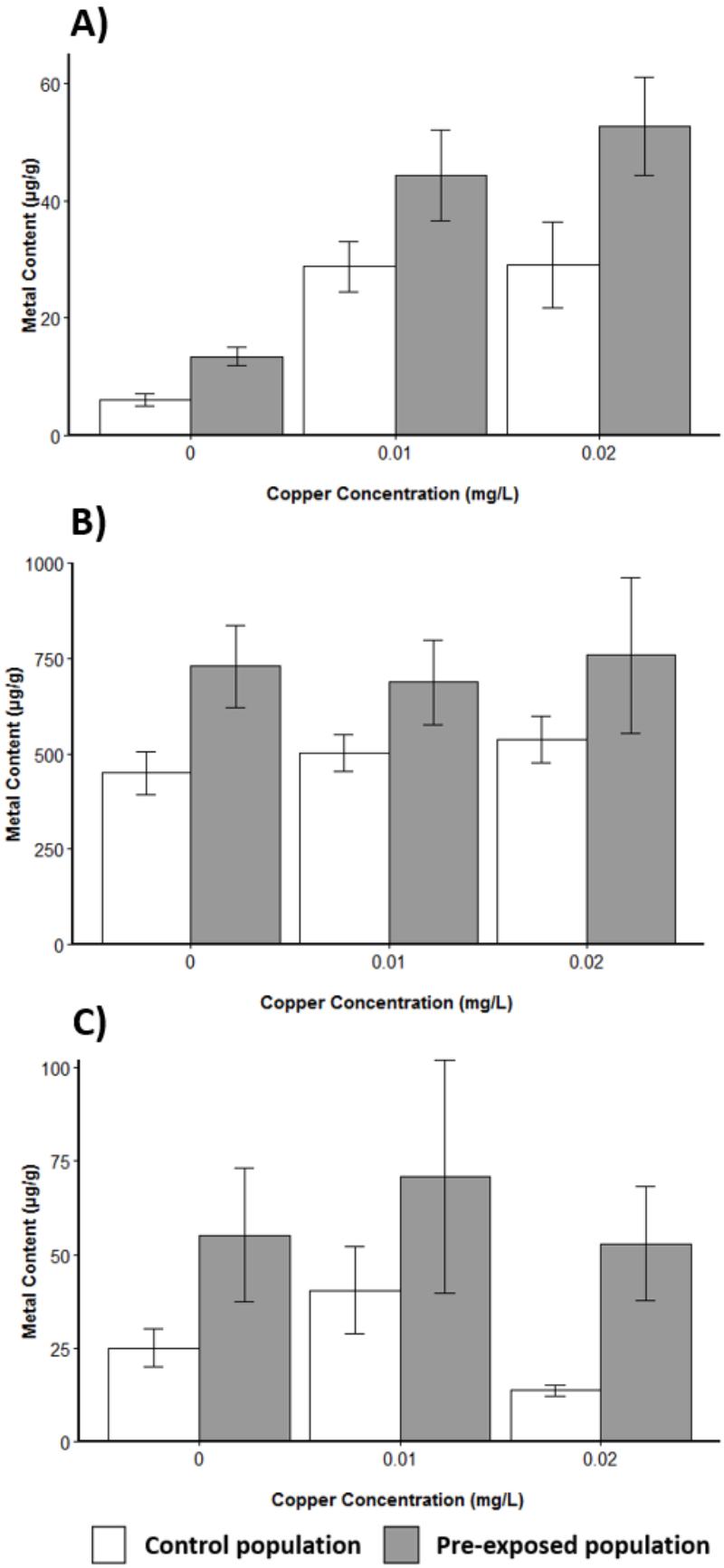
A)
Metal Content (µg/g)
60
40
20
0
0
0.01
0.02
Copper Concentration (mg/L)
B)
Metal Content (µg/g)
1000
750
500
250
0
0
0.01
0.02
Copper Concentration (mg/L)
C)
Metal Content (µg/g)
100
75
50
25
0
0
0.01
0.02
Copper Concentration (mg/L)
Control population
Pre-exposed population

Figure 3. Measured copper concentrations in the gill **(A)**, liver **(B)** and muscle **(C)** of male adult F0 stickleback pre-exposed to control conditions or 0.01 mg/L Cu during embryogenesis (1-217 hpf), and re-exposed to 0, 0.01 or 0.02 mg/L Cu for 4 days when adults (see Figure1 for details; n = 8-9 fish per treatment group). Tissue metal content was measured by ICPMS. Data is presented as mean µg Cu/g ± standard error mean. Statistical analysis were carried out using accepted minimum adequate models (analysis of variance model, R; $P<0.05$) with model details and mean values reported in Supporting Information Tables 5 and 6. **A)** In the gill, both copper re-exposure and population of origin (pre-exposure) were significant explanatory variables ($P = 6.671e-07$ and $P = 0.0029$ respectively; Supporting Information Tables 5 and 6). In the both the liver **(B)** and muscle **(C)**, population alone (pre-exposure) was found to be a significant explanatory variable determining copper content ($P = 0.0143$ and $P = 0.0303$ respectively; Supporting Information Tables 5 and 6).

Effects of pre-exposure to copper on responses to copper in F1 embryos

Fish pre-exposed to copper (0.01 mg/L Cu; copper pre-exposed population) or control (0.00 mg/L Cu; control population) during early life (1-217 hpf) were maintained in clean water until sexual maturation and F1 embryos from each population were then re-exposed to a series of copper concentrations (Figure 1). Copper caused mortalities in both the pre-exposed and control populations, and this was significant from 49 hpf until 217 hpf (P = 2e-16; Figure 4A; Supporting Information Figure 1; Supporting Information Table 7). Comparisons between the mortality curves generated for the control and pre-exposed populations revealed that significantly less mortalities were observed in embryos from the pre-exposed population, and pre-exposure was found to be a significant explanatory variable of the mortalities caused by copper exposure (217 hpf: P = 1.39e-13; Supporting Information Figure 1; Supporting Information Table 7).

Exposure to copper significantly delayed hatching during the periods of development when hatching occurs (169 hpf: P = 2e-16; 193 hpf: P = 2e-16; 216 hpf: P = 2e-16; Figure 5A; Supporting Information Figure 2 and 3; Supporting Information Table 7). Importantly, the delay in hatching was less severe in fish whose F0 parents had been pre-exposed to copper during embryogenesis (169 hpf: P = 2e-16; Figure 5A; Supporting Information Figure 2 and 3; Supporting Information Table 7). In F1 embryos originating from the pre-exposed population, premature hatching at 145hpf under high concentrations of copper was also observed (P = 0.0051; Supporting Information Figure 2 and 3; Supporting Information Table 7).

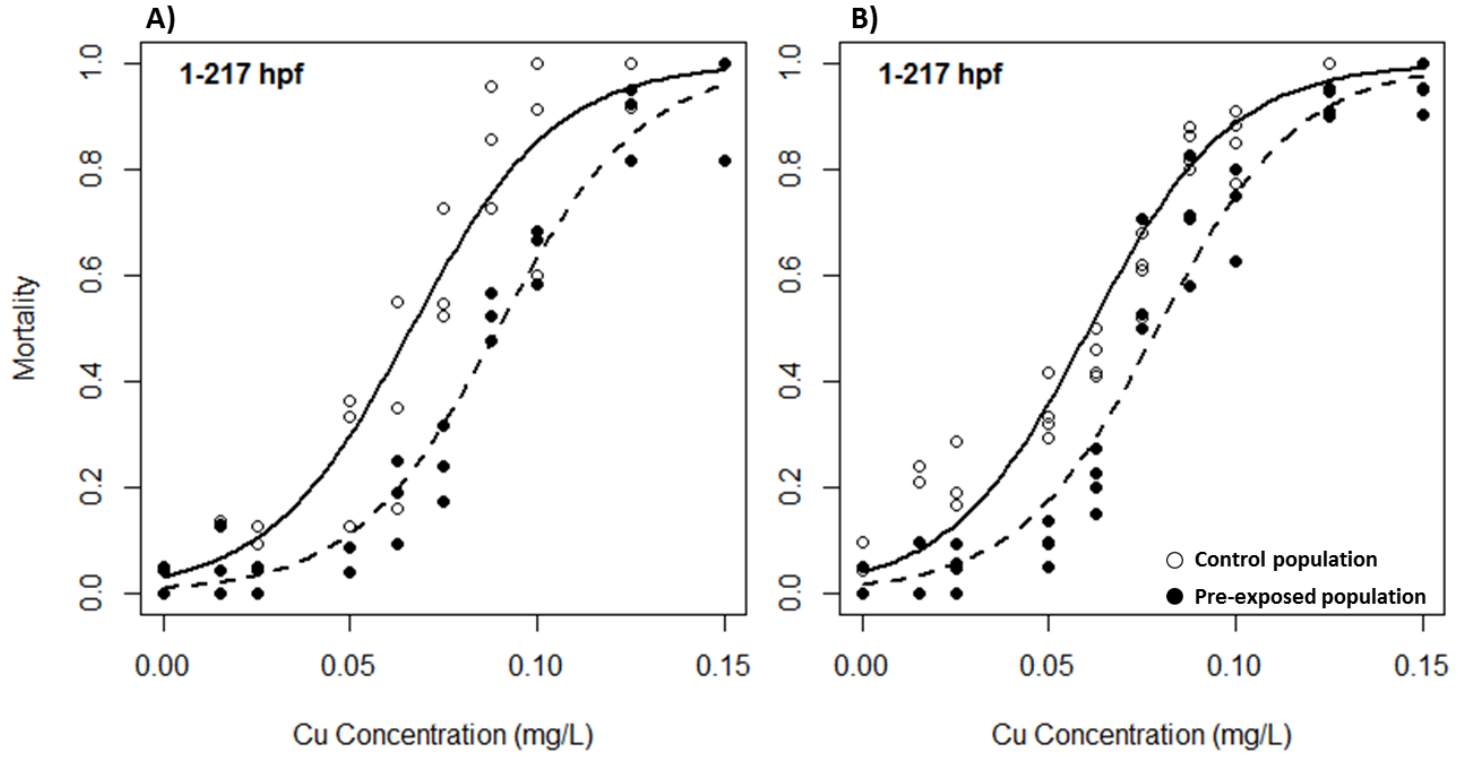

Figure 4. Embryo mortality curves for F1 **(A)** and F2 **(B)** embryos from both the control and copper pre-exposed population (n = 3-4). Embryos were exposed continuously to copper (0, 0.015, 0.025, 0.05, 0.0625, 0.075, 0.0875, 0.1, 0.125, 0.15 mg Cu/L) from 1 to 217 hpf. Black and white symbols represent groups from copper and control pre-exposed populations respectively and the lines represent the best fit model for the data, calculated using generalized linear models in R (model output summarised in Supporting Information Table 7).

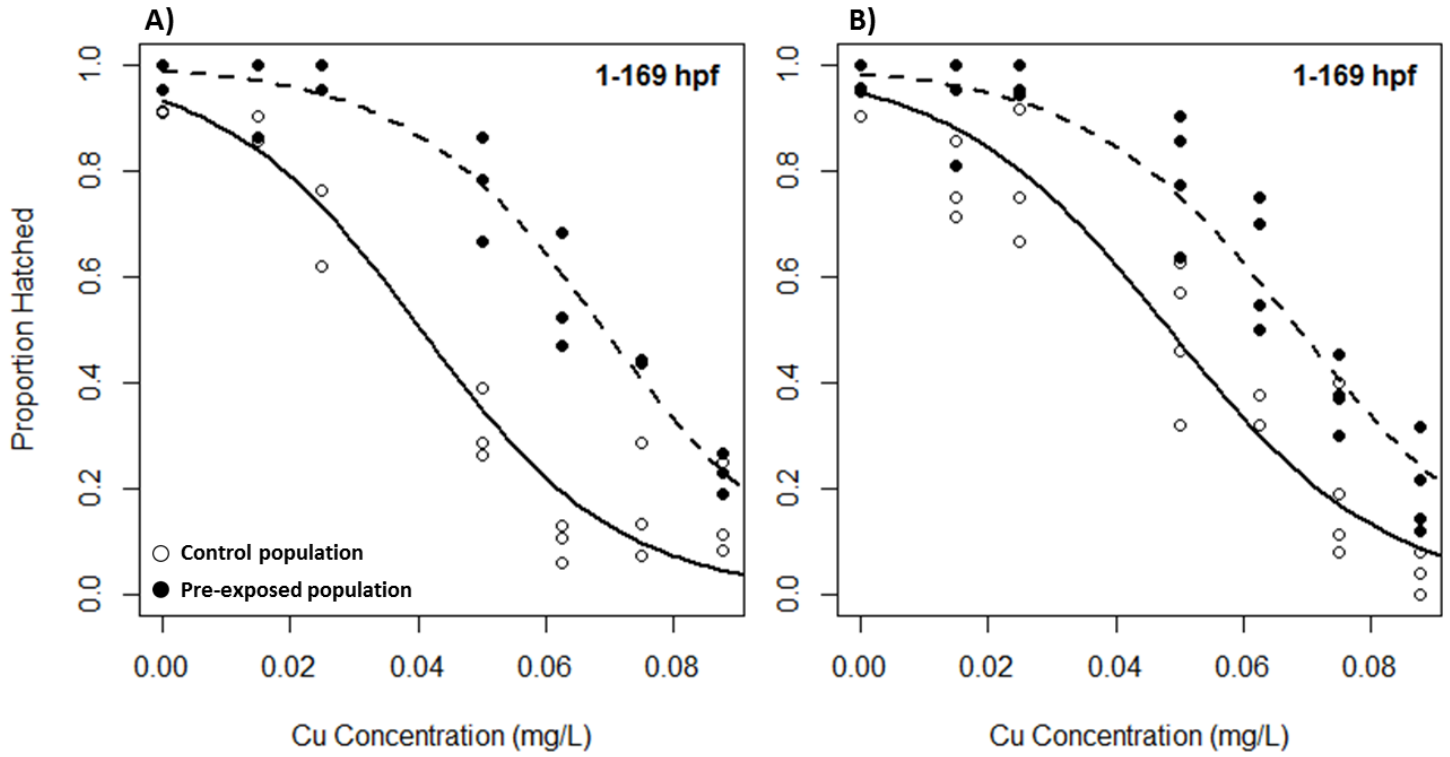

Figure 5. Proportion of hatched F1 **(A)** and F2 **(B)** embryos originating from the control and copper pre-exposed population following exposure to copper (n = 3-4). Black and white symbols represent groups from copper and control pre-exposed populations respectively and the lines represent the best fit model for the data, calculated using generalized linear models in R (model output summarised in Supporting Information Table 7).

Effects of pre-exposure to copper on tissue metal content in F1 Adults

F1 fish remained in good condition throughout life and no mortalities associated with exposure history were observed. The mean fork length, weight, condition factor, hepatosomatic index and gonadosomatic index of the female fish were 6.2300 ± 0.0733 cm, 2.2263 ± 0.0929 g, 0.9116 ± 0.0156 g/cm^3, 5.1732 ± 0.2485 and 4.0385 ± 0.8267, respectively, and there were no significant differences associated with exposure history (Supporting Information Table 8). The mean hepatosomatic index and gonadosomatic index of the male fish were 3.5143 ± 0.1081 and 0.5068 ± 0.0842, respectively, and, as was the case for female fish there were no significant differences associated with exposure history (Supporting Information Table 8). However, the fork length, weight and condition factor were significantly lower in males whose F0 parents had been pre-exposed to 0.01 mg/L Cu during embryogenesis (P = 0.0050, P = 0.0018 and P = 0.0471 respectively; Supporting Information Table 8).

