Combining population genomics and transcriptomics to identify signatures of metal tolerance in brown trout inhabiting metal-polluted rivers

Running title: Metal tolerance in brown trout

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Abstract

Industrial pollution is a major factor responsible for the degradation of ecosystems but can also act as a driver of contemporary evolution. As a result of intense mining activity during the Industrial Revolution, several rivers across southwest England are polluted with high concentrations of metals. Despite the documented negative impacts of ongoing metal pollution, brown trout (*Salmo trutta* L.) survive and thrive in many of these metal-impacted rivers. We used population genomics, transcriptomics, and metal burdens to investigate the genomic and transcriptomic signatures of potential metal tolerance. RADseq of six populations (originating from three metal-impacted and three control rivers), identified strong genetic substructuring between metal-impacted populations, and selection signatures at 122

loci, including genes involved in metal homeostasis and oxidative stress. Measuring the tissue-metal burden (cadmium, copper, nickel, zinc) in metal-impacted and control trout populations, we identified significantly higher metals in metal-impacted trout sampled from the river, and after 11 days of depuration in control water. After depuration, we used RNAseq to quantify gene expression differences between metal-impacted and control trout, identifying 2,042 differentially expressed genes (DEGs) in the gill, and 311 DEGs in the liver. Transcriptomic signatures in the liver were nuanced, but in the gill, ion transport processes were enriched, alongside the over-expression of genes involved in metal homeostasis, and response to oxidative stress, hypoxia and xenobiotics. Our results demonstrate the complexity of metal tolerance in vertebrates and offer insights into the potential adaptive processes underpinning evolution to pollution in teleost fish.

Keywords: adaptation, freshwater, pollution, RADseq, RNAseq, pollution, toxic metals

Introduction

Pollution causes long-term environmental impacts with severe implications for biodiversity and human health (Schwarzenbach et al., 2010; Travis & Hester, 1991), yet growing evidence of tolerance to toxic chemicals has resulted in a greater awareness of pollution in mediating contemporary evolution (Hendry et al., 2017; Macnair, 1987; Smith & Bernatchez, 2008; Whitehead, 2014). Whilst organisms can respond to short-term stress via phenotypic plasticity (Hansen et al., 2012), understanding the mechanisms underpinning tolerance to chronic exposure requires consideration of long-term adaptive responses (Badyaev, 2005; Ghalambor et al., 2007). However, because pollutants are often not acutely toxic by design, have wide-ranging physiological effects, and occur in complex mixtures in the environment, dissecting the role of pollutants in mediating adaptive responses is a complex task, especially in natural populations (Whitehead et al., 2017).

Metals are naturally occurring elements but are found at significantly higher concentrations in ecosystems associated with certain human activities (mining, agriculture and industrial processes (Vareda et al., 2019)), resulting in toxic levels within aquatic environments, which in some cases far exceed the thresholds for mortality of many species (see Kumar et al., 2019). Metals exert their toxic effects through different mechanisms, including the production of reactive oxygen species (ROS), oxidative stress, DNA damage, and the disruption or inhibition of enzymes, proteins, and subcellular organelles (Hartwig et al., 2002; Valko et al., 2005; Vangronsveld & Clijsters, 1994). Essential metals, such as copper, iron, manganese and zinc, are crucial for life, acting as key components for various biological processes including development, energy metabolism and immunity, but can become toxic when threshold concentrations are exceeded (Chandrapalan & Kwong, 2021). On the other hand, non-essential metals, such as arsenic and cadmium, have no known biological function, and thus toxicity manifests when such metals are present in the environment in toxic forms at lower concentrations (Wood, 2011b). Therefore, for an organism to adapt to a metal-polluted environment, various physiological mechanisms aimed at balancing the necessity and toxicity of metals are required.

