Investigating the non-genetic basis for altered susceptibility to environmental chemicals in fish as a result of exposure history

Submitted by

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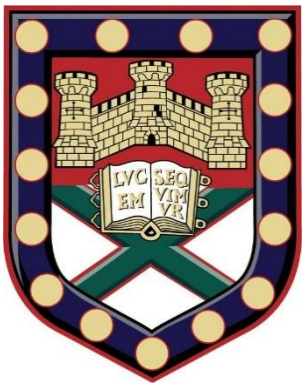

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…….. (Hannah Littler)

Abstract

Wildlife are exposed to a variety of environmental stressors, including toxic chemicals, which often occur intermittently and over long periods of time, driven by fluctuations in discharge and environmental conditions. Survival of wildlife populations is therefore critically dependent upon their ability to adapt to repeated chemical exposures. However, current chemical testing methods for environmental risk assessment generally consider only a single exposure of a previously naïve population of animals, failing to account for altered responses of organisms depending upon their exposure history. Evidence suggests that organisms may be able to acquire tolerance within a generation that persists into later life and subsequent generations, potentially driven by non-genetic mechanisms including epigenetic effects, parental effects, or a combination of these. However, the role of these mechanisms in the response of organisms to intermittent exposure scenarios is not fully understood.

During my PhD, I aimed to investigate whether exposure to common aquatic pollutants would cause altered tolerance to future exposure in later life and in subsequent generations, and explore the potential non-genetic mechanisms responsible. To address this, I used two model fish species – the zebrafish (*Danio rerio*) and the three-spined stickleback (*Gasterosteus aculeatus*) – to examine the molecular and physiological responses of fish following pre-exposure and subsequent re-exposure to a chemical both within and across generations.

First, I investigated the effects of repeated exposure to an environmentally relevant concentration of bisphenol A (BPA) – a ubiquitous endocrine disrupting chemical known to disrupt reproduction and epigenetic regulation – by comparing the response of adult zebrafish and their offspring to BPA following either a single exposure of adults or a pre-exposure followed by a subsequent re-exposure. Although no effects were observed for reproductive output, upon exposure to BPA, transcription of anti-Mullerian hormone (*amh*) – a hormone involved in oocyte maturation – was found to be significantly downregulated in the ovaries of pre-exposed fish compared to naïve fish experiencing BPA exposure for the first time. In addition, embryonic exposure of offspring demonstrated a protective effect of parental pre-exposure, with offspring of naïve parents being significantly more

susceptible to BPA toxicity compared to offspring of pre-exposed parents, which were no more susceptible than offspring of controls. These observations suggest that pre-exposure of adults to BPA led to an altered molecular response upon re-exposure. As well as altering the regulation of amh, I hypothesised that altered physiology of adults likely inferred BPA tolerance to their offspring by modifying the environment in the gonad during gametogenesis.

I then investigated the effects of pre-exposure to copper during embryogenesis on transcriptomic response to re-exposure in later life and on the copper tolerance of subsequent generations. Copper is an essential element that becomes toxic at high concentrations, and is frequently found at toxic levels in freshwater environments. Previous work had shown that pre-exposure of three-spined stickleback to sub lethal copper concentrations during embryogenesis resulted in altered copper storage in tissues in later life despite no further exposure, and caused increased copper uptake in the gill upon re-exposure, indicating lasting physiological changes as a result of historic copper exposure. To investigate this differential physiological response, I conducted RNA-sequencing in the gill of naïve and pre-exposed fish after re-exposure. Both populations exhibited a differential transcriptional response, but this response was more pronounced in naïve fish despite having a lower copper burden in their tissues. The reduced transcriptomic response in pre-exposed fish, suggests that copper caused less disturbance of homeostasis compared to naïve fish. Analysis of differential gene expression suggested that naïve fish experienced greater toxicity upon exposure to copper compared to pre-exposed fish, and may be less able to activate compensatory mechanisms effectively. This included evidence of increased oxidative stress; less effective compensatory transcriptional regulation of ion transporters to prevent ionoregulatory disturbance; and significantly greater cellular proliferation, which may suggest greater incidence of harmful epithelial thickening in the gill. I hypothesised that this differential response may be driven by epigenetic alterations induced by pre-exposure during embryogenesis – which encompasses sensitive windows of epigenetic reprogramming – leading to altered regulation of copper-responsive genes such that they are differentially expressed upon subsequent exposure to copper, inferring increased tolerance.

In addition to an altered response within a generation, previous work also showed that pre-exposure of the F0 generation led to increased copper tolerance in

offspring that was maintained to the F2 generation. In order to investigate the longevity of this effect, I exposed F3 embryos from the naive and pre-exposed populations. In contrast to previous generations, F3 embryos were less tolerant to copper exposure compared to controls. This suggests that different mechanisms may be acting in different generations, potentially including both parental effects and transgenerational epigenetic inheritance. I then repeated the F0 and F1 copper exposures in zebrafish, and found that these effects were not conserved, indicating that the mechanisms underpinning acquisition of copper tolerance differ between species.

These data highlight the potential for populations to exhibit differential responses to chemical stressors depending upon exposure history. This has implications for both chemical risk assessment and wildlife management strategies, such as restocking programmes, which should consider the potential for wild populations to become locally adapted via non-genetic mechanisms over short timescales, including via the epigenome. The emergence of latent toxic effects potentially caused by transgenerational epigenetic inheritance also advocates a need to test for potential multigenerational effects in chemical assessment that are not currently routinely investigated.

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List of Accompanying Materials

1. **Supporting information file 1 – Chapter 3 Reference Transcriptome Full Gene Description and GO Annotations.xlsx**
   A full list of gene descriptions and gene ontology annotations for the reference transcriptome used for RNA sequencing analysis in chapter 3.
2. **Supporting information file 2 – Chapter 3 Annotated DEGs.xlsx**
   A full list of differentially expressed genes, fold changes and FDR values for all comparisons conducted during RNA sequencing analysis described in chapter 3.
3. **Supporting information file 3 – Chapter 3 GO Annotations.xlsx**
   A complete list of gene ontology terms and FDR values separated by GO category for comparisons conducted during gene ontology analysis described in chapter 3.

List of General Abbreviations

ADP	Adenosine diphosphate	GR	Glucocorticoid receptor
ahcy	Adenosylhomocysteinase	GSH	Gluthatione
amh	Anti-Mullerian hormone	GSS	Glutathione synthetase
ANT1	ADP/ATP translocase 1	GSSG	Glutathione disulfide
AR	Androgen receptor	H	Hydrogen
atox1	antioxidant protein 1	HCO_3^-	Biocarbonate
ATP	Adenosine triphosphate	heri-1	HERI-1 Chromodomain Protein
atp6	mitochondrial ATP synthase 6	Hoxa10	Homeobox a10
atp7a	ATPase copper transporting alpha	hpf	Hours post fertilisation
atp7b	ATPase copper transporting beta	HPG axis	Hypothalamus-pituitary axis
bhmt	Betaine-homocysteine methyltransferase	HPI axis	Hypothalamus-pituitary-interrenal axis
BLM	Biotic ligand model	HSP	Heat shock protein
BPA	Bisphenol A	IAP	Intracisternal A particle
Ca	Calcium	insra	Insulin receptor alpha
CA	carbonic anhydrase	insrb	Insulin receptor beta
CAT	Catalse	LH	Leutenizing hormone
ccs	superoxide dismutase	MAM	Minimum adequate model
Cd	Cadmium	Mg	Magnesium
cDNA	Copy DNA	miRNA	Micro RNA
Cl^-	Chloride	Mn	Manganese
CO_3^{2-}	Carbonate	MRE	Metal response element
cox17	cytochrome c oxidase chaperone 17	mRNA	Messenger RNA
ctr1	Copper transporter 1	mt	metallothionein
Cu	Copper	Na	Sodium
cyp19a1a	Aromatase	Ni	Nickel
DDT	Dichlorodiphenyltrichloroethane	NMDR	Non-monotonic dose response
dmt1	Divalent metal transporter 1	NO_3^-	Nitrate
DNA	Deoxyribonucleic acid	PAH	Polycyclic aromatic hydrocarbon
dnmt	DNA methyltransferase	Pb	Lead
DOC	Dissolved inorganic carbon	PCB	Polychlorinated bisphenyl
DOHaD	Developmental origins of health and disease	PCNA	Prolfierating nuclear antigen
EDC	Endocrine disrupting chemical	PGC	Primordial germ cell
EE2	Ethinylestradiol	RNA	Ribonucleic acid
ERE	Oestrogen response element	ROS	Reactive oxygen species
ERR	Oestrogen-related receptor	SAM	S-adenosylmethionine
ESR	Oestrogen receptor	slc11a2	Solute carrier family 11 member 2
Fe	Iron	slc31a1	Solute carrier family 31 member 1
FSH	Follicle stimulating hormone	SOD	superoxide dismutase antioxidant
GCL	Glutamate cysteine ligase	SR	Sarcoplasmic reticulum
GGT	Gamma-glutamyltransferase	WWTP	Wastewater treatment plan
GLM	Generalized linear model	ZGA	Zygotic genome activation
GO	Gene ontology	Zn	Zinc

Chapter 1

General Introduction

1. Environmental Stressors and Exposure Scenarios

A wide range of environmental stressors are known to impact the aquatic environment; including organic and inorganic toxic chemicals and stressful abiotic environmental conditions, such as abnormal temperatures, oxygen levels and pH. Release of toxic chemicals of anthropogenic origin is a widespread and growing threat to the environment. Growth in human population, and consequent urbanisation, industrialisation, pharmaceutical developments, and intensive farming, among others, all place a pollution burden on the environment. According to the UN Global Chemicals Outlook II report (2019), production from the global chemical industry almost doubled from 2000 to 2017, and is projected to do so again between 2017 and 2030 (United Nations Environment Programme, 2019). With increased production and consumption, volume of anthropogenic chemicals released into the environment will inevitably increase. This is on top of historic sources of chemicals which continue to pollute, such as disused mines and historic landfill sites that pre-date environmental protection legislation and are awaiting difficult and expensive remediation programmes (Mighanetara *et al.*, 2009; Bird, 2016; Brennan *et al.*, 2016; Brand *et al.*, 2018). As a result, anthropogenic pollutants and adverse abiotic conditions caused by anthropogenic activity have become ubiquitous in the aquatic environment, leading to frequent exposure of wildlife that can occur continuously or intermittently over long periods of time.

Complex intermittent exposure scenarios can pose an additional challenge to organisms and environmental protection strategy. Intermittent exposure to stressors can be driven by both temporal and spatial variation of chemical concentrations or interacting abiotic conditions, and by the behaviour of the organisms themselves.

Temporal variation of anthropogenic pollution is common, linked both with temporal variation in use and discharge of chemicals and seasonal or local weather conditions that alter their release and bioavailability in the environment (Gordon, Mantel and Muller, 2012). A clear example of anthropogenic pollution with strong temporal variation is agricultural pollution. Application of pesticides, for example, is highly seasonal, leading to transient, short-term peaks of pesticides in

agriculturally influenced waters. This is particularly true for insecticides, which generally have faster activity intervals and higher intrinsic toxicity, requiring a shorter application period. It has been estimated that, despite being known to cause considerable harm to ecosystems, they may only be detectable for less than 1% of the year (Stehle, Knäbel and Schulz, 2013; Stehle and Schulz, 2015). Organisms in affected aquatic environments may receive their entire annual exposure to these chemicals within these short exposure windows, with almost a year between exposures (Stehle and Schulz, 2015).

As well as patterns in the use of toxic chemicals, variable weather patterns can have a strong impact on temporal variation of pollutant release and concentration. For example, in regions with long, distinct dry and wet periods, a phenomenon termed the “seasonal first flush” from urban storm water discharge can occur, where long dry periods facilitate a pollutant build up in storm drains which results in a dramatic increase in chemical discharge during the first heavy rainfall event that follows (Lee *et al.*, 2004).

Discharge of chemicals in effluents from wastewater treatment plants (WWTP) is also known to fluctuate seasonally due to varying efficiency of chemical removal. This process is strongly impacted by seasonal variations in temperature, light intensity, and rainfall, which affect degradation rates, occupancy time, organic sorption and dilution of chemicals. For example, a case study of a WWTP in Finland observed a reduction in the elimination efficiency of five common pharmaceuticals in winter, leading to their total concentration in effluent being 3-5 times higher in winter, resulting in higher measured concentrations in the recipient river (Vieno, Tuhkanen and Kronberg, 2005). Several studies have also demonstrated seasonal fluctuations in the concentration of estrogenic compounds and the total oestrogenicity of WWTP effluents (Rodgers-Gray *et al.*, 2000; Hemming *et al.*, 2004; Jin *et al.*, 2008; Olujimi *et al.*, 2012). For example, a study in the UK found that concentrations of steroidal oestrogens were approximately 3 times higher in a study period including colder months from November to March compared to a study period including warmer months from July to December. In contrast, the opposite pattern was seen for synthetic nonylphenolic chemicals which were present at approximately 10 times higher concentrations in the warmer study period from July to December (Rodgers-Gray *et al.*, 2000). Hemming *et al.* (2004) also found correlation between the oestrogenicity of effluents and changes

in the composition of influents due to a 30% increase in population size in the service area of the WWTP following seasonal student enrolment at local universities (Hemming *et al.*, 2004). As well as fluctuations following seasonal patterns, large transient fluctuations in effluent concentrations have been observed after extreme weather events. For example, concentrations of oestrogens in effluent of a WWTP were found to be 7.5 times higher during a drought year compared to a non-drought year (Shore, Gurevitz and Shemesh, 1993).

Environmental concentrations of chemicals from mining and landfill leachate are also known to show high seasonality due to patterns in rainfall and flooding events, which alter chemical concentration by affecting discharge rates, dissolution and dilution of pollutants (Grimshaw, Lewin and Fuge, 1976; Herr and Gray, 1996; Olías *et al.*, 2004; Tränkler *et al.*, 2005; Mangimbulude *et al.*, 2009; Brennan *et al.*, 2016). For example, in a study of seasonal variations in water quality of a river affected by acid mine drainage, Olías *et al.* (2004) found that water quality was highly influenced by seasonal weather conditions, with effects varying for different metals. Autumn rains were found to dissolve metal precipitates and cause an increase in dissolved iron (Fe), zinc (Zn), manganese (Mn), cadmium (Cd) and lead (Pb) concentrations, which were then decreased in winter when intense rains caused increased river flow and dilution of metals. This was followed by an increase in the concentration of most metals in summer when river flow reduced. This was in contrast with copper (Cu), which was present at lower concentrations in summer due to higher co-precipitation with hydroxysulphates than other metals, followed by an increase in winter months when it was re-dissolved (Olías *et al.*, 2004).

As well as affecting the concentration of dissolved metals, seasonal weather conditions can also cause changes in water parameters that impact the bioavailability of chemicals to aquatic organisms, and thus their toxicity. This is particularly relevant to metal pollutants, whose bioavailability and toxicity in aquatic environments is strongly influenced by various interacting water parameters, including temperature, salinity (Na^+, Cl^-), hardness (Ca^{2+}, Mg^{2+}), pH (H^+), alkalinity (HCO_3^-, CO_3^{2-}), dissolved organic carbon (DOC), and other organic and inorganic ligands (Smith, Balistrieri and Todd, 2015). These parameters determine the speciation of metals, affecting the proportion of the metal that exists

in a toxic bioavailable form in the water column, and the proportion that is complexed with organic or inorganic ligands, and is thus unavailable to organisms. They also affect toxic interactions of free metal species at the epithelial surfaces of organisms, such as the gill and gut, by affecting binding strength of metals and competing with them for ion binding sites at transporters and receptors. This moderates the interaction and uptake of metals at the epithelial surfaces and their ability to disrupt transport and regulation of other important ions. These factors can vary greatly according to local and seasonal weather patterns (Gundersen and Steinnes, 2001; Gundersen, Olsvik and Steinnes, 2001). For example, seasonal episodic acidification of streams and lakes is known to occur following snowmelt and heavy episodes of rainfall (Davies *et al.*, 1992). These natural events can also exacerbate the effects of anthropogenic pollution. For example, pollution of environments with acidifying chemicals, such as nitrate (NO_3^-) deposition from air pollution, that is usually chronic in nature can become acute during these events when a build-up of these pollutants on land is released rapidly into waterways during snowmelt and heavy rainfall. (Lepori, Barbieri and Ormerod, 2003). This temporal variability in water parameters can therefore lead to variability in the bioavailability and toxicity of metals in the environment, even where total metal concentration does not change.

An approach frequently used in environmental risk assessment to predict the toxicity of metals to aquatic animals in the context of these environmental factors is using a biotic ligand model (BLM)(Adams *et al.*, 2020). The BLM calculates the combined effects of these interacting factors on the efficiency with which the metal is able to bind to metal-binding sites – biotic ligands – on the gill surface and couples this with *in vivo* toxicity data to predict the toxicity of a metal in a given environmental context (Wood, 2012). When provided with the necessary water parameter data, as well as making site-specific assessments, BLMs can help to address temporal variation in metal bioavailability and toxicity, and allow season-specific assessments (Lathouri and Korre, 2015). However, prediction using BLMs still relies on toxicity data which is usually generated using naïve populations of lab reared organisms, and does not account for differential tolerance of wild populations according to their exposure history (Smith, Balistrieri and Todd, 2015).

There can also be considerable spatial variation of chemical concentration within the aquatic environment. This can be driven by various factors, such as the

distribution of point and diffuse sources of input, which may vary across the length of a river, for example. This spatial variation is then translated into temporal variation as organisms move through that heterogeneous environment, with organisms experiencing intermittent exposure as they move through polluted and unpolluted regions. For example, a study of Bustillos Lagoon in Chihuahua, Mexico by Ochoa-Rivera *et al.* (2017) split the 107km^2 water body into three zones characterised by input originating from agricultural, industrial and poor quality communal land. Analysis of water quality showed clear spatial variability, with statistically significant differences in parameters between the three zones, including: electrical conductivity – explained by differing Na^+ and Cl^- levels; and concentrations of the pesticide, dichlorodiphenyltrichloroethane (DDT), and its metabolites, which were up to 100 times higher in the zone associated with agricultural activity (Ochoa-Rivero *et al.*, 2017). Organisms living within the lagoon could experience differences in exposure of 100-fold depending upon their use of different sections of the water body.

Translation of spatial variation in pollution to temporal variation of exposure can be closely linked with the behaviour of individuals and the ecology of species. For example, species that occupy different habitats at different life stages may experience varying levels of exposure across their lifetime depending upon life stage. This can be across large spatial scales. For example, marine fish can migrate from relatively unpolluted pelagic habitats to use comparatively polluted coastal or riverine spawning grounds which are more vulnerable to anthropogenic pollution (Hendrey, 1987; Ezenwa and Ayinla, 1994; Vasconcelos *et al.*, 2007). It can also occur at small scales, such as species with both benthic and pelagic life stages, which might be exposed to different levels of pollution depending upon a chemical's relative concentrations in the water column and sediment. Animals that recurrently move between polluted and unpolluted sites; for example, an anadromous salmon spawned in a polluted river and repeatedly returning to that site to reproduce year on year; might experience intermittent exposure to the same pollutants across their lifetimes. Even where re-exposure does not occur within a single generation, for example, a species which does not return to habitats occupied in early life stages, but whose offspring recruit to the same nursery habitat; lasting effects of environment during early life of the parent could

influence the physiology of their offspring such that they might respond differently to the same environmental influences.

The prevalence of intermittent exposures in the environment and the unique challenge that they might pose to organisms is an important consideration for environmental protection. The importance of temporal variation in exposure is already somewhat considered in environmental risk assessment, with the effects of exposure duration and life stage on toxic outcomes being recognised (Calow and Forbes, 2003; Eggen *et al.*, 2004; Sumpter and Johnson, 2005). Both chronic toxicity and differential responses to exposure depending upon life stage are now well-represented in the literature, and chronic toxicity testing and early life stage tests have been incorporated into environmental risk assessment (USEPA, 2002; OECD, 2018, 2019). Although this partially addresses the temporal aspect of environmental exposures, this still only considers a simple exposure scenario of a single continuous exposure of a previously naïve individual or population; and omits the potential for organisms to respond differently according to their exposure history under intermittent or repeated exposure scenarios.

2. Exposure history can affect future toxic response

There are several mechanisms by which exposure history of individual organisms and populations can alter their response to environmental exposure in the future. This can include cumulative toxic effects of multiple exposures or processes of sensitisation due to prior exposure, leading to reduced tolerance to future exposures. Alternatively, mechanisms of acclimation or adaptation can occur, leading to increased tolerance in the future.

Altered sensitivity of wildlife to environmental stressors due to the influence of historic exposure has important implications for environmental risk assessment, which does not currently account for differential susceptibility of different populations according to their exposure history, and assumes uniform sensitivity for populations of a particular species upon exposure to a given level or concentration of a stressor for a given duration. It has previously been recognised that toxicity of intermittent exposure and continuous exposure to an equivalent time-averaged dose or peak concentration is not necessarily equal (Handy, 1994). Several studies have explored this idea and suggested methods for inclusion into

risk assessment, but the complexity of modelling time-varying exposures and producing assessment strategies that are applicable to the range of possible scenarios occurring in the environment has proven challenging (Reinert, Giddings and Judd, 2002; Butcher *et al.*, 2006; Ashauer and Brown, 2013).

One mechanism by which toxicity of a stressor to an organism or population can increase upon subsequent exposure is by cumulative effects, such as cumulative damage or bioaccumulation of toxic chemicals. For many chemicals, where toxicity of continuous and intermittent exposure to an equivalent time-averaged dose or peak concentration is found to be equal, cumulative toxic damage or bioaccumulation of chemicals can be accounted for with chronic exposure testing (Angel, Simpson and Jolley, 2010; Amachree, Moody and Handy, 2013). However, altered timing and duration of exposure and recovery periods can result in changes in total toxicity, due to differing effects on toxicokinetics of the chemical and the extent of physiological recovery and chemical depuration reached between exposure periods (Seim *et al.*, 1984; Siddens *et al.*, 1986; Reinert, Giddings and Judd, 2002; Lorenzana *et al.*, 2005; Ashauer and Brown, 2013). Intermittent exposure may also pose an additional challenge due to increased energetic costs of establishing homeostasis under fluctuating conditions. Evidence from chemical acclimation studies showing higher energetic demand early in the acclimation period, indicated by a more severe reduction in growth rate, that become less pronounced with time, suggests that establishing homeostasis upon initial sublethal exposure can be more costly than maintaining it (Dixon and Sprague, 1981), which could result in additional physiological stress under a fluctuating exposure compared to a continuous exposure.

As well as alterations in the timing of exposures, altered physiological response of organisms following previous exposure can also impact comparative total toxicity of intermittent and continuous exposures. For example, increased sensitivity to a chemical as a result of historic exposure is not always additive, and can manifest as sensitisation to a chemical, where response is greater upon re-exposure, resulting in combined toxicity that is greater than would be expected from additive effects. For example, early life exposure of wild roach to the synthetic oestrogen, ethinylestradiol (EE2), was shown to sensitize fish to future exposure, with significantly greater transcriptional response of oestrogen-responsive genes observed in pre-exposed fish upon re-exposure compared to naïve fish (Lange *et*

al., 2009). Similar sensitization as a result of historic exposure to oestrogens was also observed in zebrafish *(Danio rerio)*(Green *et al.*, 2018). Risk assessments of threats posed by synthetic oestrogens, for example, could therefore underestimate the risk posed by exposure to wild populations if previous exposure to exogenous oestrogens is not accounted for.

Exposure to environmental stressors can also result in altered physiology of animals that infers increased tolerance through acclimation or adaptation. These processes are essential for survival in stressful environments. However, their definitions are a subject of debate, and their use varies across scientific fields.

The term adaptation is most commonly associated with its definition in evolutionary biology as the process by which fitness is increased specifically through heritable genetic alterations, with increased fitness through other environmentally-induced changes in phenotype defined as acclimation (Orr, 2005). However, it is commonly used more loosely outside of evolutionary biology to describe any beneficial phenotypic alteration that increases the fitness of an organism or population (Fox *et al.*, 2019; Petitjean *et al.*, 2019; Navarro-Martín, Martyniuk and Mennigen, 2020). It has also been used in the field of epigenetics – the study of inducible changes in gene regulation that are both reversible and mitotically or meiotically heritable, discussed in more detail later in this chapter.

As the study of epigenetics has developed, distinct forms of phenotypic plasticity have been recognised, including phenotypic alterations induced by the environment during gametogenesis or embryogenesis that are stable into later life and transient plasticity induced across the lifetime of individual, dubbed by Beaman and colleagues as developmental plasticity and acclimation, respectively (Beaman, White and Seebacher, 2016). However, the term developmental plasticity implies that stable epigenetic alterations can only be induced during developmental windows, and overlooks the potential for their induction later in life. Stable adaptive epigenetic changes which, though reversible in the long-term, result in adaptive phenotypes that are fixed across the lifetime of an organism – referred to above as developmental plasticity – or those that are potentially stable across multiple generations, are considered by some to lie between irreversible genetic adaptation and other transient forms of physiological acclimation (Kristensen, Ketola and Kronholm, 2020). This has led to use of the term

adaptation to describe these stable epigenetic modifications, either due to more loose application of the term or as a way to better express the stability that distinguishes them from the transience of other inducible changes in gene regulation that the term acclimation implies (Devaskar and Raychaudhuri, 2007).

Where there is any ambiguity around terms, they should be clearly defined in the context they are being used. In this thesis it is necessary to clearly distinguish between transient and stable changes in gene regulation, and so, in the absence of universally agreed terms, I will use the term “epigenetic adaptation” to refer to stable epigenetic alterations that increase the fitness of an individual or population. This is distinct from transient alterations in gene regulation which require continuous stimulation from an environmental signal, which I will term “acclimation". Where genetic adaptation is discussed, this will be specified.

Acclimation to a sublethal exposure for a range of environmental chemicals and stressful conditions has been shown to increase tolerance upon subsequent acute exposure in numerous species. For example, acclimation has been observed for stressful abiotic conditions such as thermal and osmotic stress (Sangiao-Alvarellos *et al.*, 2005; Logan and Somero, 2010; Windisch *et al.*, 2011; Whitehead *et al.*, 2012). For toxic chemicals, it is most commonly seen in exposure to essential metals that are toxic when concentrations exceed the threshold for homeostasis, such as copper and zinc, or non-essential naturally occurring metals which mimic them.

Various studies have demonstrated increased tolerance in fish and aquatic invertebrates following acclimation to metals, including: cadmium (Benson and Birge, 1985; Klaverkamp and Duncan, 1987; Stubblefield *et al.*, 1999; Chowdhury *et al.*, 2003; Muyssen and Janssen, 2004); aluminium (Orr *et al.*, 1986; Allin and Wilson, 2000); zinc (LeBlanc, 1982; Bradley, DuQuesnay and Sprague, 1985; Hogstrand and Wood, 1995; Marr *et al.*, 1995); and copper (Dixon and Sprague, 1981; Grosell, Hogstrand and Wood, 1997; Bossuyt and Janssen, 2004; Adeyemi and Klerks, 2013; Zeng *et al.*, 2017). For example, in a study replicating the effects of variable metal bioavailability in a freshwater system, rainbow trout acclimated to a sublethal level of aluminium were significantly more tolerant than naïve fish when subsequently challenged with an acute pulse of aluminium, exhibiting significantly lower mortality and haematological disturbance, and faster

recovery (Allin and Wilson, 2000). Copper-acclimated yellow croaker also showed increase tolerance to lethal copper exposure, resulting in significantly lower mortality and immunotoxicity (Zeng *et al.*, 2017). Acclimation of rainbow trout was also shown to increase their tolerance to lethal zinc and cadmium challenge by approximately 3-fold and 20-fold, respectively (Stubblefield *et al.*, 1999).

Acclimation to metals prior to lethal exposure results in altered regulation of physiological pathways involved in metal response, such as metal uptake, storage and excretion pathways; proteins involved in oxidative stress response, such as heat shock proteins and metallothionein; and compensatory changes in the regulation of genes involved in processes disrupted by metal toxicity, such as ionoregulation, immune function, and metabolism (Silvestre *et al.*, 2006; Adeyemi and Klerks, 2013). Although organisms are protected against lethal challenges immediately following acclimation, these transient physiological changes and the tolerance that they confer can be quickly lost during recovery in clean media and have not been reported to be retained in subsequent generations (Dixon and Sprague, 1981; Bradley, DuQuesnay and Sprague, 1985).

At the population level, genetic adaptation to stressors is a well-known mechanism by which long-lasting tolerance can be acquired and inherited in subsequent generations. Evidence for local genetic adaptation to stressors in wild populations of aquatic vertebrate and invertebrate species has been observed for a variety of stressors (Wang and Guo, 2019), including: organic chemicals, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated bisphenyls (PCBs)(Weis, 2002; McMillan *et al.*, 2006); metals (Klerks and Levinton, 1989; Benton, Diamond and Guttman, 1994; Keklak, Newman and Mulvey, 1994; Lopes, Baird and Ribeiro, 2006; Bourret *et al.*, 2008; Bury and Durrant, 2009; Agra *et al.*, 2010; Paris, King and Stevens, 2015; Uren Webster *et al.*, 2017); pesticides (Coors *et al.*, 2009; Jansen *et al.*, 2011); and environmental conditions, including temperature and salinity (Belk *et al.*, 2005; Schulte, 2007; Purcell *et al.*, 2008, 2012). Genetic adaptation to environmental stress has also been demonstrated experimentally under laboratory conditions using species with short generation times (Xie and Klerks, 2003; Ward and Robinson, 2005; Lopes *et al.*, 2008).

However, genetic adaptation via selection for tolerant genotypes can take many generations to develop and can result in reduced genetic diversity (Nowak *et al.*,

2009). Reduced genetic diversity has been observed in several fish populations locally adapted to environmental contaminants (Kopp, Guttman and Wissing, 1992; Murdoch and Hebert, 1994; Paris, King and Stevens, 2015). For example, in an analysis of the genetic diversity of yellow perch populations living in regions affected by 80 years of mining pollution, within-population genetic diversity was significantly reduced at contaminated sites, and was negatively correlated with copper and cadmium accumulation in the liver (Bourret *et al.*, 2008). Reduced genetic diversity and permanent commitment to adaptive changes can reduce future phenotypic plasticity and adaptive potential in response to new environmental challenges (Ward and Robinson, 2005; Ribeiro and Lopes, 2013). Several studies that have detected reduced genetic diversity in pollutant-adapted fish populations also observed increased individual genetic diversity, suggesting that despite being eroded at the population level, heterozygosity was an important factor determining tolerance for individuals (Kopp, Guttman and Wissing, 1992; Bourret *et al.*, 2008).

Adaptation to stressors can also incur costs, which could become maladaptive in the absence of the stressor (Carriere *et al.*, 1994; Agra *et al.*, 2010). For example, in a genetic selection experiment that generated a cadmium-resistant population of killifish after 6 generations of selection, F7 fish exhibited 18% lower fecundity, 13% lower mean brood size, 7% shorter female life span, and 6% longer time to first reproduction than controls; indicating significant fitness trade-offs. Genetic adaptation via selective pressures is less likely under fluctuating environmental conditions, and could be maladaptive if genetic adaptation is no longer compatible with the environment should conditions regularly change.

There is now evidence that non-genetic mechanisms may also be involved in long-lasting physiological changes as a result of exposure to environmental stressors, including persistent disease phenotypes and altered tolerance to future exposures. These can include persistent changes in physiology via epigenetic mechanisms that persist across the lifetime of an organism. It can also include mechanisms that cause persistent changes to physiology of subsequent generations, including maternal effects, potential transgenerational epigenetic effects, or a combination of both.

3. Epigenetics

The most commonly accepted definition of epigenetics is the study of “heritable changes in gene expression without changes to the DNA sequence” (Sawan, Herceg and Vaissie, 2008; Baccarelli and Bollati, 2009; Probst, Dunleavy and Almouzni, 2009; Singh and Li, 2012; Mill and Heijmans, 2013). Mechanisms governing chromatin structure and gene expression recognised as epigenetic under this definition can include: DNA methylation; histone variants and post-translational modification of histone proteins; RNA-mediated gene silencing and activation; activity of proteins, such as autoregulatory transcription factors, which as well as regulating expression of other genes, regulate the transcription of their own gene; and structural inheritance through propagation of DNA-associated proteins, such as prions, gene-silencing polycomb complexes, and more recently, patterns in nucleosome positioning (Ptashne, 2004; Feng, Fouse and Fan, 2007; Sawan, Herceg and Vaissie, 2008; Halfmann *et al.*, 2012; Golbabapour *et al.*, 2013; Serizay *et al.*, 2020).

The most commonly investigated mechanisms in epigenetic studies to date are: DNA methylation (covalent addition of methyl groups to cytosine nucleotides, mainly at CpG dinucleotides) and histone modifications (modifications to amino acid residues in histone tails)(Sawan, Herceg and Vaissie, 2008). Both are critical in the regulation of chromatin structure – the tight packaging of DNA through association with nucleosomes made up of complexes of modified histones – and regulation of transcription, by recruiting or regulating access of transcription factors and transcriptional machinery to the packaged DNA (Sawan, Herceg and Vaissie, 2008).

There is still some disagreement among researchers over the definition of epigenetics, which has caused confusion in the field. Originating from the work of Waddington in the 1940s (Waddington, 1942c, 1942a, 1942b; Huxley, 1956), epigenetics originally described the study of the causal mechanisms driving epigenesis – the process by which different cell types are derived during development. Waddington proposed the idea of an epigenetic landscape, whose shape is determined by genetic interactions, which guides a process of canalization in which cells irreversibly commit to developmental pathways,

culminating in a specific and permanent phenotypic endpoint (Choudhuri, 2011).

As the field evolved, Nanney (1958) tied epigenetics to the idea of cellular memory and epigenetic systems of molecular regulators underpinning a state of non-genetic persistent homeostasis, distinct from, but linked to, the primary genetic systems (Nanney, 1958). This association of epigenetics with a system of molecular regulators controlling development, distinct from the DNA itself, was adopted by Holliday (1990), who defined epigenetics as “the study of mechanisms of temporal and spatial control of gene activity during the development of complex organisms” (Holliday, 1990). However, this definition omitted reference to the idea of heritability, an essential characteristic of mechanisms proposed to contribute to cellular memory. This definition could therefore encompass any mechanism, other than DNA sequence, that affects gene expression; failing to differentiate between general mechanisms of transient gene regulation and the stable regulatory mechanisms that we now deem epigenetic (Skinner, Manikkam and Guerrero-Bosagna, 2010). It also frames epigenetics only in terms of development, overlooking its established role in other processes, such as facilitating phenotypic plasticity in response to external factors.

In 1996, Riggs and collaborators, following on from Holliday, redefined epigenetics as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence” (Russo, Martienssen and Riggs, 1996). Aside from a small minority who still challenge this requirement for heritability in epigenetics (Bird, 2007), or restrict its use to describe only transgenerational meiotic inheritance of changes in gene expression (Deans and Maggert, 2015), this remains the generally accepted definition to date (Dupont, Randall and Brenner, 2009). This has been reinforced by authors including Ptashne (2007 & 2013) who assert that heritability has become a core principle underpinning the term epigenetics, which is fundamentally associated with cellular memory (Ptashne, 2007, 2013).

Despite the general consensus being that epigenetic marks must be mitotically and/or meiotically stable, there is still controversy over which marks satisfy these conditions and the mechanisms by which they are inherited, even for

commonly studied epigenetic marks such as histone modifications (Ptashne, 2007, 2013), for which this has not yet been fully determined. The mechanism by which histone patterns would be re-established following DNA replication is still unclear, with many potential mechanisms being hypothesised (Probst, Dunleavy and Almouzni, 2009). This is in contrast to DNA methylation, whose re-establishment by DNA methyltransferases using hemi-methylated DNA arising from the semi-conservative nature of DNA replication as a template is fairly well understood (Sharif *et al.*, 2007).

It is becoming increasingly clear that epigenetic inheritance of a single factor may involve the co-operation of several epigenetic mechanisms and heritable marks that are not effective in isolation. For example methyl-cytosine binding proteins have been shown to recruit histone modifying enzymes, providing a link between the inheritance of the methylome and histone-directed chromatin structure (Fragou *et al.*, 2011). It is therefore important when interpreting studies focussing on a single epigenetic mark or mechanism – which is very common given the complexity and expense of measuring even one type of epigenetic mark – to view the results in the broader context of a complex network of interacting mechanisms, and take into consideration the potential roles being played by other epigenetic factors that have not been measured.

The definition of epigenetics has evolved from being used to define a cellular property, describing a heritable cellular phenotype or transcriptional state, to being equated to molecular properties, as recently discussed by Greally (Greally, 2018). This has led to an increasing body of work which uses epigenetics to describe changes in various molecular factors regulating chromatin structure and gene expression, without confirming their heritability. This is frequently observed in studies in which changes in chromatin structure and regulatory factors – such as DNA methylation, histone modifications, or microRNAs – are investigated in an organism during or immediately following a treatment, and are reported as epigenetic effects. While alterations in these molecular factors can be epigenetic in nature, it is the specific trait of mitotic and/or meiotic heritability that differentiates these epigenetic changes from transient changes in gene regulation. Without then testing whether the molecular alterations induced by the treatment persist after the treatment is

removed, it cannot be determined whether they are truly epigenetic, and they should therefore be interpreted cautiously.

The field of epigenetics was originally founded in the context of development and cell fate, as the study of mechanisms by which gene expression is stably reprogrammed during differentiation of pluripotent progenitor cells to cement their development into different cell types (Boland, Nazor and Loring, 2014). However, the potential for environmental factors to alter epigenetic programming, facilitating environmental influence on phenotype was soon recognised, particularly concerning development of disease. Originating from the work of Barker and colleagues in the 1980s, which linked incidence of coronary disease and increased death rate in adulthood with poor nutrition and environment during gestation and early development (Barker, 2007), the Developmental Origins of Health and Disease approach (DOHaD) examines the consequences of early life environment on health and disease in later life (Wadhwa *et al.*, 2009). The contribution of environmentally-induced disruption of the epigenome were soon incorporated.

Early development is considered to encompass critical windows of susceptibility for exposure to environmental stressors, when rapid growth and epigenetically-driven cell differentiation is occurring (Marsit, 2015). Early development includes two potential epigenetic reprogramming windows in vertebrates (Morgan *et al.*, 2005), the first of which occurs in somatic cells soon after fertilisation (Oswald *et al.*, 2000; Jiang, Zhang, Wang, *et al.*, 2013; Potok *et al.*, 2013), and the second occurring in primordial germ cells (PGCs) of the developing embryo during sexual differentiation (Hill *et al.*, 2018). It is thought that the epigenome is particularly susceptible to environmentally induced alterations during periods of reprogramming, and that these changes can persist into later life and result in altered phenotype.

Many studies have examined the developmental origins of disease in humans and described a likely role for environmentally-induced epigenetic changes during early life exposure in disease susceptibility in later life (Jirtle and Skinner, 2007; Liu, Li and Tollefsbol, 2008; Feil and Fraga, 2012; Hoffman, Reynolds and Hardy, 2017). These include *in utero* and perinatal conditions, such as the link between maternal nutrition and development of metabolic disorders in offspring (Godfrey,

Gluckman and Hanson, 2010; Gali Ramamoorthy *et al.*, 2018); and developmental exposure to toxic chemicals, including cigarette smoke (Joubert *et al.*, 2016), endocrine disrupting chemicals (EDCs)(Vaiserman, 2014; Braun, 2017; Yang *et al.*, 2018), and toxic metals (Hou *et al.*, 2012; Cardenas *et al.*, 2017; Park *et al.*, 2017), associated with a range of disease outcomes in later life, such as cancer; metabolic disorders, such as diabetes and obesity; and immune, behavioural and neurodegenerative disorders. Studies investigating discordance for disease outcomes among monozygotic twins has also highlighted a likely contribution of environmental and epigenetic mechanisms to diseases previously assumed to have a purely genetic basis (Bell and Spector, 2011).

The development of epigenetic studies has allowed research to begin to investigate the non-genetic mechanisms that explain observed correlation between altered phenotype and environment, which cannot be explained by genetic differences. However, there is still much we do not understand about the interactions between the environment, epigenome, and phenotype. In particular, very little is known about the specific molecular factors that transmute environmental signals into specific molecular alterations to the epigenome, which authors including Patashne have argued must include at least transient involvement of sequence-specific regulators, such as transcription factors, which can then be followed by non-sequence-specific mechanisms of maintenance and positive feedback (Ptashne, 2013). This is a substantial knowledge gap in the field of epigenetics, which warrants future research to understand the unidentified factors playing a crucial, yet elusive, role in the epigenetic mechanisms underpinning environmental influence on phenotypic plasticity and disease.

Throughout this thesis, the term “epigenetics” will be used to describe mitotically and/or meiotically heritable changes in gene expression that occur without changes to the DNA sequence. In the context of environmental toxicology, an emphasis will be placed on environmentally-induced changes in cellular state that remain stable once the inducing environmental signal is removed, indicating the presence of cellular memory which distinguishes these changes from transient changes in gene regulation.

4. Epigenetics in Ecotoxicology

As well as being studied in relation to human disease, the contributions of epigenetics to the response of organisms to their environment are now frequently investigated in ecotoxicology, with evidence that early life exposure of other animals, including fish, can impact adult phenotype through epigenetic modifications (Jonsson and Jonsson, 2014). Numerous studies have provided evidence that environmental chemicals may alter the epigenome of organisms and cause persistent or delayed onset of disease phenotypes, including: toxic metals, such as arsenic, nickel, cadmium and copper; hydrocarbons, such as PAHs; pesticides, such organochlorine compounds; and endocrine disruptors, such as bishphenol A (BPA), EE2, methoxychlor, and vinclozolin (Uzumcu, Suzuki and Skinner, 2004; Baccarelli and Bollati, 2009; Martinez-Zamudio and Ha, 2011; Vandegehuchte and Janssen, 2011, 2014; Collotta, Bertazzi and Bollati, 2013; Alavian-Ghavanini and Rüegg, 2018). There is also evidence, reviewed by Chatterjee, Gim and Choi (2018), that other stressful environmental conditions may also alter the epigenome of wildlife resulting in deleterious effects later in life, including heat stress, hypoxia, pH, and poor nutrition (Chatterjee, Gim and Choi, 2018).

The majority of studies investigating the epigenetic effects of environmental stressors on organisms report induction of persistent deleterious phenotypes due to disruption of the epigenome (Jirtle and Skinner, 2007; Skinner, Manikkam and Guerrero-Bosagna, 2010). However, epigenetic alterations also have the potential to facilitate phenotypic plasticity and adaptation, and in this way reduce the adverse effects of environmental stressors.

Some of the best examples of within-generation plasticity have come from plants. For example, exposure of *Arabidopsis* seedlings to osmotic stress was shown to have a priming effect, which caused persist changes in histone modifications, specifically targeting transcription factors and shortening H3K27me3 islands, associated with an altered transcriptional response and increased tolerance upon subsequent exposure to osmotic stress later in life (Sani *et al.*, 2013).

Evidence for within-generation epigenetic adaptation to altered environmental conditions has also been observed in fish. Methylation profiles of genes encoding

ion transporters in marine stickleback transferred to freshwater were observed to converge over time to resemble that of freshwater stickleback, allowing adaption to different osmotic conditions within a single generation (Artemov *et al.*, 2017). Environmental programming of the methylome is also thought to play an important role in regulating differences in maturation rate between genetically similar Atlantic salmon parrs (Morán and Pérez-Figueroa, 2011).

As well as adaptation to stressful environmental conditions, examples of epigenetic adaptation to toxic environmental chemicals have also been reported in plants, with several studies providing clear evidence for molecular mechanisms of this these adaptations, including for adaptation to toxic metals such as nickel, mercury, zinc and copper (Ou *et al.*, 2012; Cicatelli *et al.*, 2014; Talukdar, 2017; Gullì *et al.*, 2018).

For a small number of environmental chemicals, there is also some evidence that epigenetic alterations may play a role in adaptive responses to chemical exposure in animals, including toxic metals, such as arsenic and cadmium (Kille *et al.*, 2013; Plautz and Salice, 2013; Ye *et al.*, 2014); endocrine disruptors, such as BPA (Wolstenholme *et al.*, 2012); hydrocarbons, such as naphthalene (Leonardi *et al.*, 2020); and fungal toxins (Babu *et al.*, 2012).

Fewer of these studies are able to report proposed molecular mechanisms underpinning phenotypic observations. However, some examples report convincing evidence for the involvement of the epigenome, including DNA methylation. For example, Ye *et al.* (2014) reported that induction of cadmium tolerance in pre-exposed human lymphoblast cells could be inhibited with application of a DNA methyltransferase inhibitor, strongly suggesting that DNA methylation changes may underlie this adaptive change in gene expression (Ye *et al.*, 2014). However, it is important to note that some of these observations came from wild populations, and could be confounded by genetic changes that have also influenced the tolerant phenotype, or undetected genetic changes in the underlying DNA sequence that have contributed to the measured changes in the epigenome. The potential for epigenetic adaptation to environmental chemicals in animals warrants further investigation with laboratory studies where genetic factors can be more easily controlled.

Despite the growing number of chemicals proven to cause epigenetic disruption, examples of induced plasticity and tolerance seem to be more frequent for altered environmental conditions and toxic concentrations of chemicals that also occur naturally in the environment or chemicals with similar toxic action. These include essential metals and others that mimic them; environmental chemicals that mimic endogenous hormones and other signalling molecules, such as oestrogens; or chemicals which stimulate common stress response mechanisms, such as oxidative stress; which cause consistent epigenetic alterations to specific molecular pathways.

This is supported by assertions by some researchers in the field of environmental epigenetics that alteration of the epigenome by environmental factors at specific genomic regions must be directed by a sequence-specific regulator, such as transcription factors, suggesting that instances of adaptive epigenetic phenotypic plasticity may rely on existence of evolved genetic pathways that facilitate the translation of environmental signals to specific epigenetic modifications (Ptashne, 2007; Deichmann, 2016).

Epigenetic disruption on the other hand can be caused by interference with general epigenetic processes. For example, Lee and colleagues (2009) propose that disruption of DNA methylation by some environmental conditions and toxic chemicals may be exerted through a common pathway of depletion of methyl donors, such as S-adenosylmethionine (SAM), through, for example, dietary deficiency or preferential synthesis of glutathione (GSH) over SAM due to oxidative stress (Lee, Jacobs and Porta, 2009). This may explain the greater range of chemicals observed to cause epigenetic disruption, compared to those observed to induce apparent epigenetic adaptation.

Many of these reported epigenetic effects and associated phenotypes are induced as a result of exposure to environmental stressors during critical windows of susceptibility in early development when epigenetic reprogramming is occurring (Uzumcu, Suzuki and Skinner, 2004; Collotta, Bertazzi and Bollati, 2013; Vandegehuchte and Janssen, 2014). As a result, epigenetic effects due to environmental exposure are often driven by alterations in the environment of the embryo during embryogenesis as a result parental effects, coinciding with early reprogramming windows.

5. Parental Effects

Many studies have demonstrated that parental environment can positively and negatively impact the phenotype of offspring (Van Cann *et al.*, 2019). During gametogenesis, a variety of factors are accumulated in gametes that direct early embryogenesis. These factors include paternal and maternal transcripts that are important for the early stages of embryo development prior to embryonic genome competency at zygotic genome activation; regulatory proteins and signalling molecules, such as transcription factors, enzymes and hormones; antioxidants, including glutathione and metallothionein; and energy in the form of energy storage molecules, such as vitellogenin (Dworkin and Dworkin-Rastl, 1990; Boerke, Dieleman and Gadella, 2007; Mtango, Potireddy and Latham, 2008).

Numerous studies have demonstrated how the parental environment can cause changes in the accumulation of these factors in gametes during gametogenesis and impact the quality of gametes and the physiological development of offspring, resulting in both toxic effects and induction of adaptive phenotypic plasticity (Brooks, Tyler and Sumpter, 1997; Timothy A. Mousseau and Fox, 1998; Mtango, Potireddy and Latham, 2008; Ritchie and Marshall, 2013; Gallo, Boni and Tosti, 2020). Epigenetic alterations can be involved in driving both the environmentally-induced physiological changes in adults that cause differential accumulation of these factors in gametes; and in driving the persistent changes in physiology of offspring caused by these differentially transferred factors.

Many studies have demonstrated negative effects of parental exposure to environmental chemicals on offspring. For example, *in utero* exposure of mice to BPA during oogenesis has been shown to reduce oocyte quality by disrupting meiosis through oestrogen receptor (ESR)-mediated endocrine disruption, leading to increased aneuploidy in oocytes and resulting embryos (Susiarjo *et al.*, 2007). Developmental exposure of female zebrafish to fluoxetine was found to cause disruption of the stress axis in F1 offspring, thought to be driven by altered transmission of maternal transcripts in oocytes encoding the glucocorticoid receptor, DNA methyltransferase 3, and miRNA components that target the stress axis (Martinez *et al.*, 2019).

However, chemical exposure history of parents could can also lead to adaptive changes in the physiology of offspring as a result of differential transfer of non-genetic factors in response to parental environment (Bonduriansky, Crean and Day, 2012; English *et al.*, 2015). For example, Weng and Wang (2014) reported evidence for maternal transfer of metal tolerance to oyster larvae from exposed parents which was concluded to be partially driven by increased metallothionein expression in eggs as a result of the maternal environment. Eggs from exposed parents were found to contain increased concentrations of metallothionein, which correlated with tissue concentration-dependent maternal transfer of copper and zinc in oocytes (Weng and Wang, 2014). Similarly, maternal exposure of tilapia to cadmium was found to cause increased cadmium resistance in F1 offspring. No maternal transfer of cadmium was observed in oocytes, however, oocytes of cadmium-exposed females contained higher levels of metallothionein transcripts, suggesting that maternal exposure led to adaptive alterations in accumulation of maternal transcripts that allowed offspring to express higher levels of protective metallothionein earlier during developmental exposure to cadmium (Lin, Hsu and Hwang, 2000). Lister *et al.* (2017) reported increased expression of antioxidant enzymes in F1 offspring of F0 PAH-exposed oysters that reflected the antioxidant status of the F0 ovary, and inferred resistance to oxidative damage to lipids and proteins (Lister, Lamare and Burritt, 2017).

Examples of the influence of parental environment on offspring phenotypic plasticity also include other environmental factors, such as temperature, nutrition and stress.

Metabolic programming of offspring according to maternal dietary conditions has been reported in numerous studies, with favourable and poor nutritional environment of the parents leading to differential expression of genes related to metabolism, altering tendency toward energy storage and energy expenditure in preparation for similar nutritional environment in the future (Rotem, Agrawal and Kott, 2003; Kuzawa, 2005; Meaney, Szyf and Seckl, 2007; Wells, 2007; Kappeler and Meaney, 2010; Vaag *et al.*, 2012). For example, Newman *et al.* (2016) demonstrated that zebrafish receiving higher dietary rations had altered maternal transcripts in oocytes related to metabolic processes and produced offspring with higher survival and increased levels of activity upon rearing in a nutrient rick environment (Newman *et al.*, 2016). Fuiman and Perez (2015) demonstrated that

differential deposition of the fatty acid, docosahexaenoic acid, in oocytes according to maternal dietary conditions caused lifelong metabolic programming of offspring that was associated with increased fitness (Fuiman and Perez, 2015).

Parental exposure to temperature has also been shown to alter developmental programming of offspring via non-genetic factors. Marine stickleback acclimated to elevated temperature produced offspring with a larger juvenile body size when reared at the same temperature. This correlated with increased mitochondrial respiration capacity which lasted in adulthood in the F1 generation, suggesting that the maternal environment led to differential transfer of mitochondria or other factors related to mitochondrial function to offspring, inferring a fitness advantage when reared in a similar environment (Shama *et al.*, 2014). Similarly, inheritance of cold tolerance was observed in blue tilapia offspring, correlated with altered expression of mitochondrial ATP synthase 6 (*atp6*) gene, which was demonstrated to be driven by non-genetic maternal inheritance (Nitzan *et al.*, 2016). A study in round goby also found alterations in maternal transcripts transferred in oocytes that correlated with temperature experienced by the mother prior to ovulation which related to temperature-sensitive gene groups. The authors suggest that non-genetic inheritance via maternal transcripts may be a mechanism by which environmental factors can induce adaptive intergenerational phenotypic plasticity in offspring (Adrian-Kalchhauser *et al.*, 2018).

Offspring reprogramming due to influence of maternal stress has also been reported in various species. Maternal stress as a result of environment and transfer of glucocorticoids, such as cortisol, in oocytes, and perinatal maternal behaviours have been shown to have both adaptive and maladaptive effects on offspring (Sopinka *et al.*, 2017). A study in lake sturgeon found that high egg cortisol led to a potentially adaptive reduction in stress response to acute stress in early life (Wassink *et al.*, 2020). High egg cortisol in rainbow trout was also found to cause altered behaviour and increased swimming speeds in response to stressful stimuli later in life (Colson *et al.*, 2015). Research in damselfish found that increased maternal cortisol levels induced by interaction with predators determined progeny size through a stress-mediated mechanism (Mccormick, 1998). Maternal behaviour during perinatal development in rats has been found to cause long term changes in functioning of hypothalamic-pituitary-adrenal axis in offspring and greater resistance to stress; with offspring of mothers exhibiting high

levels of licking and grooming behaviour shown to have increased glucocorticoid receptor (GR) expression, correlated with altered GR DNA methylation, and reduced pituitary-adrenal responses to stress (Kappeler and Meaney, 2010).

As well as endogenous factors, xenobiotics and metabolites taken up and produced by parents can also accumulate in gametes and be transferred to offspring. Transfer of xenobiotics in gametes has been observed for a variety of chemicals in numerous species, with negative impacts on offspring health (Russell, Gobas and Haffner, 1999; Bargar, Scott and Cobb, 2001; Ostrach *et al.*, 2008; Wu *et al.*, 2013; Chen *et al.*, 2016; Khadra *et al.*, 2019). For example, in fathead minnow, maternal transfer of methylmercury as a result of maternal dietary exposure was shown to cause significant disruption of neurochemical pathways in F1 embryos that persisted into adulthood (Bridges, Venables and Roberts, 2017).

Studies have shown that exposure history of organisms can alter their physiological response to exposure in the future, including uptake and metabolism of xenobiotics (Grosell, Hogstrand and Wood, 1997; Kamunde, Clayton and Wood, 2002). Exposure history therefore has the potential to alter impacts of subsequent parental exposure on offspring, by altering the internal toxicokinetics of chemicals in the body of the parent due to acclimation or adaptation, potentially leading to differential accumulation and transfer of toxic chemicals in gametes. Indirect exposure of offspring to parentally-transferred toxic chemicals has been implicated in causing epigenetic effects in developing offspring, as exposure occurs during sensitive epigenetic reprogramming windows occurring in early development (Vilahur, Vahter and Broberg, 2015; Bommarito, Martin and Fry, 2017).

Many of these effects on offspring may be mediated through altered epigenetic programming of embryos as a result of parentally transferred factors, which are then mitotically inherited across the lifetime of offspring, resulting in these long-term adaptive and maladaptive phenotypes. Similar to within-generation epigenetic changes, adaptive changes may also more common for chemicals or stressors that are either naturally occurring or mimic the activity of naturally occurring stressors, with mechanisms for parental influence of the epigenome having been genetically determined. Persistent toxic effects, on the other hand,

could arise from disruption of epigenetic mechanisms during early embryonic development that do not rely on sequence specific targeting, and may therefore occur more frequently following exposure to other anthropogenic chemicals that do not share similarities with naturally encountered stressors.

6. Transgenerational epigenetic inheritance

As well as persistence of epigenetic alterations across an organism's lifetime due to mitotic inheritance, it has also been proposed that transgenerational epigenetic inheritance may transmit alterations in the epigenome of one generation stably to subsequent generations. However, the existence of transgenerational epigenetic inheritance is highly controversial, and the term is often used inconsistently and inappropriately.

The debate highlights a clear divide between researchers with a mechanistic focus and those with a phenotypic focus (Burggren, 2016). It is broadly acknowledged that in order for an environmentally-induced epigenetic alteration to be transgenerationally inherited, the alteration must occur and be maintained in the germline cells across multiple generations to at least one generation that was not directly exposed. Phenotypically focused fields often use the terms "intergenerational" and "transgenerational" interchangeably, including in areas of ecotoxicology and animal behaviour, for example (Ho, 2014). Whereas, mechanistically focussed epigeneticists use the term "transgenerational" specifically to describe events of epigenetic inheritance through the germline, with "intergenerational" serving as an umbrella term describing all instances of effects across generations, including via phenomena such as cross-generational maternal effects. This is an important distinction that could prevent misattribution of phenomena as transgenerational epigenetic inheritance.

Transgenerational epigenetic inheritance is a field with potential for high impact, but is very immature – a crude analysis of epigenetic literature by Burggren (2016) roughly estimated that less than 7% of epigenetics papers include terms suggesting a focus on transgenerational inheritance and evolution (Burggren, 2016). However, this is probably an overestimation of the volume of work focussing on true transgenerational inheritance, as many of the

studies which claim to be cases of transgenerational epigenetic inheritance may be a misattribution of intergenerational effects. Most are reports of environmentally-induced phenotypes being inherited across multiple generations, which are reasoned to be caused by inheritance of an altered epigenome; however, potential inheritance of genetic alterations or transmission of an altered microbiome are often not investigated and properly ruled out and very few studies provide molecular or mechanistic evidence to support their claim.