For both male and female F1 fish, there was no significant difference in tissue metal content for the gill, liver, muscle or gonads of fish associated with pre-exposure in the F0 generation (Supporting Information Figure 4; Supporting Information Table 9).

Effects of pre-exposure on tolerance to copper in F2 embryos

Copper caused mortalities in both the pre-exposed and control populations, and was significant from 49 hpf until 217 hpf (P = 1.26e-05; Figure 4B; Supporting Information Figure 5; Supporting Information Table 7). Comparisons between the mortality curves generated for the control and pre-exposed populations revealed that significantly less mortalities were

observed in embryos from the pre-exposed population, and pre-exposure was found to be a significant explanatory variable of the mortalities caused by copper exposure (217 hpf: P = 7.43e-12; Supporting Information Figure 5; Supporting Information Table 7). From 49 – 145 hpf, we also detected a significant interaction between copper exposure and pre-exposure (145 hpf: P = 0.0489; Supporting Information Figure 5; Supporting Information Table 7)

Exposure to copper significantly delayed hatching during the periods of development when hatching occurs (169 hpf: P = 2e-16; 193 hpf: P = 2e-16; 216 hpf: P = 2e-16; Figure 5B; Supporting Information Figure 6; Supporting Information Table 7). However, the delay in hatching was still less severe in fish whose F0 grandparents had been pre-exposed to copper during embryogenesis (169 hpf: P = 2e-16; Figure 5B; Supporting Information Figure 6; Supporting Information Table 7).

Discussion:

We report evidence that stickleback fish pre-exposed to copper during early life show differential copper accumulation compared to a control population in later life. These effects were characterized by the ability of fish pre-exposed to copper to both maintain a higher copper concentration of this metal in liver, gill and muscle under control conditions, and an increased copper accumulation in their gills when re-exposed to copper in adult life. Importantly, F1 embryos originating from parents who were exposed to copper during embryogenesis were significantly more tolerant to copper when compared to embryos from the control population kept in parallel, and this effect was maintained in the F2 generation. Together, our data support the hypothesis that exposure to copper during early life has the potential to increase the tolerance of a vertebrate model across generations, and highlight the importance of the conditions that embryos experience during development in contributing to their susceptibility to environmental stressors across generations.

Exposure to copper during embryogenesis was unlikely to result in genetic selection

We selected a concentration of copper for the initial exposure of the F0 embryos that caused only 1.24%. This level of mortality is sufficiently low to exclude the potential of the initial exposure to cause selection for a copper tolerant genotype, but it does not exclude that potential random mutations may have occurred. In addition, a single pool was made of all embryos generated from the original stock, and assigned at random to the two treatment groups, ensuring that all parents contributed equally to both populations generated by the F0 embryo exposure. Together these facts suggest that it is likely that changes in phenotype

observed for subsequent generations are unlikely to be explained by genetic selection for tolerant genotypes.

Exposure to copper during embryogenesis caused alternations in copper accumulation in later life (F0 adults)

Under control conditions, adult male stickleback that had been pre-exposed to copper during early life, had significantly higher copper concentrations in their gills, liver and muscle when compared to adult male fish that had not been pre-exposed during embryogenesis. Similar findings to date have been reported only for continuous exposures but not for experimental designs including a long period of depuration (9 months), highlighting the importance of our study [70].

Our data is in contrast to that from previous studies in which exposures occurred only during the adult life stage. For example, our own studies in adult sticklebacks originating from the same population revealed that the concentration of copper in fish exposed to 0.02 mg/L Cu for 4 days returned to control levels after 30 days in clean water (Laing *et al*., In Preparation; Chapter 4). In addition, in a previous study rainbow trout (*Oncorhynchus mykiss*) pre-exposure to 22 µg/L copper for 28 days, followed by a depuration period of between 10 and 30 days resulted in partial recovery and declines in the acquisition of new copper in the gills of pre-exposed fish [71]. In both of these studies, the initial pre-exposure was performed on adult fish, unlike in the present study where the initial exposure was performed during embryogenesis, when significant developmental processes are occurring. Together, these data suggest that the window of development in which pre-exposure

occurs, is critical in influencing the physiological responses in fish later in life. These results observed as a consequence of exposure during embryogenesis may be explained by the disruption of epigenetic processes that occur during development, including epigenetic reprogramming [32,56,57].

Although data exists documenting epigenetic reprogramming in embryos immediately after fertilisation for zebrafish and a number of mammalian species [52,72–74], to date no studies have investigated epigenetic reprogramming events in the stickleback. However, given that this phenomenon is widely conserved in vertebrates, and associated to the wide conservation of *de novo* and maintenance methyltransferases required for the active remethylation of cytosines, and TET proteins required for DNA demethylation [75], we hypothesise that epigenetic reprogramming is likely to occur in stickleback too, but the exact timing of these events is unknown. The vulnerability of this developmental window could provide some explanation for the significant elevation in the copper content of three tissues observed for pre-exposed fish after 9 months in clean water, in contrast to studies in adult fish (Laing *et al*., In Preparation; Chapter 4) [71,76].

Owing to its essential nature [58], the machinery required for the homeostatic regulation of copper uptake, storage and excretion is tightly regulated based on environmental cues, including fluctuations in the water and dietary sources of copper [59]. The fish used in this study had been maintained in control conditions in the lab for more than three generations and during this time the primary source of copper was the diet, with ambient water concentrations of < 0.006 mg/L Cu. Due to the absence of any significant previous exposure to copper via the water, it may be that copper exposure during early life had some beneficial effects, and resulted in improved transport and storage of this essential metal via

the gills [59]. This hypothesis could explain the elevation in copper content observed in F0 adults in the absence of re-exposure.

Furthermore pre-exposed fish were found to accumulate significantly more copper in the gills when re-exposed to 0.01 or 0.02 mg/L Cu, but not in liver or muscle tissue. These data support previous studies describing the gills as the principal route of metal uptake in fish exposed to waterborne copper [59,77]. In freshwater fish copper uptake via the gill usually makes up a small proportion of the overall copper intake [59,78]. There is evidence from aquatic toxicity studies in freshwater species that the gill can contribute considerably to copper uptake when fish are under significant elevated copper exposure or dietary deficiency [59,78]. This is explained by the large gill surface area which is in continual and direct contact with the water, and the presence of metal specific carriers and other ion/metal transporters capable of transporting copper ions, including putative epithelial sodium channels (ENaC) and copper transporters (CTR1) [77].

Upon re-exposure, fish pre-exposed to copper during embryogenesis were able to accumulate more copper in their gills compared to naive fish. Although conducted in adult fish, a study exploring the role of copper acclimation on cadmium accumulation in the gills of rainbow trout (*Oncorhynchus mykiss*) exposed to 75 µg/L for 30 days followed by 3 hour gill cadmium binding experiments, demonstrated a higher capacity to accumulate new cadmium and a higher affinity for cadmium in copper acclimated fish compared to unexposed controls [79]. It was hypothesised that this increase in cadmium accumulation as a result of acclimation may be explained by the increased gill ion regulatory activity associated with copper exposure, for example, the increase in Na^+/K^+ ATPase activity to counteract copper induced Na^+ loss [79].

Similarly, we hypothesise that copper pre-exposure changes the way in which metals are handled in acclimated fish. It is important to note that in the study by McGeer *et al*, exposures were conducted in adult fish, without a depuration period prior to the final exposure experiment, therefore the molecular mechanisms controlling the effects observed were not shown to be maintained in the absence of exposure. However, these data do suggest that the pre-exposure to copper may also modulate the ability of fish to handle other metals, particularly those that use similar uptake routes [59].

In this study, the increased tissue concentration of copper after re-exposure was only observed in the gill, and not in the other tissues in which elevated copper concentration was observed in F0 adult male fish under control conditions. This is likely due to the short exposure time. Interestingly, similar findings were reported in Laing *et al*., (In Preparation; Chapter 4), where sticklebacks exposed to 0.02 mg/L Cu for 4 days had no significant differences in tissue metal content in the muscle or liver, suggesting that a longer time period may be necessary for any increases in copper tissue concentrations to be detectable in other tissues.

Exposure to copper during embryogenesis increased copper tolerance during the F1 and F2 generation

Toxicity testing in F1 embryos revealed that embryos collected from the copper pre-exposed population were more tolerant to copper than embryos collected from the control population, evidenced by significantly less mortalities, and a significantly greater proportion of embryos able to successfully hatch. In addition, we report that the differential tolerance

between the control and copper pre-exposed populations observed in the F1 generation are still present in F2 embryos. These findings were consistent and strongly significant across all replicate exposures.

These data are concordant with previous studies, where exposure of the F0 generation to chemical contaminants in a fish model has resulted in phenotypic changes that were maintained up to the F2 generation. Juvenile zebrafish (*Danio rerio*) exposed to 2,3,7,8 Tetrachlorodibenzo-p-dioxin (TCDD) in the F0 generation, had altered sex ratios and scoliosis-like axial skeleton abnormalities, in the F1 and F2 generation [80]. In addition, intergenerational effects have been reported for toxicants such as vinclozolin, BPA, phthalates and dioxin in mammalian models [46,81–83]. For metals, few examples exist where tolerance has been measured over multiple generations; for example, Bossuyt *et al*., report copper tolerance in *Daphnia magna* as a result of chronic copper exposure over multiple generations [84], and Guan *et al*., also report similar findings for *Daphnia Magna* exposed to cadmium over six successive generations[85]. However, in these studies acquired tolerance was observed after the organisms had been exposed continuously across multiple generations, meaning that the observed changes in phenotype were reported under continuous exposure. In addition, the concentrations used were likely to cause significant mortality, thus potentially selecting for a tolerant genotype.

It is important to note that in the present study only F0 embryos were exposed and only for the period of embryogenesis, while the F1 and F2 generation had not been exposed for one and two generations, respectively. In addition, we observed no significant differences in copper burden in the gill, liver, muscle or gonad of F1 adult male and female fish from the pre-exposed population. Together, these data suggest that this transgenerationally

inherited phenotype is not explained by the presence of elevated copper concentration in the tissues of parental F1 fish.

Potential mechanisms of increased tolerance to copper in subsequent generations

There are a number of hypotheses to explain the observed differential responses in later life and increase in tolerance in the future generations. Firstly, the improved ability to handle copper, in terms of being able to store more copper in adult life across a number of tissues and an increased tolerance in F1 and F2 embryos to elevated exposure concentrations could be explained by physiological changes caused by the initial F0 exposure.

For example, studies exploring the effects of acute metal exposure have reported alterations in the expression of a number of genes involved in membrane components and ion homeostasis, particularly in the gills. Previous studies reporting the acclimation of fish to copper following chronic exposure, often report acclimation to be driven by the restoration of plasma ionic balance and morphological changes in the gill structure [86,87], thereby regulating the uptake of copper from the external environment. During embryogenesis, previous studies report that populations of killifish (*Fundulus heteroclitus*) historically exposed to methylmercury (meHg) modulate meHg uptake through decreasing chorion permeability, and in this way protect themselves from toxicity through regulating toxicant uptake [30].

To date, studies have associated acclimation of fish to metal exposure with elevated expression of a number of metal binding proteins identified to play important roles in the homeostasis of metal storage. Frequently used as a classic biomarker of metal exposure,

metallothionein (MT) is a metal binding protein with a high affinity for metal ions, involved in the transport and storage of toxic metals, detoxifying, buffering and storing metal ions through sequestering and therefore reducing the amount of the free metal ions [88]. Exposure to sub-lethal concentrations of a number of essential and non-essential metals including copper, mercury, zinc and cadmium have been shown to stimulate metallothionein expression in both short term laboratory exposures and in chronically exposed wild fish [89–91]. In a study using the least killifish (*Heterandria formosa*), in fish pre-exposed to 15 µg/L copper for seven days followed by exposure to a lethal copper level (150 µg/L copper), metallothionein induction was reported to be higher for the acclimated fish compared to the controls [92]. Studies in rainbow trout (*Oncorhynchus mykiss*) pre-treated with either cadmium or arsenic were more tolerant to cadmium or arsenic upon re-exposure [31,93,94], and the acclimation to these metals was attributed to the stimulation of metal-binding proteins, involved in the storage and detoxification of metals [31]. In addition, toxicity testing in a cadmium tolerant population of *Daphnia magna* found metallothionein to be significantly up-regulated in all six generations [85]. It is important to note however, that in these studies, test organisms were exposed continuously without depuration. We hypothesis that the up-regulation of metallothionein may explain, at least im part, the effects seen. If this is the case, the maintenance of this effect throughout the life course (from embryos to mature adults) could be occurring via epigenetic mechanisms.

Physiological changes mediating the acclimation response could also involve the regulation of toxicant excretion. Grosell *et al*., report that copper acclimated rainbow trout (*Oncorhynchus mykiss*) clear a single bolus of injected of copper from their plasma more effectively than non-acclimated fish, reporting a four-fold increase in hepatobiliary copper

excretion in copper acclimated fish [95], suggesting part of the acclimation response includes the ability to remove copper from the body.

These studies suggest that in this way prior exposure or acclimation to copper can result in a number of physiological changes that can modify the ways in which fish react to metal contaminants upon re-exposure. Fish can therefore modulate the amount of copper that is taken up from the external environment, stored and excreted, and in so doing can modulate copper toxicity. Interestingly, in the present study we have detected a significantly higher basal copper load in three tissues in F0 adult fish from the copper pre-exposed population, suggesting that the physiological changes associate with prior exposure to copper are likely to still be present when the gametes to produce F1 embryos were developing. Therefore, it is likely that the gametes that resulted in F1 embryos underwent gametogenesis in a copper rich environment, which could in turn modulate the physiological responses of F1 fish to copper. However, we observed no significant differences in copper burden in the gill, liver, muscle or gonad of F1 adult male and female fish, suggesting that from the F1 to the F2 generation at least, this trans generationally inherited phenotype is not explained by the presence of elevated copper concentrations in the paternal or maternal F1 tissues.

Alternatively, the differential responses to copper exposure observed in this study in the pre-exposed population could be explained by genetic changes. Previous studies in wild and laboratory fish have associated prior exposure to a number of metals and acquired tolerance with genetic adaptation. Key examples include wild populations of brown trout (*Salmo trutta*) [13,14], three-spined stickleback (*Gasterosteus aculeatus*) [15] and wild yellow perch (*Perca flavescens*) [16] historically exposed to high concentrations of toxic metal mixtures, and laboratory studies in *Daphnia magna* successively exposed for six generations

to chronic cadmium stress [85]. However, often these studies involve chronic historic exposure over multiple generations and exposure to extremely high concentrations causing significant mortality, and therefore selection for tolerant genotypes. In contrast, the copper pre-exposed population in the present study was only exposed once, during embryogenesis in the F0 generation, these were then maintained in clean water until the end of the experiment, without re-exposure. In addition, the concentration chosen for the initial F0 embryo exposure caused less than 1.5% mortality, and no significant mortalities were record after this. This, in conjunction with the fact that embryos generated from a large parental stock were distributed equally between the two treatments during the F0 embryo exposure, suggests that it is unlikely that a copper tolerant genotype has been selected for or that the genetic background of the original stock could explain the phenotype observed in the F2 generation of fish from the copper pre-exposed population.

Alternatively, the differential susceptibility observed in F1 and F2 embryos could be explained, at least in part, by epigenetic modifications, often referred to as the epigenetic memory [32]. In mammalian models, a number of examples exist where copper exposure has been associated with changes at the DNA methylation level. For example, studies using the Cohen diabetic sensitive rats, associated changes in global DNA methylation with copper exposure, specifically hyper global DNA methylation in placental tissue, in response to copper supplementation [96]. In addition, in the Jackson toxic milk mouse model of Wilson disease, changes in hepatic copper concentration have been associated with alterations in global DNA methylation [43]. Copper exposure has also been associated with changes to epigenetic marks within chromatin. Exposure of HL-60 human leukemia cells to copper has been shown to cause a concentration dependant decrease in histone acetylation, attributed

to Cu-induced oxidative stress [97,98]. These studies are concordant with previous findings from Kang *et al*., who found that exposure of Hep3B cells to copper caused inhibition of histone acetylation both at a toxic as well as at a non-toxic concentrations [99,100].