Over the last four decades, a wealth of information has been published on the impact of metals on fish including: discerning the role of different tissues in metal uptake, storage and detoxification (*e.g.*, Bradley & Morris, 1986; Eastwood & Couture, 2002); understanding the physiological processes regulating homeostasis and toxicity (see Shahjahan et al., 2022; Wood, 2011a, 2011b); and characterising the molecular mechanisms pertaining to metal homeostasis (Hamilton & Mehrle, 1986; Srikanth et al., 2013). However, very few studies have investigated the evolutionary signatures of metal tolerance. At the genomic level, whilst a few studies have evaluated the impact of metal pollution on neutral processes, such as population structure and genetic diversity (Bourret et al., 2008; Maes et al., 2005), studies addressing potential adaptive responses are rare (although see Hamilton et al., 2017; Laporte et al., 2016). Meanwhile, at the transcriptomic level, several studies have quantified differences in gene transcription between metal-impacted and control fish (Bélanger-Deschênes et al., 2013;

Coffin et al., 2022; Pierron et al., 2009), highlighting genes and pathways potentially involved in the mitigation of metal toxicity. However, measuring gene expression from individuals sampled directly from their native sites does not allow for a distinction between short-term responses to acute stress, versus responses to chronic stress (López-Maury et al., 2008). In this regard, constitutive gene expression, *i.e.*, the higher baseline expression of certain genes or pathways, can confer protection by providing a pre-emptive adaptive response (Hendry, 2016; Rivera et al., 2021). Generally, investigations aimed at better understanding the concerted role of the genome and transcriptome in underpinning metal tolerance in fish are lacking.

Brown trout (*Salmo trutta* L.) in the southwest of England provide one of the best purported cases of metal tolerance in aquatic environments. Mining in the area dates back to the Bronze Age (ca. 2500-800 BCE), and later, during the Industrial Revolution (1850-1900), the region dominated the global output of tin, copper and arsenic (Burt et al., 2014; Timberlake, 2017). Accordingly, the area has been designated as a UNESCO World Heritage Site (The Cornwall and West Devon Mining Landscape; https://www.cornishmining.org.uk/). The longevity and intensity of mining has resulted in elevated contemporary metal concentrations in several rivers in the region (Rainbow, 2020), and consequently, negative ecological and biological effects including a reduction or total extermination of aquatic life (Brown, 1977; Bryan et al., 1987; Hart et al., 2020). Yet brown trout thrive in many of these rivers, where they experience both chronic and seasonally-varying acute exposure to metals at biologically-relevant toxic concentrations known to be lethal to naïve salmonids (see Table S1). Trout from metal-impacted rivers were previously found to contain elevated tissue-metal burdens and different transcriptional profiles when compared to control fish sampled directly from the river (Uren Webster et al., 2013). Based on neutral genetic markers, trout from metal-impacted rivers were shown to be genetically differentiated from trout sampled from nearby control rivers, and displayed significantly reduced genetic diversity (Osmond et al., 2024; Paris et al., 2015). However, these previous studies demonstrated only short-term transcriptomic responses, and the impact of metal pollution on neutral processes. To investigate the signatures of potentially adaptive responses, genetic variation should be surveyed using genome-wide markers, and transcriptional responses should be quantified in fish outside of the metal-impacted environment.

Here, we use a combination of RADseq, RNAseq, and tissue-metal burdens, to investigate the signatures of potential long-term adaptation to metal pollution in wild brown trout. First, we use RADseq to genotype six populations (three metal-impacted and three control) to assess how metal pollution has shaped the neutral and non-neutral genetic make-up of metal-impacted populations. We hypothesised that if metals have caused population-level impacts, we would observe distinctive patterns of genetic variation in metal-impacted populations. We also reasoned that if metal pollution has driven an adaptive response, we should detect selection signatures at outlier loci related to metal handling. Second, we aimed to evidence and assess, if and how metals are absorbed into the tissues of metal-impacted brown trout. We address this by quantifying differences in metal burdens across five tissues (gill, liver, gut,

kidney, and muscle) between highly metal-impacted trout and trout from a matched control site, hypothesising that if metal-impacted trout are indeed affected by metals, they would show higher metal-tissue burdens. To refine our understanding of metal handling, we quantified tissue-burdens from individuals sampled directly from the river, and after an 11-day depuration in control water. Finally, to assess the role of transcriptional pathways in shaping long-term responses to metal pollution, we quantified gene expression differences between metal-impacted and control trout following the 11-day period of depuration in two key tissues known for their roles in metal uptake and detoxification (the gill and the liver). Integrating across these methodologies provides a more powerful approach to evidence adaptation to metals, allowing us to better understand the mechanisms underpinning evolution to pollution in wild fish.