While some of these reported phenomena could be true examples of transgenerational epigenetic inheritance, a stronger weight of evidence is needed. This includes identification of specific epimutations that are consistently inherited in each affected generation; confirmation that these epimutations cause a change in gene expression which contributes to the phenotype, genetic analysis to rule out confounding changes in DNA sequence, and care to investigate other potential non-epigenetic confounding factors, such as parental effects. Where these steps have not been taken or are not feasible, caution should be exercised before confidently attributing phenotypic observations as having an epigenetic basis.

Parental effects, and most commonly maternal effects – defined as the causal influence of maternal environment, phenotype or genotype on the phenotype of the offspring, independent of their own genotype – can complicate inheritance (Seisenberger *et al.*, 2013). For example, exposure to an epigenome-altering environmental signal is often multigenerational; meaning that the generation in which inheritance of altered epigenetic states or phenotypes that can be considered transgenerational differs depending upon the life history of the organism (Mill and Heijmans, 2013).

In oviparous species, such as most birds and fish – where no embryonic development takes place within the body of the parent (F0), inherited environmentally-induced alterations following F0 embryonic exposure can usually be considered transgenerational when phenotypic effects are measurable in the F2 generation, as only the F0 animal and their germline (F1) were directly exposed to the original environmental trigger (figure 1). A commonly overlooked exception to this includes cases where accumulation

and maternal transfer of chemicals in F0 gametes leads to F1 embryonic development occurring inside a chemical-rich egg, extending direct exposure to the germ cells of the F1 embryo, which give rise to the F2 generation. This possibility is explored later in this thesis in chapter 4. However, in a viviparous species, such as mammals, maternal effects frequently alter the generational range of exposure. For example, exposure of a gestating female to an environmental trigger results in the exposure of the F0 female, the gestating F1 offspring, and the gametes of the developing F1 embryo which will become the F2 generation (Moazed, 2009)(figure 1). Exposure during gestation in mammals is often necessary for environmental epigenetic studies, in which exposure during sensitive reprogramming windows during early embryogenesis is often desirable. Therefore inheritance of an altered epigenetic state could not be considered transgenerational until the F3 generation in some instances of mammalian exposure. It is therefore important for researchers to properly rule out maternal effects before proposing that epigenetic inheritance has occurred, particularly where molecular evidence is lacking.

As well as failing to rule out alternative explanations for inherited phenotypes, such as maternal effects and genetic drift, potential epigenetic mechanisms behind observed inheritance of phenotypes are often not proposed and/or investigated. For an epigenetic alteration in germline cells to be maintained and transmitted across multiple generations, the alteration must survive erasure and reprogramming processes that occur during gametogenesis and embryonic development; and neither the mechanisms by which they escape this erasure, nor the processes of reprogramming are entirely understood (Heard and Martienssen, 2014).

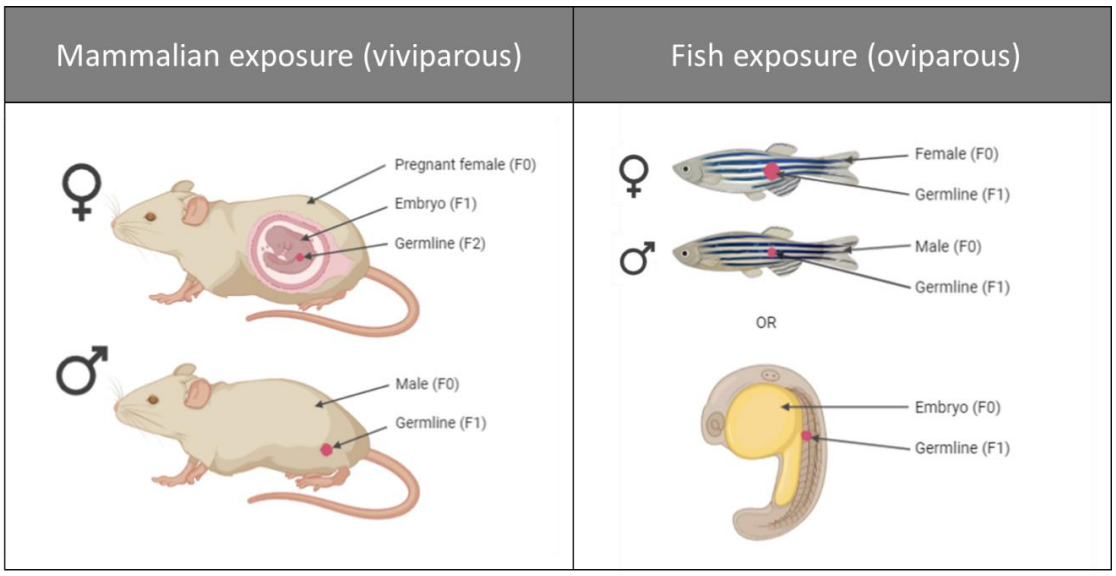


Figure 1: *Comparison of total generations directly or indirectly exposed during chemical exposure of viviparous and oviparous animals. Adult exposure of both fish and mammals results in exposure of the F0 male or female and their germline (F1). Exposure during embryogenesis – considered a sensitive period in epigenetic studies due to occurrence of reprogramming windows – results in exposure of 3 generations in pregnant female mammals due to embryonic development within the mother (F0 female, F1 embryo, and its germline (F2)) compared to 2 generations in a directly exposed externally developing fish embryo (F1 embryo and its germline (F2)).*

Removal of existing epigenetic marks acquired during development or as a result of environmental influences throughout a parent's lifetime, including in the methylome and histone code, is essential during gamete production in order to produce a totipotent cell capable of developing into the multitude of cell types encoded within their genetic blueprint (Fragou *et al.*, 2011; Tanae *et al.*, 2012). This presents an obvious challenge to epigenetic inheritance. As previously discussed, DNA methylation – the most commonly studied epigenetic mark – has a clear mechanism for replication and mitotic intragenerational inheritance through the activity of DNA methylation maintenance enzymes, but this is less clear for potential transgenerational meiotic inheritance (Richardson *et al.*, 2014).

In mammals, the methylome undergoes two rounds of global reprogramming via active and passive processes (Tanae *et al.*, 2012; Susiarjo *et al.*, 2013). The first round occurs in the early embryo, soon after fertilisation, when the paternal DNA is quickly actively demethylated and the methylation of the maternal DNA is slowly passively demethylated with each cleavage due to lack of DNA methyltransferase 1 (dnmt1) activity (Tanae *et al.*, 2012). The second occurs in the PGCs – the progenitors of sperm and oocytes – as they migrate from the genital ridge to the early bipotential gonad when their genetic material bearing the parental somatic methylome undergoes dramatic global demethylation prior to gonadal sex determination (Ptashne, 2004; Tanae *et al.*, 2012; Susiarjo *et al.*, 2013). Sex-specific re-methylation then occurs as they develop into gametes during gonadal sex determination (Ptashne, 2004).

Although this reprogramming represents a barrier to epigenetic inheritance, not all parts of the genome are equally reprogrammed and exceptions include imprinted genes – where genes are expressed in a parent-of-origin specific manner due to inherited chromatin states – which has been demonstrated in plants, insects and mammals, providing evidence that mechanisms exist for some epigenetic marks to escape erasure processes (Macdonald, 2012; Susiarjo *et al.*, 2013). Some other highly methylated genomic elements also show particular resistance to demethylation during reprogramming, such as intracisternal A particles (IAPs) – retrotransposons involved in gene regulation, which could therefore facilitate transgenerational inheritance of altered epigenetic states (Lane *et al.*, 2003).

Work by Jiang *et al.* (2013) and Potok *et al.* (2013), which has confirmed that genome-wide reprogramming of the methylome does occur in developing zebrafish embryos, also serves as evidence for possible mechanisms by which transgenerational inheritance might occur. Through analysis of genome-wide methylation patterns by whole genome bisulphite sequencing of sperm, oocyte and whole embryos at different stages up to and including the blastula stage, after zygotic genome activation (ZGA), they revealed that the paternal genome present in sperm is inherited stably without significant reprogramming during early development, whereas the maternal methylome is reprogrammed to resemble the paternal methylome (Jiang, Zhang, Wang, *et al.*, 2013; Potok *et al.*, 2013). This is thought to be partially facilitated through the action of

placeholder nucleosomes, which were found by Murphy *et al.* (2018) to occupy virtually all unmethylated DNA regions in sperm and cleavage embryos, residing particularly at promoters encoding housekeeping genes and transcription factors important for early embryogenesis (Murphy *et al.*, 2018). It is thought that these placeholders deter methylation at genes important for development, priming certain parental genes for gene-specific activation or facultative repression by other factors. Disturbance of these placeholders were found to alter methylation accumulation, corresponding with inappropriate changes in expression of these genes during development (Murphy *et al.*, 2018). The fact that the paternal epigenetic programming is retained by the embryo throughout development raises the question of whether the paternal epigenome could be inherited stably across multiple generations and whether this may serve as a mechanism for transgenerational inheritance of altered epigenetic states.

Despite some mechanistic explanation provided by discovery of these paternal placeholder nucleosomes (Murphy *et al.*, 2018), there is still much we don't know about the other mechanisms targeting this specific demethylation and reprogramming of the maternal methylome, and possible reprogramming of other epigenetic elements such as histone modifications and regulatory RNAs. Further experiments suggested that although the paternal methylome seems to dominate over the maternal methylome during development, sperm may also contribute other determinants to the embryo that are important for development, including some transcripts and small RNAs (Hackett and Surani, 2013; Jiang, Zhang, Wang, *et al.*, 2013; Potok *et al.*, 2013).

Until recently it was not known whether the second window of epigenetic reprogramming occurring in primordial germ cells in mammals also occurs in fish. However, recent studies have investigated methylation reprogramming events in the PGCs of both zebrafish and medaka (Ortega-Recalde *et al.*, 2019; Skvortsova *et al.*, 2019; Wang and Bhandari, 2019). Skvortsova *et al.* (2019) reported a lack of global demethylation in zebrafish PGCs compared to observations in mammalian PGCs, detecting instead only localised reprogramming of DNA methylation at genes, and their regulatory elements, that are related to transcriptional regulation and PGC function, indicative of differential usage of gene-regulatory regions between the developing germline

and somatic cells (Skvortsova *et al.*, 2019). This lack of global demethylation and reprogramming was also independently confirmed in a similar study by Ortega-Recalde *et al.* (2019)(figure 2). They note that the lack of global methylome reprogramming in fish compared to mammals correlates with the differences in their mechanisms of germline specification.

In mammals signals from surrounding somatic tissues reprogram epiblast cells to form PGCs, whereas zebrafish have a pre-formed germline that is continuously defined through inherited cytoplasmic determinants in the germ plasm (Johnson *et al.*, 2011). Global reprogramming in PGCs of mammals mirrors that occurring in somatic cells, whose lineage they share. Lack of global reprogramming in zebrafish reflects the fact that cellular reprogramming is not required to the same extent as in mammals, due to the continuously defined nature of the zebrafish germline (Ortega-Recalde *et al.*, 2019). Pre-formation of the germline through inherited germplasm is a PGC specification strategy that has evolved independently in numerous vertebrate lineages (Johnson *et al.*, 2011). Studies in mammals have generally concluded that transgenerational inheritance, though possible, is rare, due to two stages of comprehensive reprogramming; but it has been suggested that this lack of global reprogramming of the methylome in zebrafish PGCs may provide a potential mechanism for stable transgenerational inheritance of an altered methylome in some vertebrates that is unlikely in mammals (Ortega-Recalde *et al.*, 2019; Skvortsova *et al.*, 2019).

However, factors other than PGC specification strategy must also contribute to differences in reprogramming events, as evidence from a study of PGC methylome reprogramming in medaka – a fish species also thought to have a pre-formed germline (Herpin *et al.*, 2007; Z. Li *et al.*, 2014; Li *et al.*, 2016) – which confirmed similar PGC demethylation and reprogramming as observed in mammals (Wang and Bhandari, 2019). This highlights that epigenetic dynamics and reprogramming events are likely to vary widely across species, even within more closely related lineages – with these studies indicating that epigenetic reprogramming mechanisms cannot be generalised among fish species, and might translate to altered propensity for transgenerational inheritance to occur in diverse fish species.

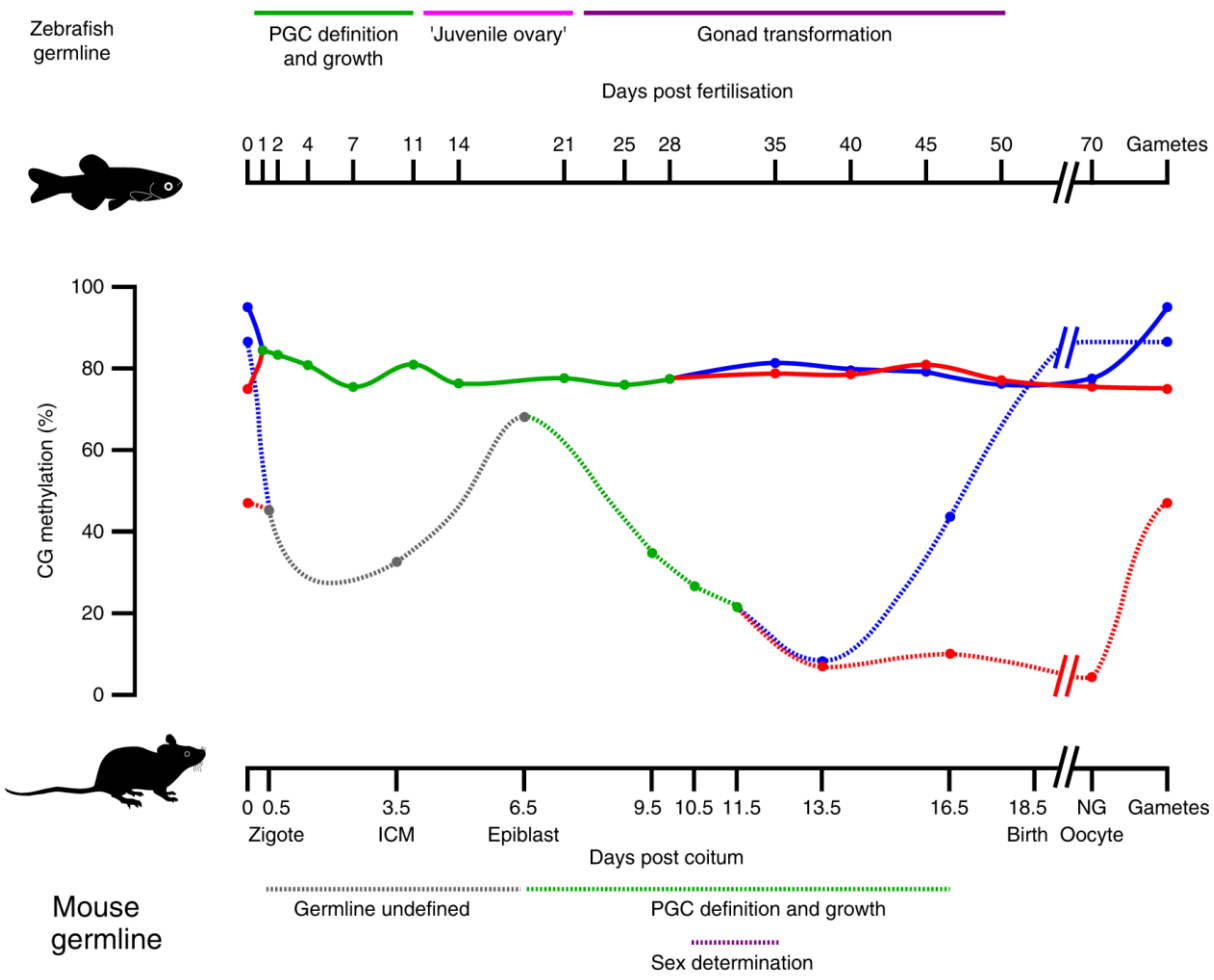


Figure 2: *Comparison of the global DNA methylation dynamics of the germline in the mouse (dashed lines) and zebrafish (continuous lines)(taken from Ortega-Recalde et al., 2019). In contrast to mammals, during primordial germ cell (PGC) specification, the zebrafish germline methylome does not undergo extensive erasure and reprogramming. Global DNA methylation in the germline remains similar in male (blue), female (red) and undifferentiated (green) fish (Ortega-Recalde et al., 2019).*

The dynamic nature of the epigenome across an organism's lifetime and its pivotal role in differentiation and development of cells and organs result in critical windows of sensitivity to epigenome-altering agents (Skinner, Manikkam and Guerrero-Bosagna, 2010). Although epigenetic reprogramming presents a barrier to epigenetic inheritance, it can also serve as a critical window of sensitivity for organisms exposed to epigenome-altering agents. Altered epigenetic programming and gene expression during sensitive developmental windows, such as reprogramming events, can lead to abnormal development

and disease in later life (Skinner, Manikkam and Guerrero-Bosagna, 2010). Studies have shown the effects of a chemical on organisms can vary greatly, depending upon the time of exposure. For example, studies on vinclozolin exposure have found that embryonic exposure during gonadal sex determination resulted in disease, however a similar exposure at a later developmental stage had no effects (Anway, Leathers and Skinner, 2006).

Strong evidence for transgenerational epigenetic inheritance has been provided by studies of invertebrates, such as the nematode worm, *Caenorhabditis elegans (C. elegans)*, with several environmentally-induced epigenomic and phenotypic alterations shown to be transgenerationally inherited in offspring, particularly via programming of histone modifications. For example, Klosin and colleagues demonstrated the transgenerational inheritance of temperature-induced changes in gene expression in *C. elegans* lasting for 14 generations, was associated with altered trimethylation of histone H3 lysine 9 at associated repeats that was transgenerationally inherited via both the sperm and oocyte (Klosin *et al.*, 2017). Manipulation of chromatin modifiers in the parental generation in *C. elegans* has also been shown to cause transgenerationally inherited changes in offspring longevity (Jeanrenaud, Brooke and Oliver, 2020). Rechavi *et al.* (2014) also found evidence for transgenerational inheritance of altered longevity via inheritance of endogenous starvation-induced small RNAs that cause heritable silencing of genes related to nutrition (Rechavi *et al.*, 2014).

Invertebrate studies have also provided evidence for active control over propagation of epigenetic effects, which partially explain why these alterations can be lost in subsequent generations – a key criticism of vertebrate studies in which apparent transgenerational inheritance is shown not to be permanently heritable. Several proteins have been identified in *C. elegans* which are thought to regulate transgenerational inheritance, such as the HERI-1 Chromodomain Protein, which is thought to negatively regulate heritable RNAi-gene silencing. Mutation of the *heri-1* gene caused transgenerational inheritance of RNAi-mediated gene silencing to last at least 20 generations longer (Perales *et al.*, 2018).

Outside of effects at specific imprinted genomic elements, convincing examples of transgenerational epigenetic inheritance are rare in vertebrates. However, there is evidence for the potential for transgenerational inheritance via both the maternal and paternal germline (Soubry *et al.*, 2014; Santangeli *et al.*, 2019). Studies of endocrine disrupting chemicals have provided some of the strongest evidence in vertebrates. For example, exposure of rats to vinclozolin during gonadal sex determination has been reported to cause reprogramming of the germline epigenome that can be transferred transgenerationally and result in adult onset of several disease phenotypes up to the F4 generation (Anway *et al.*, 2005; Anway, Leathers and Skinner, 2006; Anway, Rekow and Skinner, 2008). Other studies of endocrine disruptors have also reported transgenerational epigenetic inheritance of disease. For example, a recent study of early life exposure of medaka to atrazine during germ cell development, gonadogenesis and sex determination reported transgenerational infertility in the F2 generation, manifesting as reduced sperm count and motility, and reduced fertilisation success, accompanied by altered expression of genes involved in steroidogenesis and DNA methylation, despite no subsequent exposure (Cleary *et al.*, 2019).

Mechanistic evidence for transgenerational epigenetic inheritance in vertebrates is lacking, suggesting that transgenerational inheritance may be rare in vertebrates with reprogramming events acting as effective barriers preventing transmission of altered epigenetic states to the same extent as seen in invertebrate species. However, evidence that somatic reprogramming in fish involves greater retention of parental epigenetic states, particularly the paternal methylome, and more recent evidence that the second round of global reprogramming occurring in mammalian PGCs does not occur in fish, which do not share a common PGC determination mechanism; suggests that the efficiency of this barrier may vary between vertebrate species, with greater opportunity for germline transmission of epigenetic alterations in species, such as zebrafish, which appear to exhibit less extensive epigenetic reprogramming in both the soma and germline (Johnson *et al.*, 2011; Jiang, Zhang, Wang, *et al.*, 2013; Potok *et al.*, 2013; Ortega-Recalde *et al.*, 2019; Skvortsova *et al.*, 2019).

Efficient reprogramming could be a beneficial process in vertebrates, preventing transmission of harmful epimutations. More comprehensive epigenetic reprogramming and absence of mechanisms to support transgenerational transmission of adaptive epigenetic changes in vertebrates may also make more evolutionary sense, given that vertebrate generation times are usually substantially longer than invertebrates, reducing the likelihood that parental environment would be an accurate predictor of offspring environment. In studies that report transgenerational inheritance of consistent changes in gene expression and resulting phenotype, reported epimutations associated with these changes are often inconsistent across generations, highlighting the lack of mechanistic evidence for direct transmission of specific epimutations. A recent hypothesis suggests that inheritance of transgenerational epigenetic instability may explain propagation of altered gene expression across generations despite inconsistencies in specific epigenetic changes (Blake and Watson, 2016). However, the nature of such a mechanism, reliant on inconsistent epimutations, would make it difficult to confirm experimentally.

On the whole, for wild vertebrate species, studies on the role of epigenetic mechanisms in the long term effects of environmental stressors, including during the lifetime of an organism or during subsequent generations, are still in their infancy and require further investigation.

7. Chemicals investigated in this thesis

In this thesis, two chemicals were chosen for investigation – BPA and copper – representing two important groups of environmental pollutants – endocrine disruptors and toxic metals. Evidence exists that these chemicals can cause phenotypic effects across multiple generations, and both are thought to interact with the epigenome; making them ideal candidates for investigating the effects of exposure history on future exposure.

7.1. Bisphenol A

BPA – also known as 2,2-bis(4-hydroxyphenyl) propane (figure 3A) – is a commercially important chemical plasticiser, used in the production of polycarbonate plastics and epoxy resins (Corrales *et al.*, 2015). It is one of the most highly manufactured chemicals worldwide, with BPA production accounting for £15 billion of the global chemical market share in 2013, which was predicted to grow by 4.7% annually until 2020 (Ashfaq *et al.*, 2018). It is used widely in consumer products, including medical equipment, dental sealants, thermal receipt paper, toys, water piping, lining of cans, and in plastic food and beverage containers (Rubin, 2011).

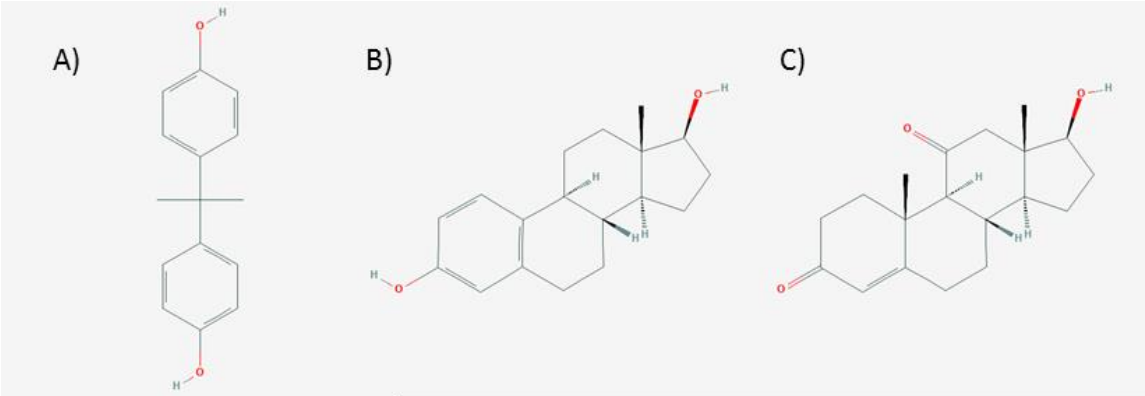


Figure 3: *Chemical structure of A) Bisphenol A (2,2-bis(4-hydroxyphenyl) propane) - $C_{15}H_{16}O_2$ (National Center for Biotechnology Information, 2020b) B) 17 beta oestradiol - $C_{18}H_{24}O_2$ (National Center for Biotechnology Information, 2020c) and C) 11-ketotestosterone - $C_{19}H_26O_3$ (National Center for Biotechnology Information, 2020a).*

Leaching of BPA from these consumer products has led to almost universal exposure of humans. In Asia and the USA, BPA has been detected in the urine of ~95% of adults (Calafat *et al.*, 2005; Zhang *et al.*, 2011). It has also been detected in breast milk, foetal plasma and placental tissue, and there is evidence that BPA exposure may be higher for children (Vandenberg, 2007; Braun and Hauser, 2011; U.S. Environmental Protection Agency, 2011; Lee *et al.*, 2018). Some evidence suggests that BPA can bioaccumulate in the foetus during pregnancy,

with one study recording 5-fold greater BPA levels in the amniotic fluid around the foetus compared to maternal serum levels (Ikezuki *et al.*, 2002). This raises concerns that BPA exposure may occur at sensitive windows of development, including during the pre- and perinatal period, early development and puberty.

Largescale manufacturing of BPA and widespread use in consumer products has led to release of BPA into the environment, particularly aquatic environments. BPA has a short half-life in the aquatic environment, being degraded or transformed by bacteria, algae, plants and by photodegradation (Im and Löffler, 2016). The half-life of BPA from industrial effluents in natural waters was estimated to be 2.5-4.5 days (Dorn, Chou and Gentempo, 1987). However, its continuous release from various sources, including industrial effluents, landfill leachates, WWTP effluents, and from the breakdown of plastic in the environment, has resulted in ubiquitous contamination of the environment with BPA (Oehlmann *et al.*, 2009).

BPA concentrations of up to 21µg/L have been reported for surface waters (Belfroid *et al.*, 2002; Kolpin *et al.*, 2002; Staples *et al.*, 2002), but can exceed this in the proximity of these sources. Concentrations of up to 17,200µg/L have been reported in landfill leachates (Yasuhara *et al.*, 1997; Yamada *et al.*, 1999; Yamamoto *et al.*, 2001; Coors *et al.*, 2003). High concentrations have also been reported in industrial effluents. For example, a study of final effluents from eight wastepaper recycling plants in Japan reported BPA concentrations up to 370µg/L, with an average of 130µg/L (Fukazawa *et al.*, 2001); and effluents from industrial facilities in Toronto, Canada, were reported to contain BPA concentrations up to 149µg/L (Hing-Biu and Peart, 2000). Efficiency of aqueous BPA removal in WWTPs has been found to range from 62.5-99.6% (Luo *et al.*, 2014); but environmentally significant concentrations of BPA can still be found in treated effluent. Analysis of reported BPA concentrations in effluents of 144 WWTPs in North America, Europe, Asia, and Australia ranged from undetectable levels to 3.1µ/L, with an average of 0.188µg/L (H. Wang *et al.*, 2019).

There is also evidence that BPA concentrations in the environment can fluctuate over time. A study of BPA levels in WWTP influents from urban communities in the US observed oscillations in BPA concentrations of up to 10-fold across a 1 year period, fluctuating between 0.8 and 10µg/L. As flow rate did not fluctuate to the same degree, they concluded that this was due to fluctuations of BPA input and

was heavily influenced by industrial release, and it appeared to show seasonality, being higher in autumn and winter (Santos *et al.*, 2016). A study of a WWTP in China also observed fluctuations in the concentration of BPA and its degradation products in both the influent and effluent of the WWTP across daily, seasonal and yearly scales, with BPA in effluent ranging from below detection limit to 0.162µg/L (Ashfaq *et al.*, 2018). They also observed seasonal changes in the concentration of BPA and its degradation products in a receiving river and its estuary, being significantly higher in the dry season due to dilution in the wet season (Ashfaq *et al.*, 2018).

Concentrations have also been shown to vary in surface waters according to depth, due to the hydrodynamics of a water body. For example, BPA concentration in the water of a river with an estuary dam was found to be 650 times higher in the relatively hypoxic bottom layer of water compared to the surface, as BPA degradation is slow under anaerobic conditions (Ike *et al.*, 2006; Funakoshi and Kasuya, 2009). BPA was also found to redissolve from sediments, where BPA has a high tendency to sorb and biomagnify due its moderate hydrophobicity and the anoxic conditions that can develop in sediments (Cousins *et al.*, 2002; Voordeckers *et al.*, 2002; Funakoshi and Kasuya, 2009; Im and Löffler, 2016). Species that occupy both the sediment and water-column, for example, at different life stages, could experience extreme fluctuations in exposure across their lifetime or generations.

BPA is an endocrine disruptor in vertebrates that exerts its toxicity by via several mechanism, including acting as both an oestrogen agonist and antagonist, and as an androgen and thyroid hormone antagonist (Moriyama *et al.*, 2002; Wetherill *et al.*, 2007; Canesi and Fabbri, 2015). In mammalian animal studies, exposure to BPA, particularly during sensitive windows of development, has been associated with a variety of toxic phenotypes (Richter *et al.*, 2007), including disruption of sexual development and reproduction (Howdeshell *et al.*, 1999; Hunt *et al.*, 2003; Richter *et al.*, 2007), interference with the hypothalamic-pituitary-gonadal axis (Akingbemi *et al.*, 2004; Ceccarelli *et al.*, 2007; Kawai *et al.*, 2007), metabolic toxicity (Rubin *et al.*, 2001), behavioural disruption (Kubo *et al.*, 2003; Seta *et al.*, 2005), compromised immune function (Alizadeh *et al.*, 2006), and increased susceptibility to cancer (Ho *et al.*, 2006; Prins *et al.*, 2008). Similarly, developmental exposure to BPA has also been associated with a variety of adult

onset diseases in humans, including metabolic disorders, cardiovascular disease, behavioural disorders, disruption of the immune system, cancer, and reproductive disruption (Melzer *et al.*, 2010; Rochester, 2013; Rezg *et al.*, 2014).

BPA exposure has also been observed to cause reproductive disruption in fish in both males and females, including reduced gonadal growth (Sohoni *et al.*, 2001), disruption of egg production and spermatogenesis (E. Haubruge, Petit and Gage, 2000; Sohoni *et al.*, 2001; Lahnsteiner *et al.*, 2005; Q. Huang *et al.*, 2018; Q. Wang *et al.*, 2019), reduced fertilisation success (Laing *et al.*, 2016), induction of feminisation and intersex conditions (Mandich *et al.*, 2007), inappropriate synthesis of vitellogenin in males (Sohoni *et al.*, 2001; Lindholst *et al.*, 2003; Larsen *et al.*, 2006; Correia *et al.*, 2007), and altered sex steroid levels (Labadie and Budzinski, 2006; Mandich *et al.*, 2007; Q. Wang *et al.*, 2019). Other toxic effects include growth suppression (Sohoni *et al.*, 2001), cardiac toxicity (Lombó *et al.*, 2015), and developmental deformities (Pastva *et al.*, 2001; Honkanen, Holopainen and Kukkonen, 2004; Lam *et al.*, 2011; Fan *et al.*, 2018).

Some of these severe effects, such as induction of intersex conditions, only occur at high concentrations in the hundreds of micrograms to low milligram per litre range, which are unlikely to be found in the environment outside exceptionally contaminated sites. However, reproductive disruption and toxicity in reproductive organs have been observed in fish at low environmentally relevant concentrations, especially after chronic exposure. For example, exposure of brown trout to concentrations as low as 1.75 µg/L BPA for ~3.5 months was reported to cause significantly reduced sperm density and motility in males and delayed ovulation in females, with 5µg/L inhibiting ovulation entirely (Lahnsteiner *et al.*, 2005). After 14 days of exposure to BPA in carp, Mandich *et al.* reported severe alterations in testis structure and atresia of oocytes starting from 1µg/L, and alterations in sex steroid levels at 1µg/L and 10 µg/L that were not significantly affected at 1mg/L (Mandich *et al.*, 2007). Vitellogenin induction in males – a biomarker of oestrogen exposure in fish, has been reported at concentrations as low as 10µg/L (Correia *et al.*, 2007). Hatef *et al.* reported reduced androgen levels and sperm motility in goldfish after 20 days exposure to as little as 0.6µg/L BPA (Hatef, Alavi, *et al.*, 2012). Continuous exposure of zebrafish to 0.228µg/L BPA from 8hpf for two generations was found to cause a range of toxic effects. These included a female-biased sex ratio in both the exposed F0 and F1 generations; reduced sperm

density and motility, and disruption of mitochondrial biogenesis and Wnt signalling in the gonad of exposed F1 males; and reduced mRNA expression of several DNA methyltransferases in F2 larvae from exposed F1 males (Chen *et al.*, 2015). Qui et al (2016) reported evidence of immune disruption in carp after chronic exposure to concentrations as low as 0.1µg/L BPA (Qiu *et al.*, 2016).

BPA is also known to cause toxicity in aquatic invertebrates, often reported at lower concentrations that those affecting vertebrates, with effects including feminisation (Oehlmann *et al.*, 2000), reproductive disruption (Aarab *et al.*, 2006; Oehlmann *et al.*, 2006), and supressed growth and development (Marcial, Hagiwara and Snell, 2003; Kiyomoto *et al.*, 2006), with certain taxa, such as freshwater molluscs, being hypersensitive to BPA (Flint *et al.*, 2012). However, the existence of low dose effects for BPA have been disputed by some studies, which failed to reproduce some of these findings, and highlighted that the mechanistic basis for reported low dose effects are not reported or are not fully understood (Vandenberg *et al.*, 2009). This remains a controversial issue surrounding regulatory assessment of BPA, and requires further investigation.

Additional controversy exists around reports that some endocrine disrupting chemicals, including BPA, may induce a non-monotonic dose response (NMDR), where high and low concentrations induce a stronger response than intermediate concentrations (Welshons *et al.*, 2003; Welshons, Nagel and Vom Saal, 2006; vom Saal *et al.*, 2007; Vandenberg *et al.*, 2012; Villar-Pazos *et al.*, 2017). This is contested in several studies (Kamrin, 2007; Rhomberg and Goodman, 2012), including the CLARITY-BPA Core Study which found inconsistencies and lack of evidence for NMDRs (NIEHS, 2018).

The activity of EDCs in organisms, including BPA, is highly dependent on context, and can be altered by changes in the physiology of organisms, such as alterations in proportion of receptor subtypes, or presence of other chemicals, such as altered competition from endogenous oestrogens for receptor binding (Vandenberg *et al.*, 2012). Timing of measurements in relation to dynamic changes in physiology occurring, for example, across circadian rhythms or at different stages of reproductive or developmental cycles, could have large impacts on detection of toxic outcomes. Lack of detection of low-dose effects in some studies may reflect that NMDRs are rare and specific to certain molecular pathways, which may go

undetected when a study's methods lack sufficient sensitivity to that endpoint; or that there is variation between studies that is due to alterations in these other contextual factors.

Non-monotonicity could pose a significant challenge to regulatory toxicology. Assessments based on the assumption that high dose effects can be used to predict low dose effects are commonplace in existing regulatory testing, but would not be valid for non-monotonic responses; making establishing safe exposure limits difficult (Vogel, 2004; Vandenberg *et al.*, 2012). Though many examples of NMDRs have been reported in the literature, mechanistic explanations are often lacking, highlighting the need for further investigation.

Another concern for regulatory toxicology is the potential for chemicals to cause multigenerational effects. Several studies have demonstrated that BPA exposure can cause effects across multiple generations in both mammals and fish (Xin, Susiarjo and Bartolomei, 2015). Parental exposure to BPA has been shown to cause toxic effects in offspring that include developmental abnormalities, hormonal disruption, impaired stress response (Birceanu, Mai and Vijayan, 2015; Thomas *et al.*, 2018), growth suppression and metabolic disruption (Aluru, John F. Leatherland and Vijayan, 2010; G. Li *et al.*, 2014), altered brain development and behaviour (Kundakovic *et al.*, 2013; Wolstenholme, Goldsby and Rissman, 2013; Boudalia *et al.*, 2014), and disrupted sexual developmental and reproduction (Susiarjo *et al.*, 2007; Mahalingam *et al.*, 2017). Mechanisms hypothesised to underpin these multigenerational effects include both parental and epigenetic effects, and a combination of these mechanisms. Parental effects can result from developmental BPA exposure *in utero* or via maternal transfer of xenobiotics in gametes or during lactation (Susiarjo *et al.*, 2007; Boudalia *et al.*, 2014). For example, simulated maternal transfer of BPA in trout eggs was shown to cause impaired stress response in offspring for two generations (Thomas *et al.*, 2018). Maternal exposure to BPA during gestation in mice also led to decreased preantral follicle numbers in F1 offspring and disrupted steroidogenesis in F1 and F2 offspring (Mahalingam *et al.*, 2017).

Many studies have provided evidence that persistent epigenetic changes in gene expression both within a generation and across generations may also underpin BPA-induced phenotypic changes. This is often as a result of exposure to BPA

during windows of susceptibility in early development, during which the epigenome is thought to be more sensitive to environmental influence.

One of the first examples was reported in the Agouti mouse model. *In utero* exposure to BPA was shown to cause hypomethylation at an upstream retrotransposon regulating the Agouti gene, leading to altered gene expression and a change in coat colour. Maternal dietary supplementation with a methyl donor was able to rescue this BPA-induced phenotype (Dolinoy, Huang and Jirtle, 2007).

Several mammalian studies have since demonstrated that maternal BPA exposure can cause lasting disruption of developmental epigenetic programming at imprinted genes in offspring (Bromer *et al.*, 2010; Chao *et al.*, 2012; Susiarjo *et al.*, 2013).

For example, *in utero* exposure of mice to BPA led to altered regulation of Hoxa10 – a homeobox gene which controls uterine organogenesis. mRNA and protein expression was upregulated in the reproductive tracts of maternally exposed mice, correlating with a parallel decrease in DNA methylation at regulatory regions of the *Hoxa10* gene (Bromer *et al.*, 2010). Susiarjo *et al.* (2015) reported sex-specific metabolic effects, including glucose intolerance, in F1 male mice following *in utero* BPA exposure, which were partially inherited by F2 males, and correlated with altered DNA methylation and mRNA expression of the imprinted gene, insulin-like growth factor 2. Unlike exposed F0 females, which exhibited glucose intolerance during gestation, the metabolic milieu of F1 females was unaffected during gestation, suggesting a role for an epigenetic mechanism underlying metabolic effects in the F2 (Susiarjo *et al.*, 2015). Similar glucose intolerant and insulin resistant phenotypes were also observed in F2 mice by Li *et al.* (2014) after exposure of F0 females during gestation and lactation. This was accompanied by reduced expression of the gene encoding glucokinase in the liver, whose promoter became hypermethylated compared to controls (Li *et al.*, 2014).

In utero exposure of mice to environmentally relevant concentrations of BPA has also been found to cause sex-specific changes in the mRNA expression of genes encoding oestrogen receptors and epigenetic regulators in the brains of male and female offspring. This included changes in *Esr1* and *Dnmt* mRNA expression that correlated with changes in DNA methylation at the *Esr1* gene (Kundakovic *et al.*,

2013). Transient developmental exposure of rats to low-dose BPA was also reported to lead to increased risk of adult-onset carcinogenesis in the prostate, which was associated with epigenetic alterations at several cell signalling genes (Ho *et al.*, 2006). As well as changes in the methylome, BPA exposure has also be linked with alterations in histone modifications. *In utero* exposure of mice was found to increase the expression of the histone methyltransferase, Enhancer of Zeste Homolog 2, in the mammary gland, accompanied by an increase in histone H3 trimethylation in later life – both of which have been linked with increased breast cancer risk (Doherty *et al.*, 2010).

Epigenetic alterations have also been observed in fish as a result of BPA exposure. For example, following exposure of zebrafish to 1 mg/L BPA, Laing *et al.* (2016) observed significantly reduced mRNA expression of DNA methyltransferase 1 (*dnmt1*) in the ovary and testis, associated with significant global hypomethylation (Laing *et al.*, 2016). Exposure of rare minnow to 15µg/L BPA for 7 and 35 days caused significant changes in the expression of several genes encoding methyltransferase enzymes in the ovary, with the direction of change differing for some genes at 7 and 35 days. This was correlated with a significant increase in global DNA methylation at 35 days, which was not significant at 7 days. Additionally, DNA methylation upstream of the aromatase (*cyp19a1a)* gene was significantly decreased and mRNA expression significantly increased after 7 days exposure, with the opposite effect observed after 35 days (Liu *et al.*, 2014). This suggests that BPA affects epigenetic regulation in the gonad, but that effects may differ over time.

Some studies have also reported evidence of multigenerational toxic phenotypes hypothesised to be caused by transgenerational epigenetic inheritance of BPA-induced transcriptional alterations. Lombó *et al.* (2015) reported significant induction of cardiac and skeletal deformities in zebrafish at 7dpf in F1 and F2 offspring following exposure of F0 males to 2mg/L BPA during spermatogenesis. Transcript abundance of two insulin receptor genes important during early development, *insra* and *insrb*, was significantly downregulated in F0 and F1 spermatozoa, as well as significant downregulation of their mRNA expression in F1 and F2 embryos. Morphants of these genes had previously been observed to develop similar deformities (Toyoshima *et al.*, 2008). This suggests that BPA-induced alterations in the mRNA contribution of F0 sperm potentially led to altered

epigenetic regulation of these genes that was transgenerationally inherited to the F2, resulting in transgenerational toxicity (Lombó *et al.*, 2015).

Manikkam *et al.* (2013) also describe potential transgenerational epigenetic inheritance of disease phenotypes via the male germline in rats; though exposure was to a mixture of plastic-derived EDCs, including BPA, bisphthalate, and dibutyl phthalate, so specific effects of BPA cannot be determined. Exposure of gestating F0 females during the period of foetal gonadal sex determination caused a significant increase in the incidence of a variety of adult onset disease phenotypes in subsequent generations, including pubertal abnormalities, testis disease, obesity, and ovarian disease, which were inherited to the unexposed F3 generation. Analysis of the F3 generation sperm methylome identified epimutations at genes previously linked to several of the observed transgenerational disease phenotypes, suggesting possible transgenerational epigenetic inheritance via the male germline (Manikkam *et al.*, 2013).

Transgenerational epigenetic inheritance of BPA-induced toxic phenotypes has also been reported via the maternal line in zebrafish. Following exposure of adult F0 females to 20µg/L BPA, Santangeli *et al.* (2019) reported heritable downregulation of anti-Mullerian hormone (*amh*) transcription and hypermethylation of its promoter starting in F1 embryos and persisting in embryos and adult ovaries across three generations up to the F3 embryonic stage (Santangeli *et al.*, 2019).

Epigenetic studies in BPA provide some evidence supporting the existence of transgenerationally inherited disease phenotypes as a result of BPA exposure; however, studies have rarely been able to identify specific epimutations experimentally confirmed to contribute to the disease phenotype that are also present in each subsequent generation, demonstrating faithful germline transmission through multiple generations to at least one unexposed generation. Further study will be required to confirm reports of transgenerational epigenetic inheritance.

Although many studies have demonstrated the ability of BPA to cause long-lasting toxic effects on the physiology of organisms, both within a generation and in subsequent generations, with evidence that some of these effects may be driven by persistent alterations to the epigenome; there has been little investigation into

how persistent effects from historic or ancestral BPA exposure might impact the response of organisms to subsequent BPA exposure. To my knowledge there are no studies investigating the potential for organisms to develop altered tolerance to BPA via acclimation, adaptation, or sensitisation. Given the widespread, long-lasting and sometimes fluctuating exposure of wildlife to BPA both within and across generations, it is important to investigate the potential for BPA sensitivity of organisms to change, in order to accurately assess the risk that repeated and chronic exposure to BPA poses to wildlife.

7.2. Copper

Toxic metals are another common group of environmental pollutants and are particularly problematic as they are chemically and biologically indestructible (Fragou *et al.*, 2011). However, some toxic metals, as well as acting as toxic pollutants at high concentrations, are also essential metals, resulting in more complex toxicological effects in organisms.

Copper is an essential micronutrient for all eukaryotes and many prokaryotes (WHO, 1998; Festa and Thiele, 2013). For example, Cu is an essential cofactor for many enzymes in the body, including enzymes involved in intestinal transport; formation of connective tissue, myelin in neurons and melanin in skin, hair and eyes; scavenging of free radicals; and production of neurological hormones (Gaetke, Chow-Johnson and Chow, 2014). Notably, it is essential for generation of ATP by acting as cofactor for cytochrome c oxidase – the last enzyme in the respiratory electron transfer chain, which catalyses reduction of oxygen to water in mitochondria (Gaetke, Chow-Johnson and Chow, 2014). However, at high concentrations copper can become toxic, and is therefore tightly regulated within organisms to ensure a balance between maintaining an adequate supply of copper for essential processes and preventing accumulation of toxic forms of copper at high levels. Due to the more complex nature of eukaryotic cells and multicellular eukaryotic organisms, complex processes of Cu homeostasis evolved in eukaryotes to control delivery at both an intracellular level – to specific intracellular compartments or proteins, and an extracellular level – to specific tissues and organs (Festa and Thiele, 2013). Organisms also control copper levels by altering its rate of uptake and excretion (Gaetke, Chow-Johnson and Chow, 2014).

Aquatic organisms are particularly susceptible to waterborne copper contamination as, in addition to dietary exposure, they are continuously exposed via the gills (Grosell and Wood, 2002). Studies in freshwater fish have shown that branchial and intestinal copper uptake interact, and can be differentially regulated according to the copper status of an animal, with increased uptake through one route reducing uptake via the other (Kamunde *et al.*, 2001, 2002; Grosell and Wood, 2002; Minghetti, Leaver and George, 2010). Although the intestine is the

major route of copper uptake under normal dietary conditions, when dietary copper availability is suboptimal, uptake across the gill compensates for this. However, under exposure to excess waterborne copper, the gill is thought to be the main route of uptake.

The exact mechanisms of branchial copper uptake have not been fully described, however, three mechanisms of uptake, including sodium-sensitive and sodium-insensitive pathways, have been hypothesised, largely based on research conducted in rainbow trout, and are reviewed by Grosell (2012).

First, copper ions can compete with sodium ions for uptake through apical sodium channels. This is supported by evidence from many studies in fish (Sola, Isaia and Masoni, 1995; Pyle *et al.*, 2003; Sloman *et al.*, 2003); including demonstration of inhibition of Cu uptake in trout gills using a sodium channel blocker (Grosell and Wood, 2002). This leads to competitive inhibition of apical sodium uptake; which, along with inhibition of other sodium transporters and increased sodium efflux, contributes to copper-induced disruption of sodium homeostasis (Grosell, 2012).

It is thought that Cu is also taken up via a copper-specific apical transport protein. A likely candidate is copper transporter 1 (ctr1), also known as solute carrier family 31 member 1 (slc31a1); whose expression in the gills has been demonstrated in numerous fish species (Mackenzie *et al.*, 2004; Minghetti *et al.*, 2008; da Silva *et al.*, 2014; Cheng *et al.*, 2017; Anni *et al.*, 2019).

A third hypothesised route of apical uptake is via divalent metal transporter 1 (dmt1)(Grosell, 2012), also known as solute carrier family 11 member 2 (slc11a2); which is known to promiscuously transport a variety of divalent metal ions and whose expression has been demonstrated in the gill (Bury, Walker and Glover, 2003).

Transport across the basolateral membrane of the gill epithelium likely involves active transport via ATPase copper transporting alpha (atp7a) (Craig, Galus, *et al.*, 2009; Minghetti, Leaver and George, 2010; Chen and Chan, 2011b).

Once taken up by cells, Cu must be sequestered by proteins, such as chaperones, storage proteins or cuproenzymes, to prevent oxidative damage from free Cu ions and deliver copper to specific cellular targets (Grosell, 2012; Gaetke, Chow-Johnson and Chow, 2014). Intracellular mechanisms of copper homeostasis have

not been entirely characterised in fish, but are thought to be similar to those in mammals. Many conserved copper chaperone proteins are known to exist in fish, including metallothioneins (mt) and glutathione, which, as well as performing antioxidant and reactive oxygen species (ROS) detoxification functions, sequester and store cytosolic copper; antioxidant protein 1 (atox1), a chaperone that delivers copper ions to transporters; copper chaperone for superoxide dismutase (ccs), which transfers copper to the copper/zinc superoxide dismutase antioxidant complex (SOD1); and cytochrome c oxidase copper chaperone (cox17), which acts as a copper chaperone for the biogenesis of cytochrome c oxidase in the aerobic respiratory chain (Grosell, 2012; Pereira, Campos and Bogo, 2016). Many of these chaperones are also known to be differentially regulated in response to copper exposure in fish (Craig, Wood and McClelland, 2007)(Chen and Chan, 2011a).

After excretion from cells, Cu is transferred to proteins in the blood, such as ceruloplasmin (Cousins, 1985), which transports copper to sites of storage or metabolism, including the liver, which is main site of Cu metabolism and excretion (Grosell, 2012). There is also some limited evidence that Cu may be excreted across the gills; however, this was inferred from detection of ambient Cu in the water around Cu infused trout that was determined not to be of hepatobiliary origin, and is yet to be verified with direct measurement at the gills (Grosell, McGeer and Wood, 2001).

Exposure to excess copper that overwhelms copper handling mechanisms can lead to toxicity. One of the main toxic effects of copper is disruption of ion homeostasis through competitive and non-competitive inhibition of several important ion transporters, leading to net loss of sodium, chloride and potassium and disruption of osmoregulation at the gill. For example, competitive inhibition of apical sodium channels and inhibition of the basal Na^+/K^+-ATPase – an important ionoregulatory enzyme that maintains the ion gradient across the gill by actively transporting Na^+ into the blood – contributes alongside increased sodium efflux, to cause net loss of sodium (Laurén and McDonald, 1985).

As well as disrupting ion balance in the gill, toxic levels of copper can also cause damage to the epithelial surface of the gill, including cell degeneration via necrosis and apoptosis; lifting, rupture, and peeling of the lamellar epithelium; lamellar

fusion; hyperplasia; and cellular hypertrophy (Mazon, Cerqueira and Fernandes, 2002). Additionally, copper exposure is known to induce epithelial cell proliferation and epithelial thickening at the gill surface. It is thought to initially be a compensatory response that attempts to increase the surface area of the gill, but as toxicity progresses this thickening increases the diffusion distance between the blood and the water, impairing gas exchange and causing respiratory distress (Wilson and Taylor, 1993b; Pelgrom *et al.*, 1995; Perry, 1997; Heerden, Vosloo and Nikinmaa, 2004).

Copper exposure has also been observed to disrupt ammonia excretion, which is thought to occur primarily across the gill, and is thought to involve disruption of carbonic anhydrase (CA) activity, which has been observed in guppies (Zimmer *et al.*, 2012). Disruption of CA activity – which catalyses the conversion of CO_2 and H_2O to HCO_3^- and H^+ - also contributes to copper-induced disruption of intra- and extracellular acid-base balance (Wilson and Taylor, 1993a; Wang *et al.*, 1998; Grosell, 2012).

When copper storage and antioxidant defences are overwhelmed, free cellular copper also causes oxidative stress by production of ROS, leading to oxidative damage of DNA, lipids, and proteins (Craig, Wood and McClelland, 2007; Bopp, Abicht and Knauer, 2008; Gaetke, Chow-Johnson and Chow, 2014). Disruption of blood chemistry, increased energy demands for processes of detoxification, repair and restoration after homeostatic disruption, and direct inhibition of metabolic enzymes also leads to copper-induced metabolic stress (Beaumont, Butler and Taylor, 2000; Carvalho and Fernandes, 2008; Oliveira *et al.*, 2008; Braz-Mota *et al.*, 2018; Kim *et al.*, 2018).

Copper is a significant pollutant of both marine and freshwater systems. In the marine environment, harbours and marinas, made up of relatively sheltered waters which experience very little mixing, are particularly vulnerable to build up of copper to toxic levels. A major source of copper in harbours is from antifouling paints, which contain copper oxide as a biocide, especially since the tributyltin ban in 2003 (Schiff, Diehl and Valkirs, 2004; Sussarellu *et al.*, 2018). These paints can contain between 20% and 76% copper as a biocide, which were shown by Schiff et al. to leach copper at a rate of approximately 4µg/cm^2/day, equating to around 25g/month for a typical 9m powerboat (Schiff, Diehl and Valkirs, 2004; Schiff *et al.*,

2007). Other studies in the US and UK report even higher levels of leaching of over 20µg/cm^2/day (Thomas *et al.*, 1999; Valkirs *et al.*, 2003). In a study of San Diego marinas, 86% of marina surface waters tested breached water quality objectives, with dissolved copper concentrations up to 13.4µg/L (Schiff *et al.*, 2007). Copper contamination of anthropogenic origin, including contamination from vessels and runoff from land, lead to considerable copper contamination of coastal water (Santos-Echeandia *et al.*, 2008). A study of costal water in California found that while pollution with other metals, such as lead and silver, were decreasing, copper contamination increased by 25% from 1977 to 1990 (Stephenson and Leonard, 1994).

In freshwater systems, naturally occurring background copper levels range from 0.2 – 30µg/L (U.S. EPA, 2007; Sevcikova *et al.*, 2016). However, due to anthropogenic input, copper levels can soar to highly toxic concentrations, which can vary widely. A meta-analysis by Donnachie *et al.* (2014) of metals threatening freshwater organisms in the UK reported copper concentrations in 1455 rivers ranging from 0.02 – 133µg/L, and ranked copper as the metal of most concern based on exposure via the water column (Donnachie *et al.*, 2014). In areas substantially affected by copper-rich anthropogenic discharges from mining, agriculture and industry, concentrations can reach 100µg/L or more, with extreme reports in mining areas of up to 200 mg/L (USEPA, 2007).

Concentration and bioavailability of anthropogenic copper can vary widely both spatially, due to distribution of point source inputs within an environment and propensity for copper and other compounds that affect its bioavailability to build up in certain portions of a habitat such as sediments (Besser, Ingersoll and Giesy, 1996); and temporally, due to temporal variation in use of copper-containing substances and the influence of local and seasonal weather patterns that impact runoff from mining and agriculture. For example, copper sulphate is a major component of the “Bordeaux mixture” pesticide, whose seasonal use leads to copper rich agricultural run-off in freshwater systems (Sussarellu *et al.*, 2018). The Environment Agency has identified mines as one of the largest drivers of pollution under the Water Framework directive, with copper being among the metals for which Environmentally Quality Standards failures were commonly linked with abandoned mines (Tipping *et al.*, 2009). Metal discharge from mines can be heavily influenced by local and seasonal weather patterns. In a study of high-

elevation headwaters in the US Rocky mountains, Sullivan and Drever (2001) found that acidic mine drainage caused an increase of at least 10-fold in levels of several toxic metals immediately downstream, including copper, which increased by a factor of almost 100-fold to nearly 0.65 mg/L, despite drainage from the mine contributing only 5% of flow in the creek even at low-flow. The metals were then diluted along the length of the creek. High seasonality of metal concentrations was observed, with decreasing metal concentrations being associated with seasonal snow-melt leading to increased flow and dilution (Sullivan and Drever, 2001).

Other sources of copper contamination are also impacted by seasonal weather patterns. A study by Gaur *et al.* (2005) assessing seasonal variation in toxic metal distribution in water and sediment in the river Gomti, India, found that concentrations of six metals (Cd, Cr, Cu, Ni, Pb, and Zn) were all higher in both water and sediment during the rainy season, compared to summer and winter. This seasonality was likely caused by increased agricultural, industrial and domestic runoff during the rainy season (Gaur *et al.*, 2005). Copper concentrations in the water ranged from 12 – 19µg/L in summer, 0–6 µg/L in winter, and 16 – 35µg/L in the rainy season. Copper concentrations in the sediment ranged from 4.36 – 79.54µg/g in summer, 4.98 – 47.14µg/g in winter, and 3.89 – 91.17µg/g in rainy season. The Gomti river water pH was found to be high (pH 7.5 – 8.5), favouring the precipitation of metals in sediment.

Like many other toxic metals, copper bioavailability in freshwater is influenced by water parameters that influence its speciation and propensity to bind to copper binding proteins and transporters of organisms, as discussed above. Fluctuations in water parameters, including river flow, DOM, DOC, salinity, hardness, alkalinity, pH, salinity, pH, and temperature, have been observed to fluctuate over time in copper-affected freshwater systems, and impact the concentration, bioavailability and toxicity of copper to aquatic organisms (Winner and Owen, 1991; Gundersen and Steinnes, 2001; Gundersen, Olsvik and Steinnes, 2001; Meylan, Behra and Sigg, 2004; Waeles, Riso and Le Corre, 2005; Martin and Goldblatt, 2007; Whitby, Hollibaugh and van den Berg, 2017). For example, copper bioavailability is generally considered to increase with decreasing pH, salinity, hardness, alkalinity and concentration of other ligands; rendering freshwater organisms much more vulnerable to copper toxicity than marine organisms, which occupy water with higher levels of these protective factors (Grosell, 2012).