It has been hypothesised that embryogenesis represents a critical window of development, which may be vulnerable to permanent changes in the epigenome that can be inherited via the germ line [32,56,57]. In zebrafish, epigenetic reprogramming has been shown to occur immediately after fertilisation [32,52,53]. This re-programming process is proposed to be highly conserved, and it has been documented in a number of vertebrate species, including fish and mammalian species [52,54]. It is thought that during re-programming, chemical pollutants such as copper may interfere with this process of re-methylation and sequences that escape reprogramming may be involved in epigenetic inheritance [32,56,57].

Our data demonstrate that the tolerant phenotype was still present in F2 fish and was not explained by the presence of elevated copper concentrations in the paternal or maternal F1 tissues. In addition to the fact that the initial exposure caused very few mortalities, we hypothesise that the differential tolerance observed in the F2 generation is most likely attributed to epigenetic mechanisms. To explore this hypothesis, in a parallel study, whole genome bisulfite sequencing is being conducted according to Booth *et al*. [101], in order to test if differentially methylated regions are present between F2 embryos collected from the control and copper pre-exposed populations. In addition, the study will also test for the presence of genetic changes, in order to eliminate genetic selection from the potential hypothesis driving this inherited phenotype.

Environmental implications

There are a number of environmental implications associated with these findings. Firstly, these effects were observed after the exposure of embryos to an environmentally relevant concentration of copper. Therefore, our data suggests that differential responses to chemical contaminants such as copper are likely to be common in wild populations, and highlights the likelihood that fish inhabiting each unique water body have acclimated to their specific environment, via a process likely to be driven by genetic, epigenetic or physiological mechanisms, alone or in combination.

These data raise important questions regarding the management of local water bodies, particularly regarding the re-stocking of fish populations. Our data suggests that embryogenesis represents a critical window of development, in which pre-exposure can significantly affect the way in which fish handle pollutants both in later life and in subsequent generations. With this in mind, these data highlight the possibility that it may be important for re-stocking populations to be maintained in conditions matching the water chemistry of the target water body during this critical period of development. This practice could represent an important strategy in order to ensure re-stocking populations are acclimated to the unique conditions present in the water body which they will re-stock. This has been further evidenced by re-stocking studies in salmon (*Salmo salar L.*) on the River Thames, UK, where genetic analysis was used to reveal that over multiple years the number of individuals from the original re-stocking population dramatically declined, and in fact the new fish colonizing the river were predominantly from other rivers in southern England, where the chemical characteristics of the water bodies is likely to be similar to that of the Thames [102].

In addition, it is important to consider the potential trade-offs associated with acclimation to a given stressor. While exposure to copper during embryogenesis resulted in an increased tolerance during subsequent generations, F1 male fish from the exposed population had significantly lower condition factor compared to males from the control population. These data highlight the potential for the physiological and/or molecular changes maintained as a result of prior exposure to copper, to result in trade-offs in energy budgets, in order the maintain a tolerant phenotype. Trade-offs can occur when the improvements in one trait may be achievable only at the expense of others [103]. There are many examples in the literature where trade-offs as a result of exposure to a stressor and /or acclimation can result in impacts to population growth rates [104], the immune system [105] and reproductive endpoints [106]. For example, a laboratory induced resistance to cadmium in a population of least killifish (*Heterandria formosa*) was associated with reduced fecundity, a shorter female life span and smaller brood sizes [106].

These data also raise important questions regarding the interpretation of laboratory testing for ERA, where the responses of laboratory bred populations is likely to be significantly impacted by the exposure history of the test population. Previous studies have highlighted the potential for prior exposure to certain chemicals to impact on a population's responses to other chemicals, for example, acclimation to copper can result in differential responses to cadmium in subsequent exposure scenarios in fish [5,19]. It is also important to consider, that in the wild organisms are exposed to multiple stressors, including changes in abiotic parameters such as dissolved oxygen levels, pH or temperature [2,3,107,108], and a vast array of toxicants from metal contaminants to pharmaceuticals and agricultural substances [13,109–111]. Therefore, each water body represents a unique environment, to which the native

populations will likely have developed specific mechanisms in order to be acclimated to the surrounding physical and chemical environment via physiological, genetic and epigenetic mechanisms. Therefore, it is essential that laboratories maintain adequate records of the chemical exposure history of their model organisms used in toxicity testing, including origin of the fish, the water chemistry of the location the fish were originally sampled from, and consider the extent of variation in susceptibility in the wild when interpreting data used for the establishment of legislation and management initiatives [5].

Conclusions

The data generated in this study provide evidence that the exposure history of fish populations can significantly affect tolerance in subsequent generations, including for environmentally relevant scenarios. We provide an exemplar dataset demonstrating that low levels of copper during early life has the potential to increase the tolerance of a vertebrate model across generations, and highlight the importance of this early life developmental stage in contributing to a further layer of information in addition to genetic adaptation, which in turn can alter responses across generations. These data also emphasise the importance of considering the exposure history of test organisms used in toxicity testing, when the data generated will be used to inform legislation, and to protect and manage wild populations.

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Supporting Information

Exposure to copper during embryogenesis caused a differential response to copper in later life and increased tolerance in subsequent generations, in a fish model.

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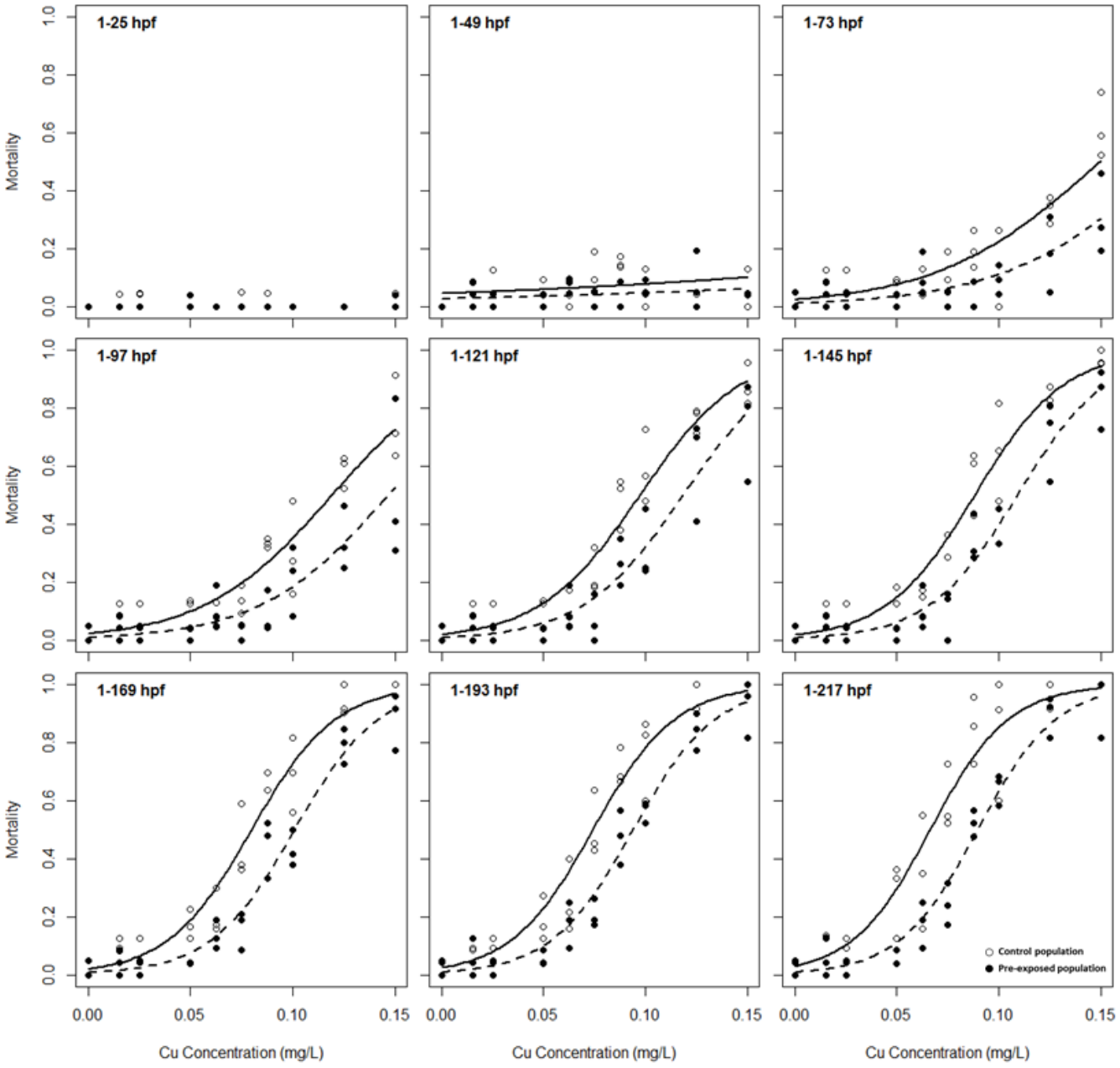
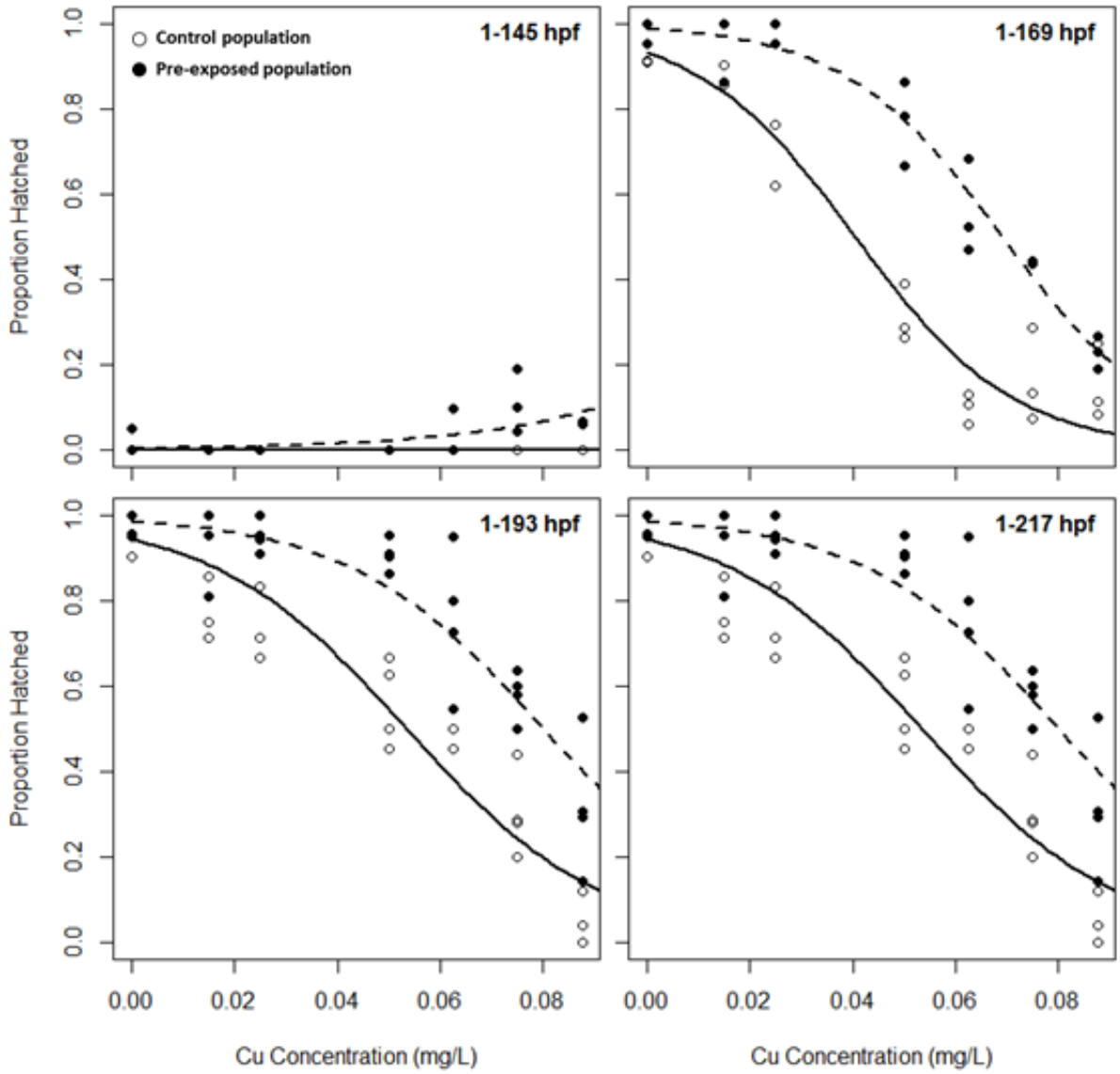
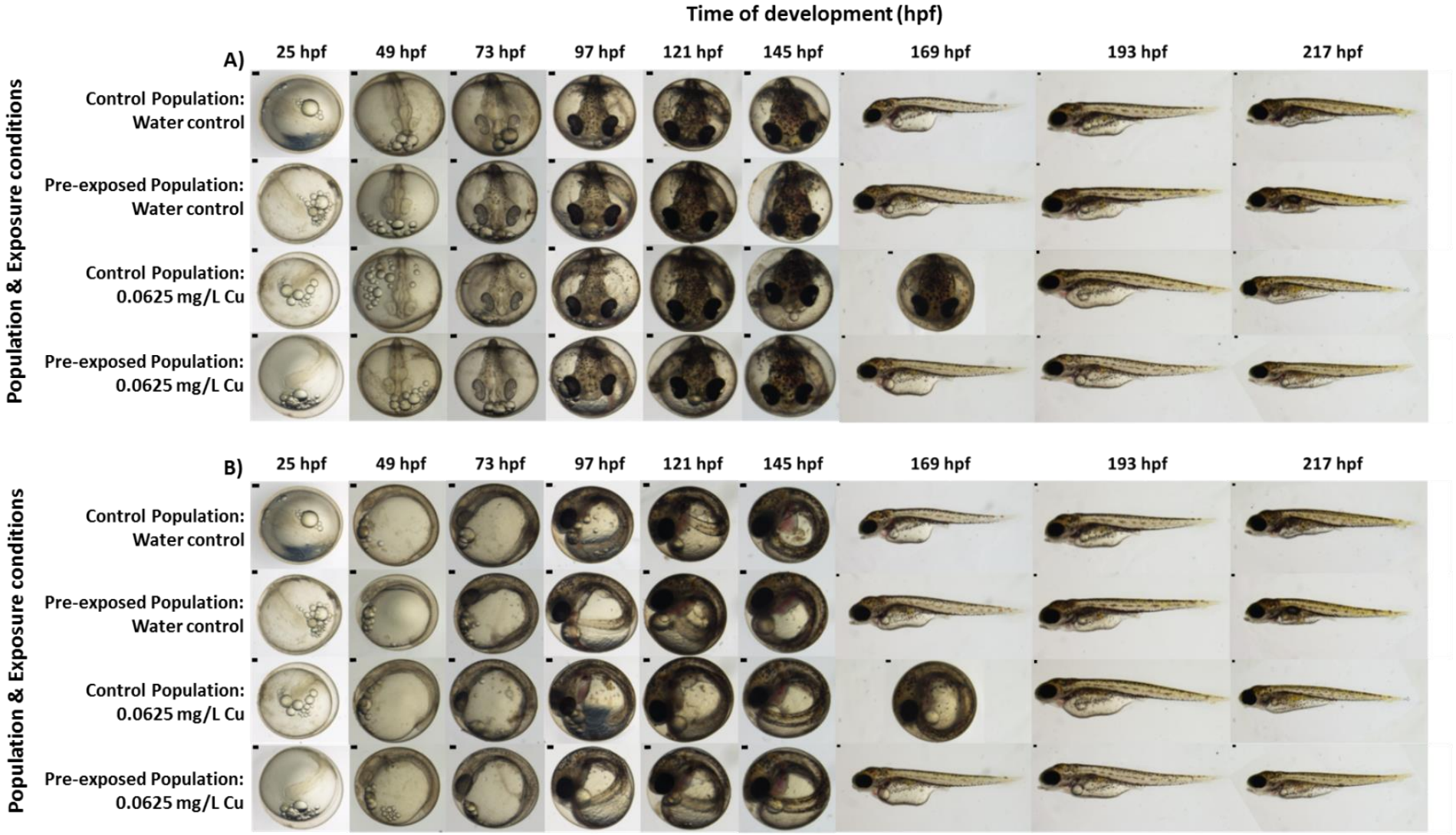