Materials and Methods

2.1 Study Sites and Sampling

The Cornwall and West Devon mining region contains many small rivers affected by metal pollution. While many rivers are affected by high concentrations of metals, individual rivers are polluted with different mixtures of metal pollutants (Figure 1A). For clarity, we term trout sampled from these rivers as “metal-impacted”. To reduce confounding effects of biogeographic history, we sampled what we term “control” trout from rivers with lower concentrations of metals sampled within the same region. Due to the nature of the bedrock and extensive history of mining in the region, we note that control rivers may also have been historically impacted by metals, but today show comparatively lower metal concentrations (Table S2).

For the population genomics analyses, a total of n=120 trout were sampled from six rivers (n=20 per river; Figure 1B; Table S3). The largest adult fish (>150 mm fork-length) were sampled to ensure resident origin (and prolonged survival) in the metal-polluted rivers. Fish were anaesthetised using benzocaine (10g/100 mL ethanol) diluted 1:2000 in river water prior to adipose fin-clip removal. Tissue was stored in 95% EToH at 4°C prior to DNA extraction.

For the quantification of tissue burdens and transcriptomics analysis, trout (putative 0+) were sampled from the River Hayle (metal-impacted, n=26) and the River Fowey (metal-sensitive, n=26). Fish from the River Hayle were chosen as representative metal-impacted trout as they have been studied previously (Paris et al., 2015; Uren Webster et al., 2013), and also showed the strongest genetic signatures in our analyses. Trout from the River Fowey were selected as control fish since the river exhibits relatively low metal contamination and the most comparable water chemistry features (which strongly determine metal bioavailability) to those of the River Hayle (Table S4).