The fluctuating copper concentrations and bioavailability in the environment pose a significant challenge to risk assessment and environmental protection, which currently does not properly account for this temporal variation. Work by Lathouri and Korre (2015), who observed significant temporal and seasonal variation in water parameters and copper bioavailability and toxicity in UK rivers, demonstrated how temporal variation could be incorporated into biotic ligand modelling to improve assessment of threats to copper environmental quality standards across time (Lathouri and Korre, 2015). However, as previously discussed, this cannot account for the effects of potential altered copper tolerance of historically exposed animals on toxic outcomes for wild populations experiencing fluctuating copper exposure.

There are numerous examples of populations of various species that have developed tolerance to elevated copper levels, including wild fish populations. These include examples of local adaptation against chronic copper exposure over many generations, via genetic selection (Gale *et al.*, 2003). Genetic adaptation against copper toxicity has been observed in population of numerous fish species (Schlueter *et al.*, 1995; Bourret *et al.*, 2008) For example, a population of trout inhabiting the River Hayle in Cornwall (UK) was reported to be occupying water with copper concentrations that would be lethal to naïve fish, thought to be facilitated by genetic adaptation of metal- and ion-homeostasis pathways (Durrant *et al.*, 2011; Uren Webster *et al.*, 2013). For example, Paris and Usher (2019) investigated different isoforms of metallothionein in these trout populations, and found that expression of specific isoforms were important factors contributing to the increased tolerance against several metals in metal-adapted populations (Paris and Usher, 2019a, 2019b). Altered transcriptional responses of a stickleback population historically exposed to copper, suggestive of adaptive changes in copper handling, were also thought to involve genetic adaptation (Uren Webster *et al.*, 2017). Copper tolerance associated with genotype variation has also been observed experimentally in a fathead minnow population (Kolok *et al.*, 2004). Genetic adaptation to copper exposure has also been reported in numerous invertebrate species, including *Daphnia* species (Agra *et al.*, 2010), earthworms (Fisker *et al.*, 2011; Fisker, Holmstrup and Sørensen, 2013), and polychaetes (Bryan and Hummerstone, 1971).

These genetic adaptations are thought to involve selection for tolerant alleles of genes (or their regulatory regions) involved in the physiological response to copper exposure. There are many mechanisms by which organisms can adapt their physiology in their response to copper exposure, including changes preventing uptake and accumulation, and increasing excretion (Grosell, McGeer and Wood, 2001; Gale *et al.*, 2003); preventing toxic damage through enhanced resistance to oxidative stress; and compensating for negative effects of copper on various important homeostatic processes (Sellin, Tate-Boldt and Kolok, 2005; Adeyemi and Klerks, 2013; Bougas *et al.*, 2016). For example, organisms can prevent uptake and increase excretion of copper by regulating expression of copper transporters in different tissues (Minghetti *et al.*, 2008; Uren Webster *et al.*, 2013; da Silva *et al.*, 2014). Organisms can increase the efficiency of sequestration of copper in non-toxic forms and upregulate factors involved in the oxidative stress response to prevent damage from free copper, including upregulating the synthesis of copper chaperones and antioxidants, such as metallothionein and glutathione (Minghetti *et al.*, 2008; Eyckmans *et al.*, 2011; Uren Webster *et al.*, 2013). Compensatory changes in regulation of ion homeostasis pathways can also combat copper-induced disruption of ion homeostasis, osmoregulation and acid-base balance (Larsen, Pörtner and Jensen, 1997; De Polo *et al.*, 2014). Many of these responses are elicited through activation of a hormonal stress response (Schreck and Lorz, 1978; Bury *et al.*, 1998).

As well as genetic adaptation of copper response pathways, increased tolerance to copper as a result of previous exposure is known to be acquired within a generation through non-genetic mechanisms, such as acclimation. Numerous studies have demonstrated acclimation of fish to low-level copper exposure that then infers tolerance to subsequent lethal challenge, by altering regulation of copper response pathways, including copper transport, ion regulation, and oxidative stress (Dixon and Sprague, 1981; Buckley *et al.*, 1982; Miller *et al.*, 1993; Taylor *et al.*, 2000; Adeyemi and Klerks, 2013). However, although fish were more tolerant to acute copper exposure immediately following acclimation, this tolerance is usually lost soon after being transferred to clean water for recovery (Dixon and Sprague, 1981).

Physiological adaptation against copper, as for other toxicants, is known to incur costs (Kwok, Grist and Leung, 2009; Hamilton *et al.*, 2017). Temporary acclimation may therefore be beneficial over permanent commitment to physiological adaptations that occur with genetic adaptation, should environmental conditions be likely to change, when the benefits of adaptation to copper may no longer outweigh the costs. However, acclimation alone may not provide lasting protection against future exposure to copper in fluctuating environments. It has been suggested that epigenetic adaptation to stressors may provide an alternative mechanism by which organisms can develop long-lasting tolerance to environmental stressors after historic exposure.

Several studies have provided evidence for interaction between copper exposure and alterations to the epigenome. Observations in a Wilson disease mouse model demonstrated that maternal methyl supplementation and treatment with a copper chelator altered global methylation patterns of offspring during gestation and restored transcription levels of genes involved in pathways dysregulated in Wilson disease to control levels, including methionine metabolism, oxidative phosphorylation and mitochondrial function (Medici *et al.*, 2016). This implication of epigenetic mechanisms in disruption caused by accumulation of copper in Wilson disease, provides evidence that excess copper is able to alter epigenetic pathways. Copper has also been observed to cause epigenetic alterations in plants. For example, exposure of rice seedlings to high concentrations of copper was shown to cause hypomethylation at several transposable elements and protein-coding genes (Ou *et al.*, 2012; Vandegehuchte and Janssen, 2014). Exposure of zebrafish to copper during embryogenesis was shown to significantly upregulate transcription of three *de novo* DNA methyltransferase genes (Dorts *et al.*, 2016). Copper exposure in PC12 cells has been shown to alter mRNA expression of several enzymes involved in epigenetic functioning, including DNMT1 and NAD-dependent deacetylase sirtuin-1 (Sun *et al.*, 2014). Several studies have also demonstrated an effect of copper exposure on histone modifications (Cheng, Choudhuri and Muldoon-Jacobs, 2012). For example, exposure of human hepatoma cells to copper was shown to cause a decrease in the overall histone acetylation and a decrease of histone H3 and H4 acetylation through direction inhibition of histone acetyltransferase activity (Kang *et al.*, 2004).

Epigenetic alterations may therefore contribute to observed copper toxicity or development of tolerance, though few studies have investigated this.

Together, the literature has provided strong support for the capacity of fish and other aquatic organisms to adapt to metal pollution, including copper, via genetic adaptation. In addition, the potential for acclimation within a short period after the initial exposure to copper and other metals is also widely supported in numerous studies for teleost species. Evidence is starting to emerge documenting the potential for copper to cause epigenetic alterations in vertebrates and linking this with modified tolerance or disease susceptibility. Therefore, there is a knowledge gap as to what extent epigenetic alterations contribute to altered susceptibility of environmental species to metals, including copper, and the relative contribution and interactions between genetic and epigenetic adaptation, and this should be a research priority in the future.

8. Aims and hypotheses

In this thesis, I aimed to investigate the effects of historic exposure to environmental pollutants on the response of organisms to future exposure both within a generation in subsequent generations. I aimed to address the following objectives and test the stated hypotheses:

1) ***Investigate how previous exposure to BPA affects the response of zebrafish upon re-exposure***:

Given the evidence that BPA affects epigenetic pathways causing persistent changes in gene expression and phenotype, particularly in relation to reproduction, and can exert effects on subsequent generations, I aimed to investigate how previous exposure to this chemical may alter the response of pre-exposed fish and their offspring to subsequent exposure. This objective aimed to address the following questions:

- Does previous exposure of adult zebrafish affect their transcriptional and physiological response upon re-exposure?

- Does parental exposure to BPA affect the response of offspring upon subsequent exposure during early development?
- Do effects of parental exposure to BPA on offspring during early life differ between offspring of naïve fish compared to fish with prior exposure to BPA?

I hypothesised that exposure of adult zebrafish to BPA would cause altered expression of target genes involved in reproduction and epigenetic signalling, and that their response upon re-exposure would be different, possibly due to stable epigenetic alterations induced during the pre-exposure period.

In addition, I hypothesised that embryos originating from exposed parents would be more susceptible to BPA than embryos originating from control parents. Further, I also hypothesised that pre-exposure of the parents would result in altered responses of their embryos compared to those originating from naïve parents experiencing exposure to BPA for the first time during gametogenesis.

2) ***Investigate the influence of copper exposure during embryogenesis on the responsiveness to copper upon re-exposure in three-spined stickleback (Gasterosteus aculeatus):***

In work by Laing (2017), stickleback embryos exposed to copper during embryogenesis were observed to have increased copper concentrations in their tissues as adults compared to naïve fish, indicating persistent alterations in physiology. In addition, pre-exposed fish also showed increased copper accumulation in the gill upon re-exposure, which did not occur in naïve fish. In this objective I utilised gill samples from the experiment conducted by Laing (2017) to measure the global transcriptome profiles and interrogated the resulting data to address the following questions:

- Do fish pre-exposed to copper during early life maintain a differential transcriptome profile into later life following depuration?
- Do fish pre-exposed to copper during early life exhibit an altered transcriptional response upon re-exposure compared to naïve fish?
- What are the differences in transcriptional response upon re-exposure between populations?

- Do they explain the altered physiology underpinning differential copper accumulation between populations?
- Do observed transcriptional changes suggest alterations in other aspects of physiological response to copper, including potential changes in copper tolerance?

I hypothesised that early life exposure to copper would likely induce changes in the transcription of genes involved in copper uptake, storage and or/excretion which are maintained throughout life via epigenetic modifications, explaining the altered copper accumulation in the tissues of pre-exposed fish. I also hypothesised that pre-exposed fish would exhibit altered transcriptional response upon re-exposure to copper compared to naïve fish, including additional alterations in regulation of similar genes involved in copper handling, reflecting the observed increase in copper accumulation upon re-exposure; as well as differential transcriptional response of genes involved in other metal response pathways and pathways known to be negatively impacted by copper toxicity. I hypothesise that the differential transcriptional response of pre-exposed fish will suggest an adaptive response in these fish, including altered regulation of pathways involved in metal detoxification and oxidative stress response, and potentially compensatory changes in the regulation of pathways known to be disrupted by copper, such as ion regulation, acid base balance, and ammonia excretion.

3) Investigate the potential transgenerational inheritance of differential responses to copper exposure in stickleback and whether these effects are conserved in other fish species:

Following on from previous work by Laing (2017), I aimed to determine whether differential susceptibility to copper induced in offspring of adult stickleback pre-exposed during early life and maintained to the F2 generation was inherited to the F3 generation, and whether this is driven by transgenerational epigenetic inheritance. I will aim to address the following questions:

- Does exposure to copper during embryogenesis cause long lasting tolerance up to the F3 generation?
- Is the multigenerational tolerance induced by early life exposure to copper in stickleback conserved across fish species?

I hypothesised that copper tolerance induced by early life exposure of F0 embryos would either be inherited to the F3, indicating potential transgenerational epigenetic inheritance, or would be lost in the F3 generation, indicating that tolerance in F1 and F2 embryos was likely driven by maternal effects. I also hypothesised that early life exposure to copper might also induce a similar multigenerational tolerant phenotype in zebrafish as observed in stickleback.

9. Model species

For this work, I chose two model fish species: the zebrafish (*Danio rerio*) and the three-spined stickleback (*Gasterosteus aculeatus*).

As oviparous species, they provide useful models in which to study multigenerational effects of chemical exposure, with fertilisation and embryonic development occurring outside the body of the parent. As previously discussed, this allows simplification of exposure designs for investigating multigenerational effects, with exposure of either parent resulting in direct exposure of only the F0 male or female and the gametes that give rise to the F1 generation. External fertilisation also allows for easier measurement of potential parental transfer of chemicals to offspring via the gametes. Reducing the number of directly exposed generations enables easier separation and interrogation of mechanisms and heritability of different toxic effects of chemicals in each generation, which that can occur simultaneously under more complicated exposure regimes, such as gestational exposure, in which the F0 female, F1 embryos and its differentiating gametes (F2) are simultaneously exposed. It is often desirable to apply chemical exposure during embryogenesis – a critical window of susceptibility during which organisms are thought to be most sensitive to environmental influences. In oviparous fish, this can be achieved through direct exposure of the externally developing embryo, allowing more control over exposure conditions endpoints than would be possible in a viviparous model, in which exposure during embryogenesis is modulated by the internal toxicokinetics of the mother. The externally developing and transparent embryos of these species are also more accessible for assessment of early developmental endpoints.

Other advantages of the zebrafish for environmental toxicology studies include its small size, low maintenance costs, rapid and well-described development, short generation time of ~3 months, high fecundity, and wide availability of genetic resources; including a fully sequenced and relatively well-annotated genome, with considerable homology with human genome identified (Dai *et al.*, 2014). Highly isogenic laboratory reared lines of zebrafish can also provide genetic stability, reducing the risk of confounding factors in studies investigating toxicological effects on epigenetic functioning. The wide availability of a variety of zebrafish mutants, including for reporter genes, also makes them convenient model for environmental monitoring and high throughput bioassays, and for mechanistic studies (Dai *et al.*, 2014).

Stickleback also offer a model with a small size, low maintenance costs, and a fully sequenced genome. Although they have slightly slower development, a longer generation time of ~9 months, and lower fecundity than the zebrafish, these traits still offer more convenience than many other vertebrate models. They also exhibit fertilisation success of almost 100% using *in vitro* fertilisation and experience low background levels of embryo and juvenile mortality, helping to prevent genetic selection occurring in multigenerational experiments. Unlike zebrafish, which are native to South Asia, the native range of three-spined stickleback includes most of the northern hemisphere, where it can occupy both the freshwater and marine environment. For this work we sourced stickleback from the River Erme, in Devon. Our laboratory reared populations remained relatively few generations from the wild compared to laboratory strains of zebrafish, which experience substantial inbreeding. This made them an environmentally relevant model for studying pollutants threatening European freshwaters, such as copper.

Chapter 2

Does pre-exposure to bisphenol A affect the susceptibility of breeding zebrafish (Danio rerio) upon re-exposure?

Declaration of contributions:

I conducted all adult in *in vivo* exposures and reproductive output assessments, and conducted embryonic exposures with assistance from undergraduate project students. Chemical analysis was conducted by Maciej Trznadel at the University of Exeter. I conducted all molecular and statistical analyses, and data interpretation, and wrote the chapter.

1. Introduction

Bisphenol A (BPA) is a commercially important chemical plasticiser, with one of the highest global production volumes of any industrial chemical, at over 3 million tons per annum (Vandenberg, 2007). BPA is used in the production of epoxy resins, which line food and beverage containers, and in the production of polycarbonate plastics, which are used in various consumer products from water piping and toys to food packaging and thermal receipt paper (Mileva *et al.*, 2014). As a result, human exposure is of great concern. Studies in Asia and the USA have reported detection of BPA in urine samples of ~95% of adults tested (Calafat *et al.*, 2005; Zhang *et al.*, 2011). It has also been detected in human adult serum, breast milk, foetal plasma and placental tissue, indicating that BPA exposure may occur during sensitive windows of development (Vandenberg, 2007; U.S. Environmental Protection Agency, 2011; Lee *et al.*, 2018). Due to its widespread use, BPA has become ubiquitous in the environment, with concentrations in surface water reaching up to 21µg/L (Staples *et al.*, 2002). Although BPA readily degrades in the environment, it is pseudo-persistent due to continual input from many anthropogenic sources, leading to frequent exposure of wildlife (Flint *et al.*, 2012).

BPA is a known oestrogenic endocrine disruptor that exerts its effects by acting as both an agonist and antagonist of oestrogen receptors (ESRs)(Canesi and Fabbri, 2015). Due to its ubiquity it has become one of the major environmental oestrogens, with two studies in North America and Japan concluding that BPA accounted for the majority of oestrogenic activity in landfill leachate detected using human cell line assays (Mileva *et al.*, 2014). It has also been shown to have anti-androgenic activity, with evidence suggesting that this is partly mediated through antagonism of androgen receptors (ARs)(Canesi and Fabbri, 2015). Although BPA is less potent than some other common environmental xenoestrogens, such as ethinylestradiol (EE2), previous assertions that BPA only resulted in physiological effects at very high concentrations and is efficiently metabolised into less potent metabolites has since been challenged (Mileva *et al.*, 2014). Efficiency of metabolism is known to differ between species, with differing proportions of oestrogenic free-BPA escaping metabolism (U.S. Environmental Protection Agency, 2011). Some studies have also found that metabolites produced from

BPA may be more toxic than BPA itself (Kovacic, 2010; Okuda, Takiguchi and Yoshihara, 2010; Nakamura *et al.*, 2011). Several studies have demonstrated low-dose effects in the ng/L range (Hatef, Alavi, *et al.*, 2012; Chen *et al.*, 2015; Qiu *et al.*, 2016); and, although controversial, some studies suggest that BPA may exhibit a non-monotonic dose response (Welshons, Nagel and Vom Saal, 2006; Rhomberg and Goodman, 2012; Villar-Pazos *et al.*, 2017).

BPA has been shown to disrupt development and reproduction in various species, including in fish (Sohoni *et al.*, 2001; Canesi and Fabbri, 2015; Laing *et al.*, 2016) and mammals (Mileva *et al.*, 2014), with effects including feminisation, altered sex hormone levels, reduced gonadal growth, reduced hatching in fish, reduced sperm quality, metabolic disruption, and disruption to cellular differentiation and brain development (Eric Haubruge, Petit and Gage, 2000; Hetheridge *et al.*, 2001; States *et al.*, 2010; Tse *et al.*, 2013; Ortiz-Villanueva *et al.*, 2017). Aquatic organisms are particularly vulnerable to BPA exposure, as they are continuously exposed via the water (Canesi and Fabbri, 2015). Reproductive effects have been readily observed in fish at concentrations as low as 1–5µg/L (Sohoni *et al.*, 2001; Lahnsteiner *et al.*, 2005; Mandich *et al.*, 2007), well within environmentally relevant levels. BPA has also been implicated in the etiology of various human diseases, including alterations in behaviour, carcinogenic effects, cardiovascular disease, metabolic disruption and reproductive disruption (Rochester, 2013; Mileva *et al.*, 2014; Canesi and Fabbri, 2015; Moreman *et al.*, 2018; Brown *et al.*, 2019).

BPA exposure can cause effects across multiple generations, with exposure of a parent having been shown to cause alterations in the behaviour and physiology of offspring (Boudalia *et al.*, 2014; G. Li *et al.*, 2014; Xin, Susiarjo and Bartolomei, 2015; Mahalingam *et al.*, 2017). Mechanisms underpinning these effects in offspring may include parental effects, such as indirect exposure of offspring through parental transfer of BPA in gametes or *in utero*; and potential BPA-induced alterations to maternal transfer of other factors affecting offspring physiology, including maternal transcripts, hormones and enzymes deposited into gametes. Several studies also implicate epigenetic alterations as a mechanism for toxic effects in offspring, either as a result of indirect exposure to BPA, or potentially through direct transgenerational inheritance of epigenetic alterations from an exposed ancestor (Singh and Li, 2012; Mileva *et al.*, 2014; Santangeli *et*

al., 2016). BPA has been reported to affect several epigenetic signalling pathways (Dolinoy, Huang and Jirtle, 2007; Singh and Li, 2012; Mileva *et al.*, 2014; Renaud *et al.*, 2019; Santangeli *et al.*, 2019), including alterations in transcription of histone modifying enzymes (Renaud *et al.*, 2019) and both *de novo* and maintenance DNA methylation enzymes (Laing *et al.*, 2016; Renaud *et al.*, 2019), and alterations to global DNA methylation (Laing *et al.*, 2016). The ability of BPA exposure to impact subsequent generations through epigenetic alterations was clearly demonstrated using the Agouti mouse model. Maternal exposure to dietary BPA was shown to cause hypomethylation of a retrotransposon upstream from the Agouti gene in F1 offspring. This led to altered expression of this gene and a subsequent change in coat colour. This epigenetic change and associated phenotypic effect could be negated by maternal dietary supplementation with a methyl donor (Dolinoy, Huang and Jirtle, 2007). The potential for BPA to cause long-lasting harmful effects in exposed individuals, and inheritance transfer of toxic effects to subsequent generations, is of great concern for environmental protection.

Much research has focussed on the effects of BPA under single acute exposure, however, less is known about the effects of fluctuating exposures to BPA on the health of exposed organisms and subsequent generations. This is important for environmental risk assessment, as the exposure scenarios wildlife are subject to may fluctuate over time. There is also little research into the potential for adaptation or acclimation of organisms to BPA exposure. This is an important consideration for the survival of wild populations which experience long-term BPA exposure over multiple generations.

This study aims to investigate the effect of pre-exposure of adult fish to BPA on subsequent responses upon re-exposure, and how parental exposure history affects the BPA tolerance of offspring during early life. To investigate this, we compared the reproductive and transcriptional response of fish following a single exposure to BPA; following a pre-exposure and subsequent re-exposure to BPA; or following maintenance in control conditions. As BPA has been shown to cause reproductive toxicity, with proposed mechanisms including oestrogenic, anti-androgenic, and epigenetic effects; I conducted transcriptional analysis in the gonad to investigate the response of genes related to reproduction and epigenetic regulation. I hypothesised that fish pre-exposed to BPA would have an altered

transcriptional response compared to naïve fish upon re-exposure. I also hypothesised that offspring of pre-exposed adults would have altered susceptibility to BPA compared to offspring of naïve adults upon exposure during embryogenesis. To investigate this, embryos were collected adult colonies from each treatment group and re-exposed to a range of BPA concentrations to generate mortality curves.

2. Methods

2.1. Fish source and husbandry

Wild type WIK strain adult zebrafish (originating from the University of Exeter) were randomly allocated into 14 breeding colonies of 5 males and 5 females, and maintained in 15L flow-through tanks. The fish were then kept according to conditions described in Laing *et al.* (Laing *et al.*, 2016). Tanks were supplied with mains tap water 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. Each tank was aerated, supplied with water at a rate of 48L/day, and the water maintained at a temperature of 28 ± 0.5°C and pH 7-7.5. The fish were maintained under a 12:12h light:dark cycle, including 30 minute dawn and dusk transition periods. Fish were fed live Artemia nauplii twice daily (ZM Premium Grade Artemia; ZM Ltd.) and TetraMin tropical flake food (Tetra; Melle, Germany) once daily, to satiation.

2.2. Exposures of breeding colonies of zebrafish to BPA

Prior to exposure (following an initial acclimation period), reproductive output was assessed over a 10-day pre-experimental period to ensure that all colonies were breeding consistently, including assessment of fecundity and fertilisation rate. Two colonies were then randomly assigned to each of 7 treatments groups (figure 1). The study consisted of two 5-day dosing periods separated by a 13-day depuration period in which fish were transferred to clean water and no dosing

occurred. BPA exposure was conducted at two nominal concentrations – a low dose of 10µg/L and a high dose of 100µg/L. Colonies either received chemical exposure only during the first exposure period; only during the second exposure period; or during both exposure periods. A control group which received no chemical exposure throughout both exposure periods was also included.

To begin each exposure period, tanks were spiked with BPA in order to achieve the desired concentration. Continuous dosing was then conducted using a flow-through system. Flow rates (48L/day) were monitored daily, and dosing stocks were replaced every 24 hours. Water samples were collected twice during each exposure period and the depuration, and stored at 4°C in a 1:1 sample to methanol ratio until chemical analysis using methods described in Laing *et al.* (2016).

Reproductive output was assessed by measuring egg production and fertilisation rate for each group each day over time. Eggs were collected daily at approximately 1hpf and transferred to petri dishes containing control water to assess egg output and fertilisation success by visual inspection using a dissection microscope (Motic DM143, Hong Kong).

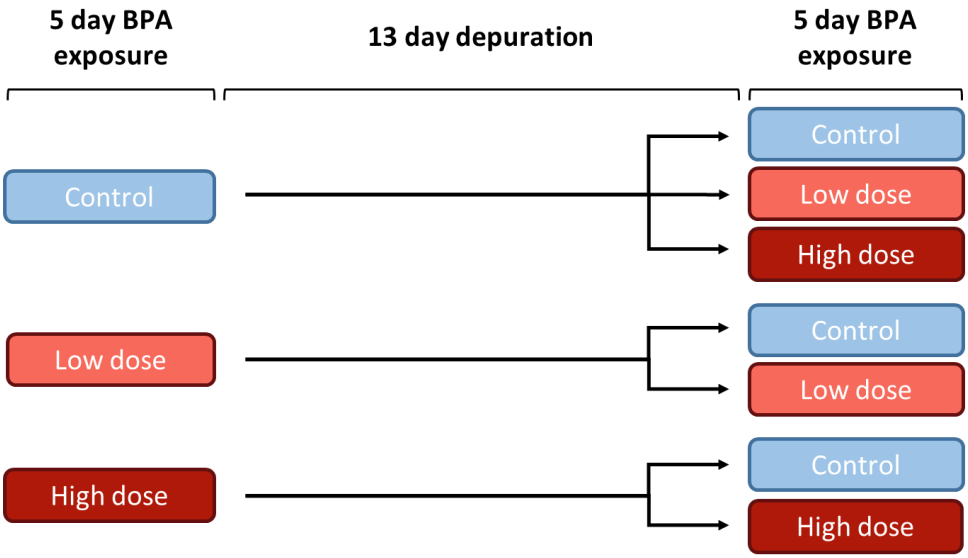


Figure 1: *Ault exposure regime. Breeding colonies of zebrafish were exposed to BPA during two 5-day dosing periods separated by a 13-day depuration period. One low dose and one high dose of BPA were tested, with colonies either receiving chemical exposure only during the first exposure period; only during the second exposure period; or during both exposure periods. A control group which received no chemical exposure throughout both exposure periods was also included.*

2.3. Exposure of embryos collected from exposed adults

Fertilised embryos were collected from the breeding adult colonies during the last 2 days of each exposure or depuration period (days 4 and 5, 17 and 18, and 22 and 23) and re-exposed to a range of BPA concentrations (0, 0.1, 0.5, 1, 5 and 10mg/L) for 72hrs to generate mortality curves (see figure 1). In each replicate tank 20 embryos were exposed from 4-76 hours post fertilisation to the desired BPA concentration in 50ml of aerated synthetic freshwater (made according to ISO-7346/3 guideline, ISO water, diluted 1:5 (International Organization for Standardization, 1996)). Mortality was assessed by visual inspection using a dissection microscope (Motic DM143, Hong Kong) and compared between offspring of different parental groups to test how parental exposure scenario affects the susceptibility of offspring to BPA exposure during early life.

2.4. Adult sampling and molecular analysis of the gonads:

At the end of the last exposure phase (day 24), all fish were sacrificed humanely in accordance with UK Home Office regulations. The fork length and wet weight were recorded, and condition factor was calculated (k) = [weight (g) x 100]/[fork length (cm)]^3. The gonads and liver were then snap frozen in liquid nitrogen and stored at -80°C.

2.4.1. RNA extraction:

RNA was extracted from the ovary of adult females per treatment group using the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A DNase I digestion (Qiagen) was conducted to remove potential DNA contamination. The NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington USA) was used to assess the purity and concentration of the extracted RNA.

2.4.2. Transcript profiling in the gonads:

cDNA was generated from 1µg of total RNA using random hexamers (MWG-Biotech, Ebersberg, Germany) and M-MLV reverse transcriptase (Promega, Madison, USA), according the manufacturer's instructions. Transcript profiling of target genes involved in epigenetic regulation and reproductive function was conducted using real-time quantitative PCR (RT-qPCR) on an iCylcer iQ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA) and SYBR Green chemistry, as previously described by Uren Webster *et al.* (2014)(Uren Webster *et al.*, 2014). These included *dnmt1, esr1, esr2a, esr2b, amh*, and *cyp19a1a* in the ovary, and *dnmt1, esr1, esr2a, esr2b, amh*, and *ar* in the testis. Each plate included a negative control in duplicate to confirm the absence of DNA contamination, and cDNA was diluted 1:2 to avoid loss of efficiency due to the presence of potential contaminants. Efficiency-corrected relative expression levels were determined after normalisation against the control gene, ribosomal protein (*rpl8*), chosen for its stable expression in the gonads following exposure to oestrogenic compounds, as demonstrated for another cyprinid fish species, the fathead minnow (*Pimephales promelas*)(Filby and Tyler, 2005, 2007). Primers for the target genes were previously optimised and published by Laing *et al.* (Laing *et*

al., 2016), and purchased from MWG-Biotech (Ebersburg, Germany). Primer specificity was confirmed by observation of a single amplification product of the expected melting temperature.

2.5. Statistical Analysis

All statistical analysis was conducted in R v3.5.0 (R Core Team, 2018).

Statistical analysis of egg production (as cumulative eggs/female/day normalised within each colony against average egg production during the 10-day acclimation period) was conducted using the lme4 package v1.1.23 (Bates *et al.*, 2015) in R to fit a linear mixed effects model within each experimental phase (exposure 1, depuration, and exposure 2). F tests were used to derive minimum adequate models. This was followed by repeated measures ANOVA to determine the explanatory power of time and BPA treatment group on egg production. For statistical analysis of fertilisation success, the regression coefficient was calculated using linear modelling in R, followed by a one-way ANOVA to determine effects of BPA compared to the control group.

Prior to analysis of offspring mortality data, replicates with background mortality above 20% were removed. Statistical analysis to test for differences in mortality between offspring of different adult treatment groups following exposure to BPA for 72hrs were conducted using a general linear model with a quasibinomial error structure in R. F tests were used to derive minimum adequate models and for posthoc testing of explanatory power of variables. A Tukey pairwise comparison was used to test for significant differences between specific parental groups.

Statistical analysis to test for differences in expression of target genes between adult treatment groups was conducted in R using Kruskal-Wallis One Way Analysis of Variance on Ranks, and where significant differences were found used Dunn's Method for All Pairwise Multiple Comparison Procedure to isolate the specific significantly different group.

3. Results

3.1. Chemistry

The mean measured concentration of BPA in the adult tank water was below the quantification limit of 1.6µg/L for the low dose groups (nominal: 10µg/L), and was 36.7µg/L (±4.9 (SD)) for the high dose groups (nominal: 100µg/L).

The measured concentration of our BPA stock was found to be 106% of nominal. It is likely that BPA was lost as a result of bacterial degradation in the exposure tanks. As fish were transferred to clean tanks at the beginning of each of the exposure periods and exposure was begun and maintained in exactly the same conditions, we are confident that any changes in BPA concentration were consistent between replicates and between the first and second exposure periods.

3.2. Effects of BPA on reproduction and morphometric parameters

Average condition factor did not differ significantly between groups for males ($p=0.0620$) and females ($p=0.912$).

During the 10 day pre-experimental period all colonies spawned consistently, and there were no differences in average fecundity ($p=0.930$) or fertilisation rate ($p=0.615$) between the colonies.

During model simplification for analysis of cumulative egg production over time for each exposure phase, treatment group was found to have no explanatory power, and was removed from the model. Only time was determined to be explanatory variable of egg production in each exposure phase (pre-exposure: $p=<2e-16$, depuration: $p=<2e-16$), re-exposure $p=<2e-16$). This indicates that cumulative egg production over time was not affected by BPA exposure for any treatment group during any of the exposure phases (figure 2).

Throughout the experiment, the fertilisation rate remained high for all colonies (average = 83.2% ±1.35% (SE)). There were no differences in fertilisation rate over time or between treatment groups during any of the exposure phases, indicating no significant effect of BPA exposure or treatment regime on fertilisation success ($p=0.342$)(figure 2).

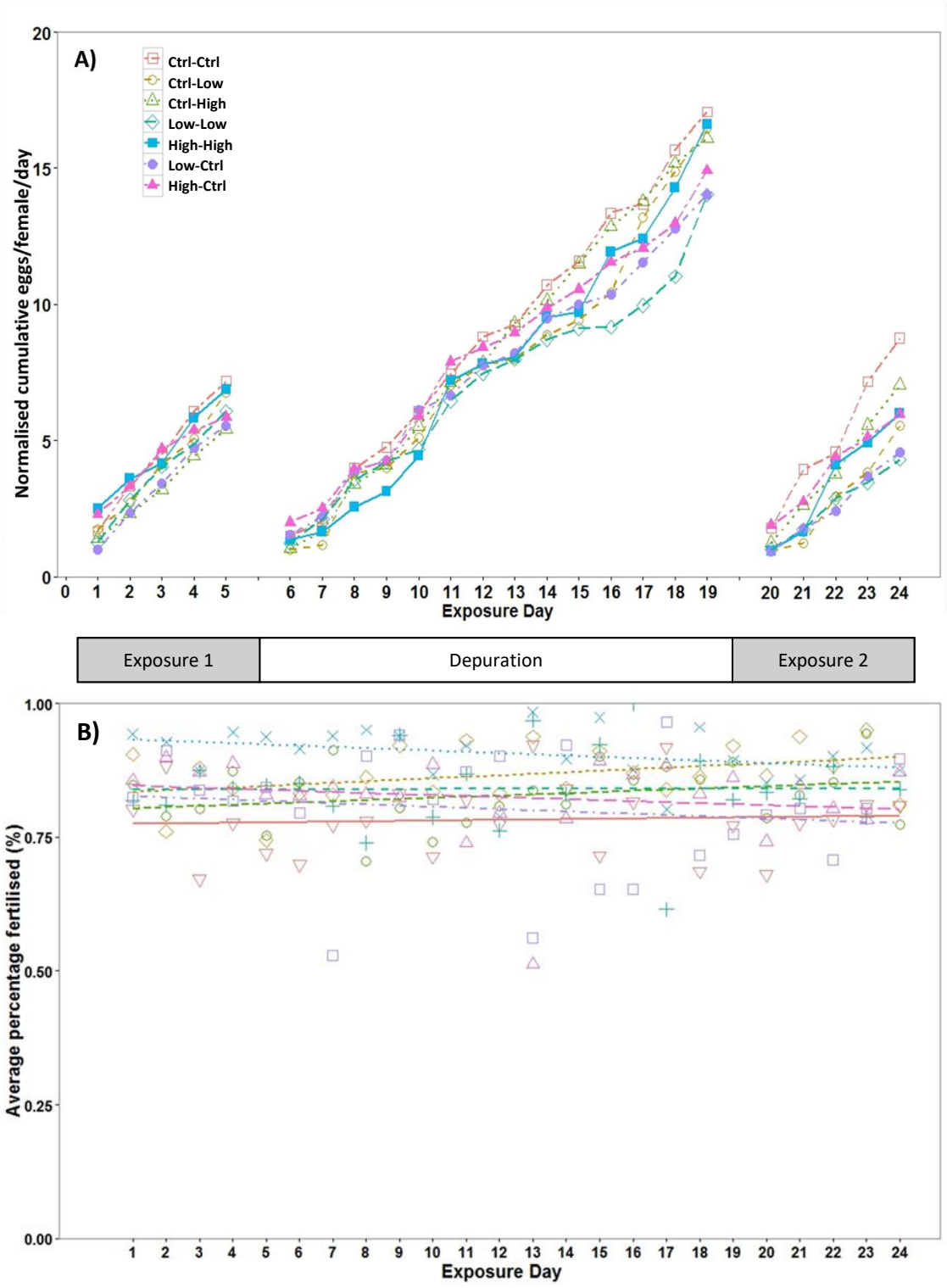


***Figure 2**: A) Cumulative number of eggs per female per day normalised within each colony against a 10 day assessment period prior to the experiment. Each point represents the normalised average number of eggs per female in two colonies exposed to the same treatment regime. Effect of treatment group and time on egg production was analysed for each exposure phase using a separate linear mixed effects model simplified using F tests, followed by repeated measures*

anova to calculate explanatory power of each variable. Treatment was found to have no explanatory power, and was removed from the model, indicating no significant effect of BPA exposure on egg production. Only time was found to have significant explanatory power (p=<2e-16 for all exposure phases). B) Mean fertilisation success (%) for breeding groups exposed to BPA in different exposure scenarios as described in figure 1. Each point represents the average fertilisation success for two colonies exposed to the same treatment conditions. A one way anova comparing regression coefficients calculated using a linear model found no significant difference between treatment groups (p=0.342).

3.3. BPA Toxicity after 72hrs for embryos from exposed adults

Following 72hrs of exposure to a range of BPA concentrations, there was no significant difference (p=0.31) between the mortality rate of embryos originating from pre-exposed parents, who received a pre-exposure to the high dose of BPA (36.7µg/L) for 5 days followed by a depuration period and re-exposure for a further 5 days (High-High), and those from control parents who received no exposure to BPA (Ctrl-Ctrl). However, embryos originating from naïve parents, who received a high dose BPA exposure for 5 days with no pre-exposure (Ctrl-High), had a significantly higher mortality rate than embryos from both pre-exposed parents (High-High)(p=0.016 *) and control parents (Ctrl-Ctrl)(p=0.014 *)(figure 3).

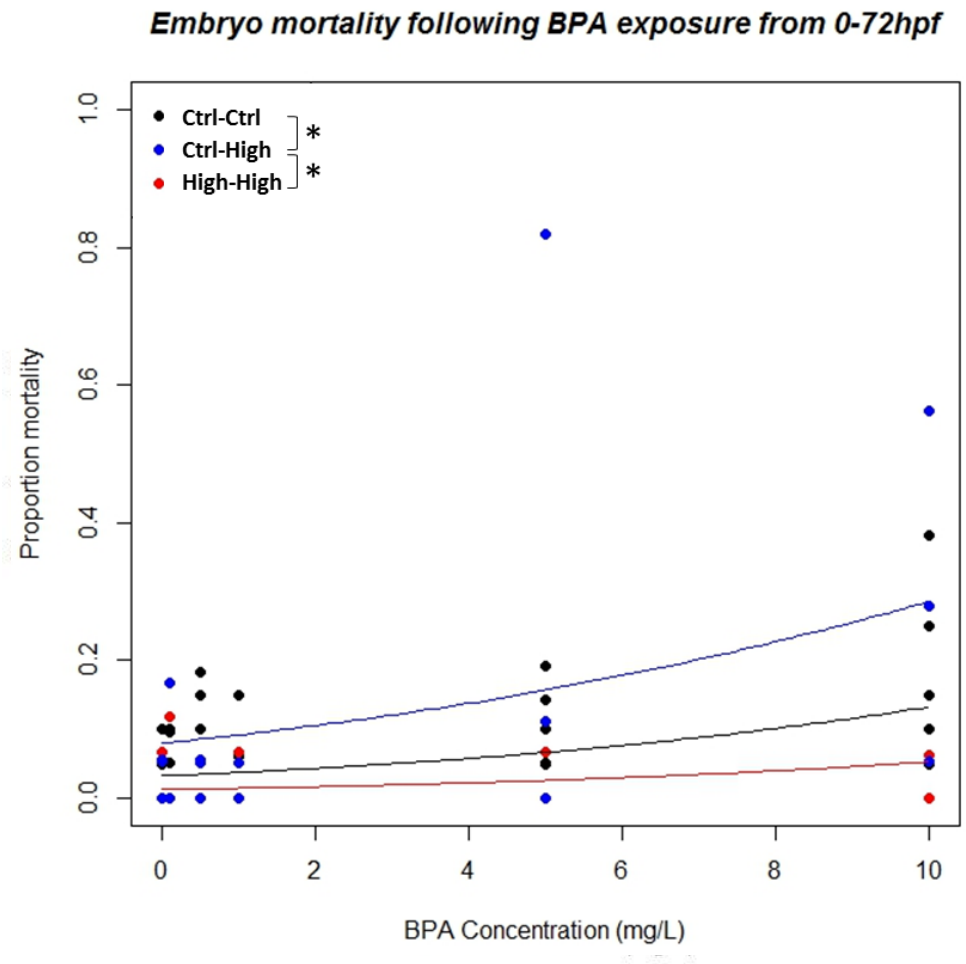


Figure 3: *Embryo mortality curves for embryos originating from parents kept in control conditions (Ctrl-Ctrl), at the end of a single 5-day exposure to BPA (Ctrl-High), and following two 5-day exposures to BPA separated by a depuration period (High-High). Embryos were exposed to a range of BPA concentrations (0, 0.1, 0.5, 1, 5 and 10mg/L) continuously from 4-76hpf. Replicate curves per treatment group: control – 6, naïve (Ctrl-High) – 3, and pre-exposed (High-High) – 3. Each point on the graph represents the proportion of mortality in an individual tank containing 20 embryos. The lines represent the best fit model for the data, calculated using a generalized linear model (mortality ~ concentration + parental_exposure_scenario). BPA-induced mortality in offspring of pre-exposed parents (High-High) was not significantly different from control offspring (p=0.31). Whereas, BPA-induced mortality in offspring of naïve parents (Ctrl-High) was significantly higher than for offspring of both pre-exposed parents (High-High)(p=0.016 *) and control parents (Ctrl-Ctrl)(p=0.014 *).*

3.4. Effects of BPA on Gene Transcription

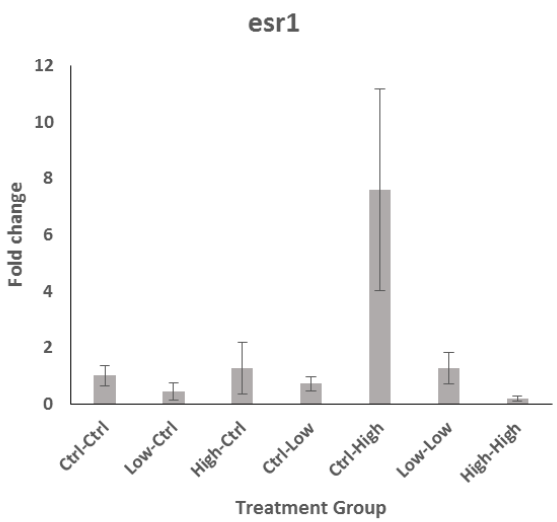
Measurements of gene transcription for several genes involved in reproduction and DNA methylation were conducted in the ovaries (*dnmt1, esr1, esr2a, esr2b, amh, cyp19a1a*) and testes (*dnmt1, esr1, esr2a, esr2b, amh*, ar) of exposed fish at the end of the final exposure period.

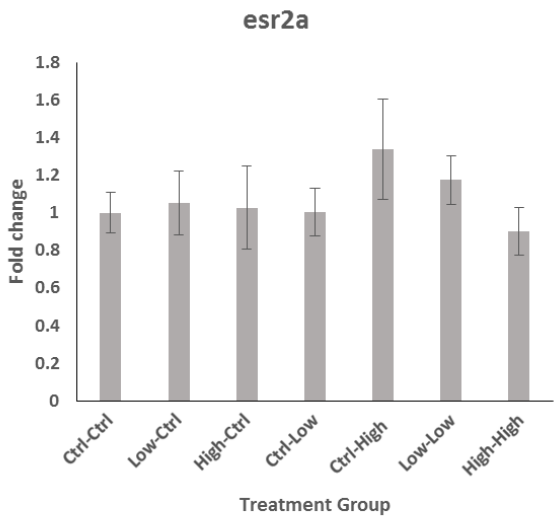
No significant effects on gene transcription were found for any of the low dose treatment groups (Low-Ctrl, Ctrl-Low, and Low-Low) compared to the control fish (Ctrl-Ctrl) in the both the ovaries and testes (figures 4 and 5).

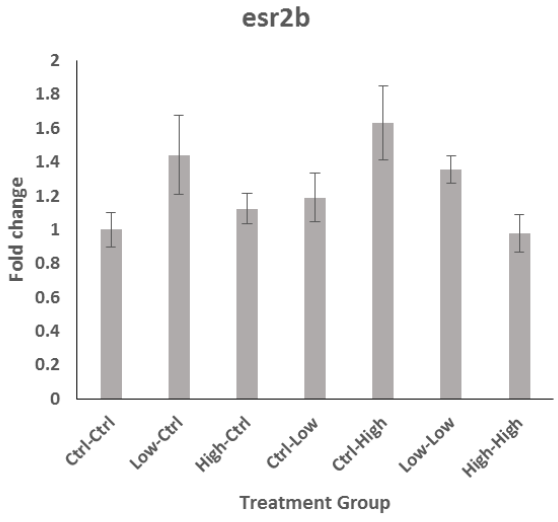
Transcription of anti-Mullerian hormones (*amh*) was found to be significantly downregulated in the ovaries of pre-exposed fish which were pre-exposed to the high dose of BPA (36.7µg/L) and re-exposed following a depuration period (High-High) when compared with control fish (Ctrl-Ctrl) ($P=0.0395$). Whereas, there was no significant change in *amh* transcription in naïve fish following a single exposure to a high dose of BPA (Ctrl-High) when compared to the control group (Ctrl-Ctrl). No significant effects were observed on the transcription of *amh* in the testis for any treatment group (figure 4).

A similar but non-significant trend was seen in the transcription of aromatase (*cyp19a1a)* in the ovary among the high dose groups, with pre-exposed fish (High-High) showing an apparent decrease in transcription compared to control fish (Ctrl-Ctrl)(figure 4).

For all treatment groups exposed to a high dose of BPA, no significant changes in transcription were found in the ovary or testis for the oestrogen receptor genes *esr1*, *esr2a* and *esr2b*, or in the epigenetic regulatory gene DNA methyltransferase 1 (*dnmt1);* and no significant change was observed in the transcription of the androgen receptor (*ar*) in the testis (figures 4 and 5).


esr1
Fold change
12
10
8
6
4
2
0
Ctrl-Ctrl
Low-Ctrl
High-Ctrl
Ctrl-Low
Ctrl-High
Low-Low
High-High
Treatment Group


esr2a
Fold change
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ctrl-Ctrl
Low-Ctrl
High-Ctrl
Ctrl-Low
Ctrl-High
Low-Low
High-High
Treatment Group


esr2b
Fold change
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
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Ctrl-Low
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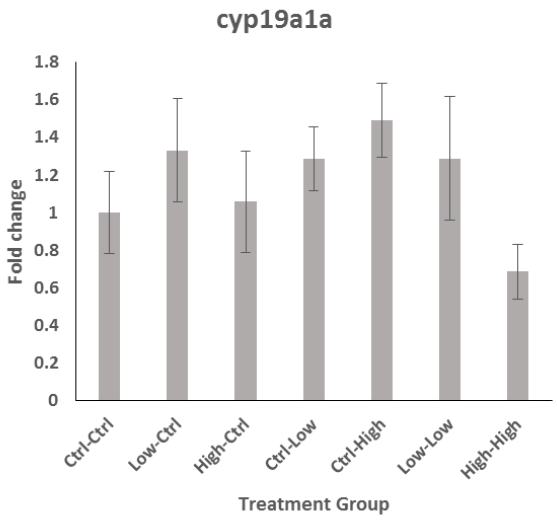
Low-Low
High-High
Treatment Group


cyp19a1a
Fold change
1.8
1.6
1.4
1.2
1
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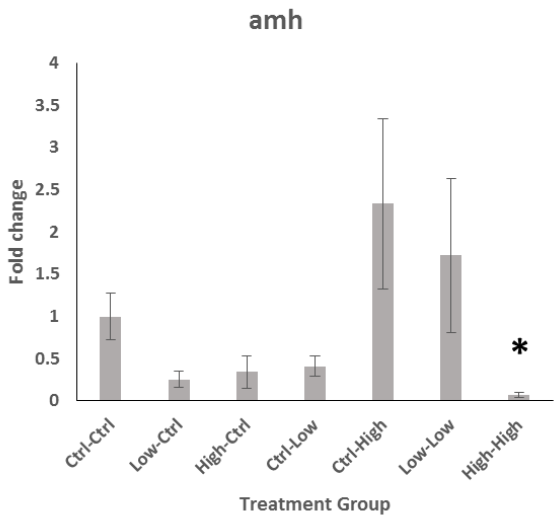
High-High
Treatment Group


amh
Fold change
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Ctrl-Ctrl
Low-Ctrl
High-Ctrl
Ctrl-Low
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High-High
Treatment Group

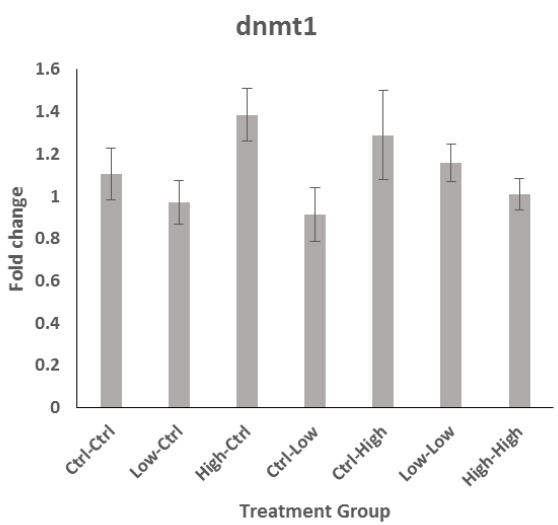
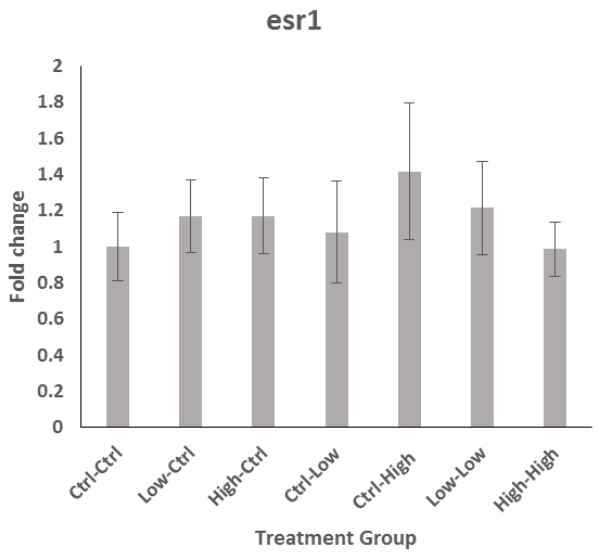

dnmt1
Fold change
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ctrl-Ctrl
Low-Ctrl
High-Ctrl
Ctrl-Low
Ctrl-High
Low-Low
High-High
Treatment Group

Figure 4: *Transcript profiles for amh, cyp19a1a, dnmt1, esr1, esr2a and esr2b in the ovaries of fish maintained in control conditions throughout the experiment (Ctrl-Ctrl) and fish exposed to BPA (low dose and high dose) during a single 5-day exposure period at the end of the experiment (Ctrl-High, Ctrl-Low), after a single 5-day exposure period followed by depuration (High-Ctrl, Low-Ctrl), and following two 5-day exposure period separated by a depuration period (High-High, Low-Low). Data are presented as fold-change relative to the control group. Relative transcription was calculated as a ratio of the efficiently corrected transcription data for amh/ efficiency corrected expression data for rpl8. For each treatment group, data were obtained for 8-10 individual fish. Statistical analysis to test for differences in transcription between treatment groups was conducted using Kruskal-Wallis One Way Analysis of Variance on Ranks, and where significant differences were found Dunn's method for All Pairwise Multiple Comparison Procedure was used to isolate specific groups where differences occurred. Asterisks represent significant differences between treatment groups compared to the control group (*P=0.0395).*


esr1
Fold change
2
1.8
1.6
1.4
1.2
1
0.8
0.6
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Ctrl-Ctrl
Low-Ctrl
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Ctrl-High
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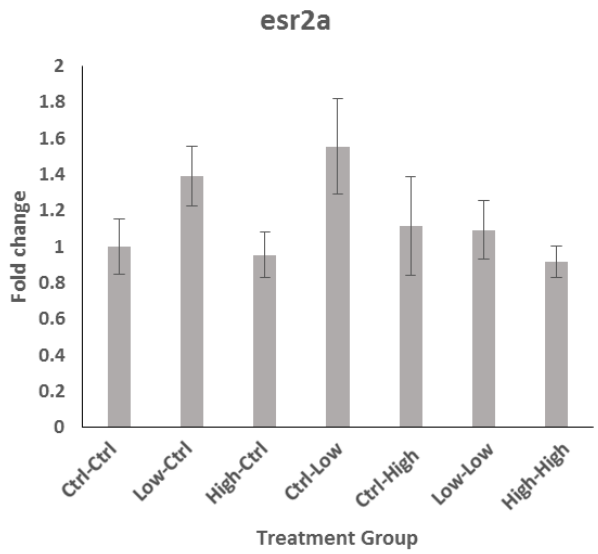
Treatment Group


esr2a
Fold change
2
1.8
1.6
1.4
1.2
1
0.8
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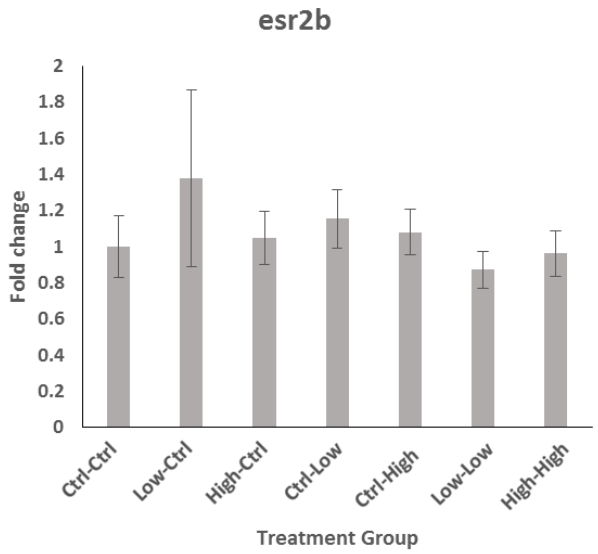
Treatment Group


esr2b
Fold change
2
1.8
1.6
1.4
1.2
1
0.8
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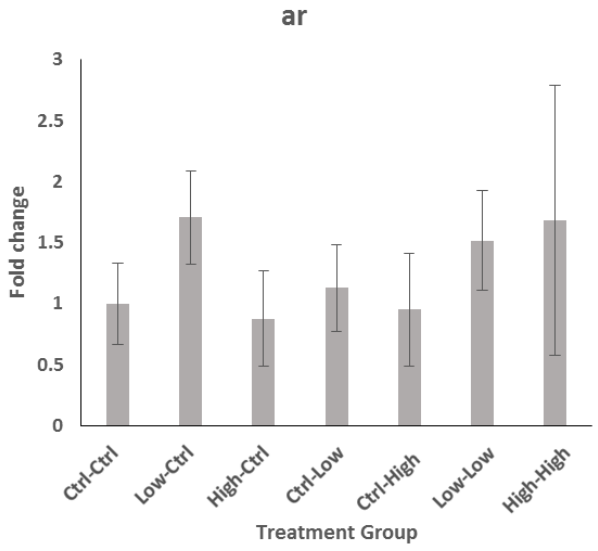
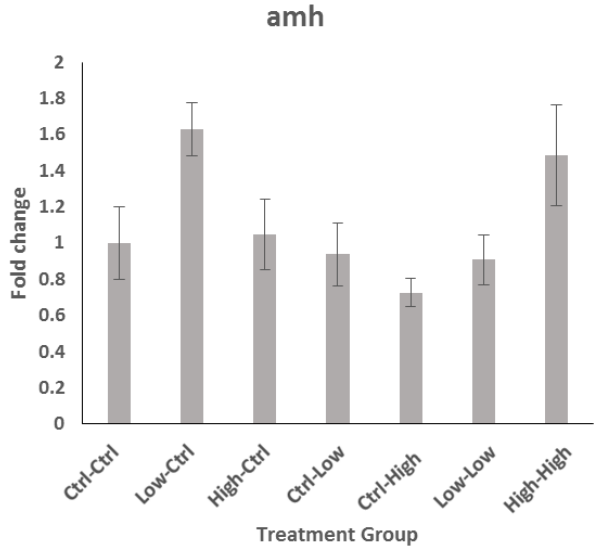
High-Ctrl
Ctrl-Low
Ctrl-High
Low-Low
High-High
Treatment Group


ar
Fold change
3
2.5
2
1.5
1
0.5
0
Ctrl-Ctrl
Low-Ctrl
High-Ctrl
Ctrl-Low
Ctrl-High
Low-Low
High-High
Treatment Group


amh
Fold change
2
1.8
1.6
1.4
1.2
1
0.8
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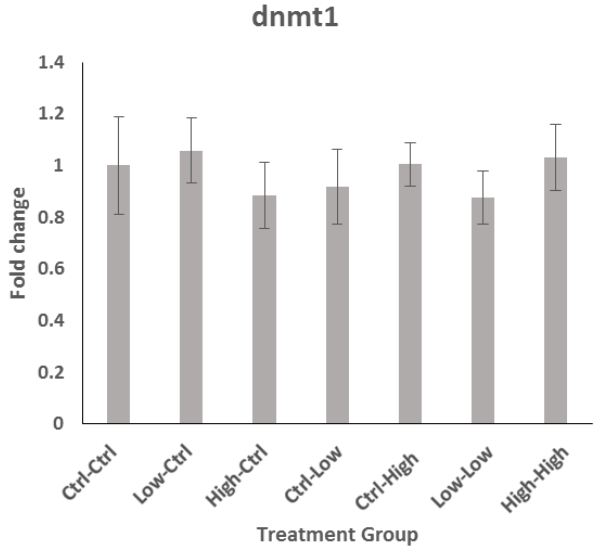
High-Ctrl
Ctrl-Low
Ctrl-High
Low-Low
High-High
Treatment Group


dnmt1
Fold change
1.4
1.2
1
0.8
0.6
0.4
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0
Ctrl-Ctrl
Low-Ctrl
High-Ctrl
Ctrl-Low
Ctrl-High
Low-Low
High-High
Treatment Group

Figure 5: *Transcript profiles for amh, ar, dnmt1, esr1, esr2a and esr2b in the testes of fish maintained in control conditions throughout the experiment (Ctrl-Ctrl) and fish exposed to BPA (low dose and high dose) during a single 5-day exposure period at the end of the experiment (Ctrl-High, Ctrl-Low), after a single 5-day exposure period followed by depuration (High-Ctrl, Low-Ctrl), and following two 5-day exposure period separated by a depuration period (High-High, Low-Low). Data are presented as fold-change relative to the control group. Relative transcription was calculated as a ratio of the efficiently corrected transcription data for amh/ efficiency corrected expression data for rpl8. For each treatment group, data were obtained for 8-10 individual fish. Statistical analysis to test for differences in transcription between treatment groups was conducted using Kruskal-Wallis One Way Analysis of Variance on Ranks. No significant differences were found for transcription between any treatment group.*

4. Discussion

Wildlife populations frequently experience repeated exposure to stressors across the lifetime of individuals and across generations. The effects of intermittent exposure on organisms is poorly understood, and the potential impact of exposure history on the response of populations to future exposure is rarely considered in environmental risk assessment. In this study I aimed to investigate whether prior exposure of adult zebrafish to BPA – an environmentally ubiquitous endocrine disrupting chemical – would alter their response, or that of their offspring, upon re-exposure. Analysis of the transcriptional response in the gonad upon re-exposure revealed altered transcription of *amh*, encoding anti-Mullerian hormone, in the ovaries of pre-exposed fish compared to naïve fish. Subsequent exposure of F1 offspring during early life also indicated that pre-exposure of F0 adults conferred increased BPA tolerance to their offspring compared to embryos of naïve parents. These observations suggest that historic exposure of fish to BPA caused lasting changes in adults that led to an altered physiological response upon re-exposure, with consequent effects on the physiology of offspring; and may involve a variety of mechanisms, including physiological, transcriptional or epigenetic changes.