1-25 hpf
1-49 hpf
1-73 hpf
1-97 hpf
1-121 hpf
1-145 hpf
1-169 hpf
1-193 hpf
1-217 hpf
Mortality
Cu Concentration (mg/L)
Control population
Pre-exposed population

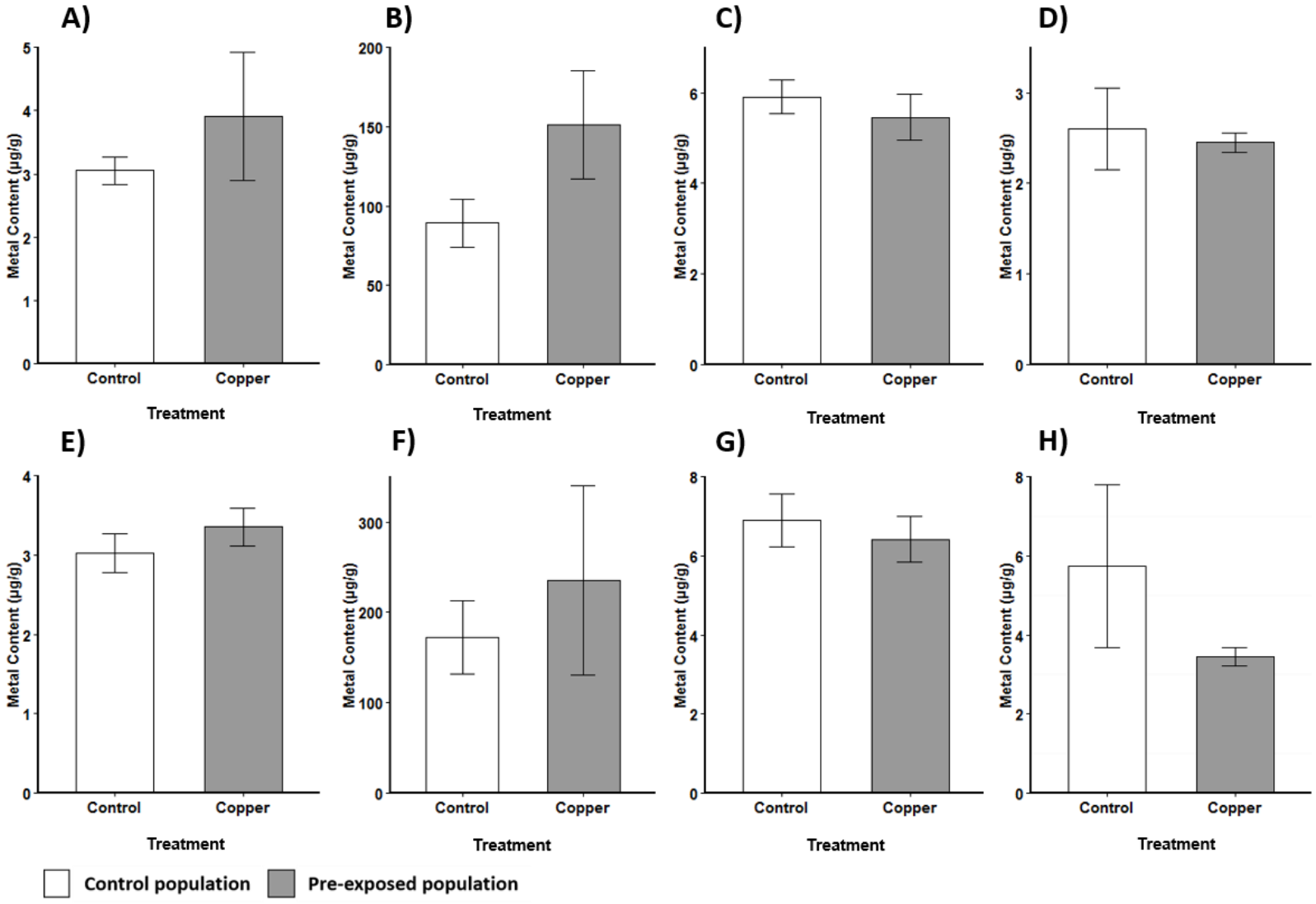
Supporting Information Figure 1. Embryo mortality curves following continuous exposure of F1 embryos from both the control and copper pre-exposed population, exposed to copper throughout development (n = 3 tanks per copper concentration tested). Each point on the graph represents the proportion of mortality in an individual replicate tank containing 20 embryos, black and white symbols represent groups from pre-exposed and control populations respectively, and the lines represent the best fit model for the data, calculated using generalized linear models in R (model output summarised in Supporting Information Table 7). From 49 hpf both copper re-exposure concentration and population of origin (pre-exposure) were significant explanatory variables (217 hpf: P = 2e-16 and P = 1.39e-13 respectively; Supporting Information Table 7).


Control population
Pre-exposed population
1-145 hpf
1-169 hpf
1-193 hpf
1-217 hpf
Proportion Hatched
Proportion Hatched
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.02
0.04
0.06
0.08
Cu Concentration (mg/L)
Cu Concentration (mg/L)

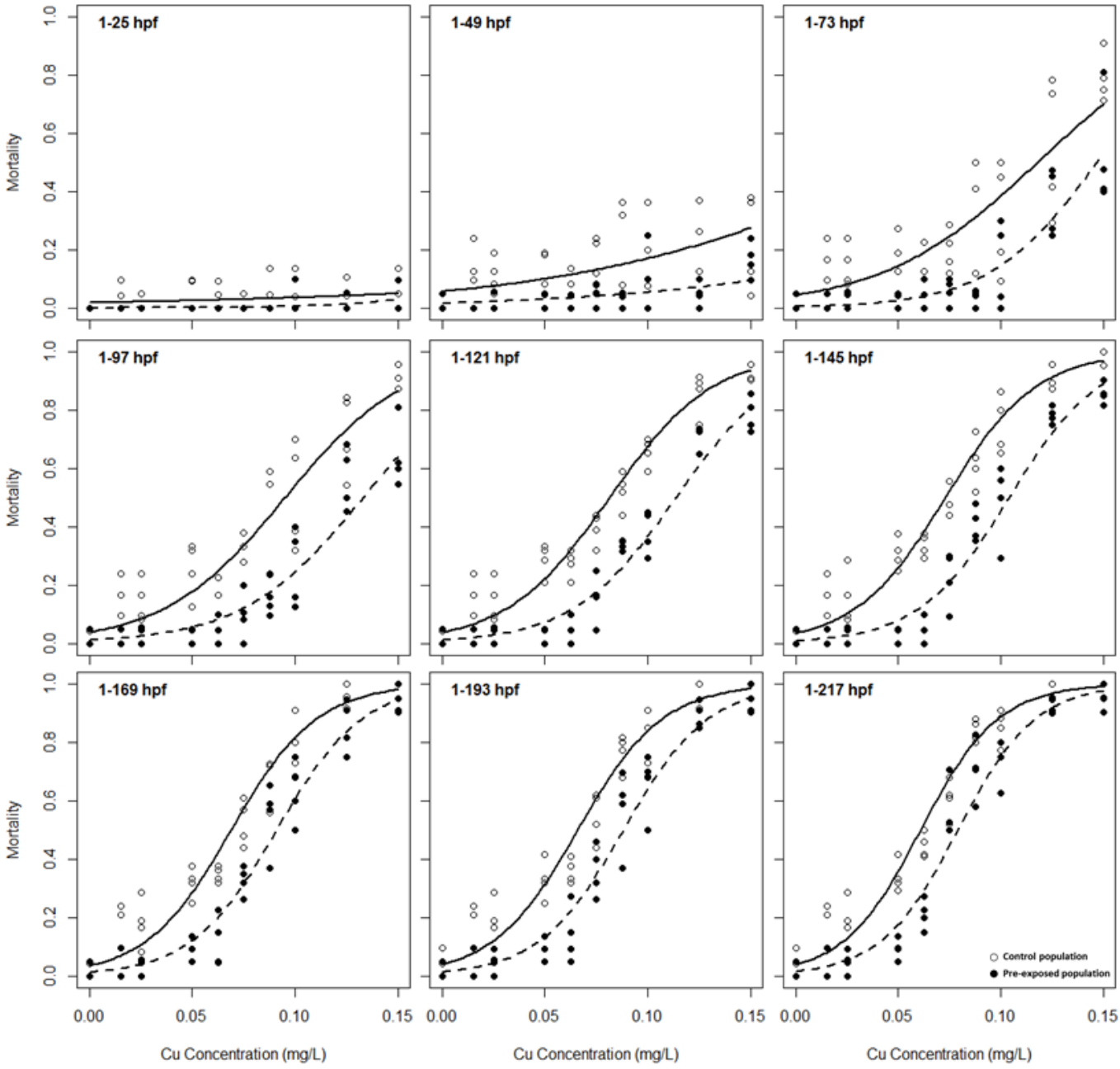
Supporting Information Figure 2. Proportion of live embryos successfully hatched following continuous exposure of F1 embryos from both the control and the pre-exposed population, exposed to copper throughout development (n = 3 tanks per copper concentration tested). Each point on the graph represents the proportion of embryos hatched in an individual replicate tank containing 20 embryos, black and white symbols represent groups from pre-exposed and control populations respectively, and the lines represent the best fit model for the data, calculated using generalized linear models in R (model output summarised in Supporting Information Table 7). From 145 hpf copper re-exposure concentration was a significant explanatory variables (217 hpf: P = 2e-16; Supporting Information Table 7). From 169 hpf population of origin (pre-exposure) was also a significant explanatory variable (217 hpf: P = 2.33e-13; Supporting Information Table 7).


Time of development (hpf)
A)
25 hpf
49 hpf
73 hpf
97 hpf
121 hpf
145 hpf
169 hpf
193 hpf
217 hpf
Population & Exposure conditions
Control Population: Water control
Pre-exposed Population: Water control
Control Population: 0.0625 mg/L Cu
Pre-exposed Population: 0.0625 mg/L Cu
B)
25 hpf
49 hpf
73 hpf
97 hpf
121 hpf
145 hpf
169 hpf
193 hpf
217 hpf
Population & Exposure conditions
Control Population: Water control
Pre-exposed Population: Water control
Control Population: 0.0625 mg/L Cu
Pre-exposed Population: 0.0625 mg/L Cu

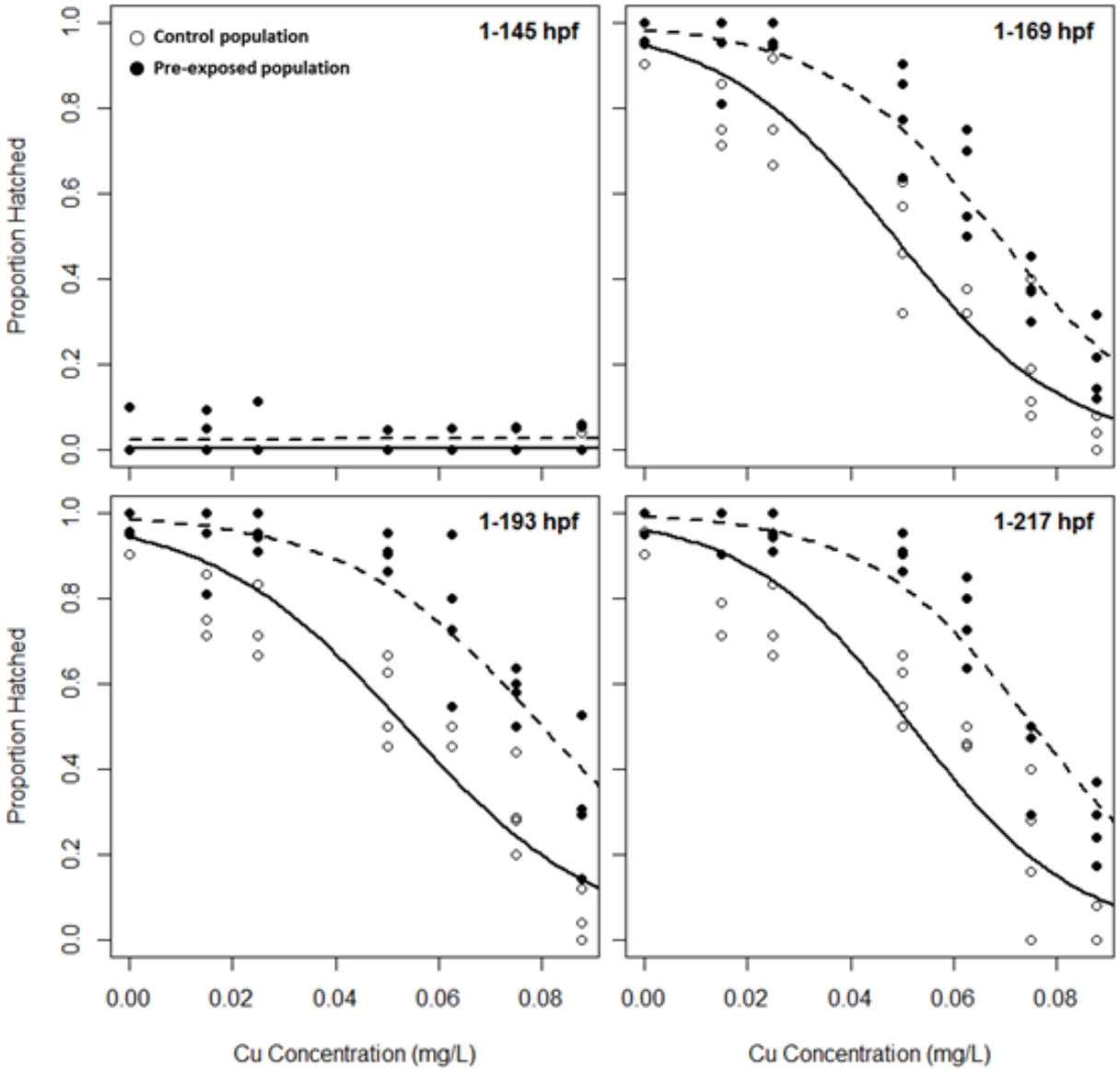
Supporting Information Figure 3. F1 stickleback embryos from the pre-exposed and control population exposed to control conditions or 0.0625 mg/L Cu throughout development. Video-captured images (in a Nikon SM21500, Japan) of embryos from 1-217hpf. Hatching was delayed in embryos originating from control F0 parents that were exposed to 0.0625 mg/L Cu, however this effect was not observed in embryos originating from pre-exposed F0 parents that were exposed to 0.0625 mg/L Cu. No other developmental delays were observed in embryos exposed to 0.0625 mg/L Cu for either population. The same effects were observed for F2 embryos from pre-exposed and control population exposed to control conditions or 0.0625 mg/L Cu throughout development.