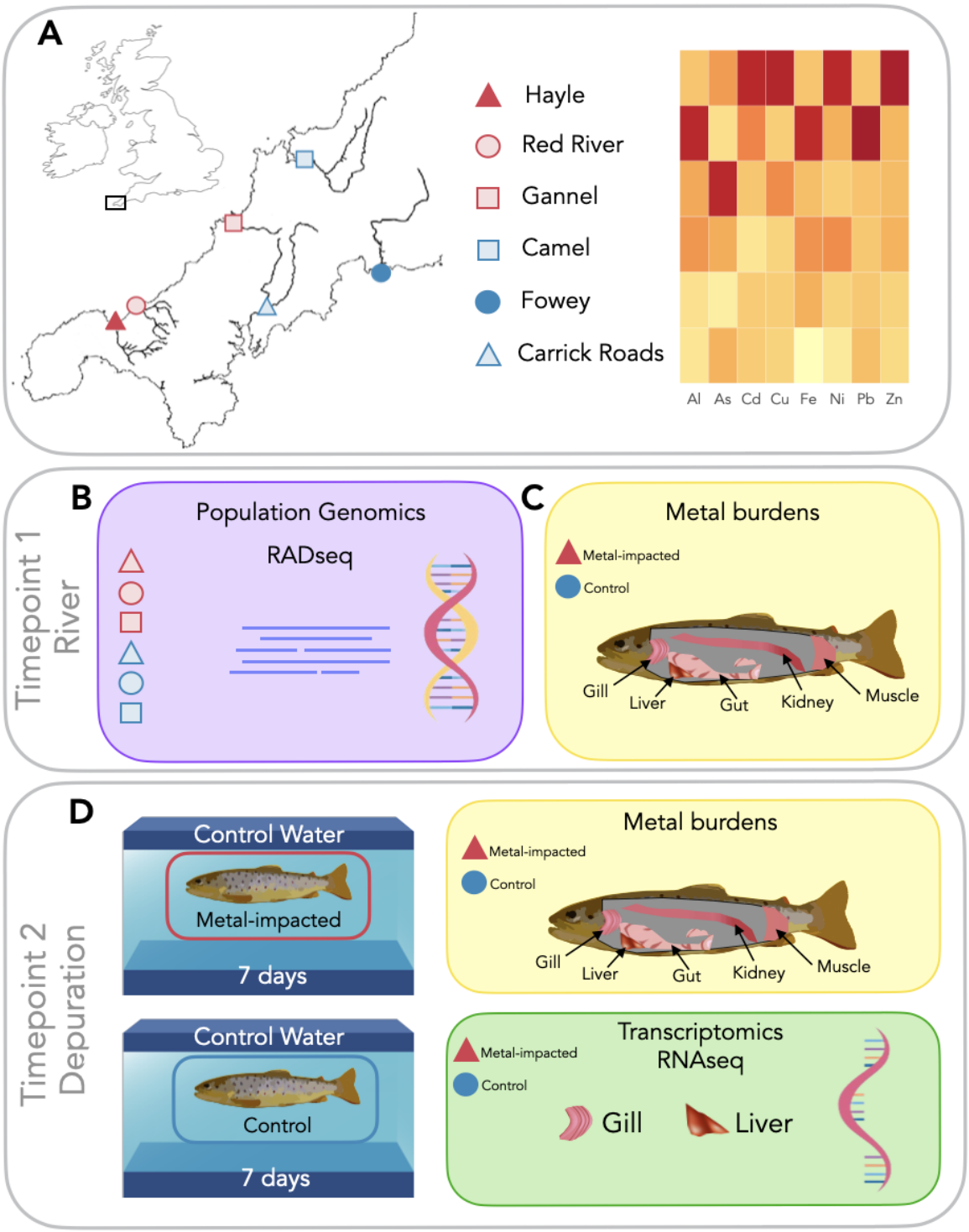

Figure 1. Schematic of collection sites and experimental design. (**A**) Location within the UK of the Cornwall and West Devon mining region and location of sampling sites. Metal-impacted sites are coloured in red and control sites are coloured in blue. Filled coloured shapes represent sites sampled for tissue-metal burden analysis before and after a period of depuration. The heatmap shows the scaled metal concentrations of each river (averaged over a 15-year period; full data available in Table S3) (**B**) Timepoint 1: River. For population genomics, trout were sampled from three metal contaminated rivers (the Hayle (red triangle; n=20); Red River (red circle; n=20); Gannel (red square; n=20)) and three control rivers the Camel (blue square; n=20); Fowey (blue circle; n=20) and Carrick Roads (blue triangle,

n=20)). (**C**) Timepoint 1: River. For tissue burden analysis, metal-impacted trout were sampled from the Hayle (red triangle; n=10) and control trout were sampled from the Fowey (blue circle; n=10). (**D**) Timepoint 2: Depuration. Trout were sampled after 11 days of depuration in control water for tissue burden analysis (n=10 per group) and transcriptomic analysis of the gill and liver using RNAseq (n=6 per group).

After sampling, trout were transported to the Aquatic Resources Centre, University of Exeter in water from their river of origin. Immediately on arrival, we randomly sampled n=10 metal-impacted and n=10 control fish to represent the status of individuals in the river: Timepoint 1: River (Figure 1C). After eleven days of depuration and acclimation to laboratory conditions (in control water), we randomly sampled n=10 metal-impacted and n=10 control fish for Timepoint 2: Depuration Figure 1D). Fish were humanely killed via overdose of benzocaine (0.5 g L^{-1}), followed by destruction of the brain. We recorded the wet weight and fork length and the condition factor was calculated (*K*) = [weight (g) x 100] / [fork length $(cm)^3$] before dissecting tissues for analysis. Due to the age of the fish, gonadal sex could not be reliably assessed, so to account for potential sex-related biases, additional muscle tissue was collected and stored in 95% EToH at -20°C for genetic sex determination (King & Stevens, 2020).

All fish were caught by electrofishing, following Environment Agency (UK) authorisation and relevant landowner permissions. Fish were humanely terminated following ASPA Home Office (UK) guidelines.

2.2 Laboratory depuration and acclimation: water chemistry and fish maintenance

Trout were maintained in the laboratory for an 11-day depuration period. Individuals were housed in 39 L glass tanks (n=13 individuals per tank). We created synthetic freshwater in the laboratory to mimic the water chemistry of the River Hayle (Minghetti et al., 2014) using salts of the following concentrations: NaCl 22.9 mg/L; $KHCO_3$ 12.8 mg/L; $MgSO_4$ 123.2 mg/L and $Ca(NO_3)_2$ 100.4 mg/L. Salts were made up to a 50x stock concentration, dosed into constant-flowing reverse osmosis purified water using a conductivity pump set to dose between 265-270 µS/cm. Water chemistry of the synthetic freshwater was assessed via daily pH and conductivity measurements. Freshwater was delivered via a flow-through system at a flow rate of 100 ml/min. The laboratory was temperature controlled at 15°C and a natural day-night 12h:12h light cycle was used. Individuals were fed daily to satiation.