4.1. Effects of BPA on reproduction and gene expression in the adult ovary

No effects on reproduction, including egg production and fertilisation success, were observed in this study after exposure to a maximum of 36.7µg/L BPA. BPA is a known endocrine disruptor which has been shown to adversely affect reproduction in numerous species, likely via oestrogenic mechanisms (Peretz *et al.*, 2011; Zhang *et al.*, 2014; Laing *et al.*, 2016), with studies showing that BPA interacts with oestrogenic signalling pathways (Peretz *et al.*, 2011; Tatarazako *et al.*, 2015). For example BPA was shown to inhibit ovulation entirely in brown trout after ~14 weeks of exposure to 5µg/L, and delayed ovulation at 1.75µg/L (Lahnsteiner *et al.*, 2005). Egg production was also inhibited in the fathead minnow after exposure to 1280µg/L of BPA for 164 days (Sohoni *et al.*, 2001). Conversely, Laing *et al.* (2016) observed significantly increased egg production in zebrafish after exposure to 1mg/L BPA for 15 days; but this was accompanied by significantly reduced fertilisation success (Laing *et al.*, 2016). This could be explained by reduced oocyte quality or impacts on spermatogenesis in males;

which is known to be inhibited by BPA, manifesting in reduced sperm count and quality (Sohoni *et al.*, 2001; Karnam *et al.*, 2015; Zhang *et al.*, 2016). The absence of effects on reproductive endpoints in this study is likely due to the short duration of exposure, maintained for two 5 day periods separated by a 13 day depuration, and the lower BPA concentration used compared to other studies.

Gene expression analysis showed no significant changes in the ovary for several genes linked with reproductive function and oestrogenic signalling, including for the oestrogen receptors *esr1*, *esr2a* and *esr2b*, which was consistent with the lack of change observed for reproductive endpoints. These results also concur with those of other studies at similar concentrations. For example, Zhang *et al.* (2014) found no changes in the expression of several steroid hormone receptors in the ovary of adult rare minnow (*Gobiocypris rarus*), including *esr1, esr2a*, *esr2b* and *ar*, after 14 days at 5, 15 and 50µg/L BPA; with changes in *esr1, esr2a* and *ar* expression only occurring after 35 days of exposure (Zhang *et al.*, 2014). After continuous exposure of adult zebrafish for 15 days, Laing *et al.* found no significant changes in transcription of *esr1, esr2a, esr2b, ar* and *amh* at concentrations up to 100µg/L BPA (Laing *et al.*, 2016). At 1mg/L only transcription of *esr2a* shown significant change (Laing *et al.*, 2016). Similarly, there was no significant change in transcription of genes related to reproductive function in the testis, including oestrogen receptors, *esr1, esr2a* and *esr2b*, and the androgen receptor, *ar*. Several studies have observed altered transcription of reproductive genes in the testis. However, these have usually occurred at higher BPA concentrations or longer exposure durations than that used in this study. For example, Laing *et al.* (2016) observed significant downregulation of *esr2b* in the testis of zebrafish after exposure to 1mg/L BPA (Laing *et al.*, 2016). Conversely, after 90 days of exposure to 0.2µg/L of BPA, Hatef *et al.* (2012) observed significantly increased transcription of *esr2b* in the testes of goldfish. In addition, 90 days of exposure to 20µg/L led to significantly increased transcription of *ar* in the testis (Hatef, Zare, *et al.*, 2012). And during exposure of Nile tilapia to 10 and 100µg/L of BPA for up to 4 weeks, Huang *et al.* (2010) also observed significantly altered transcription of several oestrogen receptor genes in the testis (Huang *et al.*, 2010).

Similar to reproductive endpoints, the lack of transcriptional changes in the ovary and testis are likely due to the short duration of exposure, and the lower BPA

concentration used compared to other studies. It is possible that after a longer exposure period or with a higher BPA concentration effects on reproduction and transcription of the genes measured may have been observed. The short duration and low concentration used in this study may not have allowed accumulation of BPA or its metabolites in tissues to the threshold required to affect the transcription in the gonad, or to overcome potential compensatory mechanisms acting to maintain homeostasis of these molecular pathways.

Although no change in transcription was observed, it is possible that BPA may be acting upon oestrogen receptors, but concentration or duration of exposure may not have reached the threshold required to affect their transcription. BPA has been shown to interact with oestrogen receptors in both a weakly oestrogenic and an anti-oestrogenic manner (Hiroi *et al.*, 1999), with the extent of their oestrogenic or anti-oestrogenic activity being dependent on contextual factors, including tissue type, ESR subtype, and presence of other oestrogens (Kurosawa *et al.*, 2002; Schmidt *et al.*, 2006; Acconcia, Pallottini and Marino, 2015). ESR transcription would be least sensitive to potential anti-oestrogenic activity of BPA, where BPA competes with endogenous oestrogens to bind ESRs in an ineffective conformation, reducing its potency on ESR targets, including its own regulation. If BPA were exerting its physiological effect on adult physiology through ESR antagonism, it may do so without changing the transcription of the affected ESRs.

Alternatively, BPA could be acting through other receptors that were not measured. Recent studies suggest that BPA activity may also be mediated through oestrogen-related receptors (ERRs), a family of nuclear receptors that is highly conserved across vertebrates and show widespread expression across tissues (Hubbard, Bland and Chaudry, 2015). It is thought that signalling through ERRs may play important roles in regulation of metabolism and energetics, including during stress (Hubbard, Bland and Chaudry, 2015). BPA has been found to have stronger affinity for ERRγ than ESRs, with binding at concentrations as low as 5.6nM (Matsushima *et al.*, 2007; Tohmé *et al.*, 2014). At least one toxic phenotype of BPA exposure in zebrafish – otolith malformation – is known to be mediated through ERRγ binding, independent of ESR binding (Tohmé *et al.*, 2014). Therefore, ERRs could be interesting targets for further investigation.

Transcription of *amh,* another reproduction-related gene which encodes the anti-Mullerian hormone, was found to be significantly decreased in the ovaries of pre-exposed fish in the high dose group (High-High)(see figures 4 and 5). Similar to mammals, zebrafish amh plays a critical role in sexual differentiation of the gonads in juveniles; and sexually dimorphic expression patterns are seen by 31dpf (Rodríguez-Marí *et al.*, 2005). Expression of *amh* is low in juvenile females, but high in males, where it acts alongside other factors to prevent the development of ovaries and stimulate testicular development (Rodríguez-Marí *et al.*, 2005). However, following sexual differentiation, amh continues to play important roles in reproductive functioning in in both males and females (Pfennig, Standke and Gutzeit, 2015). In the ovary it plays an important role in oocyte maturation, where it is expressed by somatic granulosa cells that surround oocytes in developing follicles (Rodríguez-Marí *et al.*, 2005). Secretion of amh inhibits folliculogenesis by inhibiting primordial follicle recruitment, steroidogenesis and proliferation of granulosa cells (Rodríguez-Marí *et al.*, 2005). In zebrafish, amh expression by granulosa cells begins when oocytes reach late stage I development, peaks in stage II oocytes, and decreases in early stage III oocytes, becoming undetectable in late stage III and stage IV oocytes (Rodríguez-Marí *et al.*, 2005).

Effects of BPA on ovarian *amh* transcription have been shown to vary between studies using different species, stages of development and exposure regimes, with reports of both upregulation and downregulation in fish (Rhee *et al.*, 2011; Santangeli *et al.*, 2019) and mammals (Y. Li *et al.*, 2014; Cao *et al.*, 2018). Due to the dynamic nature of hormonal control of oogenesis and ovulation, hormone levels are also likely to vary widely within individuals depending upon their stage in the spawning cycle.

The significant reduction in *amh* transcripts in the ovary of pre-exposed females could be explained by downregulation of *amh* expression from granulosa cells or reduced proportion of granulosa cells expressing *amh* compared to other cell types. BPA has been shown to reduce *Amh* transcription and Amh hormone levels in mice through depletion of granulosa cells and reduction of follicle maturation (Cao *et al.*, 2018). This reduction in Amh eventually leads to depletion of the ovarian reserve, by stimulating over-recruitment of primary follicles. In humans, reduced AMH levels are used as an indicator of reduced follicle count in patients with infertility and reproductive ageing (Barbakadze *et al.*, 2015). A study by

Migliaccio *et al.* (2018) documenting morphological changes in the zebrafish ovary as a result of BPA exposure provides evidence for a similar effect in zebrafish. They showed that BPA disrupts folliculogenesis by increasing recruitment of primary follicles, but inhibiting proper maturation, resulting in enlarged follicles that ultimately undergo atresia (Migliaccio *et al.*, 2018). Molina *et al.* (2013) also observed increased follicular atresia in zebrafish ovaries after two weeks of exposure to 100 and 1000µg/L BPA (Molina *et al.*, 2013). BPA-induced ovarian toxicity, reductions in oocyte maturation and ovulation, and increased follicular atresia have also been observed in several other fish species, including goldfish (Q. Wang *et al.*, 2019), medaka (Shioda and Wakabayashi, 2000) and carp (Mandich *et al.*, 2007). Significantly increased recruitment of primary follicles and increased number of atretic follicles would be consistent with reduced amh levels as a result of inhibition of follicle maturation and depletion of granulosa cells.

It is possible that the reduced *amh* transcription we observed upon exposure to BPA in the ovaries of pre-exposed fish could be due to depletion of granulosa cells, potentially from accumulation of damage from the pre-exposure and subsequent re-exposure, or increased BPA accumulation due to incomplete depuration. However, no significant impact on egg production or fertilisation success was observed, indicating that pre-exposed fish were not experiencing a significant increase in ovarian toxicity compared to naïve fish. This reduced *amh* expression could be as a result of toxicity beginning to occur in developing follicles, that is not yet severe enough to have a significant effect on egg output or oocyte quality. In a study of starvation-induced follicular atresia in salmon, Yamamoto *et al.* (2016) observed significant reductions in *amh* transcription that preceded increased follicular atresia (Yamamoto *et al.*, 2016). Alternatively, the number of healthy amh-secreting cells may not have changed in pre-exposed fish, and instead the reduction of *amh* transcription may be due to differential regulation through another mechanism.

At the beginning of ovarian toxicity, decreased *amh* transcription and increased recruitment of primary follicles in pre-exposed fish may serve as a mechanism to compensate for slowed folliculogenesis and maintain egg production. It may therefore initially be beneficial to alter the regulation of *amh* transcription such that *amh* response can occur sooner after exposure to chemicals that inhibit folliculogenesis. However, once inhibition of later stages of folliculogenesis

reaches a certain threshold, continuous upregulation of *amh* would become adverse by reducing the ovarian reserve of primordial follicles with no positive effect on egg production, as can be seen in severe ovarian toxicity caused by longer exposures to BPA where egg production is reduced or prevented entirely (Mandich *et al.*, 2007; Molina *et al.*, 2013; Cao *et al.*, 2018; Q. Wang *et al.*, 2019).

Regulation of *amh* expression in the ovary during folliculogenesis is not entirely understood. Folliculogenesis is known to be under endocrine control by gonadotropins as part of the hypothalamic-pituitary-gonadal (HPG) axis, with follicle stimulating hormone (FSH) broadly stimulating the growth phase of follicles, and luteinizing hormone (LH) controlling the maturation phase (Clelland and Peng, 2009; Pfennig, Standke and Gutzeit, 2015). These hormones then modulate and are modulated by a network of other hormones and growth factors produced in the ovary, including estradiol (Clelland and Peng, 2009; Pfennig, Standke and Gutzeit, 2015). These factors may play a role in regulation of *amh*. BPA exposure has been observed to interfere with the release of gonadotropins and deregulate the HPG-axis in fish and mammals (Qin *et al.*, 2013; Kurian *et al.*, 2015; Molina *et al.*, 2018). It is possible that reduced *amh* expression in the ovaries of pre-exposed fish was caused by changes in the HPG-axis. The zebrafish *amh* promoter is also known to contain oestrogen responsive elements (EREs), and may therefore be available for regulation by oestrogens (Pfennig, Standke and Gutzeit, 2015). Little information on the effect of oestrogens on *amh* transcription is available for females, but evidence from males suggests that oestrogens may inhibit *amh* transcription (Schulz *et al.*, 2007). It is possible that *amh* repression in the ovary of pre-exposed fish may be caused by direct oestrogenic action of BPA on the *amh* promoter, either as a result of higher cumulative stimulation or build-up of BPA as a result of incomplete depuration, or reduced threshold for *amh* response to BPA exposure as a result of prior exposure.

A possible mechanism for an altered threshold for *amh* activation or inhibition, in the absence of altered BPA concentrations, could be stable epigenetic changes to *amh* regulating elements during the pre-exposure that caused an altered transcriptional response upon re-exposure. BPA has been shown to cause epigenetic changes to the methylome of genes that were correlated with altered gene transcription, including genes related to reproduction (Doherty *et al.*, 2010; Kundakovic *et al.*, 2013; Santangeli *et al.*, 2017) and genes for epigenetic

regulators, such as DNA methyltransferase enzymes (Laing *et al.*, 2016). For example, BPA was shown to cause hypomethylation of the upstream retrotransposon regulating the Agouti gene in mice after in utero exposure, leading to alteration of offspring coat colour (Dolinoy, Huang and Jirtle, 2007). This effect was then shown to be negated by maternal supplementation with a methyl donor (Dolinoy, Huang and Jirtle, 2007). However, many of these changes occurred as a result of direct or indirect exposure during sensitive windows of development in early life (Dolinoy, Huang and Jirtle, 2007; Doherty *et al.*, 2010; Kundakovic *et al.*, 2013). Epigenetic effects of BPA on *amh* were reported by Santangeli *et al.* (2019), who observed significantly decreased *amh* transcription and associated hypermethylation of the *amh* promoter in F1 offspring that was maintained until the F3 generation, following exposure of F0 females to 20µg/L of BPA for four weeks. However, no changes to *amh* promoter methylation were detected in the F0 ovary, and *amh* transcription was significantly increased, indicating that the effects of BPA exposure vary according to life stage. This suggests that epigenetic effects of BPA may only occur after exposure during early life, when the epigenome undergoes periods of reprogramming that may be more vulnerable to environmentally-induced change (Santangeli *et al.*, 2019). In our study, all exposures of F0 fish occurred as adults, suggesting that epigenetic changes at the *amh* promoter may not explain the differential *amh* transcription observed in pre-exposed females. However, it is important to consider changes in *amh* transcription in the context of a network of interacting factors, including modulation by other hormones, such as those involved in the HPG-axis (van Helden, Evliyaoglu and Weiskirchen, 2019). It is possible that persistent changes in the regulation of other genes that interact with *amh,* including potentially epigenetic changes, may explain the altered transcription of *amh* in pre-exposed fish, as opposed to a direct impact of BPA on the *amh* gene.

We must also consider the possible effects of the stage of the reproductive cycle of the females at the time of sampling. Reduced transcription of *amh* might be explained by a predominance of very early or very late stage follicles, which were not yet producing high levels of *amh*. This could occur immediately prior to ovulation, for example, when the proportion of mature follicles, which do not produce amh, is highest. Given that within well-established zebrafish colonies females often synchronise ovulation, this could be a factor contributing to this

change in expression. However, egg production data indicates that the effects of recent ovulation are unlikely to explain the significant difference in *amh* expression seen in the pre-exposed adults compared to controls; as egg production in the two pre-exposed colonies (egg production: 59 and 156 eggs) was within the same range as the two control colonies (egg production: 119 and 229) on the day of adult sampling. This indicates that this reduction in *amh* transcription is driven by pre-exposure to BPA and not differences in reproductive cycle.

4.2. Effects of parental pre-exposure on tolerance of offspring to BPA during embryogenesis

Pre-exposure of adult fish to BPA altered the susceptibility of their offspring to subsequent BPA exposure during development; with embryos originating from naïve adults exposed to BPA for the first time (Ctrl-High) being significantly more sensitive to BPA exposure during embryogenesis than offspring of pre-exposed fish (High-High), whose mortality rate was equivalent to control offspring (Ctrl-Ctrl) (figure 3). This indicates that pre-exposure of parents conferred a protective effect on offspring that increased there tolerance to BPA upon subsequent exposure. I hypothesise that this is likely driven by altered physiology of adults between the first and second exposure, leading to altered conditions within the gonad during gametogenesis and altered deposition of important factors into gametes. Altered maternal transfer of a variety of factors into oocytes can affect the physiology and development of offspring, including: nutrients; lipids; enzymes, hormones, and other proteins; maternal transcripts; and xenobiotic chemicals (Mousseau and Fox, 1998). Changes to the transfer of these factors has the potential to affect the response of resulting offspring to environmental stressors. Changes in the physiology of pre-exposed adults could have affected conditions in the gonad during gametogenesis, and resulted in changes to offspring resistance through various mechanisms, discussed below.

4.2.1. Altered transfer of BPA in gametes

One possible hypothesis explaining increased embryo resistance could be altered maternal transfer of BPA or its metabolites into oocytes. BPA is a lipophilic compound, and is likely to be deposited within oocytes as they mature in the ovary of exposed females. Although BPA transfer to embryos is possible from either

parent, maternal contribution is likely to have the most impact, as BPA transfer through the relatively small volume of material deposited in sperm is probably negligible compared to the contribution of the oocyte. In this experiment, pre-exposure may have modified the molecular pathways involved in the transport, metabolism or excretion of BPA in females upon re-exposure and led to differential accumulation of BPA or its metabolites in their oocytes compared to those of naïve fish exposed to BPA for the first time. Several studies in rainbow trout showed that exposure of oocytes to BPA in ovarian fluid prior to fertilisation, simulating maternal transfer, led to several adverse effects in resulting offspring, including: growth suppression; delayed hatching; and defects in early life and long-term stress response, including disruption of cortisol profiles (Aluru, Leatherland and Vijayan, 2010; Birceanu, Mai and Vijayan, 2015; Thomas *et al.*, 2018). Similarly, studies in the red-eared slider turtle showed that BPA can inhibit *in ovo* metabolism of maternally derived steroids in embryos, which could lead to inappropriate steroid signalling during early development (Clairardin, Paitz and Bowden, 2013; Paitz and Bowden, 2015). It would be reasonable to expect that if similar adverse effects occurred in zebrafish embryos after presence of BPA in the ovarian fluid, that these effects may impair their stress response and tolerance to BPA upon subsequent exposure. If adaptive changes in adult physiology, such as increased metabolism and excretion, resulted in reduced accumulation of BPA or its metabolites in developing gametes, this could potentially prevent these adverse effects on offspring fitness, and lead to increased resistance to BPA compared to BPA-laden offspring of naïve parents. There are very few studies of potential adaptation or acclimation to BPA exposure in the literature. Most focus on toxic outcomes after a single acute or chronic exposure. There is therefore little information on how uptake, metabolism and excretion of BPA may change over the course of exposure or following historic BPA exposure. Further investigation into potential changes in BPA handling, and particularly whether this affects accumulation of BPA and its metabolites in the ovary and oocytes, could help to determine whether this is the mechanism behind the altered tolerance observed in offspring in this study.

We also considered that BPA accumulation in the oocytes could have been affected by incomplete depuration of the female between exposures, as opposed to altered physiology. As the time required for total depuration of BPA in zebrafish

is not known, the depuration period was designed to be of reasonable length such that the fish could be expected to fully depurate over the 13 days, particularly in the ovary, where oocytes potentially contaminated with BPA would have been released through several cycles of ovulation, which occurs every 4-7 days (Clelland and Peng, 2009). Following exposure to 100µg/L BPA, Lindholst *et al.* (2003) found that zebrafish undergo an initial rapid phase of depuration, eliminating over 70% of BPA and BPAS (one of its metabolites) after 2hrs, and reduced remaining BPA and BPAS to 10.4% and 4.6%, respectively, after 7 days. BPAGA, the major metabolite, was eliminated at a more steady rate, but still reached 7.5% of its original concentration after 7 days (Lindholst *et al.*, 2003). Given the fast metabolism of BPA in zebrafish and the fact that our depuration period was almost twice as long, remaining BPA was likely to be very low. Although incomplete depuration was possible, it also seems unlikely that additional BPA in gametes from pre-exposed parents due to incomplete depuration would lead to increased tolerance compared to embryos from naïve fish which would theoretically have a lower BPA content.

I hypothesise that altered BPA handling and indirect exposure of gametes is the most likely mechanism behind our observations. However, if BPA accumulation was unchanged, altered deposition of other maternally transferred factors that are important for embryo development could be responsible for the alterations in embryos resistance to BPA.

4.2.2. Altered accumulation of parental factors in gametes

During gametogenesis a variety of cytoplasmic factors are directly deposited into oocytes by the mother that can affect embryo development and physiology, including nutrients, lipids, enzymes, hormones, transcription factors, and other proteins (Mousseau and Fox, 1998). As well as direct transfer of proteins important for embryo development, parental transcripts encoding these proteins also accumulate in gametes during embryogenesis and be transferred to subsequent embryos (Pelegri, 2003). Maternal transcripts are essential for directing development of early embryogenesis prior to zygotic genome activation (ZGA) beginning at the 64-cell stage (Jukam, Shariati and Skotheim, 2017) – when the embryo is capable of producing its own transcripts – and continue to perform important functions later in embryonic development (Pelegri, 2003). While

the majority of pre-ZGA transcriptional control seems to be directed by maternal transcripts, there is some evidence that sperm also contribute RNAs (Sharma, 2019), and that altered sperm transcriptome may therefore be able to influence embryo development and susceptibility to environmental stressors.

Pre-exposure of adults to BPA could have changed their physiology such that accumulation of these cytoplasmic factors or parental transcripts into oocytes or sperm during gametogenesis was altered. Differential transfer of these factors as a result of historic exposure could lead to altered development or altered functioning of BPA-affected pathways in embryos, resulting in differential susceptibility to BPA. Parental adaptation or acclimation to BPA as a result of pre-exposure could have potentially reduced BPA-induced toxicity in the gonad and associated disruption of transfer of these factors into gametes, preventing negative effects on offspring, and increasing their resistance upon subsequent exposure to BPA. Alternatively, parental adaptation to BPA exposure could involve adaptive changes to the accumulation of these factors to actively buffer against direct toxic effects in the embryos themselves. Some of the major toxic effects known to result from BPA exposure in embryos include metabolic disruption, endocrine disruption and oxidative stress (Aluru, Leatherland and Vijayan, 2010; Wu *et al.*, 2011).

Metabolic disruption as a result of BPA exposure has been shown to interfere with energy regulation, production of proteins, and production of signalling molecules (Lam *et al.*, 2011; Ortiz-Villanueva *et al.*, 2017; Qiu *et al.*, 2019). This included inhibited transcription of enzymes involved in xenobiotic metabolism, such as carboxylesterase (Qiu *et al.*, 2019). Differential deposition of maternal enzymes or the transcripts encoding them – either as reduced disruption to this process or active upregulation of the process – could potentially improve how efficiently embryos are able to metabolise and excrete xenobiotics, such as BPA, or protect against metabolic inhibition caused by BPA; leading to increased tolerance.

Endocrine disruption is also a well-documented effect of BPA exposure in embryos (Schiller *et al.*, 2013; Mu *et al.*, 2018; Lee *et al.*, 2019). Maternal hormonal contributions to offspring via oocytes has been shown to be important for embryo development and stress response (Nesan and Vijayan, 2016). Prior to ZGA, embryos are not able to synthesise their own transcripts to produce

hormones, and instead rely on maternal transfer of these transcripts or the hormone itself. Some hormones are not produced until even later in development. For example, *de novo* cortisol synthesis does not occur in zebrafish embryos until after hatching (96hpf)(Alsop and Vijayan, 2008). Prior to this, embryos rely on a delicate balance of maternally transferred cortisol, which is essential for the proper development of hypothalamus-pituitary-interrenal (HPI) axis activity and the onset of the stressor-induced cortisol response in larvae (Nesan and Vijayan, 2016). Deficiency or excess of maternal cortisol have both been found to result in disruption of the HPI and stressor-mediated cortisol response of larvae (Nesan and Vijayan, 2016). Altered deposition of maternal hormones important for embryo development and stress response, could potentially affect susceptibility of embryos to environmental stressors such as BPA.

Exposure of embryos to BPA has also been shown to induce oxidative stress via inhibition of antioxidant enzymes and production of ROS (Wu *et al.*, 2011; Xu *et al.*, 2013). If pre-exposure of parents had led to upregulation of protective mechanisms and reduced oxidative stress in the gonads upon re-exposure, this could potentially reduce maternal transfer of damaging molecules, such as ROS, or increase maternal transfer of protective molecules, such as heat shock proteins or antioxidants. Therefore, reduced oxidative damage in gametes prior to spawning as a result of reduced oxidative stress in the gonads is also a consideration.

There is very little information in the literature exploring how BPA exposure affects maternal or paternal transfer of cytoplasmic factors in the gametes. This could be an interesting avenue for further research, given that BPA is known to affect offspring after parental exposure.

4.2.3. Potential epigenetic changes in adults underpinning inferred tolerance in offspring

As discussed above, increased tolerance in offspring of pre-exposed fish compared to naïve fish suggests that pre-exposure likely caused altered physiology in pre-exposed adults that inferred protection to offspring by altering the development of gametes and transfer of parental factors important for early development in the F1 generation.

As discussed for altered *amh* expression observed in the ovary, altered expression of other genes in pre-exposed fish upon re-exposure to copper due to persistent alterations in the epigenome induced during pre-exposure, may underpin the possible physiological changes in adults hypothesised to have affected gamete development. BPA has previously been shown to stably alter the epigenome in fish, including the methylome, correlating with changes in gene expression, including for genes encoding epigenome modifying enzymes (Doherty *et al.*, 2010; Kundakovic *et al.*, 2013; Laing *et al.*, 2016; Santangeli *et al.*, 2017). As the activity of genes can be affected both by changes in their own regulatory elements and by the activity of other genes in the same physiological pathways and networks, the potential epigenetic changes driving the altered physiology hypothesised in pre-exposed adults may not be restricted to the gonad. Pre-exposure to BPA could have altered a variety of defence pathways in different tissues of adults which could have knock-on effects for the physiology of the gonad and development of gametes. For example, altered BPA burden in the gonad and resulting transfer in gametes could occur as a result of altered BPA metabolism elsewhere in the body. The differential capacity for activation of defence pathways in other tissues should therefore considered during any investigation of mechanisms behind altered gonad physiology.

4.2.4. Epigenetic alterations during embryogenesis

The epigenome is particularly sensitive to environmentally-induced alterations during windows of reprogramming; which is known to occur in somatic cells of zebrafish embryos soon after fertilisation (Jiang, Zhang, Wang, *et al.*, 2013; Potok *et al.*, 2013) and potentially in differentiating germ cells during primordial germ cell (PGC) migration later in embryogenesis (Ortega-Recalde *et al.*, 2019; Skvortsova *et al.*, 2019). Santangeli *et al.* (2019) found evidence that maternal exposure to BPA can cause hypermethylation in the promoters of several genes in F1 offspring along with associated downregulation of these genes. These effects were then inherited to the F3 generation (Santangeli *et al.*, 2019). No changes in DNA methylation were observed in somatic cells of the F0 ovary. This suggests that BPA-induced epigenetic changes, for at least some genes, only occur during early development when cells undergo sensitive windows of epigenetic reprogramming;

which likely occurred due to maternal transfer of BPA or parental factors into gametes.

It is possible that the differential mortality observed for offspring in our study may be due to differences in BPA-induced epigenetic changes in embryos as a result of differing BPA content. Offspring of naïve parents were significantly more susceptible to BPA than offspring of pre-exposed parents, which were no more susceptible than offspring of controls. This could indicate that offspring of pre-exposed parents experienced fewer damaging epigenetic changes than offspring of naive parents, potentially due to decreased BPA transfer into eggs, reducing BPA content of embryos during the epigenetic reprogramming window happening soon after fertilisation.

4.3 Limitations and further research

The physiological changes hypothesised to be occurring in adults as a result of pre-exposure are likely driven by transcriptional changes that we did not measure, which may include organs other than the gonads. More extensive transcriptional analysis would be needed to investigate these potential changes further.

A hypothesised mechanism underpinning altered tolerance in offspring included altered BPA metabolism in adults leading to altered transfer of BPA or its metabolites in their gametes. We were unable to measure BPA content of adult tissues, gametes or embryos, which would be required to confirm this hypothesis. In addition, this chemical analysis would be required to confirm that complete depuration of adults took place between the first and second exposure periods.

In order to investigate whether altered transfer of cytoplasmic parental factors had occurred between pre-exposed adults and offspring, further investigation of gamete contents, such as parental transcripts, proteins, and hormones would be required. Limited information exists in the literature on the effects of BPA on gamete development, and would be an interesting area for further research.

Although the differential response of pre-exposed fish upon re-exposure suggests maintenance of altered gene regulation through the depuration period via epigenetic changes, this study did not include direct analysis of the epigenome, which would be required to confirm this hypothesis.

5. Conclusions

This study demonstrates that pre-exposure of adult zebrafish to BPA leads to an altered physiological response upon re-exposure, including changes in gene transcription and to the susceptibility of embryos generated by pre-exposed populations. The mechanisms underpinning these effects could include physiological, transcriptional or epigenetic changes in directly exposed adults and indirectly exposed embryos. This suggests that fluctuating exposures to BPA could lead to different responses in organisms compared to stable exposure scenarios, and should be considered in environmental risk assessment.

Chapter 3

Pre-exposure to waterborne copper during embryogenesis in the three-spined stickleback (Gasterosteus aculeatus) causes a differential transcriptional response in the adult gill upon re-exposure

Declaration of contributions:

In vivo exposures, tissue extractions for metal analysis and associated statistical analyses were conducted by Dr Lauren Laing as part of her doctoral thesis at the University of Exeter (Laing, 2017). Metal analysis was conducted at King's College London. The description of the animal experiments conducted and results obtained are included for clarity of the origin of the samples and to facilitate the interpretation of the transcriptomic data.

I conducted all RNA extractions from gill samples and prepared the libraries with Exeter Sequencing Service, who then conducted the sequencing. I performed all the bioinformatics analysis, conducted all the data interpretation and wrote the chapter.

1. Introduction

Wildlife frequently experience fluctuating exposures to toxicants due to variations in chemical release and environmental conditions. In order to protect wildlife from intermittent exposures across their lifetime it is important to understand how historic exposure can affect response in later life. If subsequent sensitivity to a chemical is altered depending upon prior exposure, regulatory testing using a single, continuous exposure of previously naïve animals will be insufficient to assess the risk posed by that chemical to the environment.

Early development is considered to encompass critical windows of susceptibility for exposure to environmental stressors, including two potential windows of epigenetic reprogramming in vertebrates (Oswald *et al.*, 2000; Morgan *et al.*, 2005; Jiang, Zhang, Wang, *et al.*, 2013; Potok *et al.*, 2013; Marsit, 2015). It is thought that the epigenome is particularly vulnerable to environmental influence during reprogramming windows, and that mitotic inheritance of epigenetic marks between parent and daughter cells can maintain epigenetic alterations into later life, causing altered adult phenotype (Jirtle and Skinner, 2007).

Many studies examining the developmental origins of health and disease in humans have described a likely role for environmentally-induced epigenetic changes occurring as a result of stressful *in utero* or perinatal conditions, including developmental exposure to toxic chemicals, in a variety of disease outcomes in later life (Barker, 2007; Wadhwa *et al.*, 2009; Godfrey, Gluckman and Hanson, 2010; Feil and Fraga, 2012; Hou *et al.*, 2012; Vaiserman, 2014; Joubert *et al.*, 2016; Cardenas *et al.*, 2017; Hoffman, Reynolds and Hardy, 2017; Park *et al.*, 2017; Gali Ramamoorthy *et al.*, 2018). Similarly, many studies have linked early life exposure of wildlife to toxic environmental chemicals with adult onset disease phenotypes. For example, brief exposure of chinook salmon to a metabolite of the widely used pesticide, dichlorodiphenyltrichloroethane (DDT), immediately after fertilisation and hatching resulted in long-term immunosuppression as adults, despite no further exposure (Milston *et al.*, 2003). A review by Weis (2014) also describes a variety of delayed adverse behavioural effects occurring in aquatic organisms later in life as a result of early life exposure to chemicals, including metals, pesticides, hydrocarbons and EDCs (Weis, 2014). Long-term reproductive disruption as a result of transient chemical exposure during early life has also

garnered much interest. Many studies have reported reproductive disruption in adults as a result of transient exposure to common environmental EDCs, such as ethinylestradiol (EE2), methoxychlor and atrazine, during critical windows of susceptibility in early development (van Aerle *et al.*, 2002; Pettersson *et al.*, 2006; Coe *et al.*, 2010; Gore *et al.*, 2011; Wirbisky *et al.*, 2016).

However, as well as playing a role in developmental origins of disease, the epigenome may also provide mechanisms by which exposure to environmental stimuli during development can lead to altered response to subsequent exposure later in life, including both reduced and increased tolerance. Many examples of adaptive “stress memory” have been identified in plants where epigenetic mechanisms likely play a role, including priming against osmotic stress, chemical stress and herbivory (Lämke and Bäurle, 2017). Fewer examples exist for animals outside of acclimation studies. While many physiological changes involved in acclimation to continuous chemical exposure are underpinned by adaptive changes in gene regulation, these transient changes are often quickly lost after removal of the chemical and as a result tolerance is attenuated. Acclimation to chemical exposure has been demonstrated in animals for a variety of chemicals, including metals, such as cadmium, arsenic and copper (Dixon and Sprague, 1981; Benson and Birge, 1985; Sellin, Tate-Boldt and Kolok, 2005; Miller *et al.*, 2007; Adeyemi and Klerks, 2013). Persistent alterations in chemical tolerance within a single generation would require a similar mechanism of “stress memory” as has been observed in plants, and would likely involve stable changes in gene regulation resulting from mitotically heritable epigenetic modifications. The potential for induction of long-term intragenerational tolerance to environmental chemicals could have important implications for wild populations living in environments where chemical exposure fluctuates, preventing acclimation.

In this study I have focused on copper, one of the most significant pollutants of freshwater systems in the UK and worldwide (Donnachie *et al.*, 2014). It is an essential element for almost all organisms, performing critical functions such as acting as a cofactor for enzymes involved in energy production and metabolism (Grosell, 2012). However, at elevated concentrations it can become highly toxic, and is therefore subject to tight homeostatic regulation (Festa and Thiele, 2013). Aquatic organisms are particularly susceptible to waterborne copper contamination as, in addition to dietary exposure, they are continuously exposed

via the gills (Grosell and Wood, 2002). Toxic effects of copper in aquatic organisms include: oxidative stress and DNA damage, leading to apoptosis; disruption of ionoregulation, particularly sodium loss, leading to osmoregulatory distress; inhibition of ammonia excretion and disruption of acid-base balance; and impaired gas exchange due to damage and thickening on the gill epithelium, leading to respiratory distress (Grosell, 2012).

During exposure to excess waterborne copper, toxic concentrations of this metal are taken up across the gill. Grosell (2012) reviews three hypothesised mechanisms of branchial apical Cu uptake (Grosell, 2012). Copper ions can compete with sodium ions for uptake through apical sodium channels, leading to competitive inhibition of sodium uptake – which along with inhibition of other sodium transporters and increased sodium efflux is one of the mechanisms by which it contributes to disrupted sodium homeostasis (Sola, Isaia and Masoni, 1995; Grosell and Wood, 2002; Pyle *et al.*, 2003; Sloman *et al.*, 2003; Grosell, 2012). Copper is also thought to be taken up via copper-specific apical transport proteins expressed in the gill (Mackenzie *et al.*, 2004; Minghetti *et al.*, 2008; da Silva *et al.*, 2014; Cheng *et al.*, 2017; Anni *et al.*, 2019), including copper transporter 1 (ctr1), also known as solute carrier family 31 member 1 (slc31a1); and via apical uptake across divalent metal transporter 1 (dmt1)(Grosell, 2012), also known as solute carrier family 11 member 2 (slc11a2)(Bury, Walker and Glover, 2003). Transport across the basolateral membrane of the gill epithelium likely involves active transport via ATPase copper transporting alpha (atp7a)(Craig, Galus, *et al.*, 2009; Minghetti, Leaver and George, 2010; Chen and Chan, 2011b). Once taken up by cells, copper must be sequestered by proteins, such as chaperones, storage proteins or cuproenzymes, to prevent oxidative damage from free copper ions (Grosell, 2012). Intracellular mechanisms of copper homeostasis have not been entirely characterised in fish, but are thought to be similar to those in mammals, and many conserved copper chaperone proteins are known to exist in fish, including metallothioneins (mt) and glutathione (gsh), antioxidant protein 1 (atox1), copper chaperone for superoxide dismutase (ccs), sod1 antioxidant complex (copper/zinc superoxide dismutase), and cytochrome c oxidase copper chaperone (cox17)(Grosell, 2012; Pereira, Campos and Bogo, 2016). Many of these chaperones are also known to be differentially regulated in response to copper exposure in fish (Craig, Wood and McClelland, 2007; Chen

and Chan, 2011a). After excretion from cells copper is transferred to proteins in the blood, such as ceruloplasmin, to be transported to sites of storage or metabolism, including the liver, which is main site of copper metabolism and excretion (Grosell, 2012).

Copper occurs naturally at low levels in the freshwater environment, ranging from 0.2 - 30 µg/L (USEPA, 2007), but can become elevated in areas affected by copper-rich anthropogenic input, such as discharge from mining, agriculture, industry and municipal wastewater treatment plants. Reported concentrations of waterborne copper in UK rivers ranges from 0.02 – 133 µg/L (Donnachie *et al.*, 2014), and extreme concentrations have been reported elsewhere from mining areas, reaching up to 200mg/L (USEPA, 2007). The toxic potential of copper in freshwater systems can fluctuate across time, influenced by local and seasonal weather patterns; which determine levels of runoff and dilution of copper from these anthropogenic sources, and lead to alterations in water parameters, such as salinity, temperature, pH and dissolved organic matter (DOM), which heavily influence bioavailability of copper to organisms, even without a change in total copper concentration (Gaur *et al.*, 2005; Lathouri and Korre, 2015). This makes aquatic environments and wildlife particularly vulnerable to fluctuating copper exposure.

There are many examples of wild and experimental fish populations developing tolerance for elevated toxic metal concentrations, including adaptation to chronic exposure to high copper concentrations over many generations, likely via selection for a tolerant genotype (Gale *et al.*, 2003; Durrant *et al.*, 2011; Uren Webster *et al.*, 2013); and transient acclimation to low-level copper, leading to increased tolerance to subsequent lethal exposure when applied immediately following acclimation (Dixon and Sprague, 1981; Buckley *et al.*, 1982; Taylor *et al.*, 2000; Adeyemi and Klerks, 2013). The molecular mechanisms underpinning altered copper tolerance are not fully understood. However, a range of adaptive physiological changes have been suggested as potential mechanisms, including adaptive changes in the activity of copper homeostasis pathways and compensatory changes in pathways affected by copper toxicity, such as ion homeostasis and oxidative stress response (Sellin, Tate-Boldt and Kolok, 2005; Adeyemi and Klerks, 2013; Uren Webster *et al.*, 2013).

As is common for many chemicals, most studies examining induction of copper tolerance within a generation are acclimation studies where both the acclimation and subsequent copper challenges were performed in adult animals, with either no depuration or a very short depuration period between the acclimation and challenge. Typically persistence of tolerance after the acclimation period was either not tested (Buckley *et al.*, 1982; Miller *et al.*, 1993), or the tolerance was found to be lost soon after being transferred to clean water for recovery (Dixon and Sprague, 1981).

Very little research has focussed on the effects of early life exposure on future response in later life, leaving our understanding of the mechanisms underpinning these effects, and their propensity to persist over an organism's lifetime, limited. The potential for animals to adapt to exposure within a single generation and respond differently upon re-exposure across their lifetime is an important knowledge gap, with implications for the protection and conservation of animals which experience fluctuating exposure to environmental stressors.

Previous research by Laing (2017) reported evidence for an altered physiological response in adult three-spined stickleback upon re-exposure to copper following pre-exposure during embryogenesis. After 9 months growing in clean water, pre-exposed stickleback were found to have significantly more copper in their tissues, and were shown to take up more copper upon re-exposure than controls. They also gave rise to offspring that were more tolerant to copper when exposed during early life (see also chapter 4 in this thesis). This indicated that pre-exposure of stickleback to copper during embryogenesis resulted in altered physiology as adults; and the increased tolerance of their offspring suggested that this altered physiology may be an adaptive response. To investigate the mechanistic basis of the altered physiological responses observed, in this study I conducted RNA sequencing in the adult gill, and compared the transcriptomic response between pre-exposed and naïve fish upon subsequent exposure to copper. I hypothesised that upon adult exposure pre-exposed fish would exhibit distinct transcriptional responses to the copper exposure compared to naïve fish, in particular for pathways involved in copper handling and response to copper toxicity.

2. Methods:

2.1. Fish source and husbandry

A freshwater population of three-spined stickleback (*Gasterosteus aculeatus*) sourced from the River Erme, Devon, United Kingdom, was used to generate fish for this study. All fish were maintained and bred in the Aquatic Resources Centre at the University of Exeter. Mixed sex tanks of 112L, containing 20 fish per tank, were supplied with synthetic ISO water according to the ISO-7346/3 guideline (International Organization for Standardization, 1996) generated from mains tap water filtered by reverse osmosis (Environmental Water Systems (UK) Ltd) and reconstituted with Analar-grade mineral salts (conductivity of 300mS: 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), and delivered from an aerated 15°C reservoir. All breeding and embryo exposures were conducted during summer months (March-October), during which fish were maintained at a photoperiod of 18:6 light/dark (with 30 minute dawn/dusk transition) with a water temperature of 15 ±1°C, being 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.) Photoperiod and temperature were gradually transitioned over a 6 week period to winter conditions, comprising a photoperiod of 6:18 light/dark and water temperature of 10 ±1°C, with fish being fed to satiation twice daily with blood worms (Chironomus sp.; Tropical Marine Centre, Chorleywood, UK).

All embryos were generated with IVF, using a method adapted from Barber and Arnott (2000). Unfertilised eggs were identified and removed by visual observation as described by Swarup (1958), using a dissection microscope (Motic DM143, Hong Kong). Fertilised embryos were incubated at 20°C in artificial freshwater (according to ISO-7346/3 guideline, ISO water, diluted 1:5 (International Organization for Standardization, 1996)), in 600ml acid washed glass exposure tanks from 1-217hpf. Under these conditions, stickleback larvae have yolk sacs up until 217hpf, and as such are not considered to be independently feeding.

2.2. Chemical exposures and sampling

Using IVF, a pool of embryos was produced from between 20-26 females and 3-5 males, and randomly assigned to 600ml acid-washed glass tanks at 1hpf (corresponding to the one cell stage (blastodermic cap)). Exposures were started

from the 1 cell stage to ensure that the exposure encompassed the critical window of epigenetic reprogramming thought to occur soon after fertilisation (Potok *et al.*, 2013). Each exposure was run in quadruplicate, and was repeated three times using different parental fish. Embryos were exposed to either a water control (0mg/L Cu) or 0.01mg/L Cu (copper, added as copper (II) sulfate, Sigma Aldrich) from 1-217hpf in order to generate a naïve control population and a copper pre-exposed population. The exposure concentration was chosen for its environmental relevance and because it caused less than 1.5% mortality, ensuring that selection for a copper tolerant genotype did not occur. Each glass tank contained 40-50 embryos in 500ml of artificial freshwater (according to ISO-7346/3 guideline, ISO water, diluted 1:5 (International Organization for Standardization, 1996)) maintained at 20°C. Water was changed daily to avoid local hypoxia, and air saturation 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%. Individual embryos were observed every 24hrs 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 217hpf, 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). Water samples were collected from each tank on day 5 and 9 of the exposure to measure copper concentration in the exposure water. At 217hpf, 200 larvae from the control and copper pre-exposed populations were transferred to holding tanks, and maintained in control conditions (as described previously) until sexual maturity (~9 months).

A subset of adult males from both the control and pre-exposed populations were subsequently exposed to 0, 0.01 or 0.02mg/L Cu in duplicate (9 fish per tank) for four days via a flow through system employing a flow rate of 144L/day for each 40L exposure tank (3.6x tank volumes per day). This resulted in six treatment groups: naïve control fish subsequently exposed to 0 mg/L Cu (control_0), 0.01 mg/L Cu (control_0.01), or 0.02 mg/L Cu (control_0.02); and pre-exposed fish subsequently exposed to 0 mg/L Cu (copper_0), 0.01 mg/L Cu (copper_0.01), or 0.02 mg/L Cu (copper_0.02). Throughout the 4 day copper exposure period, fish were fasted. This was to minimise the amount of free organic matter in the water available to bind copper, therefore helping to maintain the desired copper

concentration in the water; and to reduce the likelihood of nutritional changes acting as a confounding factor between treatments, as copper exposure has been shown to affect appetite (Waiwood and Beamish, 1978). After four days, 9 fish from each treatment group were euthanised 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 ($k = (\text{weight (g)} \times 100)/(\text{fork length (cm)})^3$). 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). Gill was also collected for nucleic acid extraction (placed in an autoclaved tube and stored at -80°C). Water samples were collected from each tank on day 1 and 3 of the exposure to measure copper concentration in the exposure water.

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.

2.3. Metal content analysis in water and tissue samples

Water samples were stored in acid washed tubes 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 fat. 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 at King's

College London by ICP-MS using a Perkin Elmer NexION 350D instrument running the Syngistix software, v1.0.

2.4. Statistical analysis

Statistical analyses were conducted in R version 3.5.0 (R Core Team, 2018).

Prior to analysis, data were tested for equal variance and for normality using the Shapiro–Wilk test. The effects of copper exposure in naïve embryos on whole organism metal content and mortality were analysed using the Student's t-Test. Generalised linear models with a binomial error structure were used to test the effects of re-exposure to copper on tissue copper content and morphometric parameters, using pre-exposure (as a categorical variable) and copper concentration (as a continuous variable) and the interaction between the two. Minimum adequate models (MAM) were derived by model simplification using F tests based on analysis of deviance. F tests reported refer to the significant of each term within the MAM. All data was considered to be statistically significant when $p<0.05$.

2.5. RNA extraction, library preparation and sequencing

Transcript profiling was conducted in the gills of six sexually mature males from four of the treatment groups: fish from the naïve population subsequently exposed to 0mg/L Cu (control_0) and 0.01mg/L Cu (control_0.01), and fish from the pre-exposed population subsequently exposed to 0mg/L Cu (copper_0) and 0.01mg/L Cu (copper_0.01). RNA was extracted from gills using an AllPrep DNA/RNA Mini Qiagen Kit (Qiagen, Hilden, Germany), and treated with DNAse I (Qiagen) to remove potential DNA contamination. The concentration, purity and integrity of the RNA were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington USA) and an Agilent 2200 TapeStation (Agilent Technologies, CA, USA). The RNA used for library preparation was of high quality, with all samples having a RIN score ≥8.6. ERCC spike-in control mix (Ambion, TX, US) was added to each RNA sample to control for technical variation during library preparation and sequencing, according to the manufacturer's instructions. cDNA libraries from all 24 samples were then prepared from 500ng of RNA using the Illumina TruSeq Stranded RNA Sample Preparation kit, multiplexed (12x), and

sequenced across 2 lanes using an Illumina HiSeq 2500 to generate 100bp paired-end reads, according to the manufacturer's instructions.

2.6. Transcriptomic analysis

All analyses were carried out on a local Linux server. All plots were produced in R v3.5.0. (R Core Team, 2018) directly or using various scripts of the Trinity RNA-Seq pipeline v2.4.0 (Grabherr *et al.*, 2011).

Prior to transcriptomic analysis, Trim Galore v0.5.0 (Krueger, 2018)(with fastqc function and default parameters) was used to trim Illumina adapter sequences and low quality bases (minimum Phred quality score of 20) from the paired Illumina sequence reads, and reads shorter than 20bp were removed. Only read pairs for which both reads passed quality trimming were retained. The quality of the reads before and after trimming was inspected using the FastQC output files generated by Trim Galore v0.5.0.; quantified using SeqKit v.12.1.0 (Shen *et al.*, 2016); and one-way analysis of variance (ANOVA) in R was used to test for significant differences between treatment groups for the number of raw reads generated and reads passing quality trimming.

A reference transcriptome was generated using all *Gasterosteus aculeatus* cDNA sequences downloaded from Ensembl (BROAD S1 v93 ftp://ftp.ensembl.org/pub/release-93/fasta/gasterosteus_aculeatus/cdna/)(Howe *et al.*, 2020), ERCC spike-in nucleotide sequences (contained in the ERCC92.fa file downloaded from the Thermo Fisher Scientific website) and the stickleback metallothionein nucleotide sequence (Laing, 2017).

In addition to the gene annotations and gene ontology (GO) IDs obtained for each transcript from BioMart (Ensembl Genes 93 BROAD S1)(Durinck *et al.*, 2009), further annotation steps were undertaken to obtain as much information as possible for the functional annotation analysis described below. InterProScan v5.31-70.0 (Mitchell *et al.*, 2019) was used to identify conserved protein motifs, including trans-membrane regions, enzymes, signal peptides and structural domains, and this information was used by Blast2GO Pro v5.2.5 (Götz *et al.*, 2008) to add additional GO terms to genes in the reference transcriptome that did not have any GO terms associated with them in BioMart. In addition, to putatively identify unannotated genes, all cDNA sequences were used to perform BLASTX searches against the NCBI non-redundant protein sequences database

(downloaded 2020/02/23) using DIAMOND v0.9.28, and results filtered with a threshold E-value of 1e-15 before annotation. Following these additional annotation steps, Blast similarity searches increased the percentage of the reference transcriptome with gene description annotations from 64.6% to 90.2%, and InterproScan analyses identified annotations for 90.0% of transcripts. GO annotations were identified for 81.8% of transcripts and used to perform GO enrichment analysis. A full list of gene description and gene ontology annotations for the reference transcriptome can be found in supporting information file 1 - Chapter 3 Reference Transcriptome Full Gene Description and GO Annotations.xlsx (see list of accompanying material).

Transcript abundance estimates were determined by mapping the quality-trimmed reads of each sample to the reference transcriptome using Trinity v2.4.0, bowtie v1.1.2 (Langmead *et al.*, 2009) and RSEM v1.2.31 (Li and Dewey, 2011)(with default parameters). Differential expression analysis (at the gene level) was performed in Trinity using edgeR v3.16. (Robinson, McCarthy and Smyth, 2009) and the following conditions were compared: copper_0.01 vs copper_0, control_0.01 vs control_0, copper_0 vs control_0, and copper_0.01 vs control_0.01. Genes were considered differentially expressed when FDR < 0.05 (Benjamini-Hochberg method).

Principal component analysis (PCA; using the first 3 Principal components) was conducted on differentially expressed genes for all groups using the PtR script supplied by Trinity (parameters used: --log2, --min_rowSums 10, --CPM, --center_rows).

Hierarchical clustering (Euclidean distance similarity metric and complete linkage method) was performed on the list of differentially-expressed genes between the re-exposed groups (copper_0.01 vs control_0.01) with the analyze_diff_expr.pl script included in the Trinity pipeline.

Enrichment analysis was performed on the lists of differentially expressed genes in Blast2Go Pro v5.2.5 (Götz *et al.*, 2008) using Fisher's Exact Test. Gene lists containing genes that were differentially upregulated and downregulated upon adult exposure were analysed for two comparisons – control_0.01 vs control_0 (naïve population) and copper_0.01 vs copper_0 (pre-exposed population), and all gene sequences that were not part of the test sets were used as reference

(background). GO terms for biological process, cellular component and molecular function were considered enriched when FDR < 0.05. Differentially expressed gene lists and GO terms for the two populations were then compared to infer differences in transcriptomic response upon re-exposure to copper between naïve and pre-exposed fish. For selected themes, comparisons of enriched GO terms were visualised as dotplots using ggplot2 v.3.2.1 (Wickham, 2016) in RStudio v1.2.1335 (Booth *et al.*, 2018).

By basing our GO analysis on these comparisons, as opposed to one direct comparison between re-exposed fish from both populations (copper_0.01 vs control_0.01) we were able to observe changes in gene expression in response to copper exposure that are common to both populations, as well as those that are different, including the magnitude of change where the same gene was upregulated in one population and downregulated in the other.

3. Results and Discussion:

3.1. Water chemistry

For the initial embryo exposures, the mean measured concentration of copper in the tank water was 0.0114 ± 0.0003 mg/L (114% of nominal) for the copper treatment and 0.0002 ± 4.1145e-05 mg/L for the control treatment. For the adult exposures, the mean measured concentration of copper in the tank water was 0.0050 ± 4.668e-05 mg/L for the control treatment, 0.0135 ± 0.0003 mg/L (135% of nominal) for the 0.01 mg/L Cu treatment, and 0.0218 ± 4.6680e-05 mg/L (109% of nominal) for the 0.02 mg/L Cu treatment (Laing, 2017).

3.2. Effects of copper exposure during embryogenesis on mortality and larval copper content

Exposure to 0.01mg/L Cu caused a small but significant increase in embryo mortality by 217hpf (1.24%; P=0.0384; figure 1A), when compared to the background mortality in embryos exposed to control conditions (0.69%; figure 1A). Larvae exposed to 0.01 mg/L Cu also had significantly greater whole organism copper concentrations when compared to controls (0.0031 ± 8.2687e-05 µg/larva and 0.0013 ± 1.7487e-04 µg/larva respectively; P=1.2e-07; figure 1B)(Laing, 2017).

This demonstrates that copper was taken up by exposed larvae, and that the exposure concentration was high enough to cause a significant and measureable effect, but low enough to guard against genetic selection.