A)
B)
C)
D)
E)
F)
G)
H)
Metal Content (µg/g)
Control
Copper
Treatment
Control population
Pre-exposed population

Supporting Information Figure 4. Measured copper concentrations in the gill **(A)**, liver **(B)**, muscle **(C)** and gonad **(D)** of male adult F1, and the gill **(E)**, liver **(F)**, muscle **(G)** and gonad **(H)** of female adult F1 fish maintained in clean water (Figure 1A, n = 10). F1 adult fish were bred from F0 adult fish which were pre-exposed to copper or kept in control conditions during embryogenesis (1-217 hpf). Tissue metal content was measured by ICPMS. Data is presented as mean µg Cu/g ± standard error mean. Statistical comparisons were conducted using the Student's t-Test in R (version 3.2.4), and statistical outputs and mean values are reported in Supporting Information Table 9. For both male and female F1 fish, there was no significant difference in tissue metal content for the gill, liver, muscle or gonads of fish whose F0 parents has been pre-exposed to 0.01 mg/L Cu during embryogenesis compared to the control population (1-217 hpf; Supporting Information Table 9).


1-25 hpf
1-49 hpf
1-73 hpf
1-97 hpf
1-121 hpf
1-145 hpf
1-169 hpf
1-193 hpf
1-217 hpf
Mortality
Cu Concentration (mg/L)
0.00
0.05
0.10
0.15
0.0
0.2
0.4
0.6
0.8
1.0
Control population
Pre-exposed population

Supporting Information Figure 5. Embryo mortality curves following continuous exposure of F2 embryos from both the control and pre-exposed population, exposed to copper throughout development (n = 4 tanks per copper concentration tested). Each point on the graph represents the proportion of mortality in an individual replicate tank containing 20 embryos, black and white symbols represent groups from copper and control pre-exposed populations respectively, and the lines represent the best fit model for the data, calculated using generalized linear models in R (model output summarised in Supporting Information Table 7). From 49 hpf both copper re-exposure concentration and population of origin (pre-exposure) were significant explanatory variables (217 hpf: P = 2e-16 and P = 7.43e-12 respectively; Supporting Information Table 7). From 49-145 hpf there was a significant interaction between copper re-exposure concentration and population of origin (pre-exposure; 145 hpf: P = 0.0489; Supporting Information Table 7).


Control population
Pre-exposed population
1-145 hpf
1-169 hpf
1-193 hpf
1-217 hpf
Proportion Hatched
Proportion Hatched
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.02
0.04
0.06
0.08
Cu Concentration (mg/L)
Cu Concentration (mg/L)

Supporting Information Figure 6. Proportion of live embryos successfully hatched following continuous exposure of F2 embryos from both the control and pre-exposed populations (n = 4 tanks per copper concentration tested). Each point on the graph represents the proportion of embryos hatched in an individual replicate tank containing 20 embryos, black and white symbols represent groups from pre-exposed and control populations respectively, and the lines represent the best fit model for the data, calculated using generalized linear models in R (model output summarised in Supporting Information Table 7). From 169 hpf both copper re-exposure concentration and population of origin (pre-exposure) were significant explanatory variables (217 hpf: P = 2e-16 and P = 2e-16; Supporting Information Table 7).

Supporting Information Table 1. Measured concentrations of copper in the exposure water during the initial exposure of naïve F0 embryos to copper. Water from four replicate tanks were pooled together for each exposure. Three repeated exposures were conducted, and therefore concentrations were measured for 3 pools on day five and nine using ICP-MS and are presented as mean values ± SEM.

Replicate exposure	Day	Nominal copper concentration (mg/L)	Mean measured copper concentration (mg/L)	SEM	% of the nominal concentration
1	5	0.00	0.0002	0.0000	100.00
1	5	0.01	0.0103	0.0001	103.31
1	9	0.00	0.0003	0.0000	100.00
1	9	0.01	0.0116	0.0001	115.65
2	5	0.00	0.0002	0.0000	100.00
2	5	0.01	0.0101	0.0007	100.71
2	9	0.00	0.0002	0.0000	100.00
2	9	0.01	0.0120	0.0005	119.67
3	5	0.00	0.0002	0.0000	100.00
3	5	0.01	0.0125	0.0007	125.36
3	9	0.00	0.0002	0.0000	100.00
3	9	0.01	0.0117	0.0001	117.09

Supporting Information Table 2. Measured concentrations of copper in the exposure water for the F0 adult re-exposure of pre-exposed (copper or control) fish (See Figure1B for details, n = 8-9). Water from two replicate tanks and concentrations were measured on day one and three of the experiment using ICP-MS and are presented as mean values ± SEM.

Population of origin (pre-exposure)	Day	Nominal copper concentration (mg/L)	Mean measured copper concentration mg/L	SEM	% of the nominal concentration
Control	1	0.00	0.0050	0.0002	100.00
Control	1	0.01	0.0140	0.0012	141.57
Control	1	0.02	0.0220	0.0001	110.02
Copper	1	0.00	0.0050	0.0001	100.00
Copper	1	0.01	0.0130	0.0003	132.10
Copper	1	0.02	0.0220	0.0001	111.17
Control	3	0.00	0.0050	0.0000	100.00
Control	3	0.01	0.0130	0.0000	134.32
Control	3	0.02	0.0210	0.0000	107.04
Copper	3	0.00	0.0050	0.0002	100.00
Copper	3	0.01	0.0140	0.0006	135.16
Copper	3	0.02	0.0220	0.0002	109.57

Supporting Information Table 3. Morphometric parameters for the F0 adult re-exposure of pre-exposed (copper or control) fish to copper (See Figure1 for details; n = 8-9 fish per treatment group). All data are presented as mean ± SEM.

Population of origin (pre-exposure)	Sex	Re-exposure copper concentration (mg/L)	Morphometric parameter	Mean	SEM
control	M	0.00	Fork length (cm)	5.6017	0.0601
copper	M	0.00		5.5832	0.0501
control	M	0.01		5.6333	0.0333
copper	M	0.01		5.6278	0.0680
control	M	0.02		5.6111	0.0718
copper	M	0.02		5.6611	0.0486
control	M	0.00	Weight (g)	1.8088	0.0881
copper	M	0.00		1.9228	0.0782
control	M	0.01		1.8397	0.1016
copper	M	0.01		1.8222	0.0818
control	M	0.02		1.8069	0.1012
copper	M	0.02		1.8725	0.0665
control	M	0.00	Condition factor (g/cm^3)	1.0242	0.0253
copper	M	0.00		1.1061	0.0485
control	M	0.01		1.0223	0.0249
copper	M	0.01		1.0182	0.0255
control	M	0.02		1.0155	0.0261
copper	M	0.02		1.0315	0.0276
control	M	0.00	HSI	2.4654	0.1638
copper	M	0.00		2.4835	0.2843
control	M	0.01		2.2703	0.1396
copper	M	0.01		2.1926	0.1475
control	M	0.02		2.2392	0.1470
copper	M	0.02		2.3266	0.1771

Supporting Information Table 4. Morphometric parameters for the F0 adult re-exposure of pre-exposed (copper or control) fish (see Figure1 for details; n = 8-9 fish per treatment group). All data are presented as mean ± SEM. Analysis of variance models for the relationships between population of origin (pre-exposure), re-exposure to copper and a population of origin (pre-exposure) / re-exposure interaction. Minimum adequate models (F value) for the each morphometric parameter are shown.

Test	Morphometric parameter	Population of origin (pre-exposure)		Re-exposure to copper		Population (pre-exposure) / Re-exposure interaction	
		F value	P value	F value	P value	F value	P value
General Linear Model	**Fork length (cm)**	NS	NS	NS	NS	NS	NS
	Weight (g)	NS	NS	NS	NS	NS	NS
	Condition factor (g/cm^3)	NS	NS	NS	NS	NS	NS
	HSI	NS	NS	NS	NS	NS	NS

Supporting Information Table 5. Measured concentrations of copper in the tissues of the F0 adult fish re-exposure of pre-exposed (copper or control) fish (See Figure1 for details; n = 8-9 fish per treatment group). All data are presented as mean ± SEM.

Population of origin (pre-exposure)	Day	Tissue	Re-exposure copper concentration (mg/L)	Metal content (µg/g)	
				Mean	SEM
Control	4	Gill	0.00	6.0404	1.1977
Copper	4	Gill	0.00	12.9502	1.5304
Control	4	Gill	0.01	31.0766	4.0564
Copper	4	Gill	0.01	45.2084	7.4072
Control	4	Gill	0.02	29.0874	7.2426
Copper	4	Gill	0.02	52.7103	6.9410
Control	4	Liver	0.00	449.3222	56.8732
Copper	4	Liver	0.00	669.6825	111.8246
Control	4	Liver	0.01	502.8988	47.7638
Copper	4	Liver	0.01	687.4111	111.8294
Control	4	Liver	0.02	537.4766	62.1633
Copper	4	Liver	0.02	758.0657	59.9669
Control	4	Muscle	0.00	23.6347	5.4842
Copper	4	Muscle	0.00	53.0742	15.7075
Control	4	Muscle	0.01	40.5180	11.6211
Copper	4	Muscle	0.01	70.8920	31.0444
Control	4	Muscle	0.02	13.6270	1.4225
Copper	4	Muscle	0.02	49.3402	14.0242

Supporting Information Table 6. Measured concentrations of copper in the tissues of the F0 adult fish re-exposure of pre-exposed (copper or control) fish (See Figure1 for details; n = 8-9 fish per treatment group). All data are presented as mean ± SEM. Analysis of variance models for the relationships between re-exposure to copper, population of origin (pre-exposure) and a re-exposure / population of origin (pre-exposure) interaction. Minimum adequate models (F value) for the each parameter are shown (Significance codes: *** P<0.001, ** P<0.01, * P<0.05).

Generation	**Time point**	**Tissue**	**Test**	**Re-exposure to copper**		**Population of origin (pre-exposure)**		**Re-exposure / Population (pre-exposure) Interaction**	
				F value	P value	F value	P value	F value	P value
F0	1 year	Gill	Generalized Linear Model	19.5395	**6.671e-07 *** **	9.8499	**0.002932 ** **	NS	NS
		Liver		NS	NS	6.3926	**0.01425 * **	NS	NS
		Muscle		NS	NS	4.9949	**0.03032 * **	NS	NS

Generation	Time point (hpf)	End point	Test	Re-exposure to copper		Population of origin (pre-exposure)		Re-exposure / Population (pre-exposure) Interaction	
				Z value	P value	Z value	P value	Z value	P value
F1	1-25	Mortality	General Linear Model	NS	NS	NS	NS	NS	NS
	1-49			2.23	**0.0261 ***	-2.16	**0.0311 ***	NS	NS
	1-73			11.16	**2e-16 *****	-4.61	**4.07e-06 *****	NS	NS
	1-97			14.69	**2e-16 *****	-5.33	**9.98e-08 *****	NS	NS
	1-121			17.42	**2e-16 *****	-5.38	**7.51e-08 *****	NS	NS
	1-145			18.08	**2e-16 *****	-5.94	**2.84e-09 *****	NS	NS
	1-169			18.41	**2e-16 *****	-6.33	**2.38e-10 *****	NS	NS
	1-193			18.61	**2e-16 *****	-6.13	**8.68e-10 *****	NS	NS
	1-217			18.72	**2e-16 *****	-7.40	**1.39e-13 *****	NS	NS
F1	1-145	Proportion hatched	General Linear Model	2.80	**0.00514 ****	NS	NS	NS	NS
	1-169			-14.30	**2e-16 *****	8.49	**2e-16 *****	NS	NS
	1-193			-9.16	**2e-16 *****	8.12	**4.77e-16 *****	NS	NS
	1-217			-8.25	**2e-16 *****	7.33	**2.33e-13 *****	NS	NS
F2	1-25	Mortality	General Linear Model	NS	NS	-2.36	**0.0181 ***	NS	NS
	1-49			4.37	**1.26e-05 *****	-4.64	**3.48e-06 *****	2.61	**0.00894 ****
	1-73			12.25	**2e-16 *****	-5.18	**2.27e-07 *****	2.74	**0.0062 ****
	1-97			13.82	**2e-16 *****	-5.50	**3.84e-08 *****	2.60	**0.00944 ****
	1-121			14.85	**2e-16 *****	-5.46	**4.77e-08 *****	2.42	**0.0154 ***
	1-145			15.35	**2e-16 *****	-5.27	**1.38e-07 *****	1.97	**0.0489 ***
	1-169			15.49	**2e-16 *****	-4.52	**6.12e-06 *****	NS	NS
	1-193			21.31	**2e-16 *****	-7.83	**4.96e-15 *****	NS	NS
	1-217			21.45	**2e-16 *****	-6.85	**7.43e-12 *****	NS	NS
F2	1-145	Proportion hatched	General Linear Model	NS	NS	NS	NS	NS	NS
	1-169			-17.70	**2e-16 *****	7.59	**3.1e-14 *****	NS	NS
	1-193			-16.13	**2e-16 *****	8.91	**2e-16 *****	NS	NS
	1-217			-16.99	**2e-16 *****	8.84	**2e-16 *****	NS	NS

Supporting Information Table 7. Analysis of variance models for the relationships between population of origin (pre-exposure), re-exposure to copper and a population of origin (pre-exposure) / re-exposure interaction in F1 and F2 embryo mortality curves. Minimum adequate models for proportion of mortality using a binomial error structure are shown. Minimum adequate models (Z value) for the each parameter are shown (Significance codes: *** P<0.001, ** P<0.01, * P<0.05).

Supporting Information Table 8. Morphometric parameters for the F1 adult fish bred using IVF from F0 adults which were pre-exposed to either copper or control conditions during embryogenesis (1-217 hpf; See Figure1 for details; n = 10 fish per treatment group). Statistical comparisons were conducted using the Student's t-Test in R (version 3.2.4). All data are presented as mean ± SEM. Asterisks indicate significant differences compared to the control population (*** P<0.001, ** P<0.01, * P<0.05).

Population of origin (pre-exposure)	Sex	Morphometric parameter	Mean	SEM	t statistic	P value
control	m	Fork length (cm)	5.3650	0.0488	2.8016	**0.005028 ** **
copper			5.1550	0.0569		
control	f		6.3000	0.0637	1.3400	0.1887
copper			6.1600	0.0828		
control	m	Weight (g)	1.7603	0.0579	3.3558	**0.001806 ** **
copper			1.4864	0.0575		
control	f		2.3130	0.0834	1.3122	0.1977
copper			2.1396	0.1024		
control	m	Condition factor (g/cm^3)	1.1343	0.0188	2.0524	**0.04709 ***
copper			1.0777	0.0575		
control	f		0.9181	0.0119	0.5797	0.5662
copper			0.9050	0.0193		
control	m	HSI	3.6266	0.1587	0.8479	0.4022
copper			3.4020	0.0575		
control	f		5.3991	0.2056	1.2668	0.2138
copper			4.9473	0.2914		
control	m	GSI	0.5474	0.1109	0.7126	0.4839
copper			0.4663	0.0575		
control	f		3.9096	0.7555	-0.2157	0.8274
copper			4.1673	0.8979		

Supporting Information Table 9. Measured concentrations of copper in the tissues of F1 adult fish, bred from F0 adult fish which were pre-exposed to copper or control during embryogenesis (1-217 hpf). These fish were maintained until sexual maturity in clean water holding tanks and sampled (n = 10 fish per population). Statistical comparisons were conducted using the Student's t-Test in R (version 3.2.4). All data are presented as mean ± SEM. Asterisks indicate significant differences compared to the control population (Significance codes: *** P<0.001, ** P<0.01, * P<0.05).