2.3 Population genomics using RADseq

2.3.1 DNA extraction and RADseq libraries

DNA was extracted using the QIAGEN DNeasy Blood & Tissue Kit (QIAGEN). DNA concentrations were determined using the QuantiFluor ONE dsDNA assay system (Promega). Single-digest *SbfI* RADseq libraries were prepared following the protocol of (Etter et al., 2011). Libraries were amplified using 12 PCR cycles and were sequenced on an Illumina HiSeq 2500 (100bp single-end) at a target coverage of 15x per individual.

2.3.2 RADseq data pre-processing

Data was processed using Stacks v2.6 (Catchen et al., 2013). Raw reads were demultiplexed and cleaned using *process_radtags*. Optimised parameters for construction of *de novo* RAD loci were determined as per (Paris et al., 2017). Seven individuals were removed due to low quality. We aligned *de novo* loci to the brown trout genome (Ensembl: version fSalTru1.1) and used *stacks-integrate-alignments* (Paris et al., 2017) to provide positional information. We used stringent parameters for filtering the loci, in order to detect top candidates for local adaptation. Loci were filtered with the following parameters in *populations*: *-r 80, -p 6, -min-maf 0.1, -max-obs-het 0.8*. For analyses sensitive to linkage, we used the *–write-single-snp* option and pruned loci in linkage (LD dataset) using Plink v1.9 (Purcell et al., 2007).

2.3.3 Population structure, genetic diversity, and effective population size

Population structure was assessed by Principal Components Analysis (PCA) and a neighbour-joining tree. PCA was conducted in Plink v1.9 (Purcell et al., 2007). For the NJ tree, the VCF file was converted to Phylip format using *vcf2phylip* (Ortiz, 2019), requiring a minimum of 100 samples per SNP. Distances were calculated using the *dist.dna* function in ape v5.6 (Paradis & Schliep, 2019), using the K80 default model and removing pairwise missing data.

Expected heterozygosity (H_E), observed heterozygosity (H_O) and the inbreeding coefficient (F_{IS}) were calculated for each population using Hierfstat (Goudet, 2005). Pairwise Weir and Cockerham's F_{ST} were computed in Genodive v2.0 (Meirmans, 2020) using 1000 permutations. To perform an assessment of fixed alleles within each population, we calculated allele frequencies using VCFtools v0.1.16 (Danecek et al., 2011) and plotted the number of fixed alleles per population in R v4.0.

Biases in estimating the effective population size (Ne) using linkage-disequilibrium methods are well-known (see Waples, 2024), especially when using RADseq methods and low sample sizes (Marandel et al., 2020). To alleviate these biases, we used the recently described methods implemented in currentNE, which performs particularly well in the small sample size scenario (Santiago et al., 2024). currentNE was run by estimating the average number of full siblings (k parameter) from the input data (a Hardy-Weinberg equilibrium and missing data filtered VCF per population), using 40 chromosomes as estimated from the brown trout reference genome. 90% confidence intervals were provided by the neural network (ANN).

2.3.4 Identification of loci associated with metal pollution and water chemistry

To identify gene candidates potentially involved in metal adaptation, we used a combination of statistical methods used to detect loci showing high allele frequency differentiation: pcadapt v4.3.3 (Luu et al., 2017) and OutFLANK .0.2 (Whitlock & Lotterhos, 2015); and genotype-environment association (GEA) tests: Redundancy Analysis (RDA; Capblancq et al., 2018)) and Latent Factor Mixed Models (LFMM v1.5; Frichot et al., 2013)). pcadapt was run using $K = 4$, as identified using the *screeplot* function. q-values were corrected using an alpha value of 0.05. For OutFLANK, we removed low heterozygosity variants (<0.1) and used a q-value threshold of 0.05.