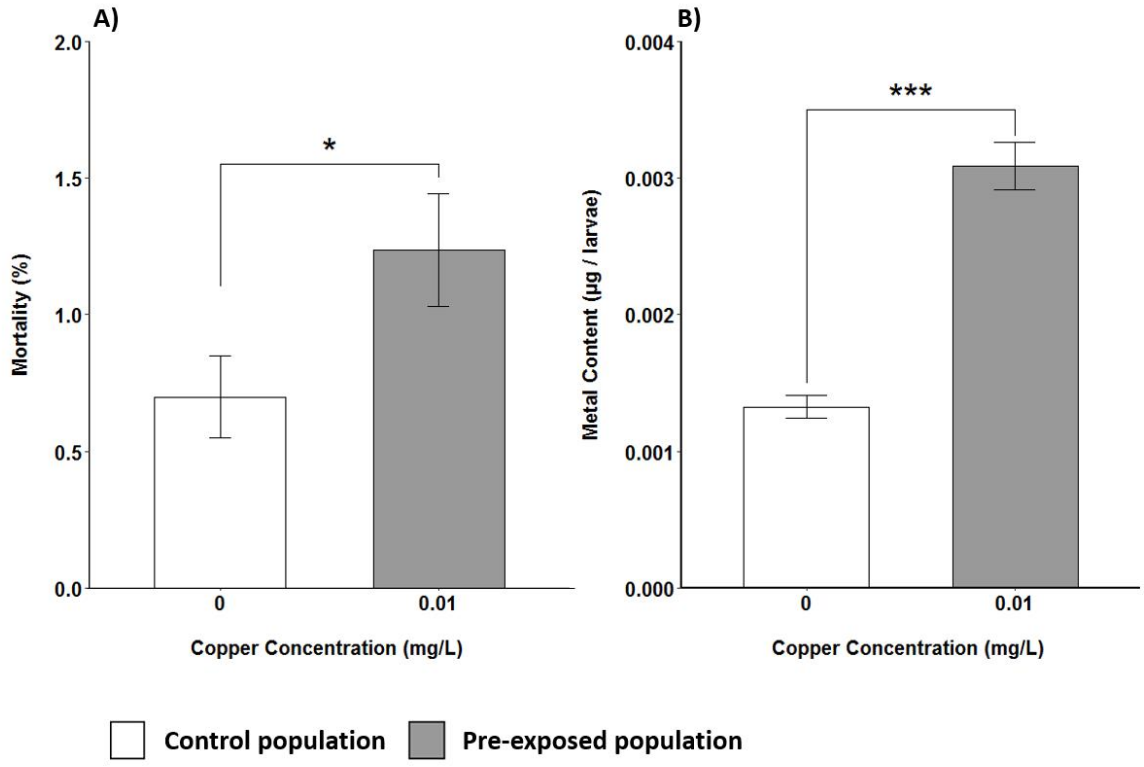


Figure 1: *A) Average mortality and B) measured copper concentrations observed in embryos exposed to 0.01mg/L copper or kept in control conditions during embryogenesis (1-217hpf; n = 3 replicate exposures, each made up of 4 replicate dishes containing 20 embryos). Statistical comparisons were conducted 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).*

3.3. Pre-exposure to copper during embryogenesis modified tissue copper content later in life and upon re-exposure

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/cm3 and 2.3296 ± 0.0223, respectively, and there were no significant differences between treatment groups (Reported in: Laing, 2017).

Copper concentration in tissues of adult fish pre-exposed to copper during embryogenesis and maintained in control conditions for 9 months until maturity was 2.14-, 1.42- and 2.54-fold higher in the gill, liver and muscle, respectively, compared to control fish (gill: P=0.0029; liver: P=0.0143; muscle: P=0.0303)(figure 2A, B and C)(Laing, 2017). The significantly higher accumulation of copper in the gill, liver and muscle of pre-exposed fish as adults prior to any subsequent exposure indicates that exposure during embryogenesis caused changes in the copper handling of the pre-exposed population that persisted into adulthood, more than 9 months after the embryonic exposure ended. The amount of copper found in the tissues of pre-exposed adults was higher than the amount provided during their embryonic exposure, showing that they continued to take up and store more copper than naïve fish during their 9 month depuration period. As there was no copper added to the water during this 9 month period, this suggests that embryonic exposure caused changes in copper handling pathways of pre-exposed fish that allowed them to take up more copper across the gut from their diet than naïve fish, despite identical feeding regimes. Consistent with the findings of Kamunde *et al.* (Kamunde *et al.*, 2001, 2002), who found evidence for interaction between dietary and branchial copper uptake, this suggests that embryonic waterborne exposure caused stable changes to the regulation of copper uptake mechanisms in the gut that persisted into later life. This maintenance of high copper concentrations in tissues over the depuration period following embryonic exposure is in contrast with other studies in adult fish, which frequently observed copper accumulation during exposure but a decline to normal levels during subsequent depuration (Laing, 2017; Ewa *et al.*, 2019). This suggests that pre-exposure during embryogenesis, which encompasses periods of putative

epigenetic reprogramming, may be central to inducing lasting changes in metal handling, which are not induced when pre-exposure occurs during later life.

Subsequent exposure of adult male fish to 0.01 and 0.02mg/L Cu caused a significant increase in copper concentration in the gills in fish from both pre-exposed and naive populations (P=6.671e-07; figure 2A). In addition, pre-exposure was found to be a significant explanatory variable determining the amount of copper detected in the gills of adult fish (P=0.0029; figure 2A). Although copper accumulation was increased in the gill for both populations upon subsequent exposure to 0.01 and 0.02mg/L Cu, the concentration of copper accumulated in the gills was 1.45- and 1.81-fold higher in fish pre-exposed to 0.01mg/L Cu during early life (1-217hpf) when compared to the naïve population, exposed in parallel. In both the liver and muscle, there was no significant effect of re-exposure on tissue copper concentration (Laing, 2017). The significantly higher rate of copper accumulation in the gills of pre-exposed fish compared to naïve fish suggests that pre-exposure to waterborne copper during embryogenesis also caused stable changes in the regulation of branchial copper handling that persisted into later life, and altered the response of copper handling pathways to subsequent exposure.

This increased accumulation rate in the gills of pre-exposed fish compared to naïve fish upon re-exposure differs from the observations of some acclimation studies. For example, upon acute exposure to 150µg/L of copper, Adeyemi and Klerks (2013) observed reduced rates of whole-body copper accumulation in least killifish that had received a 7 day pre-exposure to 15µg/L immediately prior to the challenge (Adeyemi and Klerks, 2013). This indicated physiological acclimation that reduced uptake or increased excretion of copper in pre-exposed fish. In another study, when subsequently challenged with Cu exposure, Cu-acclimated rainbow trout were shown to have reduced accumulation and increased turnover rate of Cu in plasma, which authors attributed to increased clearance via the liver and kidney, which also exhibited a higher turnover rate in Cu-acclimated fish. Cu-acclimated trout also had a slightly lower copper burden in the gills than non-acclimated fish, which reached a steady state after 3 hours of exposure, indicating that plasma clearance balanced gill uptake after this point (Grosell, Hogstrand and Wood, 1997). In a study using European eels, a 28 day sublethal pre-exposure was found to have no effect on Cu accumulation in the gills upon subsequent Cu

challenge, and resulted in reduced accumulation in the liver and muscle. They also suggest that this was driven by an increased rate of excretion, rather than reduced uptake (Grosell *et al.*, 1996).

In our study, there was no accumulation of copper in the liver or muscle upon adult exposure for either population. This indicates that changes induced by pre-exposure affecting the response of fish to future waterborne exposures may be particularly important in the gill but this does not preclude that other important mechanisms may be occurring in other tissues, such as the liver, and may not be evident in the amount of copper present in those tissues due to homeostatic processes. Based on these results, we prioritised the gills for further molecular analysis, to investigate the transcriptional pathways responsible for the differences in copper homeostasis associated with embryonic pre-exposure.

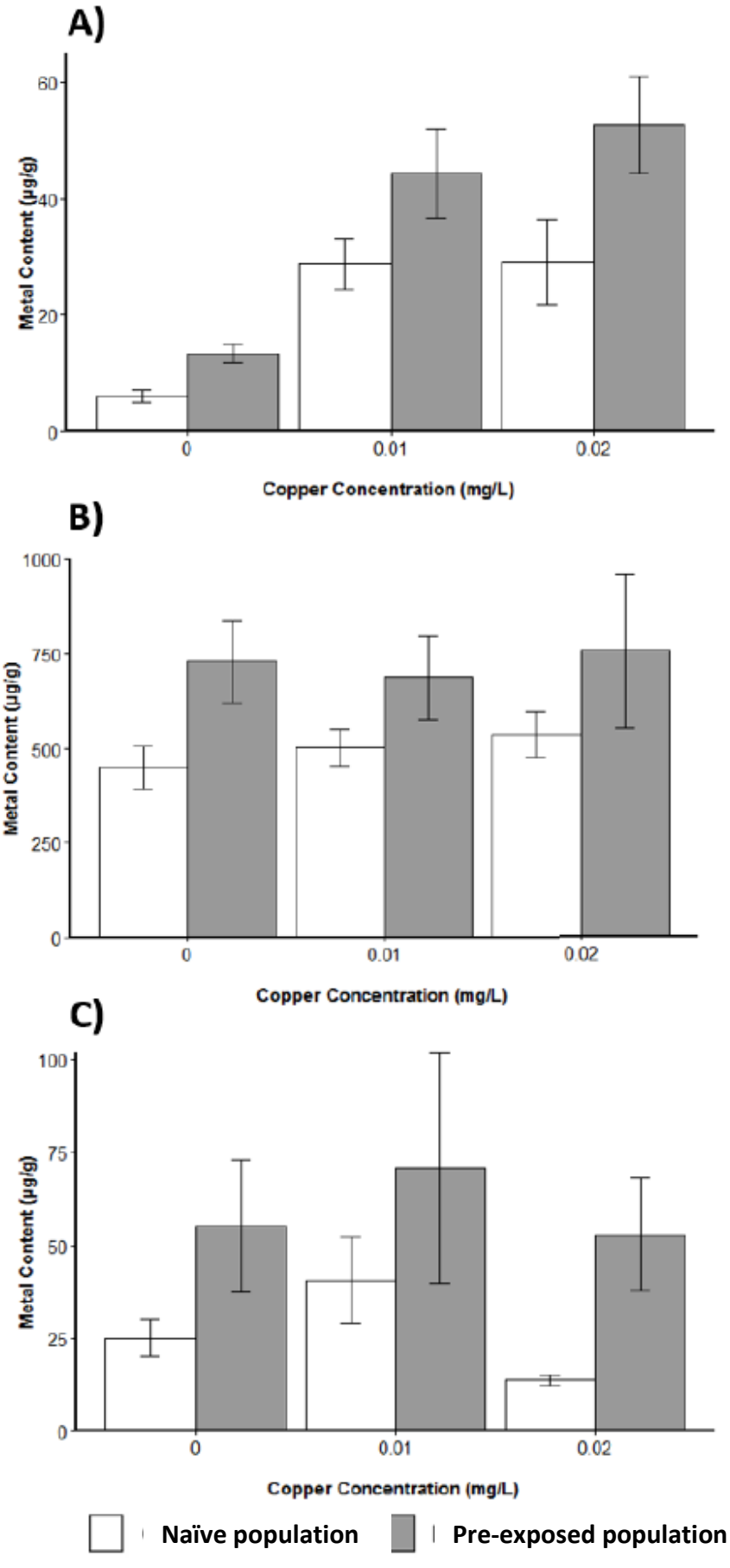

A)
Metal Content (µg/g)
60
40
20
0
0
0.01
0.02
Copper Concentration (mg/L)
B)
Metal Content (µg/g)
1000
750
500
250
0
0
0.01
0.02
Copper Concentration (mg/L)
C)
Metal Content (µg/g)
100
75
50
25
0
0
0.01
0.02
Copper Concentration (mg/L)
Naïve population
Pre-exposed population

Figure 2: *Measured copper concentrations in the gill (A), liver (B) and muscle (C) of male adult stickleback pre-exposed to control conditions or 0.01mg/L Cu during embryogenesis (1-217hpf), and re-exposed to 0, 0.01 or 0.02mg/L Cu for 4 days as adults (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 Laing (2017). 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). 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)(Laing, 2017).*

3.4. Transcriptomic Analysis

3.4.1. Quality control

Sequencing generated a total of 376.8M 100bp paired-end reads, with a mean of 15.7M reads per sample, with no significant difference between treatment groups (p=0.332). Following raw sequence read processing and quality filtering 99.9% of reads were retained and used for transcriptomic analysis, with the number of read pairs per sample ranging from 13.5M to 22.8M, with a mean of 15.7M (supplementary table 1), and no significant difference between treatment groups (p=0.332).

3.4.2. Naïve fish exhibited a greater transcriptional response compared to pre-exposed fish upon re-exposure to copper

Despite a significant difference in copper accumulation over the 9 month depuration period, in which copper pre-exposed fish accumulated significantly higher concentrations of copper compared to naïve fish in the gill, liver and muscle, there was almost no difference in transcription in the gill between populations. Only 4 genes were found to be differentially expressed between pre-exposed fish compared to naïve fish prior to re-exposure (Cu_0 vs Ctrl_0). However, given that copper uptake and storage was clearly increased in pre-exposed fish during the depuration period, this suggests persistent changes in the regulation and activity of copper uptake, storage and excretion pathways are likely to have occurred at protein level or in other organs whose transcriptomes were not

analysed. As copper was only available via the diet during the 9 month depuration period, this suggests that persistent changes in the regulation of copper uptake pathways may have occurred in the gut that led to increased copper uptake from the diet compared to naïve fish. Alternatively, if regulation of copper uptake across the gut did not differ between populations, persistent changes to the regulation of copper storage and excretion may have occurred, leading to increased storage and reduced excretion in pre-exposed fish. Together this suggests that transcriptional changes would be expected in analysis of other tissues involved in copper handling and at the protein level in the gill.

Upon adult exposure to copper, differential expression analysis identified a total of 451 differentially expressed genes (FDR < 0.05) in the gills of naïve fish (control_0.01 vs control_0). This was compared to 138 in pre-exposed fish (copper_0.01 vs copper_0)(figure 3). This suggests that although there was less copper accumulation in the tissues of naïve fish upon subsequent exposure, there was a stronger impact of exposure on gene transcription in these fish, with over three times as many genes being differentially expressed in this naïve population compared to that found for the pre-exposed population. Of those differentially expressed genes, 67 were common to both populations, suggesting a strong overlap in the affected transcripts.

Following re-exposure of adult fish to copper, direct comparison of the pre-exposed and naïve populations identified 434 differentially expressed genes (Cu_0.01 vs Ctrl_0.01), compared to only 3 genes differentially expressed between populations prior to re-exposure. This indicates that a substantial differential transcriptomic response between naïve and pre-exposed populations is only evident after copper re-exposure, and is not simply due to genes remaining up- or down-regulated since the initial exposure during embryogenesis. This suggests that early life exposure may have induced epigenetic changes to regulatory elements associated with these genes in pre-exposed fish that persisted into later life and primed these genes for a differential response compared to naïve fish upon stimulation by re-exposure to copper. This is a relatively novel approach to epigenetic study design in ecotoxicology, with re-exposure to assess differential responsiveness as a result of historic exposure in the context of stable epigenetic alterations rarely being seen in the literature. Without assessing response following re-exposure, the differential capacity for

response to copper between these populations as a result of prior exposure would not have been evident.

A list of differentially expressed genes, fold changes and FDR values for these comparisons can be found in Supporting information file 2 – Chapter 3 Annotated DEGs.xlsx (see list of accompanying material).

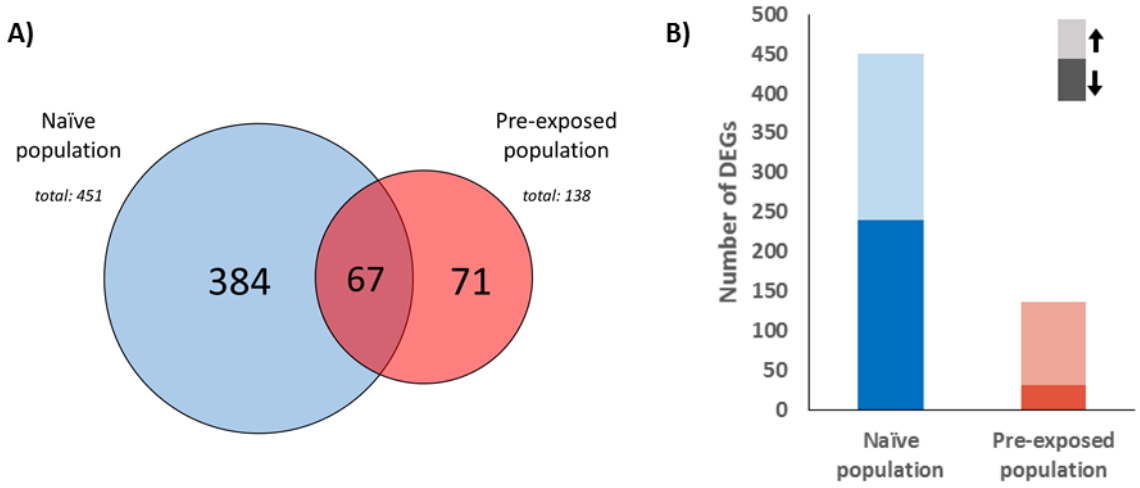


Figure 3: *A) Venn diagram showing the number of differentially expressed genes in naïve fish (blue, contrast: control_0.01 vs control_0) and pre-exposed fish (red, contrast: copper_0.01 vs copper_0) upon re-exposure to copper, including differentially expressed genes that were common to both populations (intersection). B) Bar chart showing the number of upregulated (↑) and downregulated (↓) genes in naïve fish (blue) and pre-exposed fish (red) upon re-exposure to copper.*

In a principal component analysis comparing differential gene expression for all replicates (figure 4), samples from fish originating from the pre-exposed and naïve population which received no further exposure clustered together (blue: copper_0 and red: control_0), reflecting that there were very few differences in gene expression between these groups. There was distinct clustering of re-exposed fish from both the pre-exposed and naïve population. The cluster representing re-exposed fish from the naïve population (green: control_0.01) was diverged from the control groups (blue: copper_0, red: control_0) along both PC1 and PC2, whereas re-exposed fish from the pre-exposed population (purple: copper_0.01)

only diverged from the control groups along PC2 (figure 4). This suggests that re-exposed fish from both populations exhibited a differential transcriptional response upon re-exposure, but that this differential response was substantially more pronounced for naïve fish. The unique separation of the re-exposed fish from the naïve population from all other groups along PC1, which explains 33% of the variation in gene transcription, highlights the magnitude of the distinction between their transcriptional response to copper compared to that of pre-exposed fish separating along PC2, summarising only 17.6% of variation.

The distinct transcriptional responses between the pre-exposed and naïve population upon re-exposure to copper is similarly reflected when represented in a heatmap based on differentially expressed transcripts between the re-exposed groups (copper_0.01 vs control_0.01)(figure 5).

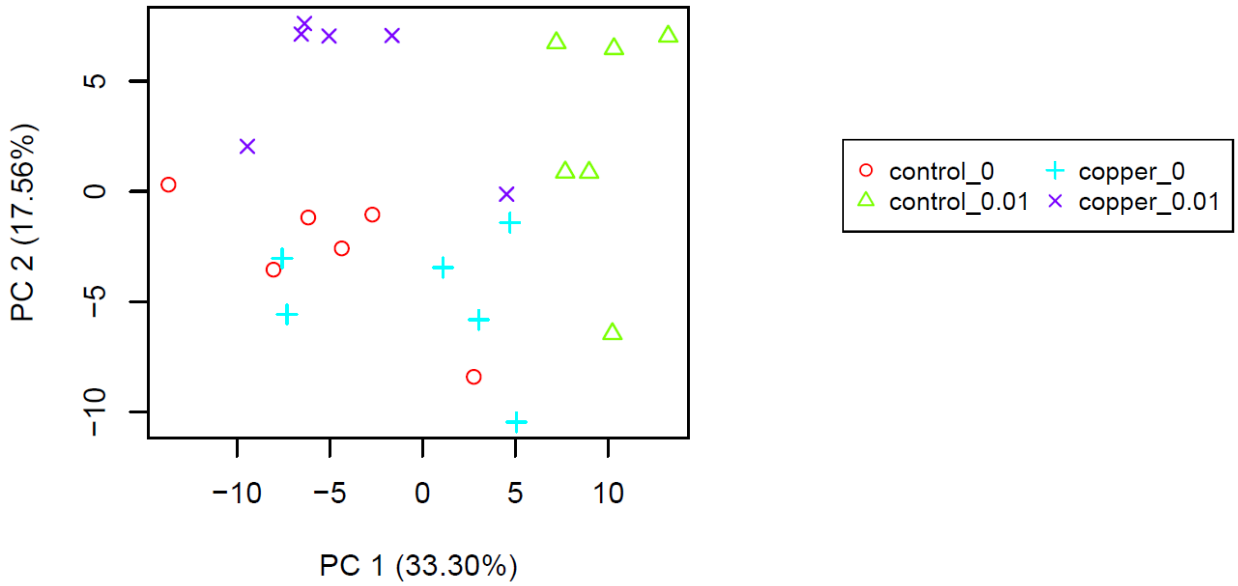


Figure 4: *Bi-plot of PC1 vs PC2 from a principal component analysis comparing differentially expressed transcript profiles between individual replicate fish for all group contrasts (no minimum threshold for FC, p<0.05, log2 transformed, centred), using Trinity v2.4.0 PtR script. Upon re-exposure, differential gene expression separated along PC1 for re-exposed fish from both the pre-exposed (copper_0.01) and naïve populations (control_0.01), explaining 17.6% of variation, compared to unexposed fish from both populations (copper_0 and control_0). Unique separation along PC1 occurred upon adult exposure for the naive population only (control_0.01), explaining 33.3% of variation.*

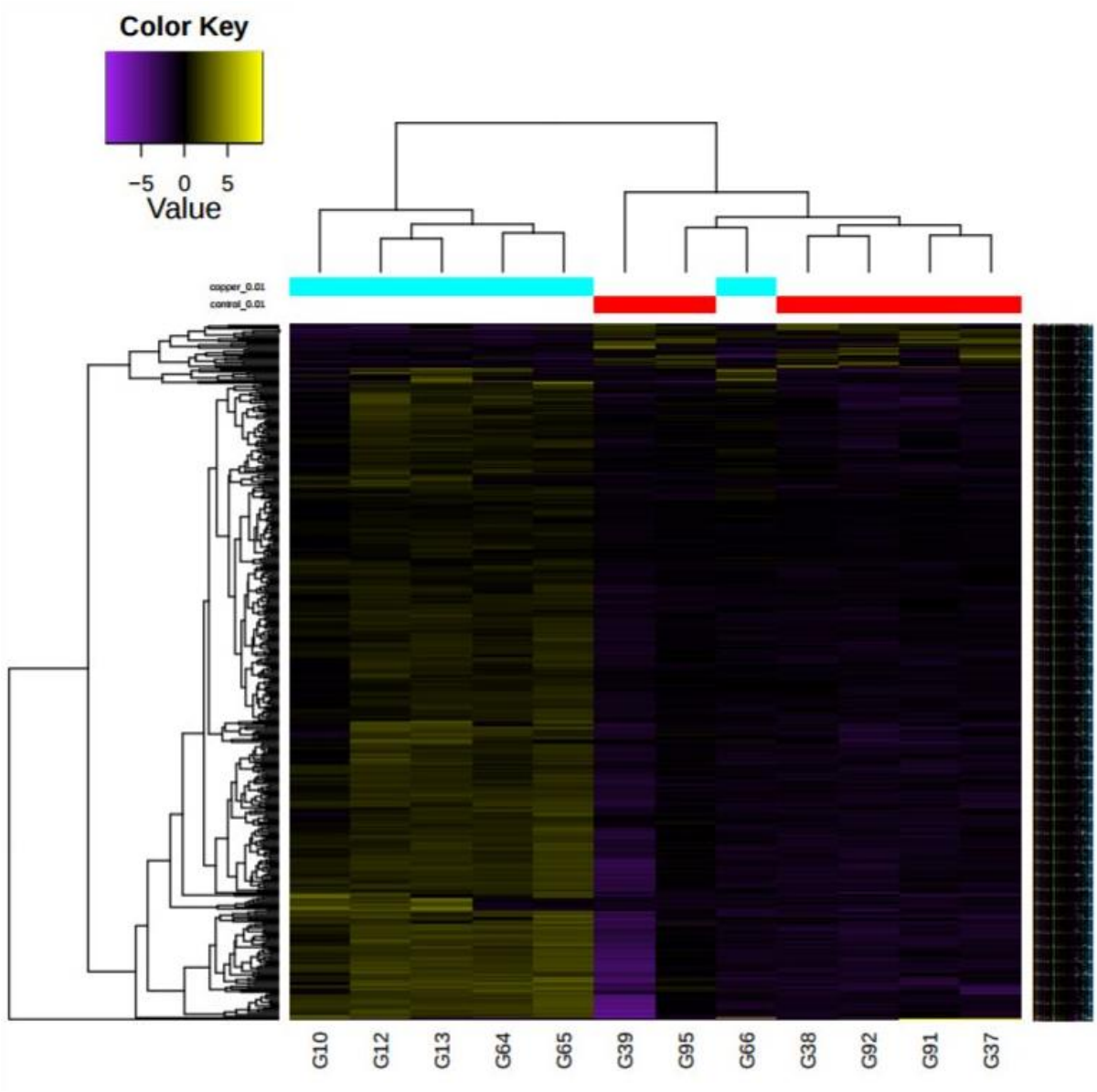


Figure 5: *Heatmap illustrating the differences between individual fish from the naïve population and pre-exposed population after re-exposure to copper (red: control_0.01; blue: copper_0.01) based on the differentially expressed genes between these two groups (log2 transformed, centred, FDR < 0.05). Each column represents an individual fish and each row represents and individual gene. Hierarchical clustering for individual fish and transcripts was performed using a Euclidean distance metric in Trinity, using edgeR.*

3.4.3. Gene Ontology (GO) Analysis:

Gene ontology enrichment analysis identified a total of 286 GO terms that were significantly enriched in the naïve population (control_0.01_vs_control_0), with 101 GO terms enriched in the list of downregulated genes and 185 in the upregulated genes. This is compared to the pre-exposed population (copper_0.01_vs_copper_0) for which there were only 62 enriched GO terms, all of which related to upregulated genes. No enriched GO terms were found for downregulated genes in pre-exposed fish. A complete list of GO terms for biological process, molecular function and cellular component is presented in Supporting information file 3 – Chapter 3 GO Annotations.xlsx (see list of accompanying material).

3.4.3.1. Copper caused upregulation of processes related to cell proliferation which was stronger in the naïve population:

Both the pre-exposed population and the naïve population exhibited alterations in gene expression consistent with upregulated cell proliferation. This is consistent with other studies where upregulation of cell cycle related transcription has been observed in gills upon exposure to copper. For example, Griffitt *et al.* observed transcriptional changes indicating cellular proliferation in the gills of zebrafish exposed to copper nanoparticles, which was accompanied by an increase in the mean gill filament width of up to 4-fold after 48 hours (Griffitt *et al.*, 2009). Similar upregulation of cell cycle related transcription was observed by Châtel *et al.* in mussels exposed to ionic copper, including upregulation of *ras* mRNA expression in the gills – proto-oncogenes involved in stimulating cell proliferation (Châtel *et al.*, 2018).

In this study, for genes upregulated following copper exposure, over-represented GO terms for both populations were predominantly related to cell cycle and the cytoskeleton, including the majority of the most significantly over-represented terms. Over-represented terms related to cell cycle and cytoskeleton common to both populations represented all three gene ontology categories. Biological processes over-represented for upregulated genes in both populations included: cell cycle (naïve – FDR: 3.52E-29; pre-exposed – FDR: 1.46E-06), mitotic cell

cycle (naïve – FDR: 8.50E-20; pre-exposed – FDR: 2.37E-05), chromosome segregation (naïve – FDR: 2.10E-16; pre-exposed – FDR: 0.00175), and cell division (naïve – FDR: 1.62E-12; pre-exposed – FDR: 5.40E-06). Molecular functions over-represented for upregulated genes in both populations included: microtubule binding (naïve – FDR: 1.67E-10; pre-exposed – FDR: 0.00800), tubulin binding (naïve – FDR: 1.58E-09; pre-exposed – FDR: 0.0129), and nucleotide binding (naïve – FDR: 6.38E-09; pre-exposed – FDR: 0.0255). Only one cellular component related to cell cycle – condensin complex (naïve FDR: 1.22E-04; pre-exposed – FDR: 0.0154) – was over-represented for upregulated genes in both populations.

Of note, cell cycle and its regulation appeared to be more strongly impacted in the naïve population, as many more terms related to cell cycle and cytoskeleton were enriched for upregulated genes, and had consistently lower false discovery rates (FDR) and a higher number of contributing genes than those for the pre-exposed population. For example, the FDRs for cell cycle, mitotic cell cycle, and chromosome segregation were approximately 23, 15 and 13 orders of magnitude lower, respectively, for the naïve population compared to the pre-exposed population. There were also important cell cycle related GO terms that were significantly over-represented in the naïve population that were absent from the pre-exposed population, including DNA replication (FDR: 3.59E-21), nucleus (FDR: 2.17E-05) – which was also under-represented for downregulated genes (FDR: 2.78E-03), chromosome (6.06E-05), and DNA polymerase activity (2.78E-02). Comparisons of the ten most significantly over-represented GO terms for upregulated genes related to cell cycle and cytoskeleton between populations for each gene ontology category are presented as dot plots in figure 7 (data also presented in supplementary table 2). There were also some over-represented GO terms related to cell cycle for downregulated genes in the naïve population. Of those terms, the majority were related to the components or functioning of the cytoskeleton, including: actin cytoskeleton (FDR: 1.98E-07), cytoskeleton organisation (FDR: 1.20E-03), and actin filament-based process (FDR: 3.51E-04). Given that over-represented GO terms for downregulated genes were dominated by terms related to muscle (discussed below), these terms may be related to the reduced expression of cytoskeleton-related genes in the specific context of muscle cells, rather than general cell cycle.

Upregulated genes underpinning these GO terms in the naïve population included genes encoding parts the DNA replication machinery that forms the replisome, most of which showed no significant upregulation in pre-exposed fish. For example, numerous genes encoding proteins involved in the CMG (Cdc45-MCM-GINs) helicase complex, which is essential for DNA replication initiation, were significantly upregulated in naïve fish. Examples included: cell division cycle 45 (*cdc45*)(FC: 2.89, FDR: 0.0183); subunits of the minichromosome maintenance (MCM) complex, such as minichromosome maintenance complex subunits 3 (*mcm3*)(FC: 2.67, FDR: 0.0327) and 6 (*mcm6*)(FC: 2.79, FDR: 0.0223); and subunits of the GINs complex, such as GINS complex subunits 1 (gins1)(FC: 2.96, FDR: 0.00440) and 2 (*gins2*)(FC: 4.05, FDR: 0.000931)(Deegan and Diffley, 2016). Several genes encoding primase and DNA polymerase enzymes were also significantly upregulated in naïve fish, such as: DNA primase subunit 1 (*prim1*)(FC: 2.44), FDR: 0.0256), primase and polymerase (DNA-directed)(*primpol*)(FC: 4.08, FDR: 0.00368), subunits of DNA polymerase epsilon (*pole* – FC: 3.19, FDR: 0.000624; *pole2* – FC: 3.79, FDR: 0.00109), and polymerase DNA directed alpha 1(*pola1*)(FC: 2.53, FDR: 0.00428).

There were also many genes related to replication machinery that were upregulated in both the naïve and pre-exposed population. These included genes encoding subunits of condensin complexes I and II, which play an essential role in the condensation and segregation of chromosomes during mitosis (Hirano, 2016). Examples included: non-condensin II complex subunits G2 (*ncapg2*)(naïve – FC: 4.00, FDR: 2.96E-03; pre-exposed – FC: 2.23, FDR: 0.0371) and D3 (*ncapd3*)(naïve – FC: 3.12, FDR: 5.29E-03; pre-exposed – FC: 2.27, FDR: 4.53E-03); and non-condensin I complex subunit H (*ncaph*)(naïve – FC: 3.48, FDR: 1.45E-03; pre-exposed – FC: 2.08, FDR: 0.0200). However, several components of these condensin complexes were only upregulated in naïve fish, including: *ncap6* (FC: 5.39, FDR: 3.16E-04), and the structural subunits *smc2* (FC: 4.59, FDR: 1.86E-04) and *smc4* (FC: 2.46, FDR: 4.08E-03). Other upregulated genes common to both populations included members of the centromere and kinetochore. The kinetochore complex assembles at the centromeres of chromatids and serves as a site of attachment for the mitotic spindle to separate sister chromatids during mitosis (Monda and Cheeseman, 2018). Examples of upregulated genes in both populations included: kinetochore complex component

ndc80 (*ndc80*)(naïve – FC: 3.53, FDR: 0.00109); pre-exposed – FC: 2.26, FDR: 0.0121), SPC24 component of NDC80 kinetochore complex (*spc24*)(naïve – FC: 4.63, FDR: 0.000624; pre-exposed – FC: 2.25, FDR: 0.0351), and centromere protein F (*cenpf*)(naïve – FC: 3.80, FDR: 0.000535; pre-exposed – FC: 2.13, FDR: 0.00517). However, similar to expression of condensin genes, many other genes related to the kinetochore and centromere were upregulated in the naïve population only.

Cell cycle regulation related GO terms were also more strongly enriched for upregulated genes in naïve fish compared to pre-exposed fish. For the pre-exposed population, only one term – regulation of cell cycle (FDR: 0.0289) – was enriched for upregulated genes. Whereas, in the naïve population over 40 GO terms directly related to cell cycle regulation were over-represented, including: regulation of cell cycle (FDR: 3.24E-10), regulation of mitotic cell cycle (1.80E-07), cell cycle checkpoint (7.58E-07), negative regulation of mitotic cell cycle (2.78E-05), and negative regulation of cell cycle process (1.97E-05).

The upregulated genes underpinning these GO terms included many genes encoding key positive cell cycle regulators, many of which were upregulated in both the naïve and pre-exposed populations. These include regulatory proteins that are considered master controllers of cell division, including cyclins, cyclin-dependent kinases, and aurora kinases (Carmena *et al.*, 2012). Examples include cyclin B1 (naive – FC: 5.3, FDR: 1.01E-05, pre-exposed – FC: 2.42, FDR: 0.00762) and cyclin-dependent kinase 1 (*cdk1*)(naïve – FC: 4.1, FDR: 9.29E-05; pre-exposed – FC: 2.54, FDR: 0.000545). Accumulation of the cyclin B1 – cdk1 complex is thought to play a major role in irreversibly triggering entry into mitosis and activating downstream cell cycle regulators (Gavet and Pines, 2010). Several other key examples included Aurora kinase A (naïve – FC: 5.0870, FDR: 0.000246; pre-exposed – FC: 2.86, FDR: 0.00762) and Aurora kinase B (naïve – FC: 4.11513591, FDR: 0.000316; pre-exposed – FC: 2.60, FDR: 0.00453) which perform essential regulatory functions during anaphase at the mitotic spindle poles and centromere, respectively (Carmena *et al.*, 2012). However, upregulation of these genes was generally stronger in the naïve population compared to the pre-exposed population, and many important positive cell cycle regulators were only upregulated in the naïve population. These included, for example, polo like kinase 1 (FC: 4.46, FDR: 1.01E-05) which has a range of essential functions throughout

mitosis, ranging from mitotic entry, to chromosome separation, cytokinesis, and mitotic exit (Vazquez-Martin *et al.*, 2011).

There was also no enrichment of GO terms or upregulated of genes related to apoptosis in either population. Apoptosis and upregulation of genes encoding apoptosis-inducing factors, such as caspase and p53 proteins, has been observed in epithelial cells of the gills upon exposure to toxic concentrations of copper (Mazon, Cerqueira and Fernandes, 2002; Luzio *et al.*, 2013). However, other studies have also observed a lack of apoptosis induction despite presence of other copper-induced damage. For example, Monteiro *et al.* (2009) found no increase in caspase mRNA expression in the gills of Nile tilapia and no increase in the volume of apoptotic cells, even at exposure concentrations up to 400µg/L, despite a significant increase in the volume of proliferating epithelial cells, consistent a concentration-dependent repair response (Monteiro *et al.*, 2009). This suggests that incidence of apoptosis as a result of copper exposure may vary between species, and supports the observed lack of apoptosis-related gene expression in our study, despite gene expression indicative of other toxic responses.

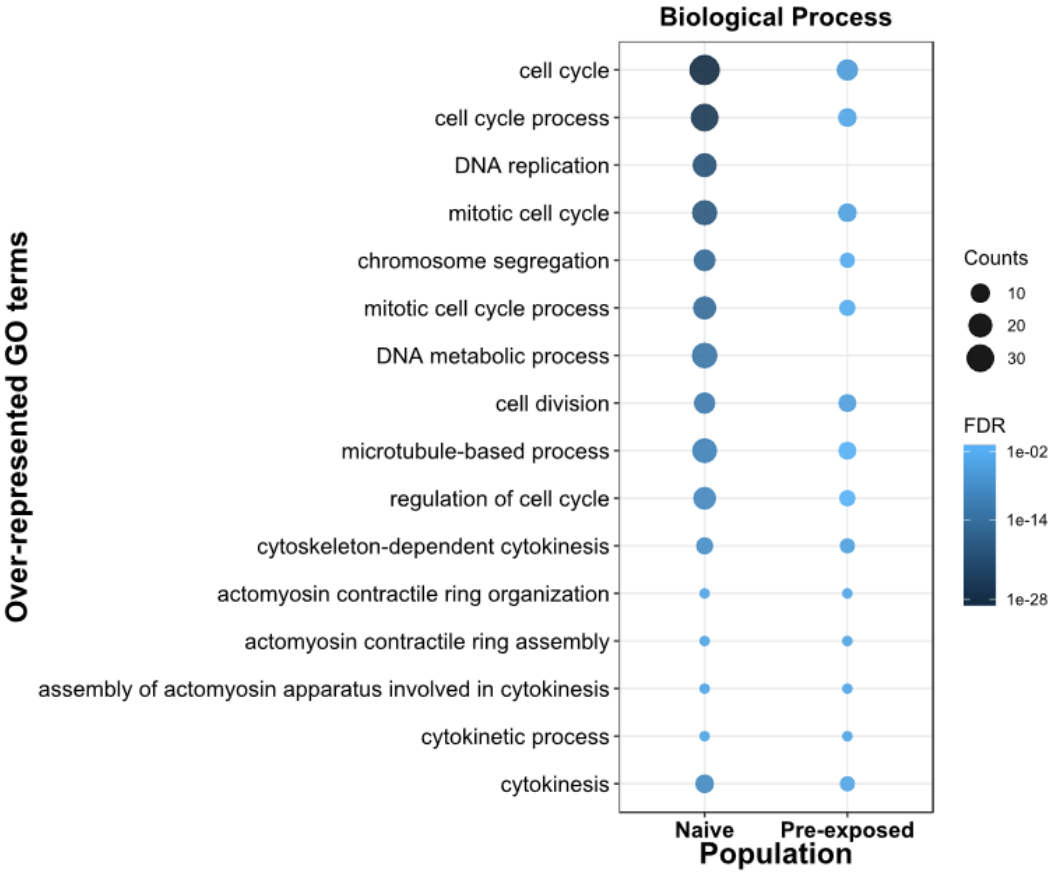
However, naïve fish also exhibited gene expression changes that were consistent with inhibition of cellular proliferation, which was not observed in pre-exposed fish. For example, E2F transcription factor 7 (*e2f7*), a transcription factor that represses cell replication, was significantly upregulated in naïve fish (FC: 4.549, FDR: 0.0338)(Hazar-Rethinam *et al.*, 2011). Several genes encoding proteins which stimulate cellular proliferation were also downregulated in naïve fish. These included Ras homolog mTORC1 binding protein *(rheb)*(FC: 0.241, FDR: 0.00512) and p21 protein (Cdc42/Rac)-activated kinase 6a (*pak6*)(FC: 0.408, FDR: 0.0140), both thought to promote cell growth and proliferation (Parmar and Tamanoi, 2010)(Liu *et al.*, 2015); and pancreatic progenitor cell differentiation and proliferation factor a (*ppdpfa*)(FC: 0.261, FDR: 0.0199), a protein predicted to be involved in cell proliferation, which, in contrast, was upregulated in pre-exposed fish (FC: 4.64, FDR: 0.0230).

Taken together with an absence of upregulated genes related to apoptosis, the general upregulation of cell cycle related genes in both populations suggests that both naïve and pre-exposed fish are experiencing an upregulation of cell

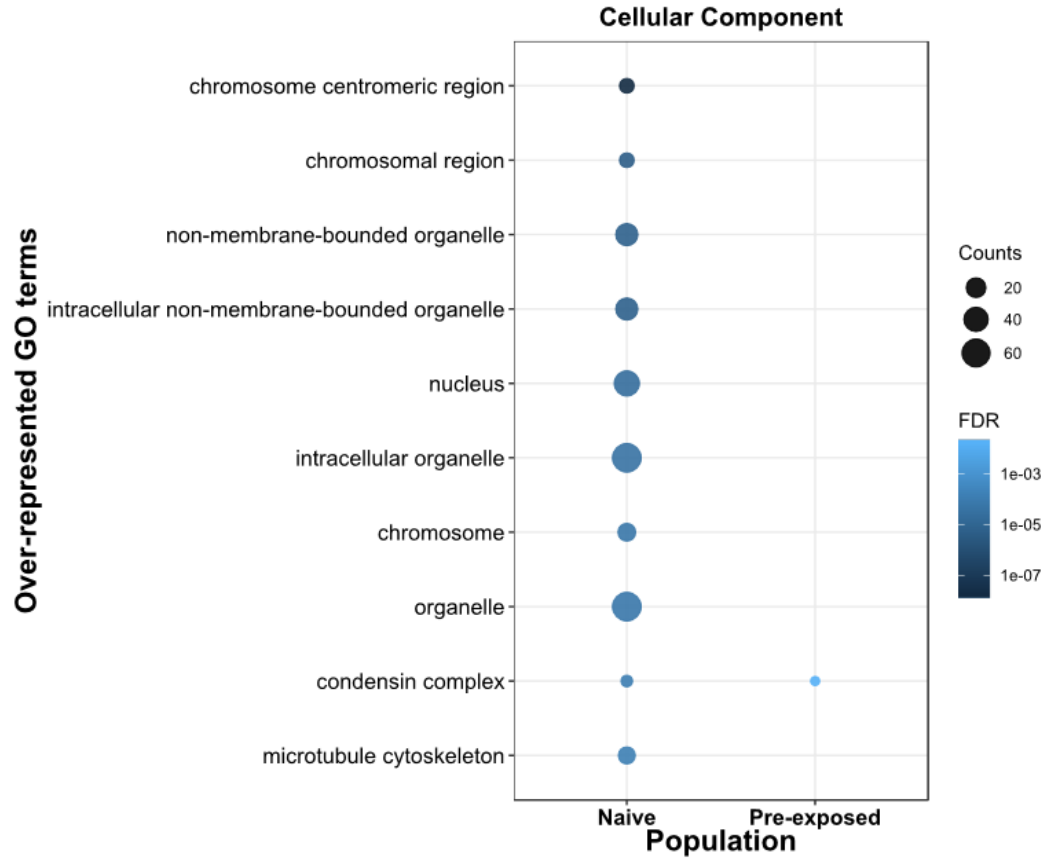
proliferation in the gills in response to copper exposure; but that this proliferation is much greater in the naïve population, which also appears to be downregulating proliferation-inducing signals in order to attenuate this potentially rapid cell proliferation in the gill.

Copper exposure has previously been shown to cause damage to the gill epithelium that includes epithelial cell proliferation and epithelial thickening (Wilson and Taylor, 1993b; Pelgrom *et al.*, 1995; Heerden, Vosloo and Nikinmaa, 2004). At high concentrations this epithelial thickening can increase the oxygen diffusion distance between the blood and water, impairing gas transfer and leading to respiratory distress (Perry, 1997; Grosell, 2012). Monteiro *et al.* (2009) reported concentration dependent proliferation in both the filament and lamellar epithelium in Nile tilapia exposed to 40µg and 400µg/L Cu, indicated by an increase in the relative volume of cells positive for proliferating cell nuclear antigen (PCNA) – a biomarker of cell proliferation (Monteiro *et al.*, 2009). In our study, PCNA mRNA expression was upregulated only in naïve fish (FC: 2.41, FDR: 0.0107) supporting the possibility that there may be a higher rate of cell proliferation in the gills of these fish compared to pre-exposed fish. The comparably high rate of epithelial proliferation in the gills of naïve fish, suggested by their stronger transcriptional response, could suggest that prior exposure during embryogenesis may have caused adaptive changes in pre-exposed fish that prevent the damaging effects of copper that lead to extensive cell proliferation, protecting pre-exposed fish from epithelial thickening and resulting respiratory distress.

A)


Biological Process
cell cycle
cell cycle process
DNA replication
mitotic cell cycle
chromosome segregation
mitotic cell cycle process
DNA metabolic process
cell division
microtubule-based process
regulation of cell cycle
cytoskeleton-dependent cytokinesis
actomyosin contractile ring organization
actomyosin contractile ring assembly
assembly of actomyosin apparatus involved in cytokinesis
cytokinetic process
cytokinesis
Over-represented GO terms
Naive
Pre-exposed
Population
Counts
10
20
30
FDR
1e-02
1e-14
1e-28

B)


Cellular Component
chromosome centromeric region
chromosomal region
non-membrane-bounded organelle
intracellular non-membrane-bounded organelle
nucleus
intracellular organelle
chromosome
organelle
condensin complex
microtubule cytoskeleton
Over-represented GO terms
Naive
Pre-exposed
Population
Counts
20
40
60
FDR
1e-03
1e-05
1e-07

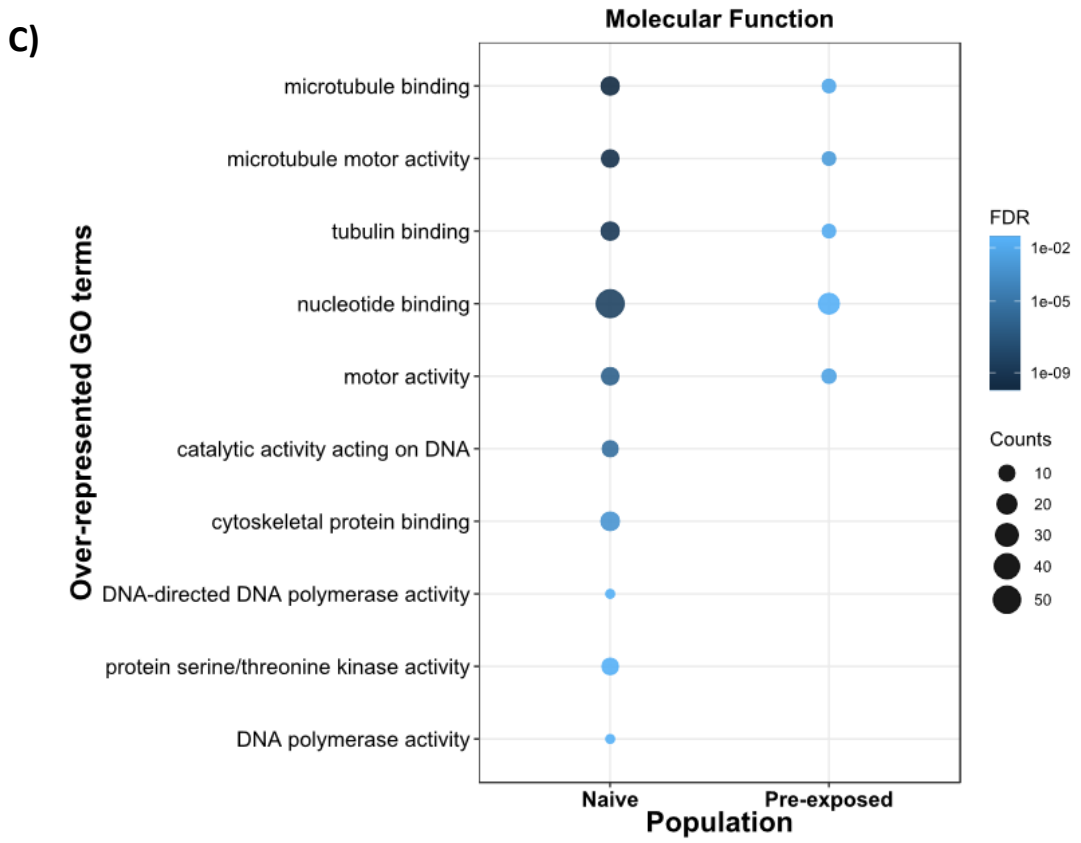


Figure 6: *Dotplots comparing Gene Ontology terms related to cell cycle and cytoskeleton for biological process (A), cellular component (B) and molecular function (C), that were among the 10 most significantly over-represented GO terms for each category in combined for both the naïve or pre-exposed population. The total combined GO terms are less than 20 where a population had less than 10 enriched GO terms for a category (and all enriched terms are included) or where common terms appeared in the top 10 for both populations. Colour gradient indicates false discovery rate (FDR), with the darkest colour representing the lowest FDR, and counts indicate the number of differentially expressed genes (threshold = p<0.05) contributing to each GO term.*

3.4.3.2. Copper caused disruption of processes related to muscle function in the naïve population:

Over-represented GO terms for downregulated genes in the naïve population were dominated by muscle-related terms. The top 23 most significant terms were directly related to muscle, and almost all enriched GO terms for downregulated genes were either related to muscle or metabolic processes. Examples included: Sarcomere (FDR: 1.31E-18), contractile fiber (FDR: 1.31E-18), muscle structure development (1.40E-09), striated muscle cell differentiation (3.72E-09), Z disc (8.28E-08), skeletal muscle tissue development (1.21E-7), and muscle contraction (9.62E-06). In contrast, there was almost no enrichment of GO terms related to muscle in pre-exposed fish, with the exception of a few terms related to contractile fiber components, which were underpinned by a small number of upregulated genes. However, this may be related to production of contractile proteins required for cell division, rather than muscle tissue.

Genes significantly downregulated in naïve fish following copper exposure included various genes related to muscle, including many of the main structural components and regulatory proteins. Examples of key structural components whose expression was significantly downregulated included genes encoding myosin and actin proteins which make up the thick and thin filaments in the myofibrils of muscle tissue. Examples included: *mylpf* (myosin light chain, phosphorylatable, fast skeletal muscle)(FC: 0.185, FDR: 0.00446), *myl1* (myosin, light chain 1, alkali; skeletal, fast)(FC: 0.238, FDR: 0.0145), putatively identified *myh* (myosin heavy chain, fast skeletal muscle)(FC: 0.204, FDR: 0.00407), and putatively identified *acta1* (actin, alpha skeletal muscle A)(FC: 0.202, FDR: 0.000624). Myosin and actin filaments are organised into sarcomeres along with a third major filament, titin, an elastic protein with kinase activity that serves as a scaffold for myofibrils and other muscle related proteins (Bhagavan and Ha, 2015). Several genes encoding proteins involved in titin filament structure and regulation during sarcomere assembly were significantly downregulated in naïve fish, including: *ttn.1* (titin, tandem duplicate 1)(FC: 0.205, FDR: 0.0133), *obscna* (obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF a)(FC: 0.211, FDR: 0.00598), *trim55b* (tripartite motif containing 55b)(FC: 0.250, FDR: 0.0435), and *tcap* (titin-cap)(FC: 0.198, FDR: 0.00997). Thin filaments also contain the

regulatory proteins troponin and tropomyosin, which power muscle contraction by regulating the interaction of actin and myosin filaments (Bhagavan and Ha, 2015). Genes encoding both of these components were significantly downregulated in naïve fish, including: *tnnt3a* (troponin T type 3a (skeletal, fast))(FC: 0.219, FDR: 0.00965), *tnnc1b* (troponin C type 1b (slow))(FC: 0.223, FDR: 0.00218), *tnnc2* (troponin C type 2 (fast))(FC: 0.228, FDR: 0.00628), and *tpm2* (tropomyosin 2 (beta))(FC: 0.253, FDR: 0.00450),

Over-represented GO terms for downregulated genes in naïve fish also included several terms related to calcium regulation, including calcium ion transport (FDR: 0.0172), cellular calcium ion homeostasis (FDR: 0.0400) and calcium ion transmembrane transport (FDR: 0.0440). No enrichment of GO terms related to calcium regulation was observed for pre-exposed fish.

Calcium (Ca^{2+}) is the key regulator and signalling molecule in muscle fibers, where calcium release from the sarcoplasmic reticulum (SR) triggers muscle contraction (Berchtold, Brinkmeier and Müntener, 2000). Muscle cells contain various calcium binding proteins and transporters involved in calcium regulation. In naïve fish, genes encoding many key proteins known to be involved in calcium regulation in muscle cells were significantly downregulated upon exposure to copper. This included many calcium-binding proteins, for example, *casq1b* (FC: 0.205, FDR: 0.00430) encoding calsequestrin 1 – the Ca^{2+} storage protein in the SR; *pvalb4* (FC: 0.223, FDR: 0.0191) encoding parvalbumin – a chaperone involved in shuttling Ca^{2+} to the SR during muscle relaxation; and *sar* (FC: 0.252, FDR: 0.00969) encoding sarcalumenin – a calcium buffering protein in the SR lumen (Berchtold, Brinkmeier and Müntener, 2000; Rossi and Dirksen, 2006). Various genes encoding calcium transporters responsible for calcium regulation in muscle cells were also significantly downregulated in naïve fish. Examples included: *ryr1a* (FC: 0.272, FDR: 0.00430) and *ryr1b* (FC: 0.300, FDR: 0.0163) encoding ryanodine receptor 1, and *cacna1sb* (FC: 0.371, FDR: 0.00598) and putatively identified *cacna1d* (FC: 0.138, FDR: 0.000793) encoding subunits of L-type voltage-dependent calcium channel alpha 1S – calcium channels involved in calcium release from the SR; and *atp2a1* (FC: 0.302, FDR: 0.0110) encoding ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 1 – a channel facilitating re-uptake of calcium into the SR (Berchtold, Brinkmeier and Müntener, 2000; Rossi and Dirksen, 2006).

Several roles of contractile tissue have been described in the gill, including: in branchial muscles associated with the gill arches and at the base of gill filaments, which are responsible for respiratory movements of the gill (Bijtel, 1939; Tumbahangfe, Subba and Prasad, 2018); and in the pillar cells – specialised vascular cells within the lamellae which possess characteristics of both endothelial and smooth muscle cells (Kato *et al.*, 2007). Though the function of the contractile tissue in pillar cells is not certain, studies have confirmed the presence of actomyosin contractile fibers (Smith and Chamley-Campbell, 1981; Mistry *et al.*, 2004; Kato *et al.*, 2007). Kato *et al.* (2007) identified actin fibers, actinin, and a phosphorylated myosin light chain of 20kDa densely located in the pillar cell cytoplasm near the collagen columns that the pillar cells surround, suggesting a contractile function (Kato *et al.*, 2007). Stensløkken *et al.* (1999) also demonstrated changes in pillar cell diameter and lamellar blood flow consistent with contraction upon application of a vasoconstrictor (Stensløkken, Sundin and Nilsson, 1999).

The cause of this strong decrease in muscle-related gene expression in naïve fish is unclear. A possible explanation is a decrease in the proportion of muscle cells in the gill. There is some evidence that copper can cause deterioration of muscle tissue. For example, two histological studies in juvenile rainbow trout (Al-Bairuty *et al.*, 2013) and adult goldfish (Vogel, 1959) describe a significant reduction in muscle fiber proportion compared to extracellular space; atrophy; and disruption of fiber organisation in skeletal muscle as a result of copper exposure. The mechanisms behind these effects are unclear. However, the concentrations used in these studies resulted in acute toxicity, evident by the increased mortality rate observed in treated groups. For example, the exposure of juvenile rainbow trout to 20µg/L Cu for 10 days led to mortality of 16.7% of treated fish, compared to 4.8% in controls (Al-Bairuty *et al.*, 2013). Muscle degeneration has also been observed in humans in conjunction with copper build-up in a case of untreated Wilson's disease (Propst *et al.*, 1995). Similarly, the concentration of copper which led to these effects was also sufficient to cause acute toxicity in several other organs (Propst *et al.*, 1995). In the present study, acute toxic effects were not observed, with no mortality in exposed adults from either population, all of which remained in good condition, suggesting that the copper concentration used may not be sufficient to cause muscle deterioration. As discussed previously, copper exposure

has been shown to cause epithelial cell proliferation in the filaments and lamellae of the gills, which is consistent with the strong upregulation of genes related to cell cycle and replication observed in naïve fish (Wilson and Taylor, 1993b; Pelgrom *et al.*, 1995). Rather than decreased proliferation of cells expressing muscle-related genes or a degeneration of contractile tissue, this decreased expression of muscle-related mRNA may be attributed to a decrease in the proportion of contractile cells, driven by potential epithelial cell proliferation in the filaments and lamellae. However, histological analysis of the gill would be needed to confirm this.

Alternatively, rather than a change in the proportion of muscle cells, downregulated expression of muscle-related genes could indicate reduced transcriptional activity in existing muscle cells in the gill. Copper has been observed to suppress metabolic activity, reduce concentrations of ATP and cause increased reliance on anaerobic energy production in a variety of tissues (Viarengo *et al.*, 1980; Carvalho and Fernandes, 2008; Santos *et al.*, 2010; Lauer *et al.*, 2012; Giacomin, Jorge and Bianchini, 2014). Reduced transcriptional activity in muscle cells could be a consequence of copper-induced suppression of metabolic activity in this energy-demanding tissue.

Many downregulated genes contributing to enrichment of muscle-related GO terms in naïve fish included genes that are also directly related to other processes that have previously been observed to be disturbed by copper exposure, including calcium regulation and metabolism (Atli and Canli, 2011; Jorge *et al.*, 2016). Disturbance of these other processes by copper exposure in naïve fish could have contributed to enrichment of muscle-related terms; and although many of the downregulated genes related to these processes were identified as muscle specific, this could be a result of the use of mammalian orthologues in annotation of fish genomes, which might overlook their role in non-mammalian tissue types, such as the gill.