Tissue	Sex	Metal content (µg/g)					
		Control population		Pre-exposed population		t statistic	P value
		Mean	SEM	Mean	SEM		
Gill	M	3.06	0.2172	3.9092	1.0166	-0.8206	0.4314
	F	3.03	0.2507	3.3572	0.2364	-0.9636	0.3481
Liver	M	89.33	14.9845	151.3921	33.9510	-0.5611	0.5867
	F	172.15	40.2770	235.3051	105.1213	-1.6722	0.1225
Muscle	M	5.91	0.3670	6.4221	0.5700	0.7207	0.4822
	F	6.90	0.6623	5.4572	0.5064	0.5497	0.5894
Gonad	M	2.60	0.4485	2.4533	0.1066	0.3227	0.7535
	F	5.74	2.0562	3.4495	0.2210	9.2080	0.2964

Chapter 6

General Discussion

6 General Discussion

6.1 Overview of findings in this thesis

Increased understanding of the role of gene function and epigenetic regulation in normal cellular development, particularly in mammalian models, has led to a greater appreciation of the potential for environmental pollutants to mediate their toxic effects via changes to epi (genetic) processes. Initial data suggests that a number of environmental pollutants may interact with epigenetic processes, and that their disruption could contribute towards toxicity, and may modulate differential responses in future exposure scenarios. Evidence also suggests that certain windows of development are particularly sensitive to chemical toxicity mediated via epigenomic modifications, for example during epigenetic reprogramming in early life.

To date, our knowledge about normal epigenetic processes in fish, as well as the potential for chemical pollutants to interact with their epigenome is sparse. In addition our understanding regarding the extent to which exposure to sub-lethal concentrations of chemical pollutants can modulate susceptibility in subsequent exposure scenarios is limited, and these areas represent significant research needs.

The overall aims of this thesis were to investigate the epigenetic and transcriptional regulation of reproduction in fish, and to establish how environmental oestrogenic chemicals (BPA) alter these processes. In addition, this thesis aimed to determine the effects of previous exposure on the susceptibility of fish upon re-exposure both later in life and in subsequent generations, and both when exposures occur during the embryonic development or in mature fish.

This thesis provides evidence for sexual dimorphism in transcription and DNA methylation profiles in the livers and gonads of an important model organism, the zebrafish, and reports novel evidence for associations between DNA promotor methylation and transcription for *esr1, amh* and *dnmt1,* and between *dnmt1* and *dnmt3* transcription and global DNA methylation in the gonads. In addition, this work documents for the first time a significant overexpression of a group of genes involved in DNA and histone modifications in ovaries compared to testis, likely associated with their role as maternal transcripts to support embryo development. Further to this, these data highlight critical considerations for investigating epigenetics in multicellular tissues, where each cell population is characterized by its own unique epigenetic signature.

With these data in mind, chapter 3 then aimed to investigate the reproductive effects of BPA, a commercially important high production chemical, in a fish model and to document its mechanisms of toxicity, including the extent to which BPA may disrupt the transcription and DNA methylation profiles for candidate genes identified in chapter 2. Overall, this chapter reports evidence that BPA caused significant disruption to reproduction in breeding zebrafish exposed to 1 mg/L BPA, likely via oestrogenic mechanisms. The potential for BPA to cause disruption of reproduction shown here supports that reported in previous studies [1–3], and raises concerns for its toxicity when organisms are exposed to BPA in the environment. This is even more pertinent in systems affected by other stressors including other environmental endocrine disruptors with similar mechanism of action that may act combination to cause reproductive disruption. Importantly, BPA also caused significant alterations in the transcription of a number of genes involved in epigenetic regulation in both hepatic and gonadal tissues, most notably a suppression in *dnmt1* transcription, which occurred in conjunction with decreases in global DNA methylation. Of note, some changes

were observed after exposure to environmentally-relevant concentrations of BPA (0.01 mg/L), corresponding to current exposure scenarios for both humans and for wildlife. These findings provide evidence of the adverse effects of BPA in a model vertebrate, and advocate for its replacement within consumer products and its reduction in the environment.

This thesis also aimed to explore the role of pre-exposure to a model chemical shown to interact with the epigenome on responses in future exposure scenarios, later in life or subsequent generations (Chapters 4 and 5). For both embryos (1-217 hpf) and adult fish, pre-exposure resulted in increased copper accumulation in the gills in subsequent exposure scenarios. However, while in adult fish just 14 days in clean water was sufficient to fully depurate copper accumulated as a result of pre-exposure, when pre-exposure was performed during embryogenesis fish pre-exposed to copper had significantly greater copper concentrations in their gills, liver and muscle after 9 months in clean water (Chapter 4). Taken together, these studies strongly suggest that embryogenesis represents a critical developmental window, in which pre-exposure to copper can elicit differential copper accumulation in later life, in a process likely mediated by altered regulation of copper uptake, transport, storage or excretion.

Chapter 4 also reports that adult fish who have received prior exposure to copper elicit a reduced transcriptional response in their gills upon re-exposure, when compared to naïve fish, suggesting that prior exposure could result in an acclimation response. Furthermore, pre-exposure to copper during embryogenesis was associated with significant increases in copper tolerance in future generations (Chapter 5). These data support the hypothesis that exposure to low levels of copper has the potential to alter responses to copper in subsequent exposure scenarios in a vertebrate model and that this effect is heritable in

subsequent generations. This suggests that wild fish populations are uniquely adapted to their local environment and supports the idea that local populations need to be locally managed given their unique physiology that is adapted to their local environment. These data also highlight the importance of considering exposure history when utilizing fish in toxicity testing which may be used for regulatory decisions and to protect wild populations.

The data generated in chapter 5 of this thesis raises a number of important questions including; how many generations can the differential susceptibility induced by the initial exposure be maintained in the absence of re-exposure? Are these fish likely to be more tolerant to other pollutants, particularly other metals which utilize similar uptake, transport, storage and excretion routes and/or have similar mechanisms of toxicity? Are these effects conserved across vertebrates? And is the ability of animals to increase their tolerance to copper following embryonic exposure conserved for other stressors?

In order to facilitate the effective management of wild populations and the use and release of chemicals into the environment, it is essential to understand the toxic effects likely to occur as a result of chemical exposure in wild populations and the extent to which prior exposure can influence responses in future exposure scenarios. These data in turn can contribute to our understanding of mechanisms of chemical toxicity, the extent to which chemicals may interact with the (epi) genome and result in differential susceptibility upon re-exposure, both within the life span of an organism or in future generations. This is particularly important considering that the amount of chemical contamination entering aquatic systems is projected to continue to increase, resulting in the increased likelihood of exposure to chemicals in both wild populations and humans.

This discussion will give a detailed view of how the research undertaken in this thesis helps in the understanding of these issues, discusses limitations and technical challenges of this work, and provides a critical synopsis of research priorities within these topics.

6.2 Choice of model species for research on responses to environmental pollutants

Choosing fish models for experiments exploring responses to environmental pollutants presents a number of challenges. Where studies aim to shed light on mechanism of chemical toxicity, it is essential that molecular tools are available for the chosen species, and the availability of a reference genome greatly facilitates mechanistic research. In addition, model species should have the characteristics necessary to allow for predictions to be made across other teleost and vertebrate species, of relevance to the species and ecosystems requiring protection or management.

In the studies presented in this thesis, the zebrafish was used in order to explore the effects of BPA exposure on reproduction in fish, and in order to shed light on the transcriptomic and epigenetic mechanisms likely to be targets of BPA toxicity. The zebrafish represent a powerful model for reproductive studies, due to their small size, ease of culture in the lab, and daily spawning activity, making the quantification of reproductive endpoints relatively easy. In addition, the zebrafish has a well annotated genome enabling the employment of targeted molecular tools such as RT-qPCR and bisulfite pyrosequencing. Previous studies have also suggested that the DNA methylation signature of the zebrafish genome is

comparable to that of mammalian genomes [4], making the zebrafish an attractive model to study the potential for chemicals to altered DNA methylation in vertebrates [5].

However, while a powerful model for exploring mechanistic endpoints, there are a number of limitations to using zebrafish as an environmentally relevant model. For example, as chapter 2 in this thesis highlights, little is known regarding the genetic regulation of sex determination, differentiation and maintenance in zebrafish, and to date no true conserved sex chromosomes have been identified for lab strains of zebrafish [6–8]. Despite this the zebrafish is a well-established model for the testing of chemicals, including those suspected to target reproductive pathways. RADseq studies in wild fish identified a single sex-linked region on chromosome 4, however for laboratory strains sex-linked loci were not detectable [8]. Taken together, these facts highlight the need for more fundamental research to be conducted on zebrafish in order to facilitate the design and interpretation of toxicology experiments to inform on environmental management and legislation. There is a need to generate fundamental data regarding sex determination, differentiation and maintenance in zebrafish fish and to conduct experiments such as those described in chapter 3 of this thesis in environmentally relevant models in order to inform the management of wild populations.

For studies investigating the potential for exposure to environmental pollutants to result in the inheritance of differential susceptibility, or the inheritance of epigenetic modifications, exposure to the toxicant of interest needs to occur during epigenetic reprogramming, a developmental window which is hypothesized to be critical for development and in influencing responses in later life. This, together with the requirements of conducting experiments over a number of generations places practical considerations on the choice of model organism, given that exposure must be initiated a precise time before completion of

the first cell division, and the life cycle needs to be sufficiently short to allow for multigenerational experiments to be conducted within a reasonable time frame. At the same time, model species are also required to be relatively easy to culture in the lab and/or allow for field based studies. For these reasons the three-spined stickleback was used as a model organism in chapter 5, as it meets all these criteria.

The research presented in this thesis demonstrates that understanding the exposure history of populations used in the lab for toxicity testing is essential in order to avoid the misinterpretation of data. In the stickleback studies presented here, I found that the prior exposure of both adult and embryo stickleback resulted in differential responses in future exposure scenarios, highlighting the potential for significant variation in susceptibility in the wild. These data suggest that each wild population is uniquely adapted to its local environment, highlighting the need to take considerable caution when designing management strategies regarding the release of chemicals into the environment and the management of re-stocking initiatives based on a single test population. In order to determine if the conclusions of my studies can be generalised and applied to other species, there is a significant research need to test if the same effects would be observed in other model species including in mammalian models, and to broaden these studies for other representative environmental chemicals.

In order to utilize model species to inform toxicity testing, it is useful in the first place to have a basic knowledge of how the systems expected to be targets of toxicity function under normal conditions. For fish models, studies exploring the potential for chemicals to interact with the epigenome are still relatively new, with the vast majority of genome wide studies being performed only in the zebrafish. A wealth of knowledge exists regarding the

epigenetic profiling of zebrafish, including DNA methylation and chromatin structure [5,9]. In particular, a number of studies have focussed on epigenetic reprogramming events described to occur immediately after fertilisation in the developing zebrafish embryo, representing exceptionally useful tools in order to aid the design of epigenetic focussed studies in the zebrafish [10,11]. To date very few studies have been carried out in order to profile the epigenome in stickleback and other fish species beyond the zebrafish [12–14], with no studies to date describing epigenetic reprogramming events in the stickleback. This represents a significant research priority in order to enable its use in environmental epigenomics.

In the present thesis, studies exploring the role of pre-exposure to copper on differential responses in subsequent exposure scenarios were conducted in the stickleback, owing to its environmental relevance and ease of culture via IVF to begin exposures from the 1 cell stage. However, there is a significant need to generate vital baseline data regarding basal epigenetic dynamics in sticklebacks and other fish species, including documenting reprogramming events, particularly for species which represent highly environmentally relevant models.

In mammalian models a second epigenetic reprogramming event occurring during germ cell development has been described, however to date studies in fish have not described a similar germ cell reprogramming event. In mammalian models, it has been hypothesized that true transgenerational epigenetic inheritance is likely to be rare, due to the requirement for alterations in epigenetic profiles in the germ line to be sustained across two reprogramming events per generation [15], germ cell reprogramming, and reprogramming immediately after fertilisation. This further highlights the need to generate more

fundamental data regarding epigenetic dynamics in fish models in order to inform the testing of potential transgenerational effects.

In addition, in this thesis Biomart was used to identify zebrafish (*Danio rerio*) orthologues for functional annotation analysis in chapter 4. This was performed due to the lack of functional annotation tools available for the stickleback, and while orthologues have been identified for the vast majority of genes between these species, there are a number of limitations associated with performing functional analysis using orthologue gene IDs from another species. For example, orthologues are not available for all genes and in some cases multiple orthologues are available resulting in a loss of information. The drawbacks associated with using models for which fewer tools have been developed highlight the need to consider model species limitations when interpreting data.

6.3 Considerations regarding the use of tissues containing multiple cell types in transcriptomic and epigenetic studies

To date, most studies exploring the role of chemical pollutants on transcriptomic and epigenetic endpoints both in mammalian and fish models conduct molecular measurements in complex tissues comprised of multiple cell types, providing averages across large numbers of cells, where each cell population is likely to be characterized by its own unique transcriptomic and epigenetic signature even when derived from an apparently homogeneous population [16]. In addition, cellular heterogeneity within cell populations is known to be widespread, and is likely to be driven by the differing biological roles of sub-populations of cells within a tissue, and variations in the cell cycle stages of individual cells within functional sub-populations [17].

This is emphasised in chapter 2, where comparisons of global DNA methylation between males and females revealed that the DNA in the testes was significantly hyper-methylated compared to that in the ovaries. I hypothesised that this was explained by the relative proportion of germ cells to somatic cells in oviparous animals differing in the male and female gonads, with testis containing a far greater proportion of maturing germ cells, including sperm, compared to ovaries, which contain large oocytes surrounded by many somatic cells [18–20]. The high levels of global DNA methylation in testes compared to ovaries, therefore, could be due to high proportion of maturing gametes, including mature sperm which are known to be hyper-methylated across vertebrates [10,21].

In the same study, the *amh* promotor region analysed was significantly hyper-methylated by 24.26% in testes compared to ovaries, despite the fact that transcription of *amh* is higher in males. In many cases correlations have been reported between transcriptional repression and methylation but causation has not been demonstrated to date [22]. *amh* is expressed predominantly in the Sertoli cells which make up a low proportion of the cells present in a whole zebrafish testis. Therefore, when conducting DNA methylation profiling on whole testis samples it is likely that the hypermethylated germ cells would have masked the DNA methylation profiles present in Sertoli cells, therefore it is not straight forward to draw conclusions regarding the epigenetic regulation of *amh* expression when measuring transcription and DNA methylation in whole testis samples. However, correlations were still observed for two of the three CpG sites measures between transcript expression and specific CpG loci methylation. These results highlight the issues of conducting molecular mechanistic studies in tissues with multiple cell types, each with unique transcriptome and DNA methylation patterns, where changes in DNA methylation on a specific cell type may not reflect the dynamics of the methylome in other cell types.

Recent technological advances promise to overcome this limitation. Pioneering single-cell assays are now available for genome, epigenome, transcriptome, proteome, and metabolome profiling [16]. Studies are now able to accurately analyse the mechanism by which transcriptome, genome and DNA methylome regulate each other by conducing measurements within the same single cell [23]. The major challenge of this approach is obtaining enough material with which to perform genome wide analysis. However, recent advances in cell sorting techniques such as the Fluidigm C1 microfluidic system allow for the isolation of individual cells, in addition to sorting cells into sub-populations in order to perform sorted cell genomics in future studies such as that described in chapter 2 of this thesis.

A further limitation to these techniques is their relatively high cost. However, as sequencing costs continue to fall, it is becoming more realistic to design and conduct studies using either sorted or single cells.