For GEA tests, we used environmental data comprising 41 water chemistry and metal contamination variables for each of the six rivers (14 sample sites; Table S2). We used the average value for each variable (data covers a 15-year period; Table S3). Due to microgeographic differences in metal contamination within each river, we extracted allele frequency data for each of the 14 sampling sites to match the environmental data (4664 loci).

RDA was run adapting the approaches of (Bourret et al., 2014) and (Forester et al., 2018). To avoid collinearity amongst the environmental predictors, the environmental data were reduced to 10 Rotated Components (RC) by running a PCA (10 factors, *varimax* rotation). RCs were reduced to a number of factors identified by a parallel analysis criterion (Horn, 1965) using 1000 bootstraps. We used a 0.01 significance threshold (0.68 loading cutoff) to associate an environmental parameter to a factor. Following the RDA, a global analysis of variance (*anova.cca*; 999 permutations) was performed to assess the global significance of the RDA and an analysis by axis was run to determine if retained RCs were significantly correlated with allele frequencies. Candidates were identified as variants in the tails of the RDA constrained axis distribution. A cutoff of 2.5 standard deviations (two-tailed p-value of 0.012) was used.

LFMM was run using the RCs generated above. The number of latent factors was estimated using the *snmf* function ($K = 4$). We ran 10,000 iterations with a burn-in of 5000. The Gibbs sampler was run five independent times on the dataset in order to estimate the appropriate average LFMM parameters (z-score values). p-values were adjusted using estimation of the genomic inflation factor (λ) for each RC variable. Significance was tested using an FDR<0.05.

Candidate loci (*i.e.*, those identified from the analyses described above) were assessed for functional gene-level effects using the Variant Effect Predictor tool in Ensembl (McLaren et al., 2016). We also classified the positional information and gene-level effects of the fixed alleles in each of the metal-impacted populations. Variants were classified as intergenic, intronic, synonymous, and non-synonymous, including the potential variant consequences.

2.4 Metal-tissue burden

2.4.1 Laboratory analysis

Five tissues (gill, liver, gut, kidney, and muscle) were dissected, dried and weighed, and stored at -20°C. Tissues were digested in 500µl of nitric acid (70%, purified by redistillation, ≥99.999% trace metals basis, Sigma Aldrich) for 48 hours at room temperature (~20°C), followed by digestion with 0.1% hydrogen peroxide (Fisher; Hydrogen Peroxide 30% (w/v) Extra Pure SLR) for a further 24 hours. Digested tissue was diluted 1:10 in ultrapure deionised H_20 and acidified with 0.1% nitric acid. A standard of 10µg/L yttrium (Y) was added to each sample to perform within-sample corrections. Metal concentrations (quantified as ng/mg) were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (E:AN 6100DRC, Perkin-Elmer, Cambridge, UK).

2.4.1 Statistical analysis

Data were checked for normality (Shapiro test) and homogeneity of variance (Levene test) prior to conducting statistical tests and were transformed when necessary. Assessment of burdens for each tissue were statistically assessed using a two-way ANOVA, with 'metal-impacted' and 'control' fish specified as Group, and time point ('River', or 'Depuration') specified as Factor. For analysis where a statistical difference was identified, post-hoc Tukey tests were performed to conduct pairwise multiple comparisons, accepting a significance threshold of FDR ≤ 0.05.

2.5 Transcriptomics analysis using RNAseq

2.4.1 RNA extraction, library construction, and sequencing

Gill and liver tissues were dissected from euthanised fish (n=6 metal-impacted; n=6 control) and were snap-frozen in liquid nitrogen and stored at -80°C prior to analysis. RNA was extracted using a QIAGEN RNA-seq extraction kit, including an on-column DNAse digestion step. RNA quantification and quality assessment (accepting a RIN>8.0) was performed using a Bioanalyser (Agilent RNA 6000 Nano). Libraries were prepared using the Illumina TruSeq Stranded mRNA library preparation protocol. Concentrations of the cDNA libraries were quantified using a Tapestation (D1000 ScreenTape) and final library pool concentrations were determined by qPCR. Libraries were sequenced (125 bp paired-end) on an Illumina HiSeq 2500.