3.4.3.3. Copper caused contrasting changes in processes related to ion regulation in the pre-exposed and naïve populations

Enrichment analysis identified relatively few enriched GO terms related to ion regulation for either population, with the exception of terms related to calcium regulation which were enriched for downregulated genes in the naïve population. The most changes were seen for downregulated genes in the naïve population, where over-represented GO terms included: ion transport (FDR: 0.0424), ion transmembrane transport (FDR: 0.0274), cation transport (FDR: 0.0195), and divalent metal ion transport (FDR: 0.0329). Upon examination of the genes contributing to these GO terms, the majority were specifically related to calcium handling.

There were surprisingly few GO terms related specifically to ion regulation for upregulated genes in either population, with the exception of two GO terms – ion binding and anion binding – which were overrepresented for upregulated genes in both the naïve population (FDRs: 0.0369 and 5.43E-06, respectively) and the pre-exposed population (FDRs: 4.21E-06 and 6.47E-05, respectively).

Few transcriptional changes related to metal storage and transport occurred in either population:

Although there was little enrichment of GO terms related to ion regulation for either population, with exception of calcium regulation, there was differential expression of some genes related to ion regulation for both naïve and pre-exposed fish. However, there was generally a lack of changes in transcription of specific copper transporters, chaperones and storage proteins, despite the differential copper accumulation in the gill observed between populations.

A notable exception was metallothionein (*mt*) – a high affinity metal-binding protein which acts as a chaperone involved in safely sequestering free metal ions and acting as a metal donor for the production of metalloenzymes (De Boeck *et al.*, 2003; Abril, Costa and Bianchini, 2018). There are four known metallothionein isoforms in teleosts, two of which – mt1 and mt2 – can be induced by metals through binding of transcription factors at metal-responsive elements (MREs) in the promoter regions of the metallothionein genes (Wood, 2012). In accordance with other studies, *mt2* expression was upregulated in both the naïve and pre-exposed populations in response to copper exposure. A slightly stronger increase

in expression was observed in pre-exposed fish, with an increase of 5.27-fold (FDR: 1.35E-11) compared to an increase of 3.50-fold (FDR: 0.002603) in naive fish, though the difference was not significant. Upregulation of *mt* transcription is a well-known response to copper exposure in fish (Buckley *et al.*, 1982; Roch and Mccarter, 1984; Heerden, Vosloo and Nikinmaa, 2004; Minghetti *et al.*, 2008; Craig, Galus, *et al.*, 2009; Minghetti, Leaver and George, 2010). For example, in a study by Uren-Webster *et al.* (2013) of metal-tolerant brown trout from a population chronically exposed to toxic metals, including copper; the authors concluded that metal- and ion-homeostasis pathways were likely to be the most important mechanisms contributing to the extreme metal tolerance observed (Uren Webster *et al.*, 2013). Following whole-transcriptome profiling, *mt2* was found to be one of the most strongly upregulated genes in the gill, gut and liver (upregulated 8.2-, 7.7-, and 5.6-fold, respectively)(Uren Webster *et al.*, 2013). Similarly, in a comparison of response to copper exposure between three freshwater fish species, the most tolerant species, the gibel carp, was found to have highest *mt* induction (De Boeck *et al.*, 2003), implying a role for *mt* in resistance to copper toxicity. Adeyemi and Klerks (2013) also observed significantly higher metallothionein levels in Cu-acclimated fish compared to controls upon acute exposure to Cu, during which these fish exhibited significantly greater survival (Adeyemi and Klerks, 2013). However, despite the upregulation of *mt2* in response to copper exposure observed for both stickleback populations in this study, the fact that it was not significantly different between populations suggests that increased sequestration of copper ions by *mt* alone is unlikely to explain the differential copper handling and accumulation in tissues observed in pre-exposed fish compared to naïve fish.

Analysis of copper-specific transporters found no significant difference in transcription in the gill for either population, including for solute carrier family 31 member 1 (*slc31a1*), also known as copper transporter 1 (*ctr1*)(naïve – FC: 1.12, FDR: 0.948; pre-exposed – FC: 0.886, FDR: 0.971), or for ATPase copper transporting alpha (*atp7a*)(naïve – FC: 0.910, FDR: 0.920; pre-exposed – FC: 0.880, FDR: 0.907). ctr1 and atp7a are thought to be important for Cu uptake across the gills, facilitating active transport of copper ions across the apical and basolateral membranes of the epithelial cells, respectively (Grosell, 2012). There were also no changes in the transcription of another apical copper transporter,

solute carrier family 11 member 2 (*slc11a2*), also known as the divalent metal transporter 1 (*dmt1*)(naïve – FC: 0.912, FDR: 0.946; pre-exposed – FC: 0.848, FDR: 0.887), which is also thought to contribute to copper transport across the apical membrane (Grosell, 2012).

Several studies have demonstrated altered transcription of copper transporters in multiple tissues of fish as a result of Cu exposure, and suggested that this is involved in compensatory responses to limit uptake of Cu and increase excretion (Craig, Galus, *et al.*, 2009; da Silva *et al.*, 2014). For example, *ctr1* transcription was downregulated in the gill of freshwater guppies after exposure to 20µg/L Cu (da Silva *et al.*, 2014). In studies of dietary and waterborne Cu exposure in sea bream, general downregulation of transcription of copper transporters *ctr1* and *atp7a* were found in the gill, suggesting adaptive changes aimed at reducing Cu uptake; while their transcription was generally upregulated in other tissues, including liver and kidney, consistent with an adaptive increase in Cu excretion (Minghetti *et al.*, 2008; Minghetti, Leaver and George, 2010).

However, many studies have also reported no change in transcription of transporters in response to copper exposure, despite other physiological effects being observed. For example, no change in transcription of *ctr1* was observed in the gills of sea bream in response to copper exposure despite significant Cu accumulation and occurrence of other Cu-induced physiological changes (Minghetti *et al.*, 2008). Craig *et al.* (2010) found no differential transcription of either *ctr1* or *atp7a* in the gills of zebrafish upon exposure to copper, but found increased *atp7a* transcription in the liver (Craig, Wood and Mcclelland, 2010). After exposure of zebrafish to 8µg/L Cu for 21 days, no change in transcription of *ctr1* was observed in the gill, despite a significant reduction in ctr1 protein expression (Craig, Galus, *et al.*, 2009). Consistent with reduced ctr1 expression, Cu uptake rate across the gill was also observed to be significantly reduced, despite overall Cu load increasing (Craig, Galus, *et al.*, 2009). Uren Webster *et al.* also found no change in transcription of specific Cu transporters in the gill of brown trout, despite significant metal tolerance and accumulation of metals being observed (Uren Webster *et al.*, 2013). Occasionally transcriptional changes inconsistent with the general hypothesis of supressing uptake and stimulating excretion were also observed, such as a study by Chen and Chan (2011), who observed increased transcription of *atp7a* in the gill of tilapia following exposure to

50µg/L of $CuCl_2$ (Chen and Chan, 2011b). However, this upregulation was weaker and non-significant in fish exposed to a higher concentration of 100 µg/L of $CuCl_2$, which coincided with the onset of significant upregulation of *atp7a* transcription in the liver, which is consistent with increased excretion (Chen and Chan, 2011b).

Although there was no significant difference in transcriptional regulation of many metal transporters for either population in response to adult copper exposure in this study, it is possible that regulation of these proteins could be occurring at the post-transcriptional level, which would not be apparent in our analysis. Post-transcriptional regulation of metal transporters is known to play a dominant role in mammalian copper homeostasis, and it has been suggested that similar mechanisms may exist in fish (Grosell, 2012). These post-transcriptional mechanisms in mammals include regulation of subcellular distribution and rate of protein degradation, controlled by protein-protein interactions and posttranslational modification of proteins (Van Den Berghe and Klomp, 2010). For example, mammalian ATP7A and ATP7B have multiple roles in copper handling, and are involved in both excretion of Cu from cells in secretory vesicles and delivery of Cu into the Golgi apparatus for incorporation into cuproenzymes (Lutsenko *et al.*, 2008). They are thought to play similar roles in fish. The balance between their various functions in mammalian cells is post-translationally regulated by shuttling of these proteins between membranes depending upon the copper status of the cell (Lutsenko *et al.*, 2008). Similarly, mammalian *Ctr1* does not seem to be transcriptionally regulated in response to copper exposure (Tennant *et al.*, 2002), and CTR1-mediated copper uptake is instead post-translationally regulated by rate of endocytosis and degradation of the transporter protein (Petris *et al.*, 2003). Similar mechanisms of post-translational regulation may exist in fish, resulting in copper-induced regulation of metal transporters independent of transcriptional changes, but this is yet to be verified (Grosell, 2012). Analysis of the zebrafish *ctr1* gene and upstream elements found no evidence that it is copper-inducible (Mackenzie *et al.*, 2004). However, this is inconsistent with several studies demonstrating altered transcription of this gene in response to copper, as discussed above.

It is possible that regulation of copper transporters in fish could involve both transcriptional and post-transcriptional regulation. In this study, there was no transcriptional response of copper transporters that might explain the differential

copper accumulation observed in the gill and other tissues; however, altered copper transporter activity at the protein level in the gill due to post-translational regulation was not investigated. Lack of detection of transcriptional changes despite changes in protein activity or expression observed in other studies could be due to different thresholds for activation of each mechanism, with post-translational regulation of transporters occurring at a lower threshold; or lack of detection could be due to insufficient sensitivity or power of transcriptional analysis. Regulation of transporters seems to vary significantly according to species, exposure conditions, and according to timing of measurement during potentially dynamic compensatory responses. The differential copper accumulation in tissues observed between pre-exposed and naïve stickleback upon re-exposure to copper clearly suggests that exposure is affecting copper handling pathways differently in these populations. Lack of transcriptional changes in copper transporters in the gill of pre-exposed and naïve fish in this study could indicate that either uptake is being differentially regulated, but only at the post-transcriptional level; or that the altered copper accumulation is caused by mechanisms other than altered uptake across the gills, such as altered metabolism and excretion in other tissues, such as the kidney and liver, as has been observed in other studies. Given that many studies have reported transcriptional regulation of copper transporters in the gill in response to exposure, altered metabolism and excretion of copper in other tissues could be an explanation for the differential copper accumulation in these populations. However, this is at odds with the chemical analysis of copper content in these fish, which showed no change in the copper content of the liver in pre-exposed fish upon re-exposure, compared to significant accumulation of copper in the gill. The role of uptake and excretion via copper transporters in the gill and other tissues in altered copper handling in pre-exposed fish remains unclear, and would require analysis of copper transporter protein expression and activity to resolve this question.

Copper caused differential transcription of genes involved in ion homeostasis, osmoregulation and acid-base balance that were distinct between populations, including changes suggesting greater disturbance in naïve fish and activation of compensatory mechanisms in pre-exposed fish:

As well as affecting transport and storage of copper in cells, copper exposure is known to affect homeostasis of several other ions, leading to disruption of osmoregulation. Transcription of several genes encoding subunits of the basolateral sodium potassium transporter – Na^+/K^+-ATPase, were differentially transcribed in both the pre-exposed and naïve populations. In naïve fish, transcription of subunits of the Na^+/K^+-ATPase was significantly downregulated upon adult copper exposure, including *atp1a2a* (ATPase Na^+/K^+ transporting subunit alpha 2)(FC: 0.246, FDR: 0.0138) and *atp1b3a* (ATPase Na^+/K^+ transporting subunit beta 3a)(FC: 0.285, FDR: 0.0183). Whereas, in pre-exposed fish, *atp1b4* (ATPase Na^+/K^+ transporting subunit beta 4) was significantly upregulated (FC: 6.13, FDR: 0.0295).

Osmoregulatory disturbance is one of the main adverse effects of copper in fish; particularly disturbance of Na^+ and Cl^- balance, even at sublethal copper concentrations, by causing net loss of Na^+ and Cl^- (McKim, Christensen and Hunt, 1970; Christensen *et al.*, 1972; Schreck and Lorz, 1978; Laurén and McDonald, 1985; Reid and McDonald, 1988; Shekh *et al.*, 2019). Net loss of Na^+ is driven by both copper-induced inhibition of Na^+ influx and increased Na^+ efflux (Matsuo *et al.*, 2004). Inhibition of Na^+ uptake occurs through both competitive inhibition due to competition for transporter binding, and non-competitive inhibition, through direct inhibition of sodium transporter activity (Lauren and McDonald, 1987; Li *et al.*, 1996; Brooks and Mills, 2003). Na^+/K^+-ATPase is a key enzyme involved in osmoregulation, which drives branchial sodium uptake by actively transporting Na^+ in epithelial cells across the basolateral membrane into the blood. This creates an ion gradient between epithelial cells and the water that results in passive uptake of Na^+ through apical sodium channels (Ay *et al.*, 1999). It is thought that the majority of Na^+ and Cl^- uptake in freshwater fish occurs via mitochondria rich cells called chloride cells or ionocytes, which contain high levels of Na^+/K^+-ATPase (Li *et al.*, 1998). Inhibition of Na^+ uptake by waterborne copper exposure is partially caused by inhibition of branchial Na^+/K^+-ATPase activity, whose sensitivity to copper has

been demonstrated *in vitro* and *in vivo* in many fish species (Lauren and McDonald, 1987; Reid and McDonald, 1988; Pelgrom *et al.*, 1995; Sola, Isaia and Masoni, 1995; Ay *et al.*, 1999; Pyle *et al.*, 2003; Chowdhury, Girgis and Wood, 2016). Measurable inhibition of branchial Na^+/K^+-ATPase activity has also been observed to occur prior to overt branchial dysfunction, highlighting its sensitivity to copper exposure (Ay *et al.*, 1999). However, several *in vivo* studies have observed compensatory increases in Na^+/K^+-ATPase protein levels and recovery of Na^+/K^+-ATPase activity during acclimation to copper (Stagg and Shuttleworth, 1982; Lauren and McDonald, 1987; McGeer *et al.*, 2002). For example, exposure to 55µg/L Cu was shown to cause a 33% inhibition of Na^+/K^+-ATPase activity in rainbow trout gills after 24 hours. However, a significant increase in Na^+/K^+-ATPase protein levels was subsequently observed, which restored Na^+/K^+-ATPase activity per milligram of tissue to normal levels by day 14 of exposure (Lauren and McDonald, 1987). This suggests that increased Na^+/K^+-ATPase expression may be a compensatory mechanism employed by fish to prevent copper-induced osmoregulatory disruption during acclimation to copper. Similar compensatory upregulation of Na^+/K^+-ATPase protein expression to combat copper-induced ionregulatory disruption in gill cells has also been observed in two euryhaline invertebrates (Boyle *et al.*, 2013). Upregulation of Na^+/K^+-ATPase expression to compensate for Cu-induced inhibition and prevent disruption of osmoregulation is therefore thought to be key adaptive response involved in copper tolerance.

A compensatory increase in Na^+/K^+-ATPase protein expression might be expected to be accompanied by a parallel increase in Na^+/K^+-ATPase gene expression. However, there are very few transcriptional studies investigating effects of Cu on this enzyme (Nogueira *et al.*, 2020). Polo *et al*. (2020) did observe a significant upregulation of genes encoding Na^+/K^+-ATPase subunits in sheepshead minnow gills after exposure to 100µg/L Cu for 19 days. Examples also exist for other tissues. Upregulation of *atp1a1* transcription (a gene encoding a catalytic subunit of Na^+/K^+-ATPase) was observed in the liver of zebrafish after chronic 21 day exposure to 8µg/L and 15µg/L of Cu, potentially as a compensatory response against the observed inhibition of the enzyme's activity in these fish (Craig, Hogstrand, *et al.*, 2009). The upregulated transcription of Na^+/K^+-ATPase subunit *atp1b4* in pre-exposed fish in our study is consistent with a compensatory

transcriptional response to increase Na^+/K^+-ATPase expression in the gill in response to Cu-induced inhibition. The fact that this upregulation only occurred in the pre-exposed population suggests that pre-exposed fish may be better able to initiate this compensatory response than naïve fish, possibly due to a stable changes in the epigenetic regulation of this gene induced during embryonic pre-exposure.

However, the mechanism behind the significant downregulation of transcription of Na^+/K^+-ATPase subunits in naïve fish is less clear. There are almost no examples of downregulation of Na^+/K^+-ATPase transcription in response to copper, with the exception of cells in the olfactory rosettes, where Na^+/K^+-ATPase performs a very different function. Here, downregulation of Na^+/K^+-ATPase activity is a compensatory mechanism against damaging sodium export from cells (Azizishirazi *et al.*, 2015) in response to copper. However, in hyperosmoregulating crabs acclimated to low salinity, low dose copper exposure for three days resulted in inhibited transcription of genes encoding the Na^+/K^+-ATPase pump in the gills, and suggest that this a potential toxic effect of Cu exposure (Martins *et al.*, 2011). No inhibition of enzyme activity levels was observed, and the authors attribute this to insertion of pre-existing Na^+/K^+-ATPase units into the basolateral membrane to compensate for the inhibitive effect of Cu. The downregulated transcription of Na^+/K^+-ATPase genes we observed in naïve stickleback upon exposure to copper could be a toxic effect of copper. However, osmoregulation differs greatly between euryhaline osmoregulators and freshwater osmoregulators, and there is little evidence of this effect occurring in other exclusively freshwater species.

If the downregulation of *atp1a2a* and *atp1b3a* transcription observed in naïve fish is not due to a direct inhibitory effect of copper, it could be due to a change in the proportion of cells actively transcribing these genes. Exposure of tilapia to 3.2µM Cu over 28 days was shown to cause a decrease in plasma Na^+ levels and branchial Na^+/K^+-ATPase activity with a parallel increase in branchial Cu content (Li *et al.*, 1998). Na^+/K^+-ATPase activity is thought to correlate with chloride cell density. However, despite proliferation and increased density of chloride cells in copper exposed fish, a parallel increase in Na^+/K^+-ATPase activity was not observed (Li *et al.*, 1998). Over the exposure, Li *et al.* observed an 8-fold increase in necrotic chloride cells (accidental cell death), and a 2-fold increase in apoptotic chloride cells (programmed cell death)(Li *et al.*, 1998). The authors suggest that

reduced chloride cell quality led to decreased Na^+/K^+-ATPase activity despite increased chloride cell number (Li *et al.*, 1998). Bury et al. (1998) also observed significantly increased rates of necrosis in gill epithelial cells after a short incubation with copper, without any effect on apoptosis (Bury *et al.*, 1998). It is possible that the reduced Na^+/K^+-ATPase transcription could be a result of increased proportion of less transcriptionally active necrotic chloride cells in the gills of naïve fish compared to pre-exposed fish. However, histological analysis would be needed to support this hypothesis. The mechanism behind this downregulated transcription observed in naïve fish therefore remain unclear. However, the differential transcription of Na^+/K^+-ATPase subunits observed between populations does suggest that they are affected by copper exposure differently depending upon their exposure history during early life.

As previously discussed, several GO terms related to calcium regulation were enriched for downregulated genes in pre-exposed fish, which included a variety of genes involved in calcium transport, storage and signalling. This was in contrast to the pre-exposed population, for which there was no downregulation of genes related to calcium handling. Many of these genes downregulated in naïve fish have been identified as muscle-specific or are known to play important roles in muscle tissue, where calcium is important for regulating muscle contraction (Berchtold, Brinkmeier and Müntener, 2000). However, the gill is also an important site regulating calcium homeostasis, which can also be disrupted by toxic copper exposure (McGeer *et al.*, 2000; Wood, 2011). Several studies in freshwater fish have demonstrated Ca^{2+} loss as a result of copper exposure (Lauren and McDonald, 1987; McGeer *et al.*, 2000). This disturbance in calcium homeostasis is likely linked to disruption of calcium transporters. For example, copper has been shown to inhibit the activity of Ca^{2+}-ATPase – the transporter responsible for driving Ca^{2+} extrusion across the basolateral membrane in the gill; as well an inhibiting activity of the Na^+/K^+-ATPase, potentially disrupting supply of ions needed for Na^+/Ca^{2+} antiporter activity (Shephard and Simkiss, 1978; Flik *et al.*, 1993; Viarengo *et al.*, 1996). Transcription of several genes involved in calcium transport, including several identified as having calcium ATPase activity, were downregulated in naïve fish only. Although many were identified as being muscle specific, as discussed above, this could be result of reliance on mammalian annotations, ignoring possible functions in the gills. Downregulation of genes

related to calcium homeostasis in naïve fish only, may therefore suggest greater disruption of calcium homeostasis in the gills of naïve fish, with embryonic pre-exposure to copper protecting the pre-exposed population from this disruption upon re-exposure as adults.

Differentially expressed genes in both the naïve and pre-exposed population also included many other genes identified as ion transporters, but were either unannotated or their specific function in relation to gills has not been entirely described. Where ion transporter genes were differentially expressed, the majority were differentially expressed in one population only, and they were generally upregulated in pre-exposed fish and downregulated in naïve fish.

In naïve fish, this included downregulation of several genes involved in chloride and bicarbonate transport. For example, *slc22a6l* (solute carrier family 22 member 6, like), which is orthologous to several human genes including SLC22A6, was significantly downregulated in naïve fish (FC: 0.251, FDR: 0.00493). It is predicted to be involved in the sodium-dependent transport and excretion of organic anions, but characterisation of the slc22 family in fish has only recently been attempted and is incomplete (Mihaljevic *et al.*, 2016). *slc4a3* (solute carrier family 4 member 3)(FC: 0.199, FDR: 0.0106) was also significantly downregulated. Its human ortholog, SLC4A3, is an anion exchanger with wide expression, and at least one of its functions involves extrusion of intracellular HCO_3^- in exchange for extracellular Cl^- in the brain (Vilas *et al.*, 2009). *slc4a1b* (solute carrier family 4 member 1b)(FC: 0.487, FDR: 0.0197) was also significantly downregulated in naïve fish. Its human ortholog, SLC4A1, is a basolateral anion exchanger known to be involved in Cl^-/HCO_3^- exchange in erythrocytes and the kidney (Bruce *et al.*, 1997). Chloride-bicarbonate exchange at the apical membrane of gill epithelial cells, where HCO_3^- is excreted in exchange for uptake of Cl^-, is essential for acid-base regulation (Grosell, 2012). Copper exposure has been shown to disrupt acid-base regulation in fish by causing elevation of HCO_3^- levels in plasma, even at sublethal concentrations causing only minor osmoregulatory disturbance (Wang *et al.*, 1998; Grosell, 2012). The mechanism of this is not entirely understood, but disruption of Cl^-/HCO_3^- exchange and carbonic anhydrase activity are thought to be involved. Downregulated transcription of genes involved in Cl^-/HCO_3^- exchange could indicate possible copper-induced disruption of acid-base regulation pathways in naïve fish. There was also significant downregulation of two genes

putatively identified an encoding proteins that form part of a sodium and chloride channel – sodium voltage-gated channel alpha subunit 4 (FC: 0.1867, FDR: 0.00620) and chloride intracellular channel 5a (FC: 0.204, FDR: 0.0183) – suggesting an impact on Na^+ and Cl^- regulatory pathways.

In pre-exposed fish, genes encoding ion transporters showed both up- and down-regulated transcription. For example, a gene putatively identified as solute carrier family 12 member 10, tandem duplicate 1 (*slc12a10.1*) predicted to encode chloride symporter was downregulated (FC: 0.272, FDR: 0.00823), whereas, two putatively identified genes, predicted to be inorganic anion transporters (potentially Cl^-), were strongly upregulated – solute carrier family 4, member 2a (FC: 100, FDR: 0.000823) and anion exchange protein 2-like (FC: 22.5, FDR: 0.0202).

There were also ion transporter genes that were differentially expressed in both the naïve and pre-exposed populations. This included, *kcnj1a.6* (potassium inwardly rectifying channel subfamily J member 1a, tandem duplicate 6) which was significantly downregulated in both populations to a similar degree (naïve – FC: 0.451, FDR: 0.00755; pre-exposed – FC: 0.451, FDR: 0.0202). It encodes an inwardly rectifying potassium channel, whose human ortholog, KCNJ1, is thought to play a major role in potassium homeostasis. This suggests that copper exposure may be having an effect on potassium homeostasis, which is common to both populations. Another gene that was differentially expressed in both populations was *cnga3a* (cyclic nucleotide gated channel subunit alpha 3a). However, it was strongly upregulated in naïve fish (FC: 25.8, FDR: 0.000225) and strongly downregulated in pre-exposed fish (FC: 0.0561, FDR: 0.0346). Its human ortholog, CNGA3, is a nucleotide gated cation transporter found in cone cells, which transports Na^+ and Ca^{2+} into cone cells (Michalakis, Becirovic and Biel, 2018). Its function in the gills has not been described.

The differential expression of ion transporter genes in the gill suggests that copper exposure may be affecting ion homeostasis and transport pathways in the gills, including homeostatic pathways of Na^+, Cl^-, K^+ and HCO_3^-. However, some of these changes differed between populations; with differential expression of genes relating to bicarbonate-chloride exchange only being observed in naïve fish, and differential expression of genes related to sodium and chloride transport varying between populations. As the functions of these genes and their products in the gill

are not fully understood, it is not possible to infer what effect their differential expression might have on the physiology of these fish in response to copper, but their differential response does suggest that the effect of copper on ion transport pathways differed between populations depending upon their exposure history.

3.4.3.4. Copper caused changes consistent with greater levels oxidative stress and DNA damage in naïve fish

Enrichment analysis identified two GO terms related to stress response that were overrepresented in response to copper exposure in the naïve population only. Both DNA repair (FDR: 2.91E-06) and cellular response to stress (FDR: 1.93E-05) were significantly overrepresented for upregulated genes in the naïve population. In contrast, there were no enriched GO terms related to stress response for either upregulated or downregulated genes in the pre-exposed population.

Induction of oxidative stress through production of reactive oxygen species (ROS) is a well-established consequence of exposure to toxic metals, including copper (Craig, Wood and McClelland, 2007; Bopp, Abicht and Knauer, 2008; Gaetke, Chow-Johnson and Chow, 2014). ROS-induced oxidative damage, such as membrane lipid peroxidation, and damage to DNA and proteins, is thought to play a major role in the cytotoxicity of copper (Bopp, Abicht and Knauer, 2008; Gaetke, Chow-Johnson and Chow, 2014). There are several conserved mechanisms by which cells can respond to oxidative stress.

One such mechanism is via production of heat shock proteins. Heat shock proteins (HSPs) are important molecular chaperones that mediate correct protein assembly, maintaining cellular homeostasis and protecting cells from damage (Mohanty *et al.*, 2018). HSPs are constitutively expressed as part of normal cell functioning; however, they also play an important role in response to stressful conditions, with stress-inducible HSPs showing strong and rapid upregulation in response to exogenous and endogenous stressors, including oxidative stress (Mohanty *et al.*, 2018).

In the present study, there was a general upregulation of heat shock protein transcription in both the naïve and pre-exposed populations; with heat shock proteins accounting for the top five most significantly upregulated genes in both populations. Many of these upregulated HSPs were common between

populations, including many annotated HSPs, such as: *hsp90aa1.2* (heat shock protein 90, alpha (cystolic), class A member 1, tandem duplicate 2)(naïve – FC: 7.4, FDR: 1.66E-11; pre-exposed – FC: 3.8, FDR: 8.23E-15); *hspa2* (heat shock protein family A (Hsp70) member 2)(naïve – FC: 22.1, FDR: 1.50E-08; pre-exposed – FC: 21.4, FDR: 3.79E-31); and several genes putatively identified as coding for heat shock proteins through sequence similarity. These included: two genes putatively identified as coding for hsp30-like (heat shock protein 30-like, also known as non-mammalian hspb11)(naïve – FC: 163.4, FDR: 7.09E-06; pre-exposed – FC: 34.1, FDR: 6.67E-08)(naïve – FC: 1597.6, FDR: 5.13E-17; pre-exposed – FC: 342.0, FDR: 9.36E-18); and one gene putatively identified as a repeat of *hspa2* (heat shock protein family A (Hsp70) member 2)(naïve – FC: 28.2, FDR: 0.000764; pre-exposed – FC: 53.5, FDR: 1.68E-17). Although many of the same HSPs were upregulated in both populations in response to copper exposure, they were generally upregulated to an equal or higher degree in naïve fish compared to pre-exposed fish, despite lower accumulation of copper in their tissues. This included two genes encoding hsp30-like, a HSP activated in response to damaged and unfolded proteins (Heikkila, 2017), whose fold changes were 4.8- and 4.7-times higher for naïve fish than pre-exposed fish. The gene encoding a member of the hsp40 family, *dnajb1b* (DnaJ heat shock protein family (Hsp40) member B1b), whose functions are thought to include stimulation of the enzymatic activity of other HSPs, including hsp70 proteins, was also significantly upregulated in response to copper exposure, but only in the naïve population (FC: 2.75, FDR: 0.000316). This suggests that naïve fish may be experiencing higher levels of oxidative stress upon exposure to copper than pre-exposed fish, resulting in greater induction of protective HSPs.

The only differentially expressed HSP gene which showed an opposite response between populations was *hspb1* (heat shock protein, alpha crystallin-related, 1), which was significantly upregulated in pre-exposed fish (FC: 3.8, FDR: 0.042241) and downregulated in naïve fish (FC: 0.31, FDR: 0.022291). However, *hspb1* is thought to play a specific role in muscle cells where it is implicated in stabilisation of the actin cytoskeleton and in protecting muscle cells from oxidative stress (Dubińska-Magiera *et al.*, 2014). The decreased *hspb1* transcription in the gills of naïve fish may therefore be a reflection of a potential decreased proportion of muscle cells in the gill, as discussed previously.

Two proteins thought to play major roles in first line defence against oxidative stress in fish are metallothionein (mt) and glutathione (gsh) (Eyckmans *et al.*, 2011). As well as its role in metal ion sequestration discussed above, metallothionein has also been shown to be inducible by ROS and function as an ROS-scavenging antioxidant (Kling and Olsson, 2000; Chiaverini and De Ley, 2010), with some studies suggesting that its role in oxidative stress response may be more important than its role in metal storage (Hogstrand, Lithner and Haux, 1991; Grosell, 2012). Many studies have demonstrated upregulation of metallothionein mRNA expression and protein levels in response to copper (McCarter and Roch, 1984; Roch and Mccarter, 1984; Minghetti *et al.*, 2008; Craig, Galus, *et al.*, 2009; Minghetti, Leaver and George, 2010). Upregulation of *mt* in both the naïve (FC: 3.50, FDR: 0.002603) and pre-exposed (FC: 5.27, FDR: 1.35E-11) populations suggests a potential activation of this first line defence against oxidative stress in both populations upon exposure to copper.

Detoxification and scavenging of oxyradicals by glutathione is also an important first line cellular defence against metal-induced oxidative stress; and differential transcription and protein expression of several enzymes involved in its production and regulation has been observed in copper-exposed fish (Minghetti *et al.*, 2008). There are three mechanisms to increase glutathione availability in cells: a 2-step *de novo* synthesis catalysed by glutamate cysteine ligase (GCL) and glutathione synthetase (GSS); regeneration of oxidised glutathione disulfide (GSSG) to reduced glutathione (GSH) by glutathione reductase (GR); or recycling of cysteine – a rate-limiting precursor for *de novo* synthesis – catalysed by gamma-glutamyltranspeptidase, also known as gamma-glutamyltransferase (GGT)(Rossi, 2014). Both the naïve and pre-exposed population showed no change in transcription of enzymes involved in *de novo* synthesis or regeneration of glutathione from GSSG, whose transcription has previously been observed to be upregulated in various organisms as a compensatory response to GSH depletion by toxic metals (Hansen *et al.*, 2007; Nair *et al.*, 2013; Jeppe *et al.*, 2014). However, a gene predicted to have gamma-glutamyltransferase activity and putatively identified as protein-glutamine gamma-glutamyltransferase 2-like was significantly upregulated in the naïve population in response to copper exposure (FC: 2.14, FDR: 0.0249). Upregulation of GGT activity has been observed in response to other xenobiotics, and may be a compensatory response to increase

availability of cysteine for glutathione production (Lee and Jacobs, 2009). Copper exposure also led to differential expression of genes involved in the methionine-homocysteine cycle in both the naïve and pre-exposed population, including adenosylhomocysteinase (*ahcy*)(naïve – FC: 2.22, FDR: 0.00932) and betaine-homocysteine methyltransferase (*bhmt*)(naïve – FC: 0.174, FDR: 0.0195; pre-exposed – FC: 4.48, FDR: 0.0432). As well as producing the universal methyl donor, S-adenosylmethionine (SAM), the methionine-homocysteine cycle also produces a precursor of cysteine (Rossi, 2014). These changes indicate an effect of copper on pathways related to glutathione metabolism, particularly in the naïve population, where they suggest a potential upregulation of pathways that provide precursors required for glutathione synthesis.

DNA damage is another well-known indicator of metal-induced oxidative stress, with many studies reporting DNA damage in fish under acute and chronic exposure to copper (Santos *et al.*, 2010). There were many genes related to both the recognition and repair of DNA damage which were upregulated in the naïve population only. For example, *rpa1*, encoding replication protein A1, was significantly upregulated (FC: 3.32, FDR: 0.000957). Its human ortholog, RPA, binds single stranded DNA at sites of damage, causing activation of DNA damage checkpoints and stimulating the homologous recombination DNA repair process (T. H. Huang *et al.*, 2018). DNA damage checkpoint activation is mediated through a key kinase signalling cascade, involving CHK1 (checkpoint kinase 1), which was also upregulated in naïve fish (*chk1* – FC: 3.25, FDR: 0.0269). Activation of this cascade prevents progression of cell cycle until DNA damage repair has occurred (T. H. Huang *et al.*, 2018). RPA is then replaced by RAD51, a recombinase involved in homologous recombination – a DNA repair mechanism involving template-mediated synthesis to replace the damaged DNA strand (Baumann and West, 1998). Genes encoding rad51 and rad51 associated protein 1 were significantly upregulated in naïve fish in response to copper (*rad51* – FC: 2.95, FDR: 0.00617; *rad51ap1* – FC: 3.17, FDR: 0.0102), as well as several other enzymes involved in various DNA repair mechanisms. Upregulation of these genes in the naïve population suggests that they may be experiencing a higher level of oxidative stress in response to copper than pre-exposed fish. However, DNA repair enzymes also play a role at the replication fork during normal DNA replication (Lambert, Froget and Carr, 2007). Their upregulation in the naïve

population could be related to cell proliferation and reflect a general upregulation of genes related to DNA replication. However, the pre-exposed population also exhibited gene expression consistent with cell proliferation, but no significant upregulation of genes related to DNA repair, suggesting that this unique upregulation of DNA repair in the naïve population may be part of a response to oxidative stress.

However, other common markers of oxidative stress, including transcriptional changes of other ROS scavenging proteins or enzymes involved in their production, were not observed for either population. For example, we observed no transcriptional changes for catalase (CAT) or superoxide dismutase (SOD) – important antioxidant enzymes employed in the oxidative stress response. Their activity and transcription has been shown to increase upon acute exposure to copper in several fish species (Vutukuru *et al.*, 2006; H. Jiang *et al.*, 2013; Simonato *et al.*, 2016). Although transcriptional changes play an important role in the oxidative stress response, the initial response of many antioxidant pathways occurs at the protein level, where altered activity of existing antioxidant proteins is rapidly induced via post-transcriptional regulation, such as covalent post-translational modification of proteins (Zhang *et al.*, 2015). Transcriptional changes occur later, when altered regulation of existing proteins in no longer sufficient to prevent oxidative stress. For example, in a study profiling dose-dependent activation of p53-mediated signalling pathways, Clewell *et al.* (2015) observed increased rates of formation of DNA-repair centers at sites of oxidative DNA damage, activated by phosphorylation of existing proteins; which occurred at concentrations lower than those causing increases in micronuclei and altered gene expression (Clewell *et al.*, 2015). This is consistent with findings in our study, where upregulation of DNA damage repair pathways was observed, despite no parallel upregulation of some antioxidant genes. The 4 day exposure to 0.01mg/L Cu conducted in these adult stickleback was relatively short and used a lower concentration of copper than many studies reporting significant oxidative stress responses. These fish may therefore be experiencing oxidative stress, but below the threshold required to cause a large transcriptional response.

Despite transcriptional changes indicative of DNA damage, there was also no upregulation of apoptotic gene expression in either population. However, a study by Bury *et al.* (1998) using gill filament cultures demonstrated that Cu was able to

directly induce necrosis of chloride cells in the gills, but not apoptosis. Apoptosis appeared to be caused by elevated cortisol levels in stressed fish; which, at low levels, protected cells from necrosis caused by ionoregulatory disturbances, but at high levels, appeared to cause apoptosis in chloride cells (Bury *et al.*, 1998). The differential expression of genes related to oxidative stress response observed in these fish may therefore represent early stages of an oxidative stress response, where exposure has been sufficient to activate some protective mechanisms but not others, including apoptosis-inducing cortisol release.

Absence of transcriptional changes in some major oxidative stress response mechanisms suggests that the copper-induced oxidative stress being experienced by fish in this study is mild; however, the differential response observed between the two populations nevertheless suggests that the effect is greater in naïve fish than pre-exposed fish. Further investigation at the protein level could potentially identify additional oxidative stress responses that were not detected in our transcriptional analysis.

3.4.3.5. Copper caused transcriptional changes consistent compensatory upregulation of metabolism in pre-exposed fish compared to suppression of metabolism in naïve fish

In pre-exposed fish, there were no significantly overrepresented GO terms related to metabolism for upregulated genes, but there were a limited number of significantly underrepresented GO terms, including: the parent term – biosynthetic process (FDR: 0.0232), and several of its 'child terms' such as cellular biosynthetic process (FDR: 0.0293), macromolecule biosynthetic process (FDR: 0.0255), and cellular nitrogen compound biosynthetic process (FDR: 0.0255). There were no significantly over- or underrepresented GO terms for downregulated genes. This general lack of enrichment of metabolism-related GO terms suggests that copper exposure had little effect on metabolic regulation in pre-exposed fish.

However, there were a small number of genes related to metabolism that were significantly upregulated upon re-exposure to copper in pre-exposed fish. These included genes involved in metabolic pathways including: *gba3* (FC: 5.254, FDR: 0.0470) encoding glucosidase, beta, acid 3 (gene/pseudogene) predicted to be involved in carbohydrate metabolic processes, and a gene putatively identified as cytochrome P450 3A27-like (FC: 2.16, FDR: 0.0351). It also included genes encoding two ion transporters – *slc6a14* (solute carrier family 6 member 14)(FC:

82.1, FDR: 0.00806) and *slc13a5b* (solute carrier family 13 member 5b)(FC: 75.8, FDR: 0.00890) – which were strongly upregulated upon re-exposure to copper. slc6a14 is a sodium-chloride-dependent transporter that co-transports Na^+ and Cl^- with amino acids, which are used in cells for biosynthetic pathways, to act as neurotransmitters, and for essential metabolic processes (Sloan and Mager, 1999). slc13a5b is sodium-dependent citrate transporter which is thought to use Na^+ to cotransport circulating citrate into cells where it can be used to generate metabolic energy or for the synthesis of fatty acids (Inoue, Zhuang and Ganapathy, 2002). Upregulation of these genes in pre-exposed fish could indicate a small upregulation of metabolism in response to copper exposure. However, upregulation of ion transporters that transport compounds involved in metabolic processes, could also be a response to potential ionoregulatory disturbance. Exposure to Cu has previously been shown to have metabolic costs for organisms and cause changes in metabolic pathways. For example, McGeer et al. observed increased metabolic load in copper exposed rainbow trout; demonstrated by increased oxygen consumption and lower critical swimming speed in forced swim test, and increased appetite, compared to control fish (McGeer *et al.*, 2000). They suggest that increased metabolic load may be a consequence of compensatory mechanisms during recovery and acclimation to copper exposure, such as the increased activity of gill Na^+/K^+-ATPase, which was observed in conjunction with recovery after a temporary disruption of Na^+ balance (McGeer *et al.*, 2000). Increased transcription of some genes encoding metabolic enzymes in pre-exposed fish could indicate possible upregulation of metabolic activity as a response to added metabolic costs of maintaining homeostasis during copper exposure, including, for example, upregulating activity of enzymes inhibited by copper, such as Na^+/K^+-ATPase, whose transcription was also upregulated in pre-exposed fish.

In naïve fish, there were two significantly overrepresented GO terms related to metabolism for upregulated genes: ATP binding (FDR: 1.39E-11) and carbohydrate derivative binding (FDR: 4.45E-08). Importantly, there were many overrepresented GO terms related to metabolism for downregulated genes. These included glycolytic process (FDR: 6.54E-04) and its parents terms, ATP generation from ADP (FDR: 6.54E-04) and pyruvate metabolic process (FDR:

9.47E-04); indicating potential downregulation of both glycolysis and oxidative phosphorylation.

There were many metabolism-related genes that were significantly downregulation in naïve fish upon exposure to copper, including genes encoding enzymes with central roles in the glycolytic pathway, the process by which glucose is broken down to provide energy for cellular metabolism. Examples, included: *gpib* (glucose-6-phosphate isomerase b)(FC: 0.269, FDR: 0.000789), *pfkmb* (muscle-specific phosphofructokinase b)(FC: 0.216, FDR: 0.00997), *aldoaa* (fructose-bisphosphate adolase a)(FC: 0.2283, FDR: 0.000225), *gapdh* (glyceraldehyde-3-phosphate dehydrogenase)(FC: 0.270, FDR: 0.0300), *pgam2* (muscle-specific phosphoglycerate mutase 2)(FC: 0.208, FDR: 0.00420), and *pkmb* (pyruvate kinase M1/2b)(FC: 0.189, FDR: 0.00112) – which encodes the last rate-limiting step of glycolysis (Gupta and Bamezai, 2010).

Copper has been shown to directly inhibit the activity of enzymes involved in the glycolytic pathway (Carvalho and Fernandes, 2008; Lauer *et al.*, 2012; Maes *et al.*, 2016). For example, Maes *et al.* observed a disturbance in cell energy metabolism of muscle cells in copper-exposed juvenile roach, with a significant decrease in ATP concentrations after just one day of exposure to 10µg/L Cu. In parallel with this metabolic disturbance they observed compensatory increases in the transcription of key genes involved in the glycolytic pathway, including phosphofructokinase, pyruvate kinase, and glyceraldehyde 3-phosphate dehydrogenase (Maes *et al.*, 2016), whose transcription decreased in naïve fish in our study.

Several genes involved in mitochondrial function and oxidative phosphorylation were also downregulated in naïve fish. Many studies have demonstrated disruption of mitochondrial function by copper, potentially due to induction of oxidative stress, leading to reduced ATP production from oxidative phosphorylation (Pourahmad and O'Brien, 2000; Krumschnabel *et al.*, 2005; Garceau, Pichaud and Couture, 2010). For example, exposure of isolated rat liver cells to ionic copper resulted in oxidative damage of mitochondria and disturbance of oxidative phosphorylation, evident from a significant decrease in ATP concentration and ATP/ADP ratio in isolated mitochondria (Sokol *et al.*, 1993; Hosseini *et al.*, 2014). Similar disruption of mitochondrial oxidative phosphorylation

has been observed in studies of Wilson disease, characterised by inability to excrete copper, leading to excessive copper levels in tissues (Zischka and Einer, 2018). Disruption of mitochondrial function has also been linked with disturbed calcium homeostasis in mitochondria (Krumschnabel *et al.*, 2005), which was also found to be affected by copper exposure in the gills of naïve fish in this study. Strong enrichment of GO terms related to muscle for downregulated genes in naïve fish, many of which were related to calcium homeostasis, as discussed above; could be partially explained by disrupted transcription of genes related to general mitochondrial function that are expressed in other tissue types but have also been linked to muscle when their gene ontology attributes were annotated.

Downregulated genes in naïve fish related to mitochondrial function and oxidative phosphorylation included genes encoding key enzymes in the electron transport chain, such as: cytochrome c oxidase subunit VIa polypeptide 2 (*cox6a2*)(FC: 0.185, FDR: 0.0180) – encoding a subunit of cytochrome c oxidase, which catalyzes the final step of the mitochondrial electron transfer chain (Li *et al.*, 2006); and mitochondrial ATP synthase 6 (*mt-atp6*)(FC: 0.451, FDR: 0.040) – a subunit of mitochondrial ATP synthase, which catalyses the phosphorylation of ADP to form ATP during the final step of the electron transfer chain (Jonckheere, Smeitink and Rodenburg, 2012). In addition, downregulated genes related to oxidative phosphorylation included genes linked with muscle tissue. For example, solute carrier family 25 member 4 (*slc25a4*), orthologous to human ADP/ATP translocase 1 (*ANT1*) found in muscle cells, was significantly downregulated in naïve fish (FC: 0.193, FDR: 0.000776). ANT1 controls exchange of ADP and ATP across the mitochondrial membrane, and plays a role in regulation of oxidative phosphorylation (Chevrollier *et al.*, 2011). Two genes encoding creatine kinase were significantly downregulated in naïve fish: creatine kinase muscle a (*ckma*)(FC: 0.247, FDR: 0.00452) and putatively identified creatine kinase S-type mitochondrial-like (*ckmt2*)(FC: 0.230, FDR: 0.000781). Creatine kinase is involved in ATP transfer from the mitochondria to the cytosol, and is particularly important in metabolically active muscle cells (Perry *et al.*, 2012), but is also widely expressed in other tissues.

Studies that have measured transcriptional response of genes involved in the electron transport chain, such as cytochrome c oxidase, in response to copper exposure have found both upregulation and downregulation of transcription (Maes

et al., 2016; Anni *et al.*, 2019), suggesting responses can include both compensatory upregulation and metabolism-limiting downregulation. Downregulation of both glycolysis and oxidative phosphorylation in naïve fish suggests a general suppression of metabolic activity. This is in contrast to pre-exposed fish, which exhibited comparatively little change in transcription of genes related to metabolism; and where transcriptional changes were observed, this was generally upregulation of genes encoding metabolic enzymes, consistent with a compensatory response against metabolic disruption.

3.5. Physiological and transcriptomic differences suggest that pre-exposure to copper during early life may infer tolerance to future exposure

The results presented indicate that pre-exposure of stickleback to copper during embryogenesis caused permanent changes to their physiology, and altered their transcriptomic and physiological response to copper exposure in the future.

The finding that pre-exposed fish accumulated more copper in their tissues as adults without any subsequent copper exposure – including in the liver, muscle and gill – indicated that waterborne exposure during embryogenesis caused lasting physiological changes that resulted in higher uptake and/or storage of copper from their diet in the gut, and potentially in other organs. The further increase in copper content observed in the gills of pre-exposed fish compared to those of naïve fish upon re-exposure to copper, but not in liver and muscle, also suggests that pre-exposure caused lasting changes in the regulation of copper uptake and/or storage that may be specific to the gills.

Although the duration of adult exposure was relatively short, at just 4 days, and the concentration of copper used was low, at an environmentally relevant 0.01mg/L, comparison of the transcriptomic responses in the gills of pre-exposed and naïve populations identified distinct differences in the response of several processes in the gill depending upon the exposure history of the population. Despite having a significantly higher copper burden in their gills upon re-exposure, the transcriptomic response of pre-exposed fish was considerably reduced compared to naïve fish, which had over three times as many differentially expressed genes. This reduced transcriptomic response in pre-exposed fish may

suggest that they experienced less disturbance of homeostasis upon exposure to copper compared to naïve fish, requiring a lower transcriptional response to restore the balance.

The process that seemed to show the greatest difference in response between populations was epithelial cell proliferation. Although both populations exhibited upregulation in the expression of cell cycle related genes, many more genes were upregulated in naïve fish compared to pre-exposed fish, and the strength of this upregulation was generally several orders of magnitude greater. Gill epithelial cell proliferation is a known response to copper in fish, and is thought to initially be a compensatory response against ionoregulatory disturbance, but eventually results in the toxic effect of gill thickening and subsequent respiratory distress. The substantially reduced proliferation implied by the modest change in gene expression seen in pre-exposed fish compared to naïve fish, suggests that pre-exposure may have resulted in adaptive changes that either directly prevented harmful proliferation or prevented the disturbances that stimulate it, such as disruption of ion homeostasis.

Copper exposure also caused strong downregulation of muscle related gene expression that only occurred in naïve fish. Although unclear, possible causes could include reduced muscle cell proportion driven by strong epithelial proliferation in naïve fish, or downregulation of other cellular processes that are also linked to muscle function. These could include altered transcription of genes related to metabolism and calcium homeostasis – processes that are key to muscle function – which were both downregulated in naïve fish in response to copper, consistent with toxic disruption of calcium homeostasis and suppression of metabolism observed in other studies. This was in contrast to pre-exposed fish, in which calcium homeostasis was largely unaffected and metabolism related gene expression was upregulated, suggesting a compensatory response against metabolic disruption in pre-exposed fish.

The transcriptomic response of genes involved in several other processes known to be affected by copper also differed between pre-exposed and naïve fish. This included differential expression of ion transporters involved in the regulation of sodium, chloride, potassium and bicarbonate, suggesting impacts of copper exposure on ion regulation in the gills of both populations. Although the specific

functions of these transporters in the gills has not been fully described, making it difficult to infer the physiological effects of these transcriptional changes, there was a general downregulation of expression in naïve fish compared to a general upregulation in pre-exposed fish, indicating that copper exposure impacted ion transport differently in these fish depending upon their exposure history. However, upregulation of one well-described ion transporter in pre-exposed fish that is essential for sodium uptake and is known to be inhibited by copper – Na^+/K^+-ATPase – does suggest that pre-exposed fish may activate compensatory mechanisms against copper-induced disruption of ion regulation sooner than naïve fish, potentially helping them to tolerate copper exposure more effectively. Both populations appeared to upregulate genes involved in response to oxidative stress, but this effect was stronger in the naïve population, which also upregulated genes consistent with DNA damage response, suggesting that naïve fish were experiencing a greater level of oxidative stress.

Taken together, the differences observed between the transcriptional responses of each population suggest that pre-exposed fish were more tolerant to copper exposure than naïve fish, experiencing fewer toxic effects, including oxidative stress, harmful epithelial thickening and metabolic suppression, and activating compensatory responses more quickly.

There was a surprising lack of change in transcription of copper transporters and storage proteins for both populations. Given that chemical analysis indicated clear changes in copper handling in the gills that differed between populations, it is likely that there were changes in the activity of copper transport and storage pathways that did not involve changes at the transcriptional level, and would require further investigation at protein level.

3.6. Potential epigenetic mechanisms underpinning altered transcriptional response to copper exposure in pre-exposed fish

The differential expression observed in the gills upon adult exposure to copper between fish that received a pre-exposure during embryogenesis and naïve fish being exposed to copper for the first time suggests that copper exposure during

embryogenesis caused lasting changes in gene regulation that persisted throughout development and altered response to copper in later life. In studies investigating altered chemical tolerance via acclimation in fish, the chemical challenge used to assess a change in tolerance is usually conducted immediately following pre-exposure to a sublethal dose during the acclimation period (Dixon and Sprague, 1981; Benson and Birge, 1985; Sellin, Tate-Boldt and Kolok, 2005; Miller *et al.*, 2007; Adeyemi and Klerks, 2013). While this is useful for investigating mechanisms by which organisms can acquire tolerance to acute chemical exposure after continuous low-level exposure – an important scenario for wildlife living with persistent long-term exposure, this can only identify changes in gene regulation present after continuous stimulation from chemical exposure. These studies generally do not investigate how long these changes and the tolerance that they confer to the animal persist (Buckley *et al.*, 1982; Miller *et al.*, 2007). Where persistence of tolerance was tested after a period of depuration, most of these studies found that tolerance was lost soon after being transferred to clean water (Dixon and Sprague, 1981). This indicates that any changes in gene regulation induced during the acclimation period are transient, and require continuous stimulation from the chemical in order to be maintained. This kind of transient acclimation could protect animals experiencing chronic and stable long-term exposure, but would not offer protection for populations which encounter fluctuating exposure to chemicals across their lifetime.

In comparison, in our study, changes in gene regulation induced during embryonic pre-exposure to copper, which resulted in differential gene expression upon re-exposure, remained stable for over 9 months in the absence of continuous copper stimulation. Interestingly, rather than causing permanent up- or downregulated expression of copper-responsive genes, embryonic pre-exposure appears to have altered the regulatory status of these genes such that altered expression in pre-exposed fish compared to naïve fish is only observed upon stimulation from re-exposure to copper. This was evident from the fact that there were only 4 genes that were differentially expressed when pre-exposed and naïve fish were directly compared prior to any subsequent exposure (copper_0 vs. control_0). Whereas, upon stimulation with copper during the re-exposure, direct comparison of the pre-exposed and naïve populations (copper_0.01 vs. control_0.01) identified 343 differentially expressed genes.

In order for an environmental factor, such as copper exposure, to cause a permanent change to the regulation of a gene even after the environmental stimulus is removed, a “memory” of the stimulus must be retained between the initial exposure and subsequent exposures, which must be faithfully transmitted to new cells during mitosis as growth and cell renewal occur.

One mechanism by which environmental factors can cause persistent changes to gene regulation and an associated change in phenotype, is through epigenetic changes, which are defined as mitotically or meiotically heritable changes in gene expression occurring without a change in DNA sequence (Probst, Dunleavy and Almouzni, 2009). These are changes to the various regulatory elements that make up the epigenome, which include DNA methylation, histone modifications and variants, RNA-mediated gene regulation, and the activity of other DNA binding proteins, such as autoregulatory transcription factors and polycomb-group proteins (Ptashne, 2004; Grimaud, Nègre and Cavalli, 2006; Feng, Fouse and Fan, 2007; Sawan, Herceg and Vaissie, 2008). What distinguishes epigenetic changes from other environmentally-induced changes in gene regulation is that alterations to the epigenome are mitotically heritable, resulting in long-term persistence across the life of an organism, in the absence of the original environmental stimulus.

The differential transcriptomic response of pre-exposed fish compared to naïve fish upon re-exposure to copper, despite 9 months in the absence of waterborne copper exposure, suggests that embryonic pre-exposure may have caused epigenetic changes to gene regulation that remained stable during development of pre-exposed fish, and primed genes to be differentially expressed upon future exposure to copper. The embryonic exposure was conducted during a period of early development that encompasses windows of susceptibility to environmental influence, including a period of epigenetic reprogramming in somatic cells soon after fertilisation (Oswald *et al.*, 2000; L Jiang *et al.*, 2013; Potok *et al.*, 2013). It is thought that the epigenome is particularly sensitive to environmental influences during reprogramming, and chemical exposure during this period has the potential to cause long-lasting phenotypic changes (Faulk and Dolinoy, 2011). Much research on the influence of early life exposures to environmental chemicals on the epigenome has focussed on their ability to cause adverse epigenetic effects that lead to long-lasting disease phenotypes (Vandegehuchte and Janssen, 2011; Maccari *et al.*, 2014; Stel and Legler, 2015; Hoffman, Reynolds and Hardy, 2017;

Barouki *et al.*, 2018). However, new evidence has suggested a potential role for the epigenome in facilitating phenotypic plasticity and rapid adaptation to changes in environmental conditions within a single generation, with potential inheritance to future generation likely to encounter similar conditions (Angers, Castonguay and Massicotte, 2010; Mirbahai and Chipman, 2014). I hypothesise that the differences in transcriptomic response observed between populations may be underpinned by copper-induced epigenetic changes during embryonic pre-exposure that persisted into adulthood and altered the responsiveness of the transcriptome upon re-exposure, in particular for molecular pathways involved in preventing copper toxicity.

3.7. Limitations

In this section I will discuss some of the uncertainties that are associated with this study.

Firstly, it is important to consider the effects of potential changes in the proportion of cell types when conducting RNAseq studies. Given that the results of this study suggested that copper exposure caused epithelial proliferation in the gill which have resulted in altered proportion of cell types; it is possible that some changes in transcript abundances may reflect a change in the proportion of cell types producing those transcripts, as opposed to a change in gene regulation and expression within individual cells. Methods that could be used to help address this include histological analysis, cell sorting and single cell RNA sequencing. It is also important to remember that although transcriptional studies can provide valuable evidence for potential mechanisms underpinning observed physiological changes, transcriptional changes do not always correlate with changes at protein level, and should therefore be interpreted cautiously in the absence of protein level analysis.

Although the persistence of transcriptomic changes across the lifetime of these fish suggests that epigenetic alterations may underpin the altered response observed, we cannot rule out the potential contribution of other factors. In particular, it is possible that early life exposure could have caused changes to the microbiome of the gill that could also have persisted into later life and resulted in altered physiology of adults.

4. Conclusions:

In this study we have provided evidence that the exposure history of fish populations can significantly affect transcriptional responses upon future re-exposure. Here we present evidence that exposure to an environmentally relevant concentration of a common aquatic pollutant during early life resulted in altered gene regulation that persisted into later life, resulting in adaptive transcriptional and physiological changes in adults upon re-exposure. This highlights the potential for wild fish populations to become locally adapted within a generation as a result of embryonic exposures, with implications for environmental protection and wildlife management strategies, such as restocking programs. The persistence of these changes into later life suggests the presence of stable epigenetic changes to the regulation of copper-responsive genes, and raises question as to whether this persistent altered response to future copper exposure may be inherited in subsequent generations.

Chapter 4

Lack of inheritance of copper tolerance following embryonic exposure in two fish species

Declaration of contributions:

The work reported in this chapter follows from an experiment conducted by Lauren Laing and included in her PhD thesis. I used existing three-spined stickleback populations created by Lauren Laing as part of a multigenerational experiment to generate adult F2 stickleback in order to investigate whether the copper tolerance that she observed up to the F2 generation following embryonic exposure of the F0 generation would be inherited by subsequent generations.