6.4 Epigenetic mechanisms in response to environmental pollutants and recent advanced in sequencing technologies

Throughout the thesis, a number of molecular techniques have been utilised in order to identify changes in transcription and DNA methylation associated with exposure to BPA or copper. RT-qPCR was used as a method to investigate the transcriptional profile of target genes hypothesised to be involved in the regulation of reproduction and to be targets of BPA toxicity (Chapters 2 and 3). RT-qPCR is a frequently used method in ecotoxicology, due to the fact that assays for target genes can be designed and accurately validated, and it can be relatively quick and inexpensive to perform analysis of selected genes. This targeted

approach is ideal for investigating specific hypotheses regarding the mechanisms of toxicity of a given chemical.

RNA-seq was employed in chapter 4, to perform genome-wide measurements of transcription, providing a comprehensive assessment of the molecular mechanisms of toxicity of a given chemical, including the potential for revealing novel or unexpected target genes and biomarkers of exposure. The generation of mechanistic data can aid in the development of predictive models (such as adverse outcome pathways [24]), which can inform regulation and management.

Bisulfite pyro sequencing was used to chapters 2 and 3 to investigate the DNA methylation profiles in the promotor region of target genes hypothesised to be involved in the regulation of reproduction and to be targets of BPA toxicity. This technique allows for detailed and high resolution analysis of DNA methylation at specific genomic regions, and is frequently used in diagnostic medicine. In addition, the LUMA assay was performed (Chapters 2 and 3), a method which utilises a luminometric technology to quantify methylation sensitive and insensitive restriction digestions in order to analyse global genomic DNA methylation.

To date, the majority of examples of studies exploring the role of chemical contaminants and environmental stressors on the epigenome in fish, including those presented in this thesis have focussed on identifying changes in transcription and DNA methylation. This choice of endpoint has likely been driven by the ease of its analysis relative to other epigenetic modifications. While these studies and the data generated in this thesis make a significant contribution to the literature, some studies, particularly in mammalian models, have identified alternative potential epigenetic targets of stressors and highlight a significant research need to document if these effects are conserved in fish models.

For example, recent advances in bisulfite treatment technologies now allow for the simultaneous analysis of genome wide 5-methlcytosine (5mC), 5-hydroxymethlcytosine (5hmC) and 5-formylcytosine (5fC) DNA methylation [25–27], shown to be play important roles in regulatory and developmental processes [25,27–29]. Previous bisulfite conversion assays, such as that which is used in the present thesis, cannot distinguish between 5mC and 5hmC DNA methylation. 5mC and 5hmC DNA methylation have very different functions within the cell, and their presence in the genome is significantly influenced by developmental stage and cell type [30–33].

Few studies have been performed to measure 5fC to date, however there has been a significant research effort in mammalian models into the potential for 5hmC to play an important role in early life development, ageing, disorders of the human brain, and studies have found 5hmC to be preferentially enriched in the gene bodies of expressed genes, with 5hmC levels of many protein-coding genes being positively correlated with transcription [30–33]. DNA methylation is dynamic [34], particularly during development and the onset of disease phenotypes where methylation patterns are subject to sweeping changes [22]. Given their emerging roles in health and disease, there is a significant research need in order to profile and quantify 5hmC and 5fC dynamics in fish models in order to assess the extent to which these modifications may be targets of chemical contaminants.

The data generated in chapters 4 and 5 of this thesis highlight the potential for differential responses to copper to be induced in stickleback exposed both during adult life and embryogenesis, in the absence of continuous exposure or genetic selection. These data strongly suggest that the exposure history and local environment of each wild population is likely to drive acclimation responses which are not driven by genetic selection. This thesis

hypothesized that these differential responses may be regulated, at least in part, via epigenetic mechanisms. It is hypothesized that epigenetic modifications are likely to occur at a much faster rate than genetic mutations, and a high level of genome-wide epigenetic divergence between individuals occupying distinct habitats is predicted [13]. New advantages in the techniques such as bisulfite-converted restriction site associated DNA sequencing (bsRAD-seq) now allows for the quantification of the level of DNA methylation differentiation across multiple individuals. This technology and associated bioinformatics pipeline accurately distinguishes between SNPs and methylation polymorphism (SMPs), and provides the opportunity for genome-wide epigenetic investigations of ecological relevance in non-model species, with or without a reference genome [13]. Advances in molecular techniques such as this, provide essential tools in order to allow environmental researchers to begin to shed light on the relative contributions of genetic and epigenetic changes in modulating acclimation of fish species to their unique environment.

Recent advances in Nanopore technology now offer the possibility of a label-free, single-molecule approach to measuring DNA modifications [35], avoiding disadvantages such as short read lengths and amplification biases associated with short read sequencing technologies, such as Illumina, 454 and Ion Torrent [36]. This sequencing technology allows for the direct detection of modified nucleotides in the DNA sequence, including N6-methyladenine, 5-methylcytosine and 5-hydroxymethylcytosine [37], which can ordinarily only be inferred, and bypasses the usual necessity of enrichment or chemical conversion. In addition, PacBIO's single molecule, real-time sequencing technology (SMRT), can also be used to identify RNA base modifications. SMRT sequencing opens a novel window for describing DNA and RNA modification in fish and other vertebrates which may have a dramatic effect on our

understanding of their biology and the toxic effects of chemical pollutants associated with disruption of epigenetic processes.

In addition to DNA methylation, studies in mammalian models have highlighted the potential for contaminants such as BPA and copper to target chromatin structure via the modification of histones [38–40]. Advances in a number of techniques have allowed for the accurate quantification of histone modifications and changes in chromatin structure. For example, Chromatin immunoprecipitation followed by sequencing (ChIP-seq) is a well-established method able to detect protein/DNA-binding interactions and histone modification sites across an entire genome [41,42]. However, a limitation of ChIP-seq analysis is the requirement for large amounts of starting material (~10^5 cells) [43]. More recent techniques such as Assay for Transposase-Accessible Chromatin with high throughput sequencing (ATAC-seq), enable the examination of open chromatin regions, where active regulatory elements such as enhancers, promoters or insulators are found, which may be targets of chemical toxicity [44]. In addition, this technique has low input requirements, and as few as 50,000 cells are sufficient for this technique.

A further recent technique designed to allow for the accurate detection of chromatin interactions in the nucleus is the chromatic conformation capture technique, Hi-C seq [45]. Ligation products contain information of not only where they originated from in the genomic sequence but also where they reside, physically, in the 3D organization of the genome [45]. Using this method, researchers are able to explore the biophysical properties of chromatin as well as the implications of chemical induced changes in chromatin structure for the biological functions of the nucleus [45]. These advances in chromatin sequencing techniques, coupled with the continually falling cost of genome wide sequencing provide

significant opportunity for ecotoxicological studies to begin to generate a more mechanistic understanding of what may be driving chemical toxicity, and the potential for chemical exposure to have consequences for future exposure scenarios in wild populations, mediated by epigenetic mechanisms.

Epigenetic studies to date, particularly those performed in mammalian models and drosophila have also identified the potential for a number of other epigenetic factors to be important in development and disease including RNA modifications and ncRNAs. For example, we now know of more than 150 RNA modifications, often referred to as the 'epitranscriptomics', and the biological functions of a few of these is currently emerging as the consequence of a leap in NGS detection technology [46,47]. The need to document these epigenetic modifications and their potential to be targets of chemical toxicity in fish models is highlighted by the lack of literature on these topics for teleosts.

It is likely that a combination of these epigenetic modifications discussed above may be targets of chemical toxicity. Therefore, there is a significant research opportunity for the community to begin documenting the role of these epigenetic factors in normal development in model organisms and the potential effects of chemicals on the global epigenome, instead of just 5mC DNA methylation which is frequently measured in ecotoxicological studies.

In the data chapters presented in this thesis, a number of molecular endpoints including transcription, global DNA methylation and promotor site specific DNA methylation were employed. However, protein levels were not measured. Proteomics is important to obtaining a complete picture of the biological processes occurring in living cells. The abundance of proteins and their activity within cells is not only regulated at the

transcriptional level, but translational and post-translational regulation are also important contributors to these at the cellular level [48]. A number of studies have reported non-linear relationships between detected mRNA and protein levels [49,50], and therefore in order to have a more complete picture of the mechanisms of chemical toxicity, proteomics should be utilized in ecotoxicological studies. A key example in the present thesis where this could aid in validating the conclusions is in data chapter 4. Adult male stickleback were exposed to 0.02 mg/L Cu for 4 days, followed by 30 days depuration in clean water and a subsequent re-exposure to 0.02 mg/L Cu for 4 days. In the gill, a significantly reduced transcriptomic response was observed upon re-exposure for fish which had been pre-exposed to copper compared to naïve fish exposed under the same conditions. I hypothesized that this decline in the number of differentially expressed genes observed in fish re-exposed to copper may be as a result of an acclimatisation response, perhaps mediated through epigenetic mechanisms. However, without measuring changes at the proteomic level, it is impossible to completely rule out the potential for this decline in the number of differentially expressed genes after a second exposure to copper, to be attributed to altered protein levels still present within the tissues as a direct result of exposure 1.

6.5 Experimental considerations for designing studies to investigate transgenerational inheritance of phenotypic endpoints or epigenetic marks

When designing studies in order to address questions surrounding the role of exposure to a model chemical on phenotypic responses and epigenetic modifications in subsequent generations, a number of considerations must be applied.

In general, examples of differential susceptibility reported in the wild to date are likely to be explained by genetic selection, owing to the fact that concentrations reported in these studies are generally extremely high, and organisms are exposed over multiple generations [51–57]. Studies have also been performed in order to address questions regarding the possibility to induce differential susceptibility in laboratory populations. Many of those studies make valuable contributions toward further understanding the role of genetic selection in establishing differential susceptibility within a population, but do not consider or account for the potential for other factors, such as epigenetics, in the regulation of differential responses.

There are a number of limitations for the studies available in the literature. Firstly, laboratory studies to date have frequently exposed test organisms to extremely high concentrations causing significant mortality rates, with some studies reporting between 20-30% mortality [58,59]. These mortality rates suggest that it is likely that genetic selection is occurring, and the phenotypes observed in future generations are explained, at least in part, by genetic changes. The choice of test concentration can significantly influence the phenotypic results observed. For example, in Schultz *et al*., high concentrations of silver were observed to cause a decrease in tolerance in subsequent generations, while low concentrations resulted in an increase in silver tolerance across generations [59]. Due to the differences in phenotypes observed as a result of these two exposures, this suggests that different molecular mechanisms are at play and supports the need for testing a range of concentrations to understand the dynamics of the effects caused by a given chemical.

While high concentrations and subsequent high mortality levels are likely to influence the relative contribution of genetics to modulating altered susceptibility in future exposure

scenarios, it is also likely that concentration may also influence the relative contribution of epigenetic processes in susceptibility. For example, data presented in chapter 5 of this thesis reports that exposure to an environmentally relevant low concentration of copper, causing less than 1.5% morality, induced a significant increase in tolerance to copper in later life and subsequent generations. These data suggest that in these fish, this low concentration of copper could be having a beneficial effect, stimulating the molecular machinery required to transport and store copper. Other studies which also do not report high mortality rates, but expose to concentrations high enough to cause a number of adverse effects in F0 embryos, often report decreased tolerances in later life or subsequent generations. For example, a recent study in zebrafish, exposed F0 embryos to levels of methylmercury (MeHg) previously shown to cause adverse effects but not to cause high levels of mortality, report these adverse effects in the F1 and F2 generation, associated with changes in DNA methylation [60]. In addition, wild and captive-reared Atlantic salmon (*Salmo salar*) exposed to 50% hypoxia during embryonic development, a level of oxygen known to cause adverse effects in this species, report evidence that while significant mortalities were not caused during the embryonic exposure, there was some evidence that hypoxia tolerance was decreased in later life [61]. Together, these data suggest that choice of concentration is extremely important in the design of studies aimed to address the relative contribution of both genetic and/or epigenetic processes, and in order to utilise such studies to address environmentally relevant questions, concentrations must reflect that which is present in the environment.

In addition, laboratory studies to date often expose test organisms over multiple generations; therefore it is impossible to attribute any observed changes in transcription, epigenetic endpoints or phenotype to the original exposure or to demonstrate heritability.

While the majority of examples of differential susceptibility to environmental stressors observed in wild populations are likely explained by a combination of genetic and epigenetic factors, in order to fully address questions regarding the role of epigenetics in these phenotypes in the absence of genetic influence, studies must control for genetic changes and ensure that test organism are not re-exposed to the toxicant of interest, in order to assess if alterations in a phenotype or epigenetic marks are truly inheritable.

In chapter 5 of this thesis, a single pool of F0 embryos produced from a large founder population were divided randomly between treatment groups; half of the embryos produced were maintained in control water, and the remainder was exposed to 0.01 mg/L Cu from 1-217 hpf. This was done in order to ensure that the genetic makeup of the parental fish would not be a confounding factor in the study, and that copper tolerance observed in this study is unlikely to be driven by differences in the allelic frequencies between fish in the copper pre-exposed and control populations. In addition, a low environmentally relevant copper concentration was chosen, which caused less than 1.5% mortalities. A concentration causing few mortalities was chosen in order to reduce the probability of genetic selection occurring in F0 copper exposed fish. In addition, fish were then maintained under control conditions, without re-exposure. Terminal toxicity testing was performed on F0 adult male fish, F1 and F2 embryos, which were then not returned to the population of origin. In this way, fish were not re-exposed, and differences in phenotype observed in future generations could be directly attributed to the initial exposure alone performed on F0 embryos from 1-217 hpf.

In order to attribute inheritance of differential responses such as those observed in chapter 5 to epigenetic mechanisms in fish, differential epigenetic modifications (such as differential

DNA methylation profiles) need to be detected in the F2 generation, as this represents the first generation that was not directly exposed. In the ecotoxicological community, a large proportion of studies hypothesizing the potential for epigenetic modifications to be driving differential responses over multiple generations do not actually measure epigenetic endpoints. In addition, previous studies have received considerable criticism for not performing population genetic testing, in order to assess the possibility for genetic selection or copper induced mutations to have occurred, and whether findings may be attributed to a combination of genetic and epigenetic changes, rather than the inheritance of epigenetic marks alone [62–65].

A study being conducted in parallel to this thesis, will measure differences in 5mc and 5hmc profiles in F2 embryos from the control and copper pre-exposed populations generated in chapter 5, and test for potential genetic differences between the populations. In this way, it will be possible to precisely identify some of the mechanisms driving the inherited phenotype observed in this study, and the likelihood of non-genetic acclimation such as epigenetic modifications, or genetic adaptation driving this phenomenon. This study will describe 5hmc profiles in the stickleback for the first time. In addition, together with the phenotypic data presented in this thesis, this data will begin to explore the hypothesis that changes in methylation patterns may be driving the inheritance of the differential tolerance to copper observed.

In addition to identifying the presence of differential epigenetic profiles, the gold standard for studies exploring potential examples of epigenetic inheritance would be to test for the presence of epigenetic modifications within the gametes themselves. In this way, it may be possible to shed light on the relative contributions of maternal and paternal factors in

inheritance. There is an increasing body of literature suggesting the potential for significant maternal contributions to the transmission of a phenotype to future generations [66–68]. However, recent studies on a number of contaminants, nutrition, and lifestyle-related conditions in mammalian models also suggest that paternal inheritance may play a significant role [69]. It would be, therefore, interesting to document the epigenetic landscape of the gametes when establishing the exact mechanisms of epigenetic inheritance, to isolate the role or the maternal and paternal epigenome to epigenetic inheritance.