All zebrafish work included in this chapter is my own.

1. Introduction

In recent decades, there has been increasing interest in the potential for exposure to environmental chemicals to cause phenotypic alterations across multiple generations. In order to accurately assess the risk chemicals pose to the environment, effects lasting or even emerging in future generations, long after the initial exposure, must be included. Studies in mammals, fish and invertebrates have demonstrated multigenerational toxic effects of numerous endocrine disrupting chemicals, including: bisphenol A (BPA)(Mahalingam *et al.*, 2017), vinclozolin (Nilsson *et al.*, 2018), perfluorobutanesulfonate (Chen *et al.*, 2018), phthalates (Li, How and Liao, 2018), and polychlorinated bisphenyls (PCBs)(Mennigen *et al.*, 2018). A range of toxic outcomes were observed in subsequent generations, including endocrine, reproductive, metabolic and behavioural disruption. There are also many examples of multigenerational toxic effects from metal exposure. For example, exposure of animals to metals such as cadmium, zinc, lead and mercury, have been linked to a variety of toxic outcomes in subsequent generations, including: inhibited growth and behaviour (Yu *et al.*, 2013), endocrine and reproductive disruption (Li *et al.*, 2018), reduced longevity (Jeanrenaud, Brooke and Oliver, 2020), abnormal cardiovascular development (Ronco *et al.*, 2011), and cognitive impairment (Cronican *et al.*, 2013; Carvan *et al.*, 2017). However, some of these findings are surrounded by controversy as direct evidence for the mechanisms underpinning these multigenerational effects and the repeatability of the findings are often lacking.

As well as causing multigenerational toxic effects, parental chemical exposure can also alter the sensitivity of subsequent generations to future exposures. This altered sensitivity to environmental chemicals as a result of ancestral exposure could be a significant factor determining population level effects of pollutants. Genetic adaptation to metal exposure is a well-documented phenomenon in wild populations living in heavily contaminated environments (Mulvey and Diamond, 1991; Morgan, Kille and Stürzenbaum, 2007). For example, a locally adapted population of brown trout in the River Hale in the South-West of England was reported to be living in water containing copper and zinc concentrations that would be lethal to naïve fish (Bury and Durrant, 2009; Uren Webster *et al.*, 2013). Single nucleotide polymorphism analysis of wild yellow perch populations chronically

exposed to metal pollution for 85 years also showed evidence of selection for tolerant alleles (Bélanger-Deschênes *et al.*, 2013). Gene duplications and functional polymorphisms in genes related to metal tolerance, such as metallothionein, were also found in metal-adapted invertebrates, including springtails and fruit flies (Janssens, Roelofs and Van Straalen, 2009); and evolution of genetically heritable cadmium tolerance was also found in midge populations inhabiting historically metal-polluted sites (Pedrosa *et al.*, 2017). However, genetic adaptation can take many generations to develop; and under selective pressure, genetic diversity can be reduced (Bourret *et al.*, 2008), with potential costs for the sustainability of adapted populations. Further, studies investigating genetic adaptation often did not consider other potential mechanisms that may lead to tolerance and may act in conjunction with selection of favourable alleles, including changes in epigenetic gene regulation, physiology and/or microbiomes of individuals, as well as dynamics of food chains.

Non-genetic mechanisms of adaptation could play an important role in the ability of populations to adapt quickly to environmental change, and in a way that preserves their genetic variability and phenotypic plasticity in the future. Examples of altered sensitivity in offspring as a result of ancestral exposure to xenobiotics or adverse conditions, occurring without genetic adaptation, include both reduced tolerance or sensitisation to stressors, including titanium nanoparticles (Bundschuh *et al.*, 2012), silver ions and silver nanoparticles (Schultz *et al.*, 2016), alcohol (Finegersh and Homanics, 2014), and opiates (Vassoler, Wright and Byrnes, 2016); and increased tolerance to stressors, including cadmium (Plautz and Salice, 2013; Reátegui-Zirena *et al.*, 2017), nickel (Pane, McGeer and Wood, 2004), zinc (Jegede, Hale and Siciliano, 2019), polycyclic aromatic hydrocarbons (PAHs)(Krause, Dinh and Nielsen, 2017), and heat stress (Norouzitallab *et al.*, 2014). There are several studies that provide examples of altered sensitivity across multiple generations following exposure to metals, mostly conducted in invertebrate species. For example, clonal *Daphnia pulex* showed increased tolerance to cadmium after continuous exposure for 50 generations, but this was rapidly lost within 1-2 generations upon transfer to clean media (Shaw *et al.*, 2019). Multigenerational exposure of *Enchytraeus crypticus* to $CuCl_2$ resulted in increased tolerance in F3 and F4 progeny, which was also rapidly lost after transfer to clean media (Bicho *et al.*, 2017). In addition, a study on the effects of

chronic multigenerational exposure to nine toxic metals in *Diptera* flies, including copper, zinc, chromium, magnesium, nickel, mercury, lead, manganese and cadmium, indicated an increase in tolerance to all metals from the F1 to F3 generation, and further increases in tolerance up to the F6 generation for most of the metals. Similar to other studies, this tolerance was rapidly lost after breeding in clean water from generations F7 to F9 (Vedamanikam and Shazilli, 2008), revealing a lack on long lasting, inheritable alterations in susceptibility.

Many studies investigating instances of altered tolerance in future generations do so under multigenerational exposure scenarios, with continuous exposure over many generations (Rossbach *et al.*, 2019; Shaw *et al.*, 2019). Whilst studying effects of continuous multigenerational exposure is important for informing environmental protection strategy, it can be difficult to deduce the mechanisms behind altered tolerance using these designs, as continuous exposure across lifecycles and generations could lead to numerous mechanisms acting simultaneously. For example, Tariel *et al.* showed that grandparental and parental exposure to predator cues had independent effects on offspring response to predator cues in a freshwater snail, which were not necessarily cumulative or concordant (Tariel, Plénet and Luquet, 2019). It therefore would be difficult to unpick these mechanisms from observations in offspring after continuous exposure of both grandparents and parents.

There are many mechanisms by which exposure of an organism to environmental chemicals can impact future generations. A major mechanism is via indirect exposure of subsequent generations due to transfer of environmental chemicals from the parent to offspring, including through *in utero* exposure (Curi *et al.*, 2019), exposure of germ cells within the gonad of the exposed parent (Wu *et al.*, 2015), chemical transfer to externally fertilised embryos via the gametes (Birceanu, Mai and Vijayan, 2015), or transfer to neonates during lactation in mammals (Somogyi and Beck, 1993). These transferred chemicals can then elicit direct effects in the indirectly exposed offspring.

Chemical exposure of parents can also affect the transfer of other non-genetic factors from parent to offspring via the germ cells and gametes. For example, during oogenesis the oocyte accumulates a range of factors important for the growth and development of the early embryo, including nutrients, proteins,

enzymes, hormones, transcription factors and maternal RNAs. These factors can regulate many essential processes in the developing embryo, including ion homeostasis, metabolism, cell cycle, DNA repair and epigenetic regulation (Mtango, Potireddy and Latham, 2008). Maternal factors are particularly important prior to zygotic genome activation, the point at which the embryo becomes capable of producing its own transcripts, which is itself initiated by cytoplasmic factors originating from the parent (Mtango, Potireddy and Latham, 2008). Though maternal contributions of cytoplasmic factors are thought to be larger, sperm are also known to contain paternally transferred factors important for embryo development, including paternal RNAs (Guo *et al.*, 2017). Parental exposure to environmental stressors has the potential to cause alterations in the accumulation of these factors in gametes, with physiological consequences for resulting offspring.

Another mechanism by which parental environment may impact offspring phenotype is through inheritance of an altered epigenome. Exposure to some environmental chemicals is known to cause epigenetic alterations and associated changes in phenotype. These changes can persist for long periods within a generation, through mitotic inheritance of epigenetic alterations; and there is also evidence that some environmentally induced changes may escape erasure during epigenetic reprogramming and be meiotically inherited by subsequent generations (D'Urso and Brickner, 2014). Transgenerational inheritance of environmentally induced epigenetic alterations is a well-established phenomenon in some invertebrate species. For example, developmental exposure of the nematode worm, *Caenorhabditis elegans*, to a variety of environmental stressors in the F0 generation, including toxic metal exposure, hyperosmosis and fasting, was shown to increase resistance to oxidative stress and proteotoxicity in subsequent unexposed generations up to the F2 and F3 generation, respectively. The inheritance of this adaptive phenotype was shown to be mediated through germline inheritance of histone modifications (Kishimoto *et al.*, 2017). Examples of transgenerational inheritance of environmentally-induced phenotypes, predominantly disease, has also been reported in vertebrates after exposure to environmental chemicals (Nilsson, Sadler-Riggleman and Skinner, 2018). One of the first reports came from exposure of rats to the fungicide, vinclozolin, during gonadal sex determination, leading to reduced male fertility in later life that was

transferred to subsequent generations up to the F4 generation. This inherited disease phenotype was found to correlate with altered DNA methylation patterns in the germline (Anway *et al.*, 2005). Germline inheritance provides a potential mechanism by which epigenetic changes and associated phenotypic changes induced by exposure to environmental chemicals or stressors may be inherited by subsequent unexposed generations. However, the mechanisms underpinning reports of transgenerational inheritance of environmentally-induced phenotypes in vertebrates remain unclear, with the majority of mechanistic studies only reporting correlations between epigenetic alterations and multigenerational phenotypes. It has also proved difficult to design transgenerational experiments that ensure no influence of confounding factors, particularly parental effects and undetected changes in DNA sequence. This has led to fierce debate on the plausibility of transgenerational epigenetic inheritance in vertebrates, with some concluding that it is likely to be rare and restricted to imprinted genes; and where it does occur, is likely to be inheritance of environmentally-induced epigenetic aberrations, and is unlikely to be adaptive (Daxinger and Whitelaw, 2012; Heard and Martienssen, 2014).

In order to interrogate the mechanisms behind the effects of parental exposure on future tolerance of subsequent generations, and the potential longevity of these effects after exposure; it is useful to simplify study systems to include direct and indirect exposure of as few generations as possible. A single exposure event often results in direct and indirect exposure of multiple generations. The number of generations affected is dependent on sex and differences in reproductive life history traits. Fish are a useful model for multigenerational toxicology studies as they are oviparous, with fertilisation and embryonic development occurring outside the body of the parents. Whereas, in viviparous species, such as mammals, both occur within the body of the mother. When conducting toxicity testing, it is often desirable to study effects at the most sensitive life stages, which are generally during early development. This is particularly relevant for exposure studies where an epigenetic mechanism is suspected, as embryogenesis encompasses periods of epigenetic reprogramming, during which it is thought that the epigenome is more vulnerable to change or disruption (Cavalli and Heard, 2019). Externally developing fish embryos offer a convenient model for studying effects of exposure during embryogenesis, as the chemical can be applied via the water and taken up

directly into the embryo. This enables more control over the amount of chemical being taken up, and reduces direct and indirect exposure to only the F0 embryo and its germ cells, which will give rise to the F1 generation. This is in contrast to exposure during embryogenesis in viviparous species, where the gestating mother (F0), developing embryo (F1) and its germ cells (F2) are all directly or indirectly exposed during a single exposure event, with metabolism of the chemical by the mother also adding a layer of complexity when trying to determine the exact level of exposure experienced by the embryo.

In this study, copper was chosen as the model stressor for the reasons discussed in chapters 1 and 3 of this thesis. Briefly, copper is an essential element to life and participates in many biochemical processes in the cell. The same reactive properties that make it fundamental for life render it highly toxic when in excess and when its intracellular concentrations overwhelm the cellular copper binding and excreting capacity and result in free copper ions which can cause widespread damage to cellular components including lipids, proteins and DNA. Toxic effects in aquatic organisms include oxidative stress, genotoxicity, disruption of ionoregulation, disruption of larval development, hatching inhibition, compromised branchial function, abnormal behaviour, and mortality (Bury, Walker and Glover, 2003; Bopp, Abicht and Knauer, 2008; Wood, 2012; Haverroth *et al.*, 2015; Dorts *et al.*, 2016). Freshwater habitats are particularly vulnerable to metal pollution. A study risk-ranking metals found copper to be the metal of most concern threatening freshwater organisms in the UK (Donnachie *et al.*, 2014); and with the exception of mercury, copper was found to be the metal most frequently responsible for water quality impairment in the US according to the USEPA (Reiley, 2007). It occurs naturally in surface waters in the range of 0.2-30µg/L (Wood, 2012; Stehle and Schulz, 2015); but as a result of anthropogenic input concentrations frequently rise to toxic levels. Local and seasonal weather patterns can lead to spatial and temporal variations in both copper concentrations and bioavailability, due to fluctuating input from anthropogenic sources and fluctuating water parameters that influence the bioavailability of copper, such as pH, salinity, and complexing ligands (Winner and Owen, 1991; Meylan, Behra and Sigg, 2004; Batty, Auladell and Sadler, 2010). These fluctuations then result in intermittent exposure of wildlife.

With widespread occurrences of aquatic wildlife living with long-term, and sometimes fluctuating, copper exposure over many generations, alterations in copper tolerance as a result of historic exposure of ancestors could have a significant impact on the fitness of wild populations. Few examples of induced copper tolerance in offspring as a result of parental exposure exist outside of studies of wild populations, where genetic adaptation is likely to be involved. A few examples exist for fish. Peake *et al.* found induced copper tolerance beginning immediately after fertilisation in F1 larvae after a 5 day sublethal pre-exposure of F0 females (Peake *et al.*, 2004). Another study found similar induction of tolerance in F1 fathead minnow larvae following maternal exposure, but reported that it was short-lived, with tolerance being lost where larval exposure was initiated at 8hpf compared to 0hpf (Sellin and Kolok, 2006). To my knowledge, there are currently no published examples of altered copper tolerance in fish as a result of ancestral exposure, which persists beyond the F1 generation, and in which genetic differences or multigenerational acclimation after continuous exposure of multiple generations has been excluded.

Work by Laing *et al.* (Laing, 2017) demonstrated that exposure of three-spined stickleback (*Gasterosteus aculeatus*) to copper during embryogenesis caused increased tolerance in two subsequent generations. Following early life exposure to 0.01mg/L of copper from 1-217hpf (causing less than 1.5% mortality to ensure genetic selection did not occur) and maintenance in control conditions until sexual maturity, pre-exposed adult F0 fish were found to have differential copper accumulation in their tissues compared to naïve fish (see also chapter 3 in this thesis). Despite receiving no subsequent aqueous exposure, adult F0 fish pre-exposed to copper during embryogenesis had higher concentrations of copper in the muscle, liver and gills (reported in chapter 3)(Laing, 2017)**.** F1 and F2 adults were generated from the pre-exposed and naive populations, with no subsequent exposure to aqueous copper. Altered accumulation of copper in adult tissues was not sustained in the F1 generation, with no significant difference found between tissue copper concentrations for F1 fish from the pre-exposed and naive populations (Laing, 2017). Mortality curves on F1 embryos showed that embryos originating from parents pre-exposed to copper during embryogenesis were significantly more tolerant to copper exposure than those originating from naive

parents, and this increased tolerance was maintained in the F2 generation (Laing, 2017)(supplementary figure 1).

In this chapter I aimed to determine whether altered tolerance to copper would continue to be inherited in subsequent generations. Following from the work reported by Laing *et al.*, I hypothesised that tolerance may continue into the F3 generation if stable epigenetic inheritance is occurring. I conducted mortality curves on F3 embryos descended from copper pre-exposed F0 fish without any subsequent exposure and on F3 embryos from the naive control population. Maintenance of tolerance in the F3 generation would support a transgenerational epigenetic mechanism; and loss of tolerance would support a mechanism underpinned by parental effects.

In order to determine whether the induction of copper tolerance observed in stickleback would be conserved in other fish species, I also repeated the experiment in zebrafish (*Danio rerio*).

2. Methods

2.1. Chemicals

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

2.2. Stickleback

2.2.1. Fish source, culture and husbandry

In previous work by Lauren Laing (Laing, 2017) a copper pre-exposed population of three-spined stickleback (*Gasterosteus aculeatus*) was generated. F0 embryos were produced from a freshwater population of three-spined stickleback sourced from the River Erme, Devon, UK. These embryos underwent embryonic exposure to 0.01mg/L of copper (as copper (II) sulfate) from 1-217hpf (encompassing developmental period from 1 cell stage to independent feeding stage). This caused a significant but small increase in mortality of ≤1.5%, demonstrating a significant adverse effect of copper exposure, whilst ensuring genetic selection did not occur. This was followed by maintenance in control conditions. An F1 generation was then bred and raised in control conditions, without further exposure to copper, in order to generate F2 embryos. A naïve population of control fish were maintained in parallel, following F0 embryonic exposure to a

water control. Mortality curves were conducted on F1 and F2 embryos from both the pre-exposed and naive population, and indicated increased tolerance to copper exposure in both F1 and F2 embryos, as described above.

Using methods identical to those used by Laing *et al.* (Laing, 2017), I subsequently bred and raised F2 fish from both populations in control conditions in order to produce F3 embryos originating from copper pre-exposed and naive F0 ancestors. I then conducted mortality curves for F3 embryos from both populations using the same method as used for the F1 and F2 generations by Laing *et al.* (Laing, 2017).

Fish were bred in the Aquatic Resource Centre at the University of Exeter and maintained in mixed sex tanks of 112L, containing 20 fish per tank, supplied with synthetic water (mains tap water filtered by reverse osmosis (Environmental Water Systems (UK) Ltd) and reconstituted with Analar-grade mineral salts (conductivity of 300mS: 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) from an aerated 15°C reservoir. All breeding and embryo exposures were conducted during summer months (March-October), during which fish were maintained at a photoperiod of 18:6 light/dark (with 30 minute dawn/dusk transition) with a water temperature of 15 ±1°C. The 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.). Photoperiod and temperature were gradually transitioned over a 6 week period to simulate winter conditions (November-February), comprising a photoperiod of 6:18 light/dark and water temperature of 10 ±1°C.

Embryos for rearing each new generation and for conducting mortality curves were obtained by *in vitro* fertilisation (IVF)(method adapted from Barber and Arnott (2000))(Barber and Arnott, 2000). Unfertilised eggs were identified and removed by visual observation as described by Swarup (1958) (Swarup, 1958), using a dissection microscope (Motic DM143, Hong Kong). Fertilised embryos were incubated at 20±1°C in aerated artificial freshwater (according to ISO-7346/3 guideline, ISO water, diluted 1:5 (International Organization for Standardization, 1996)), in 600ml acid-washed glass exposure tanks containing 500ml of water.

Embryos being reared to adulthood were maintained in these conditions from 1-217hpf, before being transferred to adult conditions described above.

2.2.2. F3 Stickleback Embryo Exposures:

Assessment of copper tolerance in F3 stickleback embryos originating from a naive population and a population pre-exposed to copper during embryogenesis in the F0 generation:

F3 embryos were continuously exposed from 1-217hpf to a range of copper concentrations (0, 0.015, 0.025, 0.05, 0.0625, 0.075, 0.0875, 0.1, 0.125, and 0.15mg/L of copper) added as copper (II) sulphate. Each treatment was run in quadruplicate. Exposures were started at 1hpf (one cell stage/blastodermic cap) to ensure that the exposure encompassed critical windows of epigenetic reprogramming during early development (Swarup, 1958; Hackett and Surani, 2013). Using IVF (as previously described), pools of embryos were produced from each population. Approximately 20 embryos were then randomly assigned to 600ml acid-washed glass exposure tanks containing the desired concentration of copper in aerated artificial freshwater (according to ISO-7346/3 guideline, ISO water, diluted 1:5 (International Organization for Standardization, 1996)), with 3-4 males and 3-4 females contributing equally to each replicate exposure. Sperm from all males in each replicate exposure experiment was pooled to ensure that genetic variability was maintained, and then used to fertilise the oocytes generated by each female individually. For females where oocytes appeared to be of lower quality (for example because the females had passed the optimal maturation stage or fertilisation was less than 98%) were removed from the experiment. The remaining fertilised eggs were then randomly allocated to treatment groups. Exposure water was replaced daily to maintain copper concentration and prevent alterations in water quality. Temperature was monitored daily and found to be within the range of 19.4-20.3°C. Embryos were examined every 24hrs using a dissection microscope (Motic DM143, Hong Kong) and the proportion of mortality.

2.3. Zebrafish

In order to investigate whether the alterations in tolerance observed across multiple generations in stickleback as a result of early life exposure were conserved in other teleost species, a similar exposure regime was conducted in zebrafish.

2.3.1. Fish source, culture and husbandry

F0 embryos were collected from a wild-type WIK strain zebrafish stock maintained in the Aquatic resources Centre at the University of Exeter, and used to generate a pre-exposed and naive population of fish. Fish were maintained in 8L tanks fed with mains tap water 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 heated to 28°C in a reservoir, before it was supplied to each aquarium using a flow-through system. Tank water was maintained at 28 ± 1°C and pH 7-7.5 and fish were maintained under a 12:12h light:dark cycle, including dawn and dusk transition periods of 30 min. Fish were fed live Artemia nauplii twice daily (ZM Premium Grade Artemia; ZM Ltd.) and TetraMin tropical flake food (Tetra; Melle, Germany) once daily, to satiation. Breeding to generate embryos for the experiments described below was performed in small groups of 2 females and 1 male, to maximise oocyte production and fertilisation. The night before embryo collection, adults were placed into divided breeding tanks separating males and females. The next day dividers were removed immediately after dawn, and the fish were allowed 15 minutes of natural breeding before being transferred back to holding tanks to allow embryo collection.

2.3.2 Embryo exposure procedure

All embryo exposures were conducted in 600ml acid-washed glass exposure tanks containing the desired concentration of copper (as copper (II) sulfate) in aerated 1:5 diluted ISO water at 28 ± 1°C (as previously described) with 25 embryos per replicate tank. All embryos exposures were conducted from 0.5hpf (one cell stage/blastodermic cap) to ensure that the exposure encompassed critical windows of epigenetic reprogramming during early development (L Jiang *et al.*, 2013; Potok *et al.*, 2013). At 0.5hpf, 50 embryos from the desired population were randomly assigned to each tank. Fertilisation rate was assessed at 1.5hpf and excess embryos removed to leave 25 fertilised embryos in each exposure tank. Exposure water was replaced immediately after removal of unfertilised and excess embryos to prevent a decrease in copper concentration due to the large number of embryos in the exposure water during these first stages.

2.3.3. Preliminary experiment

Before generating a pre-exposed and naïve population, preliminary mortality curves were conducted to identify a concentration which caused a significant phenotypic effect on zebrafish embryos without causing substantial mortality, which would risk confounding genetic selection. Zebrafish embryos were exposed to a range of copper concentrations (0, 0.025, 0.03, 0.0325, 0.035, 0.0375, 0.04, 0.045, 0.05, 0.055, 0.06, 0.07, 0.08, and 0.09mg/L Cu) from 0.5-24.5hpf. Embryos were examined every 24hrs using a dissection microscope (Motic DM143, Hong Kong) and proportion mortality recorded.

2.3.4. Generation of naïve and copper pre-exposed zebrafish populations

F0 embryos from 21 successful 2:1 female:male (F:M) spawns were pooled and randomly assigned to replicate tanks. A naïve and pre-exposed population was generated by exposing embryos from 0.5-96.5hpf to control conditions or 0.0325 mg/L copper (concentration chosen based on preliminary data). Average fertilisation rate was 96.5% ± 0.6% (SE). Embryos were examined every 24hrs using a dissection microscope (Motic DM143, Hong Kong) and proportion mortality and hatching recorded. At 96hpf, 15 larvae from each tank were randomly selected and pooled with another replicate exposure dish to form groups of 30 larvae, which were then transferred to rearing tanks and maintained until adulthood in control conditions as described above for the breeding population.

F0 survival at 50dpf and F0 adult fecundity and fertilisation success were assessed to determine whether the embryonic exposure to copper affected these parameters and whether selection for a tolerant genotype may have occurred during rearing. F0 fecundity and fertilisation success were assessed by randomly selecting 10 groups of 2:2 M:F fish from each population and placing them into spawning chambers, as previously described, and comparing fertilisation success and fecundity (as number of eggs per female) between the two populations. For groups that did not spawn, this was included in fecundity calculation as 0 eggs per female.

F1 Zebrafish Embryo Exposures

Assessment of copper tolerance in F1 zebrafish embryos originating from a naive and a copper pre-exposed population:

Mortality curves were conducted for F1 offspring of the pre-exposed and naïve populations of zebrafish, using a similar method as that used for stickleback, adapted to account for the higher relative copper toxicity in zebrafish compared to stickleback.

Embryos from both the pre-exposed and naïve population were collected from 7-10 2:1 F:M spawning groups per population, which were pooled, randomly assigned to exposure tanks, and continuously exposed to a range of copper concentrations (0, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.10 mg/L of copper) from 0.5-24.5hpf, using the method described above. Preliminary data showed that these chosen concentrations would capture the full range of mortality in zebrafish and previous work by Fitzgerald *et al.* (2016) indicated that zebrafish are most sensitive to copper during the first 24 hours of development, during which the majority of mortality occurs (Fitzgerald *et al.*, 2016). Each experiment was run in quintuplicate. Embryos were examined after 24hrs of exposure using a dissection microscope (Motic DM143, Hong Kong) and proportion mortality recorded.

2.4. Statistical Analysis

All statistical analyses were conducted in R v3.5.0. (R Core Team, 2018). Prior to the analysis, data was screened and replicates with more than 10% mortality in the control were excluded from the analysis, as according to validity criteria set out in OECD guidelines for the testing of chemicals (OECD, 2013).

The preliminary copper mortality curve conducted for zebrafish was analysed using a log-logistic model using drc package v.3.0-1 (Ritz *et al.*, 2015), and effective doses calculated for 1%, 2%, 5% and 10% mortality.

During generation of the pre-exposed and naïve zebrafish populations, effects of copper exposure on F0 embryo mortality after 24hrs of exposure, F0 hatching rate after 96hrs of exposure, survival at 50dpf after subsequent rearing in control conditions, and F0 adult fecundity and fertilisation success were analysed using independent two sample t-tests.

Effects of pre-exposure to copper on tolerance of F3 stickleback embryos and F1 zebrafish embryos when exposed to a range of copper concentrations was analysed using generalized linear models (GLMs), with a separate model for each 24hr time period after fertilisation in stickleback (1-9 days). The GLMs used a quasibinomial error structure to test for effects of copper concentration (as a continuous variable) and population of origin (pre-exposed vs. naïve, as a categorical variable), and the interaction between the two, on the proportion of mortality in F3 stickleback and F1 zebrafish embryos (as a continuous variable). Minimum adequate models (MAM) were derived by model simplification using F tests based on analysis of deviance.

All data was considered statistically significant when $p<0.05$.

2.5. Ethics declaration

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.

3. Results:

3.1. F3 Stickleback Embryo Exposures

Effects of F0 pre-exposure on copper tolerance in F3 stickleback embryos:

Copper exposure resulted in increased mortality in both the pre-exposed and naive populations, and concentration was a significant explanatory variable of mortality from 49hpf ($P = 0.014$) until 217hpf ($P = 2.2e\text{-}16$)(figure 1, table 1). Comparison of the mortality curves generated from the naive and pre-exposed populations indicated that copper caused significantly higher cumulative mortality in F3 embryos originating from the pre-exposed population from 49hpf until 121hpf. This is indicated by the interaction between concentration during re-exposure and population of origin being a significant explanatory variable of mortality from 49hpf ($P=0.019$) until 121hpf ($P=0.031$). Following this, from 145hpf to 217hpf, there was no significant effect of population of origin on mortality, either as an additive variable or as an interaction with copper concentration during re-exposure, and it was removed from the model during model simplification (figure 1, table 1).

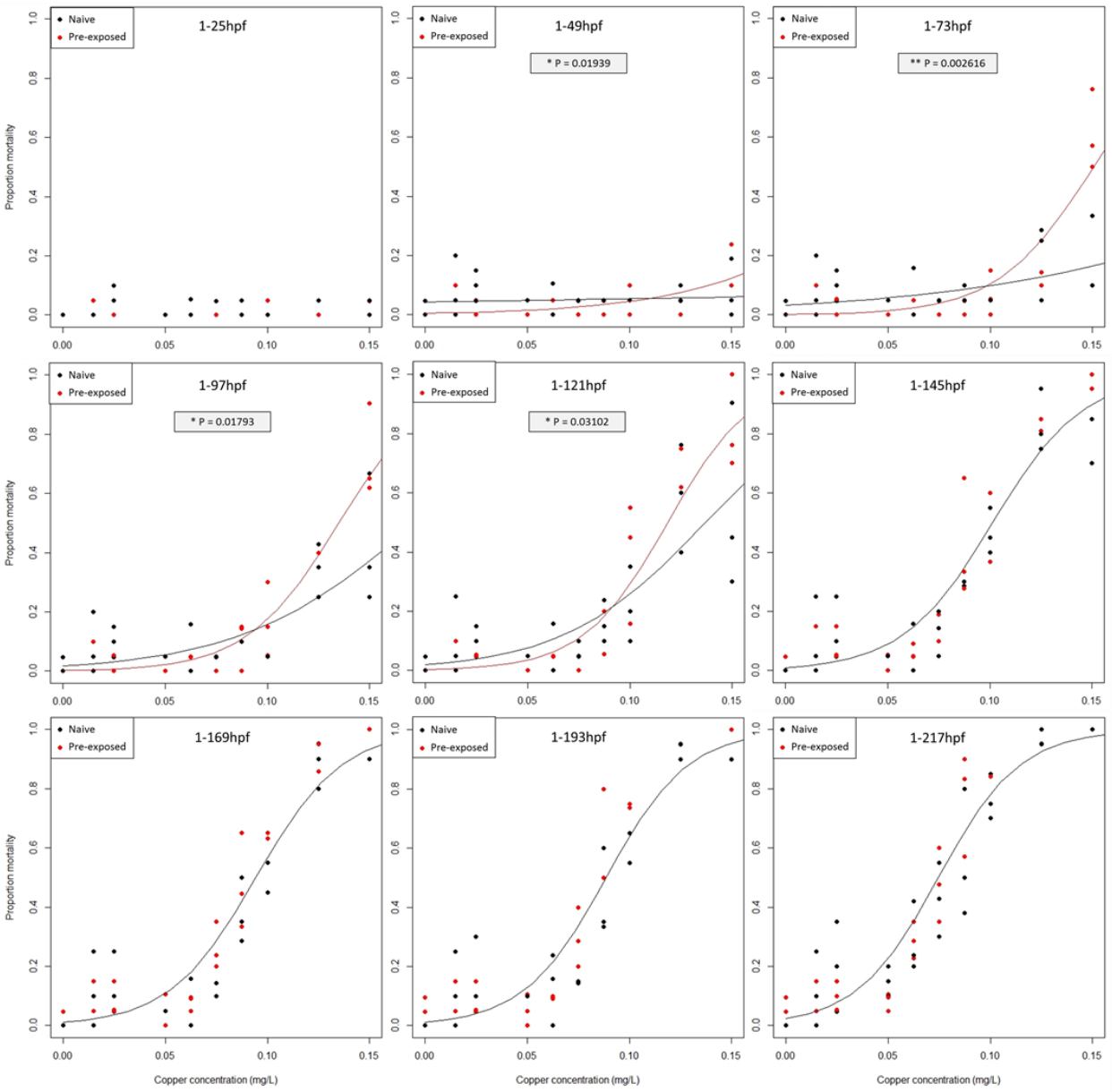


Figure 1: *Embryo mortality curves for F3 stickleback embryos from both the naive and copper pre-exposed populations (n=4 replicate tanks per dose and per population). Embryos were exposed continuously to copper (0, 0.015, 0.025, 0.05, 0.0625, 0.075, 0.0875, 0.1, 0.125, 0.15mg/L Cu) from 1-217hpf and cumulative mortality assessed every 24hrs. Graphs show cumulative mortality at 25, 49, 73, 97, 121, 145, 169, 193, and 217hpf. Black and red symbols represent replicate tanks of F3 embryos from the naïve population and the population pre-exposed to copper during F0 embryogenesis, respectively. Lines represent the best fit model for the data, calculated using generalized linear models in R (model output summarised in table 1). Two lines indicate a significant difference between populations. Significantly higher mortality was found for embryos from the pre-*

exposed population compared to embryos from the naïve population, with a significant interaction between the effects of population of origin and copper concentration upon re-exposure at 49hpf (p=0.01939), 73hpf (p=0.002616), 97hpf (p=0.01793) and 121hpf (p=0.03102).

Time point	Exposure concentration		Population of origin (naïve or pre-exposed to copper during F0 embryogenesis)		Exposure concentration/ Population of origin interaction	
	F value	P value	F value	P value	F value	P value
1-25 hpf	NA	NA	NA	NA	NA	NA
1-49 hpf	6.5070	0.01351 *	1.0249	0.31570	5.7955	0.01939 *
1-73 hpf	36.5312	1.288e-07 ***	0.6352	0.428823	9.9254	0.002616 **
1-97 hpf	83.9445	9.875e-13 ***	0.9411	0.33617	5.9482	0.01793 *
1-121 hpf	116.9628	2.477e-15 ***	0.4621	0.49944	4.8956	0.03102 *
1-145 hpf	171.49	2.2e-16 ***	NA	NA	NA	NA
1-169 hpf	219.47	2.2e-16 ***	NA	NA	NA	NA
1-193 hpf	213.02	2.2e-16 ***	NA	NA	NA	NA
1-217 hpf	366.36	2.2e-16 ***	NA	NA	NA	NA

Table 1: *Analysis of deviance models for the relationships between exposure concentration (during the re-exposure period), population of origin (naive or pre-exposed to copper during the F0 generation), and a population of origin / exposure concentration interaction for F3 stickleback embryo mortality curves. The results for minimum adequate models (MAMs) for proportion of mortality using generalized linear models with a quasibinomial error structure are shown. Minimum adequate models (F value) for each parameter are shown (Significance codes: *** P<0.001, ** P<0.01, * P<0.05). "NA" indicates that the variable had no significant explanatory power and was removed from the model during model simplification.*

3.2. F1 Zebrafish Embryo Exposures

3.2.1. Preliminary experiment

Following exposure of zebrafish embyros from 0.5-24.5hpf and fitting of a log-logistic model, effective doses were calculated for the following mortality rates – 1%: 0.0313mg/L; 2%: 0.0345mg/L; 5%: 0.0395mg/L; and 10%: 0.0439mg/L (figure 3). The steepness of the curve and small difference in copper concentration between doses causing 1-5% mortality indicated that choosing a dose that would consistently cause a small mortality rate that was significantly higher than expected background mortality rates would be difficult, and would risk genetic selection. For this reason, a low concentration of 0.0325mg/L, below that expected to cause significant mortality, was used for generating the pre-exposed population, and hatching rate used as a non-lethal endpoint to indicate an effect of copper exposure as an alternative to mortality.

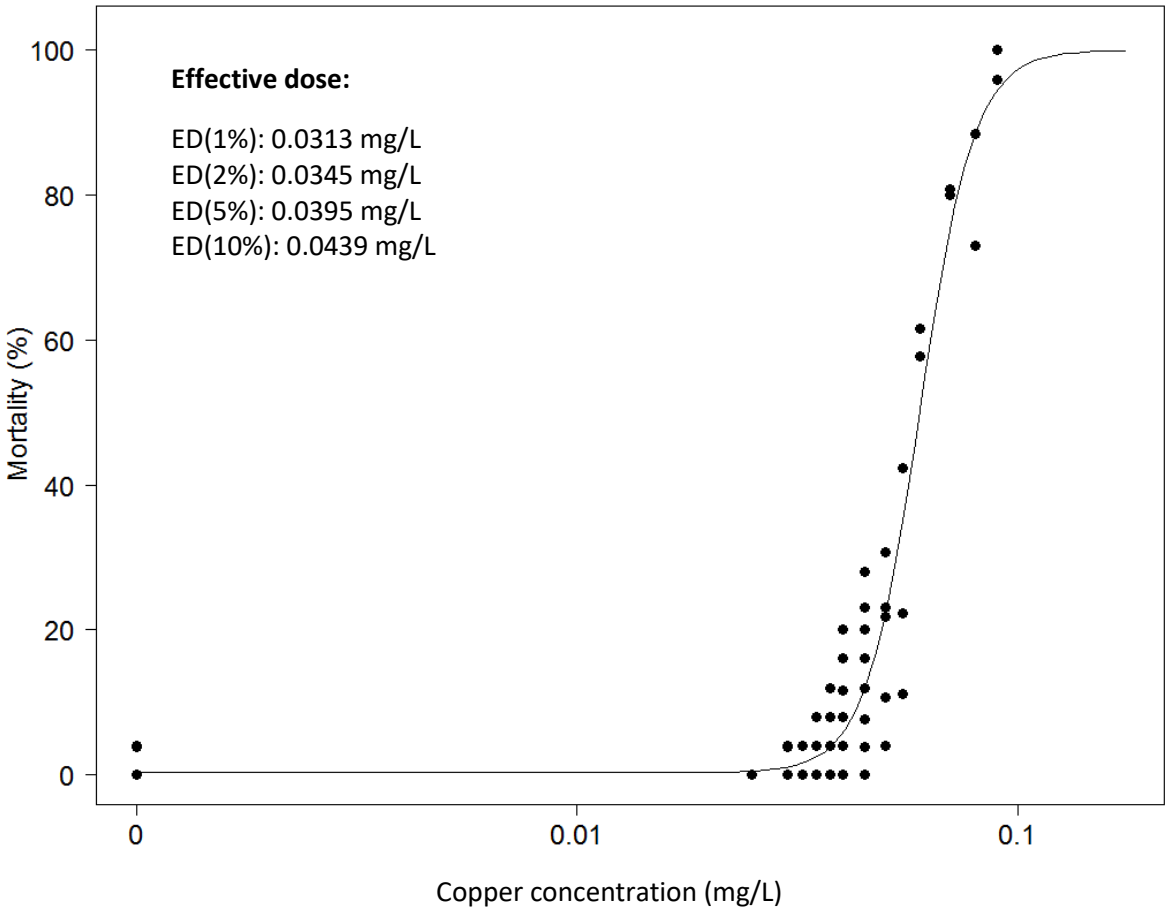


Figure 2: *Preliminary mortality curve for F0 zebrafish embryos exposed to a range of copper concentrations (0, 0.025, 0.03, 0.0325, 0.035, 0.0375, 0.04, 0.045, 0.05, 0.055, 0.06, 0.07, 0.08, and 0.09 mg/L Cu) from 0.5-24.5hpf (n= 3-75 replicate tanks per concentration containing 25 embryos; total no. tanks = 191). A log-logistic model fitted to the mortality data in R predicted effective doses of Cu for the following target mortality rates: 1%: 0.0313mg/L; 2%: 0.0345mg/L; 5%: 0.0395mg/L; and 10%: 0.0439mg/L.*

3.2.2. Generation of pre-exposed and naive zebrafish populations

After 24hrs of exposure to 0 and 0.0325mg/L Cu, an independent two-sample t-test showed no significant difference between mortality rates for each population (p=1). Mean mortality after 24hrs of exposure was 0.33% ± 0.013% (SE) in both control and copper exposed tanks (figure 3A).

A significant effect of exposure was demonstrated by a significant delay in hatching for copper-exposed embryos compared to control embryos after 96hrs of exposure (p=3.35E-14). Mean hatching rate was 90.02% ± 3.27% for unexposed control embryos and 0.42% ±0.02% in copper-exposed embryos (figure 3B).

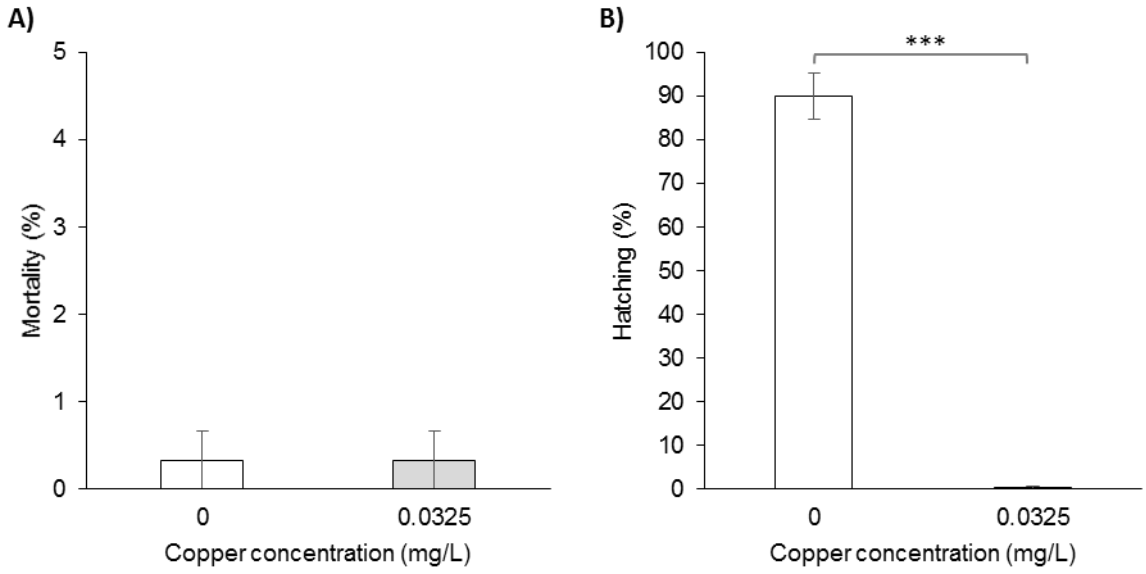


Figure 3*: A) Average mortality after 24hrs of exposure and B) average hatching rate after 96hrs of exposure observed in zebrafish embryos exposed to 0 and 0.0325 mg/L copper during embryogenesis from 0.5-96hpf (n=12 replicate exposure tanks per treatment containing 20-25 embryos per tank). Statistical comparisons were conducted using an independent two-sample t-test in R. All data are presented as mean ± SEM. Asterisks indicate significant differences compared to the control population (*** p<0.001). There was no significant difference in mortality after 24hrs between control and copper-exposed embryos (p=1). After 96hrs, hatching was significantly reduced in embryos exposed to copper (p=3.35E-14).*

After being raised in control conditions, the pre-exposed population (exposed to copper during embryogenesis) and the naïve population (maintained in control conditions during embryogenesis) showed no significant difference in survival to 50dpf (naïve mean mortality: 8.34% ±0.16%, pre-exposed mean mortality: 8.83% ±0.26%, p=0.86)(figure 4).

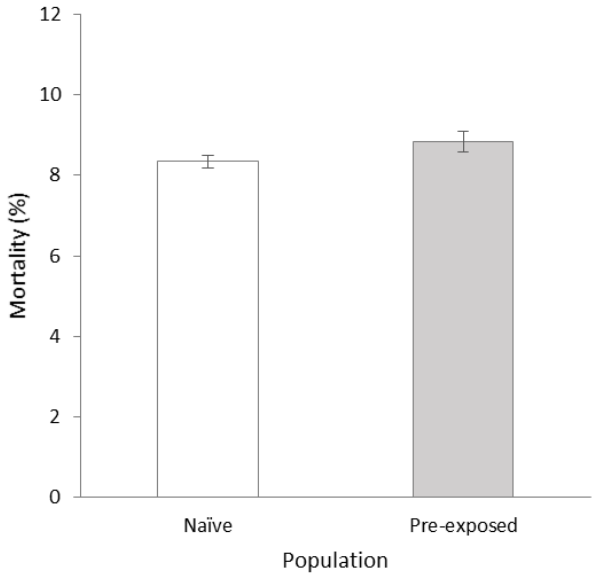


Figure 4: *Average mortality of naïve and pre-exposed fish at 50dpf following maintenance in control conditions (n = 5-6 replicate exposure tanks per treatment containing ~30 fish; one tank from the naïve population was lost due to water supply failure). Statistical comparisons were conducted using an independent two-sample t-test in R. All data are presented as mean ± SEM. Asterisks indicate significant differences compared to the control population. There was no significant difference in mortality at 50dpf between the naïve and pre-exposed population (p=0.86).*

F0 fecundity and fertilisation success also showed no significant difference between the pre-exposed and naïve population. Mean fertilisation success was 98.163% ±0.730% (SEM) for naïve fish compared 95.736% ± 1.45% (SEM) for pre-exposed fish (p=0.142)(figure 5A). Mean fecundity (expressed as number of eggs per female) was 107.3 ±11.6 (SEM) eggs for naïve fish compared to 109.4 ±20.1 (SEM) eggs for pre-exposed fish (p=0.929)(figure 5B).

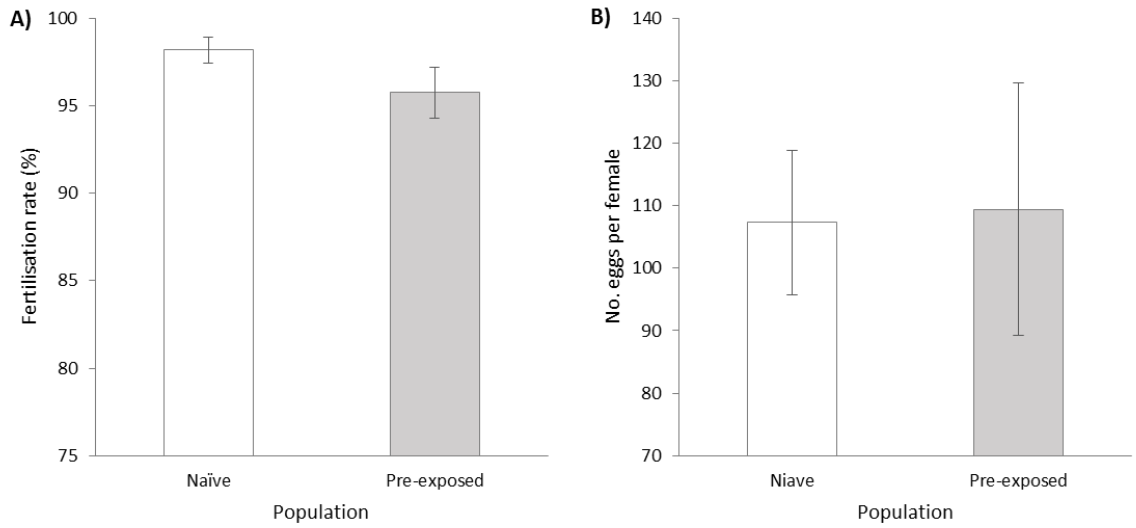


Figure 5: *A) Average fertilisation success and B) average fecundity (expressed as number of eggs per female) for F0 adult zebrafish from the naive population (white) and the pre-exposed population (grey), exposed to 0.0325mg/L copper during embryogenesis (n= 10 spawning chambers of 2:2 male:female per population). Fish in all test chambers spawned successfully, except one chamber from the pre-exposed population. This was included in fecundity calculations and excluded from calculation of fertilisation success. Statistical comparisons using two-tailed Student's t-Test revealed no significant difference in fertilisation success (p=0.142) or fecundity (p=0.929) between the two populations. All data are presented as mean ± SEM.*

3.2.3. Effects of pre-exposure of F0 fish on copper tolerance in F1 embryos

Copper induced mortality in both the pre-exposed and control populations, and dose was a significant explanatory variable of mortality after 24h of exposure (p=2.2e-16). Population of origin had no significant effect on mortality, and was removed from the model during model simplification (figure 6).

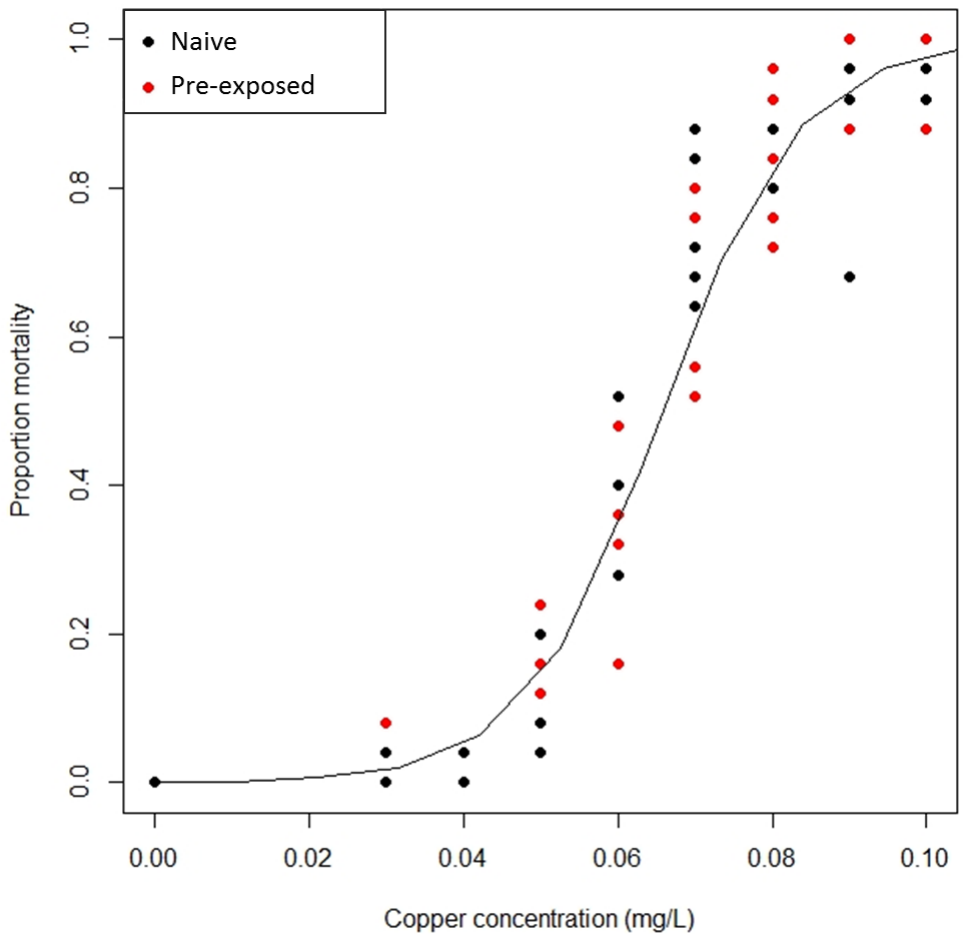


Figure 6: *Embryo mortality curve for F1 zebrafish embryos from both the naive and copper pre-exposed populations (n = 5 replicate tanks per dose per population). Embryos were exposed continuously to copper (0, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1mg/L Cu) from 0.5-96.5hpf. Mortality was assessed at 24.5hpf, after 24hrs of exposure; and is represented in the graph. Black and red symbols represent replicate tanks containing 20 F1 embryos from the naïve and copper pre-exposed zebrafish populations, respectively. The line represents the best fit model for the data, calculated using a generalized linear model with a quasibinomial error structure in R, modelling the relationships between exposure concentration (during the re-exposure period), population of origin (naïve or pre-exposed to copper during the F0 generation), and a population of origin/exposure concentration interaction. During model simplification to produce the minimum adequate model (MAM), population of origin and the population of origin/exposure concentration interaction were found to have no explanatory power and were removed from the model. Only exposure concentration was found to have a significant effect on mortality (F=975.64, p=2.2e-16). This is represented by a single line indicating no significant difference between populations.*

4. Discussion:

Here we report evidence that early life exposure to copper in a model fish species – the three-spined stickleback – leads to increased tolerance to future copper exposures up to the F2 generation, but not the F3 generation. Previous data from Laing *et al.* (Laing, 2017) has shown that pre-exposure of stickleback to copper during early life caused F0 fish to accumulate high levels of copper in their tissues in later life. This copper accumulation did not occur in F1 adults. Together, these findings support the hypothesis that this tolerance is potentially underpinned by maternal effects which led to secondary exposure of the germ cells that gave rise to the tolerant F1 and F2 generation, but not the F3 generation. In contrast with the tolerance exhibited by F1 and F2 embryos, we observed a transient decrease in copper tolerance in F3 embryos from the pre-exposed population, possibly pointing to secondary inherited effects of copper pre-exposure that had been masked by maternal effects in the F1 and F2 generation. Transgenerational inheritance of epigenetic alterations is one potential explanation for the decreased tolerance emerging in the F3 generation. These findings highlight the potential for the environment experienced during early development to influence response to environmental stressors in future generations, potentially through several simultaneous mechanisms resulting in varying effects across generations. However, these effects were not conserved in zebrafish after a similar early life exposure to copper, indicating that the mechanisms underpinning these effects likely differ between species. The variation in response between fish species highlights the challenge that such effects pose to environmental risk assessment, which often uses species, such as the zebrafish, as models to characterise risk for large groups of species, which are assumed to have similar responses to the same toxicants.

4.1. Increased copper tolerance in the unexposed F1 and F2 generations caused by F0 pre-exposure was not maintained in the F3 generation, which conversely exhibited reduced tolerance

Previous work by Laing *et al.* (Laing, 2017) revealed that pre-exposure of F0 stickleback to low level copper during embryogenesis led to significantly increased tolerance to copper exposure during embryogenesis in their F1 offspring; and this was maintained in the F2 generation. Here I tested whether the tolerance to

copper observed up to the F2 generation was maintained in subsequent generations and found that not only was this tolerance abolished in the F3 generation, but it was reversed. F3 embryos from the pre-exposed population exhibited a transient decrease in tolerance compared to the naïve population during the early stages embryogenesis, followed by attenuation of this effect by the end of embryogenesis.

Comparisons of the experimental data shown here with studies in the literature is difficult due to the differences in experimental design. Studies investigating multigenerational effects of toxic chemicals on future tolerance come mainly from invertebrate studies and often continuously expose multiple generations (Bossuyt and Janssen, 2004; Muyssen and Janssen, 2004; Kille *et al.*, 2013; Plautz and Salice, 2013; Zhou *et al.*, 2016). While it is important to investigate the cumulative effects of chemical exposures across multiple generations, which could have important implications for wild populations experiencing long-term pollution, it is difficult to untangle the mechanisms behind these cumulative effects, which could involve a mixture of physiological, parental, epigenetic, and genetic mechanisms. This is particularly important for studies investigating acquired tolerance to exposure. Many of these studies involve exposure of multiple generations before testing the tolerance of subsequent unexposed generations (Zhou *et al.*, 2016). Where exposure causes significant mortality or reproductive disruption in earlier generations, there is a high chance of causing selection for tolerant genotypes, which is difficult to untangle from other contributing mechanisms when genetic sequencing is not conducted alongside other analyses.

In our experiment, we ensured that the mortality caused by copper exposure in the F0 generation was less than 1.5%, in order to guard against genetic selection. It is possible that reproductive output may have differed between populations, with more tolerant females producing eggs more frequently, for example; which might lead to genetic selection. It was not possible to assess this for individual females when housed in colonies, whilst maintaining a constant egg supply for mortality assessment. However, fertilisation success remained above 98% in the F3 generation from both the control and pre-exposed populations, suggesting that it is unlikely that reproductive output was impaired by F0 copper pre-exposure.

Lack of inheritance to the F3 generation suggests that this increased tolerance to copper exposure observed in F1 and F2 embryos is not due to transgenerational epigenetic inheritance, as there would have to be some mechanism by which epigenetic changes were faithfully transmitted in the germline to the F1 and F2 generation but were lost or removed during transmission to the F3 generation. Similarly, the transience of these effects, and the care taken during breeding of each generation to avoid genetic selection, also makes it unlikely that genetic alterations can explain the copper tolerant phenotype.

The most likely hypothesis to explain the inherited copper tolerance in the F1 and F2 generations, which became reversed in the F3 generation, is that the tolerance is mediated by parental effects that last up to the F2 generation. Exposure could affect many non-genetic parental contributions to F1 and F2 offspring. It is thought that the majority of non-genetic cytoplasmic inheritance occurs via the maternal line. A variety of maternally transferred factors accumulated in oocytes during oogenesis can influence the development, physiology and fitness of offspring, including: nutrients, proteins, lipids, specific enzymes and hormones, maternal transcripts, and environmental chemicals; and transfer of these factors is known to be affected by the maternal environment (Mousseau and Fox, 1998). For example, Ruuskanen *et al.* found that sub-lethal copper exposure of adult stickleback led to alterations in maternally deposited thyroid hormones in oocytes (Ruuskanen, Mottola and Anttila, 2019). An example of increased metal tolerance in offspring as a result of altered deposition of maternal factors into oocytes was described by Lin *et al.* (2000), who observed induction of cadmium tolerance in tilapia larvae after maternal exposure. They found significantly higher levels of metallothionein (*mt*) mRNA in the oocytes of pre-treated females and the resulting larvae, and hypothesise that increased maternal transfer of metallothionein mRNAs to offspring led to protection from subsequent cadmium exposure by allowing earlier synthesis of metallothionein in embryos (Lin, Hsu and Hwang, 2000). A similar protective effect was seen by Peake et al., who observed increased copper tolerance in fathead minnow larvae following sublethal maternal exposure to 100µg/L of copper for 5 days, and hypothesised that similar maternal effects were responsible (Peake *et al.*, 2004). Altered maternal transfer of protective factors, including maternal transcripts, enzymes, or hormones, could be responsible for the protective effects on F1 and F2 stickleback embryos observed in our study.