6.6 Conclusions

The data presented in this thesis, in conjunction with data generated from other labs around the world in the last few years has started to deliver a valuable contribution to our understanding of the potential for chemical pollutants to interact with the epigenome. The papers presented in this thesis report evidence that two different chemicals, in two different model species can cause changes at both the transcriptomic and potentially also at the epigenetic level. In addition, the findings highlight the potential for chemical exposures during development to induce inheritable phenotypes, potentially mediated via epigenetic processes.

The mechanisms mediating differential responses to the copper in future generations reported in this thesis remain unclear and the techniques currently available to measure epigenetic endpoints present a number of challenges; rapid advances in sequencing technologies means that tools to measure epigenetic endpoints are becoming more accessible. This increased accessibility provides significant opportunity for future research to approach ecotoxicology from a genome wide mechanistic perspective, generating further understanding of the transcriptomic and epigenetic effects of chemical toxicity and tolerance in fish, and other vertebrate models.

These data highlight the fact that individual wild populations are likely to be highly unique in their responses to chemical contaminants as a consequence of the unique environment in which they have undergone development, including vulnerable developmental stages such as during embryogenesis. As a result these data raise a number of important questions regarding the development of strategies to manage wild populations and the formulation of legislation. My findings point towards the possibility that effective population management

will likely require an in-depth understanding of the exposure history of a given population in order to manage restocking initiatives, and to inform conclusions drawn from toxicity testing studies conducted using individuals originating from wild populations. In addition, these data suggest that it is likely that both epigenetic and genetic changes can contribute to the adaptation of individual populations to their local environment.

In addition to fish, other vertebrates including humans, have been shown to be exposed to the chemicals tested in this thesis. Therefore, this highlights the potential for these chemicals to also cause toxic effects in humans, potentially via epi (genetic) mechanisms, and advocate the testing of the potential for inheritable phenotypes, such as those described in this thesis, to occur in mammalian models.

Finally, the data generated in this thesis, highlight that in order to manage wild populations effectively, and to produce robust data of use for the protection of the environment and human health, studies should employ suitable methodologies, appropriate model organisms, thorough experimental design, and be conscious of the exposure history of test populations and the influence this may have on results obtained from subsequent toxicity testing.

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Chapter 7

Appendix

R code

This section provides the R code written for the purpose of this thesis where default parameters or plotting packages were not used.

Chapter 3: *Bisphenol A causes reproductive toxicity, decreases dnmt1 transcription and reduces global DNA methylation in breeding zebrafish.*

Mixed effects linear modelling of egg production data (Figure 1A):

```
# install required packages
Install.packages("lmer")
Install.packages("car")

# perform MAM testing to identify the minimum adequate model (MAM)
# testing tank as a random effect
Model_A<-lmer(number_of_eggs ~ treatment  + day + (1|tank),
data=eggs_f_day, REML = TRUE, na.action = na.omit)

Anova(Model_A, type="II")
#Anova() is for type 2 repeated measure test. This is part of the 'car' package


# Model A null : day excluded
Model_A_null<-lmer(number_of_eggs ~ treatment  +  (1|tank), data=eggs_f_day,
REML = TRUE, na.action = na.omit)


#compare models
anova(Model_A, Model_A_null, test="LRT")

# continue in this way until the minimum adequate model (MAM) is identified

# Post hoc testing on MAM
summary(glht(Model_MAM, linfct=mcp(treatment="Tukey")))
```

Regression analysis of fertilisation success (Figure 1B):

```
#Regression analysis
#load packages
library(foreign)
library (MASS)


# Repeat  analysis for each treatment group
lm_output_control<-lm(RAW_DATA$day~RAW_DATA$control,data=RAW_DATA)
summary(lm_output_control)
```

Principal component analysis (Figure 3):

```
# log transform
log.ir <- log(data[, 2:13]) # expression levels for all genes
ir.gene <- data[, 1] # treatment groups

ir.pca <- prcomp(log.ir, na.action=na.omit,
             center = TRUE,
             scale. = TRUE)

# plot method
plot(ir.pca, type = "l")
biplot(ir.pca)
# summary method
summary(ir.pca)

# Predict PCs
predict(ir.pca,
      newdata=tail(log.ir, 2))

# load packages
library("devtools")
library("ggbiplot")

g <- ggbiplot(ir.pca, obs.scale = 0, var.scale = 0,
           groups = ir.gene, ellipse = TRUE,varname.adjust = 1.5,
           var.axes =  FALSE,
           circle = FALSE)
g <- g + scale_color_discrete(name = '') +
  geom_point(aes(colour=ir.gene), size = 2.8) +
  theme_bw()
g <- g + theme(legend.direction = 'horizontal',
            legend.position = 'top')
print(g)
```

Binomial generalised linear modelling of genes where amplification was detected in less than 70% of individuals:

```
# perform MAM testing to identify the minimum adequate model (MAM) for presence vs absence of signal. Repeat for each gene.

model1<-glm(pres~treatment,family=binomial)
summary(model1)

model1_null<-glm(pres~1,family=binomial)
summary(model1_null)

anova(model1, model1_null, test="Chisq")

# continue in this way until the MAM is identified.
```

Chapter 4: *Pre-exposure to copper caused a reduced transcriptional response and increased copper accumulation upon re-exposure in adult male three-spined stickleback (Gasterosteus aculeatus).*

Analysis of variance models to test the effect of multiple independent variables (including exposure concentration for exposure 1 and for exposure 2) on morphometric endpoints and tissue metal concentrations:

```
#perform MAM testing to identify the minimum adequate model (MAM). Repeat for all endpoints

model1<-aov(X~Treatment*Population,data=Fork.length)
anova(model1)
TukeyHSD(model1)

model2<-aov(X~Treatment+Population,data=Fork.length)
anova(model2)
TukeyHSD(model2)

anova(model1,model2,test="LRT")

# continue in this way until the MAM is identified.
```

Chapter 5: *Exposure to copper during embryogenesis caused a differential response to copper in later life and increased tolerance in subsequent generations, in a fish model.*

Analysis of variance models to test the effect of multiple independent variables (including exposure concentration and if prior exposure had occurred in F0 embryos) on morphometric endpoints and tissue metal concentrations in adult fish:

```
#perform MAM testing to identify the minimum adequate model (MAM). Repeat
for all endpoints

model1<-aov(X~Treatment*Population,data=Fork.length)
anova(model1)
TukeyHSD(model1)

model2<-aov(X~Treatment+Population,data=Fork.length)
anova(model2)
TukeyHSD(model2)

anova(model1,model2,test="LRT")

# continue in this way until the MAM is identified.
```

General linear modelling to test the effect of pre-exposure on survival and hatching success in F1 and F2 embryos:

```
#perform MAM testing to identify the minimum adequate model (MAM). Repeat
for all endpoints and time points.

Model_1<-glm(y~Cu*Pop,binomial)
summary(Model_1)

Model_2<-glm(y~Cu+Pop,binomial)
summary(Model_2)

anova(Model_1,Model_2,test="LRT")

# continue in this way until the MAM is identified.
```

List of Publications

- Uren Webster, T. M., **Laing, L. V.,** Florance, H. & Santos, E. M. 2014. **Effects of glyphosate and its formulation, Roundup, on reproduction in zebrafish (*Danio rerio*).** *Environmental Science & Technology* 48, 1271-1279, Citations: 33, Impact Factor: 5.330.

- **Laing, L. V.,** Viana, J., Dempster, E. L., Trznadel, M., Trunkfield, L. A., Uren Webster, T. M., van Aerle, R., Paull, G. C., Wilson, R. J., Mill, J. & Santos, E. M. 2016. **Bisphenol A causes reproductive toxicity, decreases *dnmt1* transcription and reduces global DNA methylation in breeding zebrafish (*Danio rerio*).** *Epigenetics* 11, 526-538, Citations: 18, Impact Factor: 4.780.

- **Laing, L. V.,** Viana, J., Dempster, E. L., Trunkfield, L. A., Uren Webster, T. M., van Aerle, R., Paull, G. C., Mill, J. & Santos, E. M. **Sex-specific transcription and methylation profiles for reproductive and epigenetic genes in the livers and gonads of breeding zebrafish.** *Under Review - Epigenetics*, Impact Factor: 4.780.

List of Conference Presentations

- **Lauren V. Laing**, Jennifer A. Fitzgerald, R. van Aerle, J. Mill, Eduarda M. Santos. **Exposure to copper during embryogenesis caused increased tolerance in subsequent generations, in the Three-spined stickleback.** Invited oral presentation – FSBI Fellow Competition, American Fisheries Society 147th Annual Meeting, 20-24th August 2017, Florida.

- **Lauren V. Laing**, Jennifer A. Fitzgerald, R. van Aerle, J. Mill, Eduarda M. Santos. **Exposure to copper during embryogenesis caused increased tolerance in subsequent generations, in the Three-spined stickleback.** Invited oral presentation, Fisheries Society of British Isles Conference 2017, 3-7 July 2017, Exeter, UK

- **Lauren V. Laing**, Joana Viana, Emma L. Dempster, Maciej Trznadel, Laura Trunkfield, Tamsyn M. Uren Webster, Ronny van Aerle, Gregory Paull, Robert Wilson, Jonathan Mill and Eduarda M. Santos. **Bisphenol A (BPA) causes reproductive toxicity, decreased *dnmt1* transcription, and reduces global DNA methylation in breeding zebrafish (*Danio rerio*).** Poster presentation, Fisheries Society of British Isles Conference 2017, 3-7 July 2017, Exeter, UK.

- **Lauren V. Laing**, Joana Viana, Emma L. Dempster, Maciej Trznadel, Laura Trunkfield, Tamsyn M. Uren Webster, Ronny van Aerle, Gregory Paull, Robert Wilson, Jonathan Mill and Eduarda M. Santos. **Transcriptional and epigenetic responses to Bisphenol A in breeding zebrafish.** Poster presentation, Abcam: Chromatin and epigenetics: from mechanism to function, 5-7 April 2017, Munich, Germany.

- **Lauren V. Laing. Mechanisms of genetic and epigenetic responses to industrial pollutants in fish.** Invited seminar, 7th February 2017, Institute of Experimental Genetics, Helmholtz Zentrum, Munich.

- **Lauren V. Laing**, Jennifer A. Fitzgerald, Robert J. Wilson, Eduarda M. Santos. **Exposure to copper during embryogenesis caused increased tolerance in subsequent generations, in the three-spined stickleback.** Oral presentation, SETAC/iEOS Joint Focused Topic Meeting, Environmental and (eco)toxicological Omics and Epigenetics, 12-15 September 2016, Ghent, Belgium.

- **Lauren V. Laing**, Hannah Littler, Viana J, Dempster EL, Trznadel M, Trunkfield LA, Webster TM, Fitzgerald, JA, van Aerle R, Paull GC, Wilson RJ, Mill J, Santos EM. **Transcriptional and epigenetic responses to Bisphenol A in breeding zebrafish.** Poster presentation, SETAC/iEOS Joint Focused Topic Meeting, Environmental and (eco)toxicological Omics and Epigenetics, 12-15 September 2016, Ghent, Belgium.

- European Centre for Ecotoxicology and Toxicology of Chemicals Workshop: The role of Epigenetics in Reproductive Toxicity. Invited participant, November 12-13, 2015, Belgium.

- **Lauren V. Laing**, Joana Viana, Laura Trunkfield, Tamsyn M. Uren Webster, Jonathan Mill and Eduarda M. Santos. **Mechanisms of reproductive toxicity following exposure to Bisphenol A in Zebrafish (*Danio Rerio*).** Invited oral presentation – FSBI Student Representative, Canadian Conference for Fisheries Research, January 8-11, 2015, Canada.

- **Lauren V. Laing**, Joana Viana, Laura Trunkfield, Tamsyn M. Uren Webster, Jonathan Mill and Eduarda M. Santos. **Mechanisms of reproductive toxicity following exposure to Bisphenol A in Zebrafish (*Danio Rerio*).** Poster presentation, International Congress on the Biology of Fish, August 3-7, 2014, UK.

List of Other Presentations

- **Lauren V. Laing. Genome wide cytosine methylation: How to measure it?** Exeter Sequencing Service Seminar Series: Genomics Seminar on Epigenetics. 3rd July 2017, Exeter, UK.

- Eduarda M. Santos, **Lauren V. Laing,** Ronny van Aerle, Jennifer A. Fitzgerald, Nick Bury. **Global transcriptional responses to repeated copper exposures in the three-spined stickleback (*Gasterosteus aculeatus*).** *Oral presentation*, SETAC/iEOS Joint Focused Topic Meeting, Environmental and (eco)toxicological Omics and Epigenetics**,** 12-15 September 2016, Ghent, Belgium.

- **Lauren V. Laing. The use of pyrosequencing in understanding how chemical exposure can influence DNA methylation profiles both globally and at specific genomic regions.** Advanced methods and techniques for environmental research seminar series, 10th August 2016, University of Exeter, UK.

- Eduarda M. Santos, Tamsyn M. Uren Webster, **Lauren V. Laing**, Jennifer A. Fitzgerald. **Molecular mechanisms of reproductive disruption in fish.** Society for Reproduction and Fertility Annual Conference, 11-13 July 2016, Winchester, UK.

- Eduarda M. Santos, Tamsyn M. Uren Webster, **Lauren V. Laing,** Joana Viana, and Jon Mill. UKEMS 38th Annual Conference, Plymouth University, Plymouth, 12th-14th July 2015. Invited Talk.

- Eduarda M. Santos, Tamsyn M. Uren Webster**, Lauren V. Laing**, Jennifer A. Fitzgerald, Victoria Fowler, Ronny van Aerle, Emma L. Dempster, Joana Viana, Robert J. Wilson, Jonathan Mill. **Using genomics to determine the mechanisms of toxicity of environmental stressors in fish.** Annual Main Meeting of the Society of Biology (SEB), 30th June-3rd July 2015, Prague, Czech Republic. Oral presentation.

- Eduarda M. Santos, Tamsyn M. Uren Webster**, Lauren V, Laing**, Joana Viana, and Jon Mill. **Using genomics to understand the mechanisms of chemical toxicity in fish model systems.** SETAC Europe 25th Annual Meeting, Barcelona, Spain, May, 2015. Oral presentation.

- Eduarda M. Santos et al. **Epigenetics and systems biology in endocrine disruption. Workshop on Molecular Mechanisms of Radiation Toxicity at Chronic Low Dose Levels** - Dec 2014 - Oxford, UK. Invited oral presentation.

List of Grant Awards

- FSBI Student Exchange Scholarship: American Fisheries Society 147th Annual Meeting– FSBI Student Representative - 20-24th August 2017, Florida.

- FSBI Small Research Grant (£5000): The role of 5-Hydroxymethylcytosine (5hmc) DNA methylation in mediating increased tolerance to copper in three-spined stickleback (*Gasterosteus aculeatus).* February 2017.

- AR Training Workshop (£500): Online engagement: communicating animal research via social media and websites. November 2016.

- FSBI Small Research Grant (£5000): The role of DNA methylation in the differential susceptibility to copper in three-spined stickleback populations exposed during embryonic life. July 2016.

- FSBI Travel Grant (£880): SETAC/iEOS Joint Focused Topic Meeting, Environmental and (eco)toxicological Omics and Epigenetics, 12-15 September 2016, Ghent, Belgium.

- Primer Design Silver Level Sponsorship: February 2016.

- FSBI Student Exchange Scholarship (£1400): Canadian Conference for Fisheries Research – FSBI Student Representative - Ottawa, Canada, January 8-11, 2015.

- NERC Software Carpentry Short Course Fellowship Award (£500): Leeds University, January 2015.

- BBSRC Genotyping by Sequencing Course Grant (£800): The genome analysis centre, Norwich, November 2014.

- EOS Keystone Skills in Bioinformatics Course NERC Fellowship (£1700): CEH Wallingford, February 2014.

- Fisheries Society of the British Isles PhD Studentship Competition: September 2013.