However, no difference in *mt* transcription was observed between pre-exposed and naïve F0 adults (see chapter 3), suggesting that increased maternal transfer of *mt* mRNAs is not responsible for F1 and F2 tolerance. However, RNA sequencing was conducted for the F0 male gill only. Although we cannot rule out the possibility that *mt* regulation differs between the gonad and gill; the fact that pre-exposure to copper did not cause differential transcription of *mt* in the gill, an organ that plays a direct role in copper uptake, suggests that no significant change would be expected in other organs, such as the gonad, but it is difficult to be conclusive given the differences in gene regulation between cell types. Work by Munkittrick and Dixon (1988), who observed induction of copper tolerance in F1 offspring of white suckers (*Catostomus commersonii*) following pre-exposure of F0 females only, indicated that a yolk-associated factor was a likely mechanism by which tolerance was transmitted, as the tolerance in F1 offspring was found to be attenuated over time, being lost by the time larvae were capable of independent feeding (Munkittrick and Dixon, 1988). Differential transmission of a variety of factors in gametes as a result of altered gametogenesis in the F0 and F1 adult gonad could be playing a role in the altered tolerance observed in F1 and F2 offspring in our study.

Previous analysis of the copper burden in tissues of adult F0 fish by Laing *et al.* revealed that copper burden was significantly higher in the tissues of fish pre-exposed to copper during early life compared to naïve fish. Copper burden of the gonad was not measured at the time. However, increased copper burden in all other tissues measured, including fold increases of 2.14, 1.42, and 2.54 in the gill, liver, and muscle, respectively, indicates a widespread increase in copper storage in later life as a result of pre-exposure (Laing, 2017)(chapter 3, figure 2). I hypothesise that this increase may also have been present in the gonad, and this likely led to deposition of copper in gametes, resulting in increased copper burden in subsequent F1 embryos. Maternal transfer of environmental chemicals in gametes is a well-established mechanism by which parental exposure can indirectly impact externally developing offspring (Chen *et al.*, 2016). Metals have been shown to bind to vitellogenin - a major proportion of the oocyte (Ghosh and Thomas, 1995). This is an important mechanism for transfer of essential metals to developing offspring, but can also result in transfer of toxic concentrations of metals from adult to embryo (Ghosh and Thomas, 1995). There are many

examples in the literature demonstrating transfer of metals at elevated concentrations in gametes as a result of parental exposure, including: chromium (Chen *et al.*, 2016); zinc (Lacoue-Labarthe *et al.*, 2008; Weng and Wang, 2014); magnesium (Lacoue-Labarthe *et al.*, 2008); selenium (Covington *et al.*, 2018); and copper (Weng and Wang, 2014). These include studies that also observed increased metal tolerance in offspring of exposed adults following metal transfer. For example, Weng and Wang (2014) observed maternal transfer of zinc and copper from pre-exposed oysters to their larvae, and increased resistance of larvae from pre-exposed parents to subsequent exposures; both of which were correlated with metal concentration in the tissue of adults (Weng and Wang, 2014). Though this was observed in wild oyster populations, and so a genetic component is possible, high gene flow between populations makes this less likely. They also consider that the increased metallothionein content of larvae, which they hypothesise is expressed in larvae in response to maternally transferred metal and underpins their increased resistance, could potentially be a result of maternal transfer of either the metallothionein protein itself or maternal transcripts encoding it (Weng and Wang, 2014). A similar pattern of maternal metal transfer and increased offspring tolerance was observed in lab-reared tilapia after parental exposure to cadmium (Wu, Lin and Yang, 2008). However, other examples suggest that metal transfer is not always the driving mechanism. A study in fathead minnow observed induced copper tolerance in F1 offspring following maternal exposure, but reported no significant increase in copper content of the larvae of pre-exposed females compared to controls (Sellin and Kolok, 2006). This suggests that parental transfer of copper ions may not be the cause of induced tolerance in offspring, or may be one of several contributing mechanisms.

Uptake of copper into F0 embryos during the initial exposure and maternal deposition of copper into F1 embryos will have resulted in indirect exposure of germ cells within both F0 and F1 embryos during early development, coinciding with a period of epigenetic reprogramming occurring during primordial germ cell (PGC) specification. Epigenetic reprogramming in PGCs of mammals is well documented, especially for the methylome (Hackett and Azim Surani, 2013). Though less is known about the dynamics of the epigenome during this period in fish, recent evidence confirms the existence of a reprogramming window during PGC specification, and gives some insight into the extent of this reprogramming,

which appears to be less extensive than in mammals but variable between species (Ortega-Recalde *et al.*, 2019; Skvortsova *et al.*, 2019; Wang and Bhandari, 2019). Epigenetic reprogramming windows represent a window of susceptibility when the epigenome is more malleable and vulnerable to environmentally induced change (Ly, Chan and Trasler, 2015). Changes during this window have the potential to persist into later life and influence development and physiology of the individual (Hanson and Skinner, 2016). Early life copper exposure in the F0 generation and accumulation of copper in their adult tissues is likely to have resulted in F0 and F1 embryos containing elevated copper levels compared to controls. The germ cells developing in the potentially copper rich environment inside F0 and F1 embryos then gave rise the copper resistant F1 and F2 generations. The first generation that was not descended from germ cells potentially exposed to copper while developing inside a copper-rich embryo is the F3 generation, which did not inherit copper resistance. This supports the hypothesis that the tolerance observed in the F1 and F2 generations is caused by indirect exposure due to maternal transfer of copper into gametes. It also suggests that stable epigenetic alterations in germ cells as a result of indirect exposure during embryogenesis of the parent may persist during early development and result in physiological differences in larvae from the pre-exposed population which increased their tolerance for subsequent copper exposure.

Another possible explanation is inheritance of an altered microbiome. Though embryos are thought to develop in a sterile environment inside the chorion (Phelps *et al.*, 2017), they soon acquire a microbiome on external surfaces during the larval stage (Pratte *et al.*, 2018; Talwar *et al.*, 2018). The microbiome of surfaces in contact with the external environment, including the gut and gill – which are also sites of metal uptake; are thought to be heavily influenced by the microbiota of their environment (Pratte *et al.*, 2018; Talwar *et al.*, 2018). It is possible that exposure of F0 fish during early life, including the period immediately after hatching, may have caused alterations in the microbiome at sites involved in metal uptake or excretion, and that these alterations may have been transmitted to larvae in subsequent generations through contact with adult tank water and adherence to the chorion. Studies have indicated that the microbiome of fish changes with life stage, and becomes more influenced by host-associated factors later in life (Pratte *et al.*, 2018; Talwar *et al.*, 2018). Multigenerational inheritance

of a distinct microbiome cannot be ruled out as a contributing factor, but it is also possible that if tolerant generations were found to have a distinct microbiome from controls and intolerant generations that this could also be a consequence of altered host physiology, and not a cause.

In contrast with the F1 and F2 generation, embryos in the F3 generation were less tolerant to copper than those originating from the naïve population. This differential response suggests that different mechanisms are underpinning the effects seen in F1 and F2 embryos compared to F3 embryos. The maternal effects hypothesised to be driving altered tolerance of the F1 and F2 generations, described above, would theoretically end after the F2 generation – the last generation of fish to originate from germ cells affected by altered environment inside the F0 gonad and developing F1 embryos.

One possible explanation for the emergence of this toxic effect in the F3 generation is transgenerational epigenetic inheritance. Deleterious epigenetic changes laid down during direct or indirect exposure of a previous generation may have been inherited by subsequent generations, but masked by protective maternal effects in F1 and F2 fish. Examples of transgenerational inheritance of environmentally-induced epigenetic changes are rare and controversial.

In mammals, two rounds of extensive epigenetic reprogramming of the methylome are known to occur. The first occurs in somatic cells soon after fertilisation, and the second occurs in developing PGCs, involving global demethylation to achieve cell pluripotency (Tanae *et al.*, 2012; Seisenberger *et al.*, 2013; Susiarjo *et al.*, 2013). These reprogramming windows are thought to provide an effective barrier against transmission of epimutations except at a limited number of regions which escape this reprogramming, such as imprinted genes and intracisternal A particles (Lane *et al.*, 2003; Morgan *et al.*, 2005).

In contrast, both of these epigenetic reprogramming windows have been found to differ in zebrafish. During somatic reprogramming soon after fertilisation, the methylome undergoes less extensive reprogramming compared to mammals, in which the maternal methylome is demethylated and reprogrammed to resemble the paternal methylome prior to zygotic genome activation, resulting in inheritance of the paternal methylome in a process thought to be driven by placeholder nucleosomes which occupy and preserve regions of hypomethylation (L Jiang *et*

al., 2013; Potok *et al.*, 2013; Murphy *et al.*, 2018). Additionally, it has recently been independently confirmed in two studies that the second window of global epigenetic reprogramming occurring in developing mammalian PGCs does not occur in zebrafish, which experience only localised reprogramming of DNA methylation (Ortega-Recalde *et al.*, 2019; Skvortsova *et al.*, 2019). This suggests that there may be greater opportunity for transgenerational inheritance of epialleles or epimutations and associated phenotypes via germline transmission in zebrafish. However, Wang and Bhandari (2019) have confirmed that extensive reprogramming of the methylome, similar to that in mammals, does occur in the developing PGCs of medaka (Wang and Bhandari, 2019), highlighting that epigenetic reprogramming events can differ greatly even between teleost species. Epigenetic reprogramming of the methylome has not been studied in stickleback, so the extent to which alterations of the methylome might be transmitted across generations is unknown. There has also been little research into potential reprogramming events occurring for other epigenetic factors, such as histones or regulatory RNAs, for any species; which may also represent a barrier to transmission of epigenetically-driven phenotypes across generations.

If the hypothesised copper-induced epigenetic changes initially occurred in the F2 generation as germ cells inside copper-laden F1 embryos, these changes would have to persist through at least one and potentially two rounds of epigenetic reprogramming in the soma and germline during F2 development. If these changes occurred during exposure of an earlier generation, they would have had to persist through these reprograming windows in multiple generations.

Transgenerational epigenetic inheritance is a controversial and poorly understood phenomenon, whose study is at high risk of confounding factors. As would be expected of a population only a few generations removed from the wild, there was a high amount of individual variation in the response of these stickleback to copper challenge. In order to be confident in the reliability of the effects we observed and overcome this variation, independent replication and investigation of the molecular mechanism of these observations would be required.

4.2. Multigenerational effects of early life exposure observed in stickleback were not conserved in zebrafish

The inherited copper tolerance observed in F1 and F2 stickleback embryos as a result of F0 pre-exposure was found not to be conserved in zebrafish. No significant difference was found in susceptibility of F1 zebrafish embryos originating from the pre-exposed and naïve populations.

The fact that the increased tolerance observed in stickleback was not conserved in zebrafish after a similar exposure regime suggests that the effects of exposure during early life are likely to differ between species. Zebrafish and three-spined stickleback diverged 60-80 million years ago (Pfister *et al.*, 2007), leaving ample opportunity for differences in metal handling mechanisms and epigenetic functioning to evolve.

A hypothesised mechanisms behind the increased tolerance observed in F1 and F2 stickleback was an altered physiological response of pre-exposed fish to adult copper exposure leading to an altered environment within the F0 gonad and F1 embryo that could impact the development of PGCs giving rise to the F1 and F2 generation. The absence of altered offspring tolerance in zebrafish suggests that the physiological response of pre-exposed F0 zebrafish may differ to that of stickleback. This could be due to differences in both genetically encoded metal response pathways or in the epigenetic pathways that regulate them.

Stickleback are characteristically fast to adapt to new environments or changes in environmental conditions through both genetic adaptation (Terekhanova *et al.*, 2014) and high phenotypic plasticity (Morris *et al.*, 2014). A notable example is their ability to quickly adapt to different salinities, leading to distinct ecotypes occupying both the marine and freshwater environment. Artemov *et al.* have shown that, as well as long-term genetic adaptation to differing salinities, an immediate epigenetic response may also be involved in their ability to adapt to new salinities in the short-term (Artemov *et al.*, 2017). After transfer of marine stickleback to freshwater, they observed partial convergence of their DNA methylation profile to that of freshwater stickleback, including for genes potentially involved in osmoregulation (Artemov *et al.*, 2017). Interestingly, the genes showing epigenetic and genetic changes were not the same, suggesting that

epigenetic changes may act as a complementary adaptive mechanism alongside genetic selection (Artemov *et al.*, 2017). A comparison between the epigenetic and genetic variation of the marine and freshwater ecotypes revealed that stickleback genetically adapted to freshwater showed lower genetic variation than marine stickleback, but higher epigenetic variation, suggesting that increased epigenetic variation may act as a compensatory mechanism in stickleback to allow phenotypic plasticity in the absence of genetic variation (Artemov *et al.*, 2017). It is possible that stickleback have evolved a greater capacity for epigenetic adaptation than zebrafish, and were thus able to adapt more quickly to copper exposure, while zebrafish were not.

Epigenetic mechanisms can differ greatly between species. Epigenetic reprogramming events in somatic cells and germ cells during early development have been shown to differ substantially between mammals and zebrafish (Lan Jiang *et al.*, 2013; Potok *et al.*, 2013; Ortega-Recalde *et al.*, 2019; Skvortsova *et al.*, 2019). However, epigenetic machinery can also differ even among teleost species, as discussed previously for medaka and zebrafish (Wang and Bhandari, 2019). Epigenetic reprogramming events have not been studied in stickleback, and may differ from those in zebrafish, medaka, and other teleost and vertebrate species. For example, though much putative enzyme activity was found to be conserved, a phylogenetic comparison of chromatin-modifying enzymes between species found many differences between teleost species with respect to number of genes, gene loss/duplication events, identified conserved domains, and putative protein lengths (Fellous and Shama, 2019). Differences in somatic or germline reprogramming windows between zebrafish and stickleback could explain the lack of altered tolerance in zebrafish; which in stickleback, may have been driven by both altered physiology of F0 adults due to exposure during somatic reprogramming, and altered physiology of offspring due to exogenous influences during PGC reprogramming. In addition, in contrast to stickleback, which are thought to exhibit genetic sex determination similar to the XX female/XY male system (Peichel *et al.*, 2004), sex determination in zebrafish is more complex, and is thought to include both genetic and environmental components (Santos, Luzio and Coimbra, 2017). The process of sex determination in zebrafish begins at 25dpf and is complete by 60dpf, during which initial development of an immature gonad can be followed by sex reversal during final differentiation into a mature

ovary or testis. The occurrence of a sexual differentiation period later in development, and the ability of environmental factors to influence sex determination when applied during embryogenesis and/or during sexual differentiation at 25-60dpf, implies that additional epigenetic reprogramming programming windows may exist in the zebrafish gonad later in development after embryogenesis (Santos, Luzio and Coimbra, 2017). If similar epigenetic alterations had occurred in zebrafish PGCs during embryonic copper exposure that have been hypothesised for stickleback, it is possible that an additional period of reprogramming occurring later in development could act as a barrier for maintenance of any epigenetic alterations occurring during embryogenesis. The lack of conserved effects between stickleback and zebrafish in this study, in addition to the many studies demonstrating differences in epigenetic regulation between fish species, suggests that activity of genes regulating epigenetic modification observed in model fish species might not always be applicable to other related species (Fellous and Shama, 2019).

Epigenetic adaptation is also restricted by the genetic resources of an organism. The stickleback used in this study originated from a wild population inhabiting the river Erme in the south west of England. Zebrafish, on the other hand, are native to Asia, and this particular population originated from an inbred strain reared in the laboratory for many generations. Though they share many similarities, these species have evolved in very different environments under different selective pressures, and thus may have acquired genes or alleles related to copper handling that the zebrafish do not possess, affording them greater opportunity for phenotypic plasticity through differential expression of these genes.

Although the absence of induced alterations in copper tolerance in zebrafish in this study suggests that they respond differently to early life copper exposure, practical constraints prevented exact replication of the conditions experienced by stickleback. Preliminary data indicated that mortality could not be used as an endpoint for assessing the effects of early life copper exposure when generating the pre-exposed and naïve F0 populations; as the sharpness of the response in zebrafish made it difficult to choose a concentration that would not cause overt mortality and risk genetic selection occurring. Hatching rate was therefore substituted for mortality as an endpoint to assess the effect of embryonic copper exposure in F0 fish. Similar to stickleback, great care was taken to prevent genetic

selection, including use of hatching as an F0 endpoint, and assessment of survival, fecundity and fertilisation success at 50dpf, which showed no significant difference between populations. Although a very significant inhibition of hatching rate was observed in F0 fish, indicating a significant physiological effect of copper, and the concentration chosen was just below the concentration observed to cause mortality; we cannot rule out the possibility that a higher concentration of copper may have reproduced the effects observed in stickleback.

4.3. Implications

The potential for effects persisting across multiple generations after exposure of a single generation could have important implications for both the protection of wild populations under threat from environmental pollution and for the use of animals in research.

This lab-reared stickleback population were able to mature by 9 months under optimal conditions, however, maturation in wild populations often takes longer, ranging from 1-3 years for females; and they usually inhabit strongly seasonal environments, leading to annual reproduction (Baker *et al.*, 2008). Intragenerational epigenetic adaptation in fish spawned into a polluted environment could be a highly beneficial strategy for quick adaptation to a new environmental challenge, as is hypothesised for the F0 generation in chapter 3 of this thesis. Since the generation time of stickleback could be as little as 1 year, inheritance of tolerance to subsequent generations could give offspring a survival advantage if they are likely to grow under similar environmental conditions. However, in a quickly changing environment, long-term or indefinite commitment to this adaptive change in a species that can reproduce for up to 3 years could be detrimental if the pollutant is no longer present, as this adaptive phenotype is likely to incur a cost (Auld, Agrawal and Relyea, 2010). Development of a strategy that infers tolerance to stressors experienced by the parent through transient parental effects – as is hypothesised for the F1 and F2 generations in this study – might therefore be beneficial. Under continued exposure the population would retain a copper tolerant phenotype that protects them during early development, as well as in later life. However, should copper pollution stop, the tolerant phenotype would disappear in two generations, along with its potential associated costs. This could be highly beneficial for wild populations encountering new or fluctuating toxic

exposures by providing them with the phenotypic plasticity to quickly adapt to new challenges and increase the fitness of their offspring without committing to a costly strategy that would no longer be beneficial should the environment change in the future.

However, the study also suggests that exposure to copper may cause adverse effects that could persist in future generations, potentially through transgenerational epigenetic inheritance, resulting in long term harm to wild populations even after removal of harmful pollutants. The latency of these effects, which only emerged three generations after the initial exposure, are also a cause for concern, as it demonstrates the need for studies of the long-term, latent multigenerational consequences of exposure that are not currently part of routine chemical risk assessment, if we are to understand the long term risks of chemical exposures for populations.

There are also implications for use of animals in research. This study demonstrates that exposure history of organisms can have a significant impact on their response to future exposures for multiple generations, and that some of these effects may not emerge until several generations after the initial exposure. It is therefore essential that animals used in research come from a population whose exposure history is known. It would also be advisable to breed animals whose exposure history is not certain to at least the F3 generation before using them in experiments, in order to ensure that transient parental effects are lost, the population's phenotype is stabilised, and that it is truly naïve to the conditions being tested. It also raises an important issue for the study of heritable effects of chemical exposure. It is often assumed that in externally developing species transgenerational epigenetic inheritance can be demonstrated in the F2 generation following embryonic exposure of the F0. However, when studying toxic exposure to chemicals that are also essential trace elements this is not necessarily true, due to altered metabolism and transmission of the chemical to offspring. The potential for transgenerational epigenetic effects implied by decreased tolerance in the naive F3 generation are more problematic, as these could possibly persist for an unknown number of generations, and be difficult to detect or mitigate in experimental populations.

The fact that these effects observed in stickleback were not conserved in zebrafish also highlights the potential for multigenerational response to chemical exposure to differ between species, and even among teleosts. This could pose a challenge for inclusion of multigenerational testing into environmental risk assessment, which often uses data from model species to predict effects in other wild species. Included in these differences are potential differences in epigenetic functioning between species. Currently, investigation of epigenetic functioning in vertebrates has mainly been limited to a small number of model species. If we are to understand the influence of environment on the epigenome and the consequences of this for wild species experiencing environmental stressors, study of basic epigenetic functioning must be extended to include a wider range of species.

4.4. Limitations

Though these data suggest that damage from copper exposure could be inherited for up to 3 generations, further investigation would be required to validate these observations, and to explore the mechanisms and potential longevity of this inheritance. For studies reporting potential transgenerational epigenetic inheritance of an altered phenotype this would include identification of specific epimutations in each generation following the initial F0 exposure and a mechanism by which these epimutations are stably transmitted through the germline; or evidence of induction of heritable epigenetic instability causing propagation of inappropriate changes in gene expression in each affected generation, as has been hypothesised by Blake and Watson (2016). This would need to be followed by confirmation that identified epimutations cause the inherited phenotype via gene knockouts or manipulation of the epigenome to reproduce the phenotype. Care should also be taken to investigate the many other potential mechanisms that may explain phenomena which might otherwise be attributed to transgenerational epigenetic effects, or that might be acting alongside epigenetic mechanisms; which could include inheritance of an altered microbiome or genetic selection.

As discussed above, exact replication of experimental conditions for zebrafish and stickleback was not possible. When drawing conclusions from this study about conservation of effects between species, the possibility that these effects may

have been replicated in zebrafish under different exposure conditions should be considered.

5. Conclusions

This study provides evidence that exposure of a model fish species to a common aquatic pollutant may cause effects lasting for at least 3 subsequent generations in the absence of continued exposure. We hypothesise that they are underpinned by a mixture of mechanisms, including parental effects, intragenerational epigenetic effects, and potential transgenerational epigenetic inheritance, resulting in opposing effects that differed between generations. This study suggests that early life exposure to low level copper can infer multigenerational tolerance in offspring that is limited to two generations, after which latent toxic effects become apparent. This study highlights the need for caution in studies of multigenerational effects, in which multiple mechanisms may be occurring simultaneously. It also indicates that unless analysis is undertaken to confirm that maternal transfer of chemicals or their physiological effects is not occurring, transmission of an altered phenotype to at least the F3 generation is required before tentative attribution to a transgenerational epigenetic mechanism of inheritance. This study also suggests that these effects may be species specific, as they were not conserved in zebrafish – another well-studied teleost model. This could potentially be explained by differences in physiological metal response pathways and/or differences in epigenetic regulation. However, a better understanding of the fundamental epigenetic regulation of both species must be established before this hypothesis can be addressed.

Chapter 5

General Discussion

1. General discussion

Anthropogenic stressors are known to occur in the environment continuously and/or intermittently over long periods of time (Gordon, Mantel and Muller, 2012), including anthropogenic pollutants, other stressful abiotic environmental conditions, and their interactions (Raptis, Van Vliet and Pfister, 2016; Cook *et al.*, 2018; Hamilton *et al.*, 2018; Hasler *et al.*, 2018; Estévez *et al.*, 2019; UN, 2019; Häder *et al.*, 2020). In order to accurately assess and mitigate the risk that these intermittent stressors pose to wildlife, we must determine how organisms respond to intermittent exposures over their lifetimes.

There are several known mechanisms by which the exposure history of an individual or population may alter their sensitivity to future exposure. As discussed in chapter 1, there are many examples of altered stressor tolerance in wildlife at the population level due to irreversible genetic adaptation acquired over generational timescales; as well as various examples of short-term tolerance to stressors induced within a generation via transient physiological acclimation to continuous exposure (Demmig-Adams *et al.*, 2008). Despite this, exposure history is given little consideration in environmental risk assessment, which still generally relies on toxicity data generated from naïve laboratory-reared populations of model organisms following a single continuous exposure and assumes that effects of a given environmental exposure will be consistent for different populations of a given species (Janssen *et al.*, 2000; Barata *et al.*, 2002; Millward and Klerks, 2002).

There is now evidence that non-genetic mechanisms may also play a role in long-lasting modification of stressor tolerance following sublethal exposure, including epigenetic modifications, parental effects, and their interactions (Wang, Liu and Sun, 2017). In comparison to genetic adaptation and transient physiological acclimation, epigenetic modification is both inducible within a single generation and able to facilitate adaptations that are both reversible and intermediately stable across the lifetime of an organism or potentially several generations – qualities that may be advantageous under intermittent exposure scenarios.

However, epigenetic mechanisms are still poorly understood, and little is known about how the functioning of the epigenome differs between species and life

stages, with the majority of *in vivo* studies being restricted to a small number of model organisms, limiting applicability to wild species. Additionally, though the role of epigenetic alterations in causing long-lasting changes in phenotype is well-established, particularly in developmental origins of disease (Bollati and Baccarelli, 2010; Hoffman, Reynolds and Hardy, 2017; Barouki *et al.*, 2018); few studies have conducted repeated exposures to investigate their potential role in altering response to environmental exposures as a result of exposure history.

There is also controversy over the longevity of environmentally induced epigenetic alterations. Intragenerational epigenetic studies frequently report alterations in gene regulation immediately following an exposure, which gives little information on their persistence and cannot distinguish between transient changes in gene expression and mitotically stable epigenetic alterations. There is also intense debate over the existence of transgenerational epigenetic inheritance, particularly in vertebrates, for which there are few studies that have accounted for possible confounding factors and provide mechanistic evidence (Horsthemke, 2018). The potential for altered tolerance to be transmitted across generations via epigenetic inheritance could have substantial implications for wildlife populations living in polluted environments, and requires further study.

Multigenerational epigenetic studies are also complicated by parental effects. Though the role of parental effects in altered offspring tolerance has been well-documented, few studies have focussed on altered chemical tolerance, with the majority investigating response to other environmental and parental factors such as temperature, nutrition and stress (Kuzawa, 2005; Wells, 2007; Kappeler and Meaney, 2010; Vaag *et al.*, 2012; Shama *et al.*, 2014; Fuiman and Perez, 2015; Nitzan *et al.*, 2016; Sopinka *et al.*, 2017; Adrian-Kalchhauser *et al.*, 2018; Wassink *et al.*, 2020). There has also been relatively little work on the interaction between epigenetic and parental effects on stressor tolerance in offspring; and epigenetic studies frequently discuss parental effects only in the context of their role as confounding factors rather than as contributing mechanisms of interest. It is likely that multigenerational influences of exposure history on future tolerance involve the interaction of several simultaneous mechanisms, which are currently difficult to unravel.

In this thesis I aimed to address some of these knowledge gaps by investigating how historic exposure to two common environmental pollutants may alter the physiological and molecular response of fish upon re-exposure later in life and in subsequent generations. To do so, I investigated whether pre-exposure of adults to Bisphenol A caused altered responses during subsequent exposures in breeding zebrafish and what the consequences were for the resistance of their offspring (Chapter 2). Further, I investigated whether pre-exposure to copper during embryogenesis resulted in long-lasting alterations in tolerance within a single generation (chapter 3) and up to the 3rd non-exposed generation (chapter 4) in the stickleback. I then repeated the copper exposure in a different model species (the zebrafish) in order to examine whether the findings reported for the stickleback were conserved across teleost species (chapter 4). A summary of the key findings discussed within a broader context are detailed below.

2. Exposure of adult zebrafish to a model environmental oestrogen mimic, Bisphenol A, caused alterations in their molecular responses upon re-exposure and increased the tolerance of their offspring to subsequent exposure.

In chapter 2, I demonstrated that pre-exposure to a toxic chemical during adult life can cause an altered response upon re-exposure, and modify the susceptibility of subsequent offspring.

I found that offspring produced from adult fish which had received a pre-exposure and subsequent re-exposure to low-level BPA were no more susceptible to BPA exposure during embryogenesis than offspring of unexposed control colonies; and were in fact more tolerant to BPA than offspring of naïve adult fish that were experiencing BPA for the first time immediately prior to breeding.

This indicated that pre-exposure of adults had likely resulted in an altered physiological response upon re-exposure, potentially underpinned by stable epigenetic alterations acquired during pre-exposure, that inferred protection to offspring by altering conditions in the gonad during gametogenesis. Transcriptional analysis in the ovary revealed altered expression of *amh* – a gene encoding anti-Mullerian hormone – which is involved in folliculogenesis.

These findings are consistent with previous studies showing that parental exposure to environmental stressors can cause adaptive changes in physiology of offspring through altered accumulation of cytoplasmic factors in gametes, including hormones, enzymes, antioxidants, parental transcripts and environmental chemicals or their metabolites (Kuzawa, 2005; Wells, 2007; Kappeler and Meaney, 2010; Bonduriansky, Crean and Day, 2012; Weng and Wang, 2014; English *et al.*, 2015). However, the majority of such studies focus on environmental or innate factors, such as temperature, nutrition and stress. This study adds to the evidence base for the ability parental effects to facilitate physiological adaptation in offspring against toxic chemicals following parental exposure, for which few studies exist outside of toxic metals.

The majority of previous studies of parental effects on stressor tolerance in offspring are also limited to the effects of parental environment immediately prior to reproduction or, where there is a degree of parental care, during the perinatal period. This study's novel repeated exposure approach demonstrated that exposure history of the parent can influence the response of the parent to future exposure leading up to reproduction, and impact parentally-driven modification of offspring physiology, with consequences for their tolerance during early life.

3. Exposure to copper during embryogenesis caused alterations in global transcriptional responses in adult fish

Having established that adult exposures can influence the outcomes of subsequent exposures during a short time frame of 3 weeks, I then asked the question of whether including the period when epigenetic reprogramming putatively occurs would have a significant effect on global transcriptome responsiveness upon subsequent exposure later in life. To explore this question I used the three-spined stickleback as a model organism to investigate whether pre-exposure to copper during early life would result in an altered transcriptional response upon re-exposure in adult fish compared to naïve fish being exposed for the first time.

Strikingly, despite significantly greater copper accumulation in tissues across the 9 month depuration period following early life pre-exposure, there was almost no significant difference between the transcriptome of pre-exposed and naïve fish.

However, upon exposure to copper as adults, both populations exhibited a differential transcriptional response; but despite a greater increase in copper accumulation in the gills of pre-exposed fish compared to naïve fish, this transcriptional response was stronger in naïve fish, which showed evidence of increased oxidative stress, DNA repair and epithelial thickening. This suggests that pre-exposed fish may have experienced less toxicity and disturbance of homeostasis upon exposure to copper compared to naïve fish, requiring a lower transcriptional response to restore the balance.

Differential transcription of ion transporter genes also suggested differences in the effects of copper exposure on ion homeostasis and acid-base regulation depending upon exposure history. Of note, transcription of genes encoding the Na^+/K^+-ATPase – a critical ion transporter involved in osmoregulation in the gills, that is known to be inhibited by copper – was significantly upregulated in pre-exposed fish, compared to downregulation in naïve fish. This suggests that pre-exposed fish may have an increased capacity for compensatory response against disruption of ion homeostasis, protecting them against copper-induced inhibition of osmoregulation.

Although many examples of genetic adaptation and physiological acclimation to copper have been reported in fish in the literature, very few studies have provided evidence for persistent non-genetic adaptation to copper, which I hypothesise is likely caused by persistent alterations to the epigenome; and should be explored in further studies.

Here I employed a novel experimental design in which long lasting effects of embryonic exposures were tested by measuring the responsiveness of populations upon re-exposure. An important finding was that a differential transcriptional response between populations was only observed after re-exposure to copper, indicating that rather than causing persistent up- or down-regulation of genes, pre-exposure likely caused changes in regulatory processes such that upon re-exposure the responsiveness of pre-exposed fish was altered. As this approach has not been readily taken in studies reporting non-genetic adaptation to chemical stressors, it is possible that long-lasting changes in responsiveness may have been missed in other studies which reported attenuation of physiological effects observed after a single exposure.

4. Copper tolerance induced by embryonic exposure is reversible and not conserved across species

In the final part of my thesis, I investigated whether embryonic exposures to copper during embryogenesis, encompassing the period of putative epigenetic reprogramming, caused long-lasting tolerance in future generations. Further, I addressed the fundamental question of whether such tolerance is conserved across fish species.

In order to address the first question, I utilised populations of stickleback that had been generated during a previous study and for which an approximately 2-fold increase in copper tolerance had been observed in unexposed F1 and F2 offspring (Laing, 2017), and I examined whether this effect was inherited in the F3 generation.

I found that the increased tolerance observed in F1 and F2 offspring of F0 stickleback pre-exposed to copper during embryogenesis was not inherited to the F3 generation, which was in fact less tolerant to copper exposure during embryogenesis compared to control fish. This is an important finding, demonstrating the reversible nature of the tolerance acquired by the pre-exposed populations.

This indicated that the increased tolerance observed in F1 and F2 offspring was likely a result of parental effects. One potential mechanism for this is maternal transfer of copper from the F0 to the F1 generation. If this was the case, the germ cells that would give rise to the F2 generation would develop within F1 embryos in a copper rich environment, resulting in the indirect exposure of the F2 generation to copper via parental effects. However, contribution of other parental effects, such as altered accumulation of a variety of parentally-transferred factors in gametes, such as transcripts, enzymes, and signalling molecules, which would impact the developmental environment of F1 embryos and their germ cells, should also be considered. Further investigation to identify the specific parental effects driving this inherited tolerance is required.

Most studies investigating potential transgenerational inheritance of environmentally-induced epigenetic alterations in oviparous species, in which embryonic development occurs outside the body of the parents, interpret

maintenance of an altered phenotype to the F2 generation following embryonic exposure of the F0 generation as evidence for transgenerational epigenetic inheritance. Here I have demonstrated that where there is potential for parental transfer of chemicals or altered physiology of adults, this might influence the developmental environment of F1 embryos and result in direct impact on the development of PGCs giving rise to the F2 generation. To avoid these confounding factors, I propose that epigenetic experiments must continue until at least the F3 generation before concluding that this is evidence for transgenerational inheritance, as this would be the first generation that does not arise from germ cells directly impacted by the F0 exposure.

However, the significantly reduced copper tolerance of F3 embryos from the pre-exposed population compared to the naïve population suggested that pre-exposure of F0 fish during embryogenesis may have led to transgenerational inheritance of adverse epigenetic alterations induced during the F0 pre-exposure, whose effects were previously masked in the F1 and F2 generation by protective parental effects. Although this observation is in agreement with other studies in the literature which have reported transgenerational epigenetic inheritance of adverse effects in fish as a result of historic exposure of previous generations to toxic chemicals; a greater weight of evidence would be required before this hypothesis can be confirmed.

When the experiment was repeated with a similar exposure regime in zebrafish, no inherited copper tolerance was observed in F1 offspring. This suggests that the multigenerational effects of copper exposure are not necessarily conserved between teleost species, at least for the conditions tested in my experiment. This has important implications for the applicability of research in model organisms to other species; and therefore, impacts the feasibility for inclusion of multigenerational effects driven by parental or epigenetic effects into risk assessment, which relies on use of toxicity data in model organisms to predict the impact of environmental stressors on groups of similar species.

5. Implications of these findings

The findings of my thesis are of fundamental importance to understanding the risk posed by environmental stressors to wild populations of fish. More broadly, they can help to inform how we measure toxicity, and suggests that repeated exposures should be included in chemical testing. In addition, these findings inform on approaches for accounting for differential responsiveness and susceptibility of different populations and species, according to their exposure history. As well as being relevant to ecotoxicological risk assessment and regulation of chemicals, the findings presented in this thesis also have broader implications for conservation practices, sourcing of animals used in research, and epigenetic study design outside of toxicology.

Based on my findings, I propose novel principles for the experimental design of studies searching for long lasting epigenetic effects. My findings demonstrate that as well as persistent phenotypic changes that remain measurable in the absence of the initial environmental exposure, long-lasting epigenetic alterations can also result in persistent changes in gene regulation whose effects can only be observed upon re-exposure, manifesting as altered responsiveness later in life. Such studies should include re-exposure to the environmental factor in question in their design in order to detect these potential changes in responsiveness. Altered responsiveness – which could include both adaptive and maladaptive changes – could have profound implications for the fitness of organisms experiencing intermittent environmental exposures at both the individual and population level, and has likely been overlooked in previous epigenetic studies, the majority of which lack a re-exposure step.

The differential gene expression observed within a generation upon re-exposure to the chemical toxicants tested in my studies, along with increased tolerance in subsequent generations, suggests the induction of an adaptive epigenetic priming effect in pre-exposed individuals. This could serve as an important adaptive mechanism for animals living in rapidly changing environments, with advantages over acclimation – in which capacity for tolerance is quickly lost after removal of the stressor, or genetic adaptation – which can result in permanent commitment to an irreversible and costly adaptive change, and can lead to loss of genetic

diversity. In comparison, epigenetic adaptation that primes genes for rapid differential expression might allow animals to quickly develop the capacity for tolerance to stressors within a generation and maintain this capacity in later life, whilst limiting costs until the adaptive response is stimulated in the future. In particular, the reversibility of epigenetic adaptations may be a key element in the robustness of some species living in highly variable environments by preserving their potential for adaptation against other stressors should the environment change.

As well as adaptive epigenetic changes, this thesis presents evidence for maladaptive epigenetic effects that may be inherited across multiple generations, and that these effects may not become apparent until several generations after the initial exposure. This has important implications for environmental risk assessment, and advocates for the inclusion epigenetic toxicity in chemical risk assessment, including testing for the appearance of latent epigenetic effects in subsequent generations.

The layers of interacting parental and epigenetic effects hypothesised to underpin the various multigenerational alterations in tolerance observed in three subsequent generations after early life pre-exposure of stickleback to copper in chapters 3 and 4 further highlights the importance of considering all possible contributing mechanisms and confounding factors when designing experiments, so that they can either be measured or accounted for when investigating potential epigenetic effects caused by environmental stressors. Studies investigating potential transgenerational effects of environmental exposures are particularly vulnerable to parental effects as confounding factors, and, as discussed above, should continue analysis up to at least the F3 generation if these factors cannot be eliminated or accounted for.

The observation that effects of exposure can potentially last up to at least the F3 generation also highlights the broader issue of considering the provenance and exposure history of animals used in scientific research. Though numerous studies have demonstrated the potential impact of factors such as genetic background and husbandry conditions experienced by experimental animals on research outcomes (Stokes, 2004; Brown *et al.*, 2009; Schauwecker, 2011), epigenetic integrity has received little attention. In recognition of these factors, initiatives such

as the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments) and equivalents, have been developed to improve reporting of *in vivo* experiments, including source, husbandry and genetic background of animals in order to promote reproducibility (National Research Council (US) Institute for Laboratory Animal Research, 2011; du Sert *et al.*, 2020). However, there is still a lack of consideration and effective strategy for reporting and mitigating against epigenetic variation in experimental animals.

Epigenetic variability between experimental animals from different sources due to varying environmental conditions could include both permanent differences in epigenotype between populations and transient effects of source environment on epigenetic background. The potential for stock animals to harbour long-lasting epigenetic and phenotypic alterations as a result of source environment that persist for multiple generations in subsequently founded laboratory populations, but are ultimately transient once maintained in their new environment, could result in unstable epigenetic backgrounds during the first few generations of newly established laboratory populations.

Though initiatives such as the ARRIVE guidelines have currently been in place for over a decade, their impact has been deemed limited (du Sert *et al.*, 2020); with continued under-reporting of animal background. It is also common for animals to be sourced with little information on the background factors described in such guidelines being provided, making it difficult to ensure that animals do not have an exposure history that could impact experimental outcomes if these have caused long-lasting epigenetic or physiological effects.

The findings of this thesis advocate for breeding of newly sourced experimental animals to at least the F3 generation, to reduce the likelihood that effects of known or unknown historic exposures will interfere with experimental work through, for example, interference with physiological pathways common to the experimental condition tested. This would also allow for stabilisation of potentially unstable epigenetic states in newly established experimental stocks while the population acclimatises to the particular environmental conditions of the receiving laboratory.

The finding that exposure history can modify future responsiveness in animals also has implications for conservation practices and farming. For example, re-stocking programs are a common conservation practice in aquatic environments.

Such programs may need to consider how fish are raised and mimic the rearing conditions to those of the environments they are intended to re-stock (including for water chemistry and even for the presence of stressors) in order to ensure that the environment during their development equips individuals with appropriate responsiveness to the stressors they might encounter upon release and give them the best chance of survival and reproduction. A similar approach could potentially be applied to improve yields in aquaculture, by matching conditions in hatcheries with those in adult rearing pens, where conditions are often suboptimal in comparison.

6. Limitations

It was not possible to demonstrate the epigenetic mechanisms or confirm causality hypothesised in my experiments within the timeframe and resource limitations of this thesis. This would be an important element of proving evidence for an epigenetic basis for the reported effects. This is a common shortfall in studies of this nature, which limits the strength of interpretation of this data. However, this does not detract from the fact that pronounced, long-lasting and reversible effects were observed.

It is still very challenging to attribute causation of certain effects to epigenetic mechanisms because, unlike genetic studies, targeted and precise addition and removal of epigenetic modifications is not readily achievable for all epigenetic mechanisms. Currently, such technology is mainly limited to the CRISPR-Cas9 system, discussed later in this chapter. This is a considerable limitation in the field, as well as this thesis, that future technological developments need to resolve.

It was also not possible to investigate the numerous other non-genetic mechanisms that might underpin some of these observations, including the variety of possible parentally-transferred factors. This is also a common limitation of multigenerational studies in ecotoxicology, due to time and resource limitations. However, in lieu of being able to investigate them, identifying these possible mechanisms and taking them into consideration during interpretation is important; and something that this thesis has attempted to be thorough in.

Although it is a powerful tool, use of RNA sequencing in this thesis also comes with its limitations. As is the case for all high throughput transcriptomic studies, RNA sequencing is prone to false positive and false negative results at the level of individual genes. Differential expression of single genes should therefore be interpreted cautiously. To overcome this, I performed gene ontology (GO) enrichment analysis and based my interpretation mainly on expression changes in gene sets rather than individual genes, which are much more robust against statistical errors and allow interpretation along meaningful biological themes.

Another limitation is the confirmation of whether the effects observed are conserved in other species and for other chemicals, which this thesis alone cannot provide. The importance of this was revealed by the lack of concordance between the effects of copper exposure in stickleback and zebrafish in chapter 4. However, given that for each species only a single concentration was tested, this does not exclude the potential for different results to be obtained at different concentrations; but highlights the issues that not all findings will be conserved across the diversity of teleosts and environmental conditions. This in itself is an important conclusion, as it has direct impacts on the difficulties that might be involved in incorporating such effects into environmental risk assessment.

7. Priorities for future research

Interpretation of epigenetic studies has been made difficult by the gaps in our understanding of how the epigenome functions and the exact mechanisms by which environmental factors can induce epigenetic changes, including the specific molecular factors that target these alterations. Most research reporting alteration of the epigenome and phenotype following exposure to environmental stressors can only make correlative associations between these measures. The epigenome consists of a complicated network of numerous interacting molecular factors, but the interaction between these factors is not well-understood, and it is becoming increasingly clear that a variety of epigenetic mechanisms likely work in concert to direct changes in gene expression (Atlasi and Stunnenberg, 2017; Cuvier and Fierz, 2017; Klemm, Shipony and Greenleaf, 2019). As changes in gene expression may rely on several simultaneous epigenetic mechanisms, measurement of alterations in a single epigenetic factor – a common approach

given the current expense of epigenomic technologies – may not be sufficient to detect functional epigenetic changes underpinning altered phenotypes (Klemm, Shipony and Greenleaf, 2019). As a result, inferring causal relationships between epigenetic changes and phenotypic outcomes remains a challenge. Further research is needed to investigate how the various components of epigenetic machinery function across the lifetime of an organism, including in reprogramming windows, how they interact with each other, and how they cause altered gene expression.

Establishing causality between changes to the epigenome and changes in gene expression will require new methods that facilitate specific manipulation or editing of the epigenome. Within the last decade the CRISPR-Cas9 system has been repurposed to allow direct epigenetic modification at specific genomic regions, through fusion with epigenome-modifying enzymes that can be targeted to alter epigenetic marks at specific regulatory regions of the genome (Dominguez, Lim and Qi, 2016). As this method, and similar technologies, advance and become available for wider use in research, knowledge gaps in the functioning of the epigenome can be addressed.

Similarly, it will be important to assess how epigenetic functioning differs between species. Currently, extensive analysis of the epigenome in vertebrates has been restricted to a small number of model species, such as humans, mice, and zebrafish; and important differences in epigenetic functioning between vertebrate species has already been identified (Head, 2014; Zhang, Hoshida and Sadler, 2016; Hanna, Demond and Kelsey, 2018). Epigenetic assessment of a broader range of species will be needed if the findings of epigenetic studies in fields such as ecotoxicology can be applied to environmental species not included in this small number of model organisms.

Greater understanding of the functioning of various components of the epigenome will be essential for future studies of transgenerational epigenetic inheritance, which remains controversial. Most studies currently lack mechanistic evidence behind reported observations, which would be easier to attain with development of technologies to experimentally manipulate the epigenome. Many are also criticised for failure to account for potential undetected genetic contributions to multigenerational phenomena. Cryptic genetic mutations could underlie

observations attributed to epigenetics in many studies (Heard and Martienssen, 2014). Altered sequence at regulatory elements of genes would have direct impacts on epigenetic factors closely associated with the DNA, such as the methylome and histone proteins. It could also affect binding of other epigenetic regulators such as non-coding RNAs and transcription factors by altering the epigenetic or genetic landscape at binding sites, or by altering chromatin conformation. As well as genetic alterations at genes of interest and their regulatory genomic regions, genetic changes at genes encoding epigenetic regulators themselves, could also produce changes in the epigenome that had a genetic origin. As sequencing costs fall, it will be possible for studies to generate sequence data to inform on genetic and epigenetic modifications for the same samples, to rule out genetic changes as confounding factors (Cavalli and Heard, 2019).

Reduced sequencing costs would also allow investigation of multiple epigenetic mechanisms in the same study. This would greatly improve epigenetic research, given that it has become increasingly clear that epigenetic phenomena may be driven by complex interactions of multiple epigenetic factors. Current studies that can only investigate a single mechanism due to cost restraints cannot capture the full mechanistic story that likely underlies phenotypic observations.

Future studies should also aim to investigate other non-genetic mechanisms that may contribute to multigenerational phenomena, including a variety of parental effects and impacts of alterations to the microbiome (Heard and Martienssen, 2014). Where these mechanisms cannot be investigated, their potential contribution should be carefully considered during interpretation of studies to prevent misattribution of effects to measured changes in other pathways.

The effects of historic exposure of organisms on future response both within a generation and across generations should be incorporated into environmental risk assessment. Failure to account for altered tolerance of populations according to their exposure history could lead to inaccurate assessment of the risk posed by anthropogenic stressors. As well as altered tolerance to subsequent exposures to the same stressors, there are examples that demonstrate that previous exposure to one stressor may also affect cross-tolerance to other stressors that share similar toxic response pathways (Dixon and Sprague, 1981; Plautz *et al.*, 2013;

Pestana *et al.*, 2016; Russo, Becker and Liess, 2018). The ability of simultaneous exposure to multiple stressors to cause interactive effects on toxic outcomes has been addressed in the field of mixture toxicology (Altenburger *et al.*, 2013). However, given that environmental chemicals can cause lasting changes in physiological pathways involved in response to toxic chemicals after the chemical stimulus is removed, these lasting changes could potentially alter the response to future exposure to other chemical stressors. These observations suggest that as well as assessing the effects of simultaneous exposure to chemical mixtures, research should also investigate the effect of sequential exposure to different toxic chemicals.

8. Conclusions

This thesis has demonstrated the ability of the exposure history of an organism to alter its response to environmental stressors in the future, both within and across generations, and across multiple timescales. Further, it has demonstrated that these effects are reversible and are not necessarily conserved across species. It implicates several potential non-genetic mechanisms in these phenomena, including parental, physiological, and epigenetic effects, and their interactions.

Some significant knowledge gaps remain, and I have identified further research into the basic functioning of the epigenome, the mechanisms by which environment influences the epigenome, and the extent of environmental influence on future stressor tolerance of populations as key research priorities in this area for the future.

The findings of this thesis have broad implications in topics ranging from environmental protection – including in design of chemical testing protocols, risk assessment, and conservation strategies – to reproducibility in animal research and study design in the field of epigenetics. Importantly, the ability demonstrated in this thesis for organisms to acquire rapid and reversible tolerance to stressors via epigenetic adaptation may partially explain the resilience of species currently observed in the face of unprecedented anthropogenic change, and give hope for their continued survival as future challenges such as climate change and anthropogenic pressures from increased human population inevitably intensify. In light of this, in the same way that genetic diversity is recognised by scientists and

policy makers as a key characteristic of populations that should be protected to preserve species' ability to adapt to these challenges, capacity for epigenetic adaptation should be given similar consideration as a biological characteristic to be managed and protected.

Supplementary Information

Sample	Well number	Total raw read pairs	Read pairs passing adapter trimming and quality filtering
Control_0	A1	17260062	17235402 (99.86%)
Copper_0	A2	15216204	15194976 (99.86%)
Control_0	A3	14070937	14054653 (99.88%)
Control_0	B1	14810948	14791485 (99.87%)
Copper_0	B2	13877414	13858791 (99.87%)
Copper_0.01	B3	14684089	14660718 (99.84%)
Control_0.01	C1	22776785	22755761 (99.91%)
Control_0.01	C2	14741878	14726327 (99.89%)
Control_0.01	C3	13520831	13504278 (99.88%)
Copper_0.01	D1	21720706	21694758 (99.88%)
Copper_0.01	D2	14296132	14275753 (99.86%)
Control_0	D3	14046237	14025016 (99.85%)
Copper_0	E1	14739668	14722631 (99.88%)
Copper_0	E2	14053982	14039945 (99.90%)
Copper_0.01	E3	20840838	20814837 (99.88%)
Control_0	F1	14338573	14323242 (99.89%)
Control_0.01	F2	20534728	20512133 (99.89%)
Copper_0	F3	14940887	14915394 (99.83%)
Copper_0.01	G1	14830760	14815048 (99.89%)
Copper_0	G2	14639796	14625309 (99.90%)
Control_0.01	G3	14130882	14112412 (99.87%)
Control_0.01	H1	14144320	14126827 (99.88%)
Copper_0.01	H2	14816817	14789797 (99.82%)
Control_0	H3	13793778	13776349 (99.87%)

Supplementary table 1 – Chapter 3: *Total number of raw read pairs generated for each sample during Illumina RNA sequencing, and number and percentage of read pairs retained after adapter trimming and quality filtering using Trim Galore v0.5.0 (Krueger, 2018)(with fastqc function and default parameters, minimum Phred quality score of 20, and removal of reads shorter than 20bp)) quantified using SeqKit v.12.1.0 (Shen et al., 2016).*

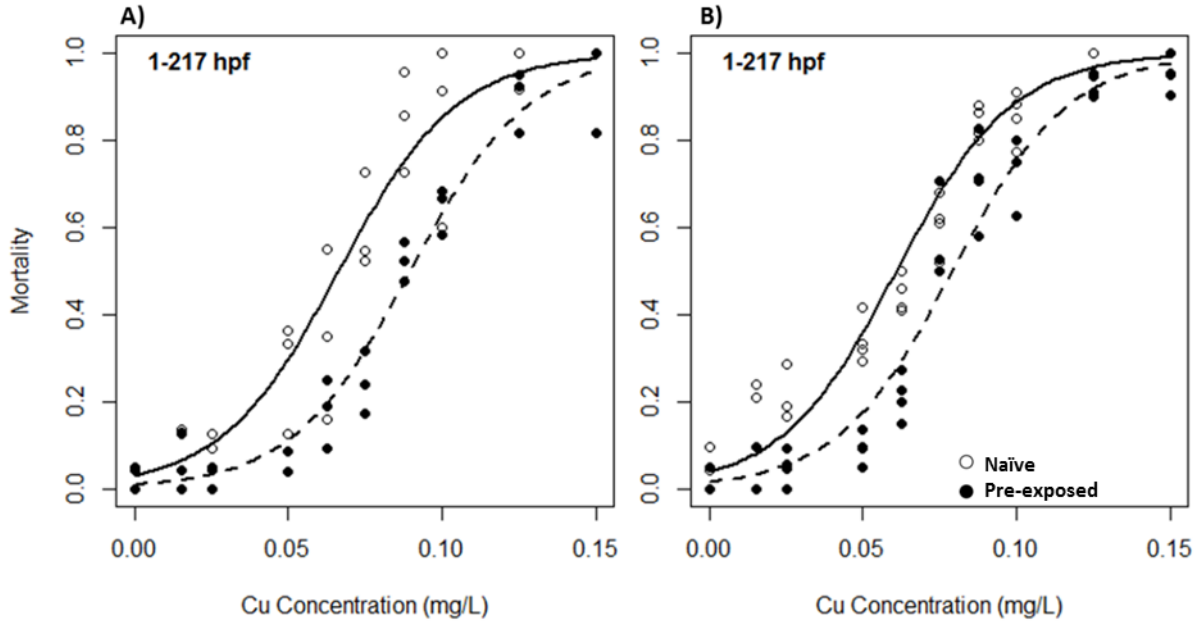
Biological process	Naïve population		Pre-exposed population	
	FDR	No. genes	FDR	No. genes
cell cycle	**3.52E-29**	**39**	**1.46E-06**	**14**
cell cycle process	**2.00E-26**	**30**	**1.31E-04**	**9**
DNA replication	**3.59E-21**	**20**		
mitotic cell cycle	**8.50E-20**	**23**	**2.37E-05**	**9**
chromosome segregation	**2.10E-16**	**15**	1.75E-03	5
mitotic cell cycle process	**1.58E-15**	**18**	3.34E-03	6
DNA metabolic process	**1.40E-13**	**24**		
cell division	**1.62E-12**	**14**	**5.40E-06**	**8**
microtubule-based process	**3.65E-11**	**22**	1.54E-02	8
regulation of cell cycle	**3.24E-10**	**17**	2.89E-02	6
cytoskeleton-dependent cytokinesis	9.97E-09	7	**1.82E-05**	**5**
actomyosin contractile ring organization	1.22E-04	3	**1.31E-04**	**3**
actomyosin contractile ring assembly	1.22E-04	3	**1.31E-04**	**3**
assembly of actomyosin apparatus involved in cytokinesis	1.22E-04	3	**1.31E-04**	**3**
cytokinetic process	1.22E-04	3	**1.31E-04**	**3**
cytokinesis	6.85E-10	9	**1.39E-04**	**5**

Cellular component	Naïve population		Pre-exposed population	
	FDR	No. genes	FDR	No. genes
chromosome, centromeric region	**1.79E-08**	**7**		
chromosomal region	**4.52E-06**	**7**		
non-membrane-bounded organelle	**6.67E-06**	**30**		
intracellular non-membrane-bounded organelle	**6.67E-06**	**30**		
nucleus	**2.17E-05**	**44**		
intracellular organelle	**3.79E-05**	**64**		
chromosome	**6.06E-05**	**14**		
organelle	**6.25E-05**	**64**		
condensin complex	**1.22E-04**	**3**	**1.54E-02**	**2**
microtubule cytoskeleton	**1.33E-04**	**12**		

Molecular function	Naïve population		Pre-exposed population	
	FDR	No. genes	FDR	No. genes
microtubule binding	**1.67E-10**	**15**	**7.95E-03**	**6**
microtubule motor activity	**4.17E-10**	**13**	**1.75E-03**	**6**

tubulin binding	**1.58E-09**	**15**	**1.29E-02**	**6**
nucleotide binding	**6.38E-09**	**53**	**2.55E-02**	**23**
motor activity	**5.74E-07**	**13**	**4.44E-03**	**7**
catalytic activity, acting on DNA	**4.34E-06**	**10**		
cytoskeletal protein binding	**6.41E-04**	**16**		
DNA-directed DNA polymerase activity	**2.34E-02**	**3**		
protein serine/threonine kinase activity	**2.42E-02**	**11**		
DNA polymerase activity	**2.78E-02**	**3**		

Supplementary table 2 – Chapter 3: *A comparison of the ten most significantly over-represented Gene Ontology terms in each GO category related to cell cycle and cytoskeleton for upregulated genes combined for the naïve and pre-exposed fish. The total combined GO terms are less than 20 where a population had less than 10 enriched GO terms for a category (all enriched terms are included) or where common terms appeared in the top 10 for both populations. Categories include: biological process, cellular component, and molecular function. Values for GO terms that appeared in the top ten most significantly over-represented terms for that population are in bold font. Values reported for each GO term include the false discovery rate (FDR) and the number of differentially expressed genes (threshold = p <0.05) contributing to the GO term (No. genes). Cells are coloured based on FDR values, with the darkest colour representing the lowest FDR.*



Supplementary figure 1 – Chapter 4*: Embryo mortality curves for A) F1 and B) F2 stickleback embryos from both the naïve and pre-exposed populations conducted by Laing et al (2017). Embryos were exposed continuously to copper (0, 0.015, 0.025, 0.05, 0.0625, 0.075, 0.0875, 0.1, 0.125, 0.15mg/L Cu) from 1-217hpf (n=3-4 replicate tanks per dose and per population). Black and white symbols represent replicate tanks from the pre-exposed and naïve populations, respectively. Lines represent the best fit model for the data calculated using generalized linear models in R. Two lines indicate a significant difference between populations. Significantly higher mortality was found for both F1 and F2 embryos from the naïve population compared to embryos from the pre-exposed population, with a significant effect of population of origin on mortality in F1 (p=1.39e-13) and F2 (p=7.43 e-12) embryos (see: Laing, 2017 for more information).*

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