2.4.2 RNAseq data pre-processing

Raw reads were cleaned using Trimmomatic v0.27 (Bolger et al., 2014) and aligned to the brown trout reference genome (Ensembl version fSalTru1.1) using STAR v2.5.4 (Dobin et al., 2012). Transcript abundances were calculated at gene regions using featureCounts v2.0.1 (Liao et al., 2014). Low expressed genes were filtered by only including genes with more than 10 counts in ≥ 6 samples. This reduced the initial dataset of 43,946 expressed genes to 28,261 genes in the gill and to 21,820 genes in the liver.

2.4.2 Differential gene expression analysis

Analysis of differentially expressed genes (DEGs) was performed using DESeq2 v1.40.2 (Love et al., 2014). For assessment of biological replicate clustering, the counts matrix was normalised using a vst transformation, and proportion of the explained data was explored using a PCA. Size factors and dispersion were estimated using the default DESeq2 parameters. A negative binomial GLM was used to test for differentially expressed genes (DEGs), retaining only those genes with a significance threshold FDR ≤ 0.05 and a log2foldchange 1 | -1.

All genes (including non-significant DEGs, but with outliers removed) were used to test for a statistical enrichment in Gene Ontology terms via Gene Set Enrichment Analysis (GSEA) using clusterProfiler v3.16.1 (Yu et al., 2012). Lists containing GO categorisation of brown trout terms (AH85410) were downloaded from Ensembl using BioMart v2.44 (Smedley et al., 2009). Genes were ranked by a single ranking metric by multiplying the log fold-change by the

negative logarithm (base 10) of the adjusted p-value. This was done to ensure that genes with both large fold-changes and low p-values receive higher combined ranks. Minimum and maximum gene set sizes of 20 and 200 were used. GO terms with a Benjamini–Hochberg adjusted p-value ≤0.25 were classified as significant.

Results

3.1 Population genomics

3.1.1 RADseq libraries and loci construction

We generated an average of 2,279,882 million reads (SEM ± 119,860) per individual at an average of 20x coverage, assembling 10,115 SNPs in 8,718 loci genotyped across 80% of the individuals from each of the six rivers. Details of the RADseq libraries, including number of reads and coverage per individual can be found in Table S5.

3.1.2 Population genetics characterisation of metal-impacted and control trout

Overall, we observed strong genetic sub-structuring with the highest pairwise F_{ST} values detected in comparisons to metal-impacted trout, in particular those sampled from the Hayle (F_{ST}>0.1 in all comparisons) and the lowest pairwise F_{ST} values observed between control trout (Figure 2A).

PCA separated the metal-impacted trout populations from one another, and also from the control trout (Figure 2B). PC1 (18.8%) defined trout from the Hayle, PC2 (8.97%) separated the Red River and Gannel trout, and PC3 (8.09%) further separated the Gannel trout. In the NJ tree, we observed that metal-impacted trout showed three distinctive branches, with the Hayle and Red River individuals originating from a shared branch in the NJ tree, whereas the Gannel formed its own group (Figure 2C). The Hayle and Red River showed particularly long branches, indicative of higher substitution rates. We also observed genetic substructuring within the Hayle (upstream and downstream of a highly metal-contaminated middle region) and the Red River (by tributary) in the NJ tree.

We observed differences in the genetic diversity and effective population size (N_e) estimates between metal-impacted and control trout (Figure 2D). Compared to control sites, heterozygosity was significantly lower in metal-impacted trout sampled from the Red River (H_O=0.28, Mann-Whitney U p=0.005) and the Hayle (H_O=0.23, Mann Whitney U p<0.001), but not in the Gannel (H_O=0.31, Mann-Whitney U p=0.322). Heterozygosity also showed a more even distribution in the control trout, whereas long tails were observed in the Hayle and Red River, indicative of high variance among the individuals sampled from these sites. Further investigation of the heterozygosity per sampling site showed that this high variance was not caused by microgeographic differences in the upstream and downstream reaches of the River Hayle, or by the two sampled tributaries in the Red River. Differences in observed heterozygosity were reflected in the inbreeding coefficient (F_{IS}), which was overall higher in metal-impacted trout compared to control trout, being highest in trout sampled from the Hayle (F_{IS}=0.25), and lowest in the Fowey (F_{IS}=0.04).

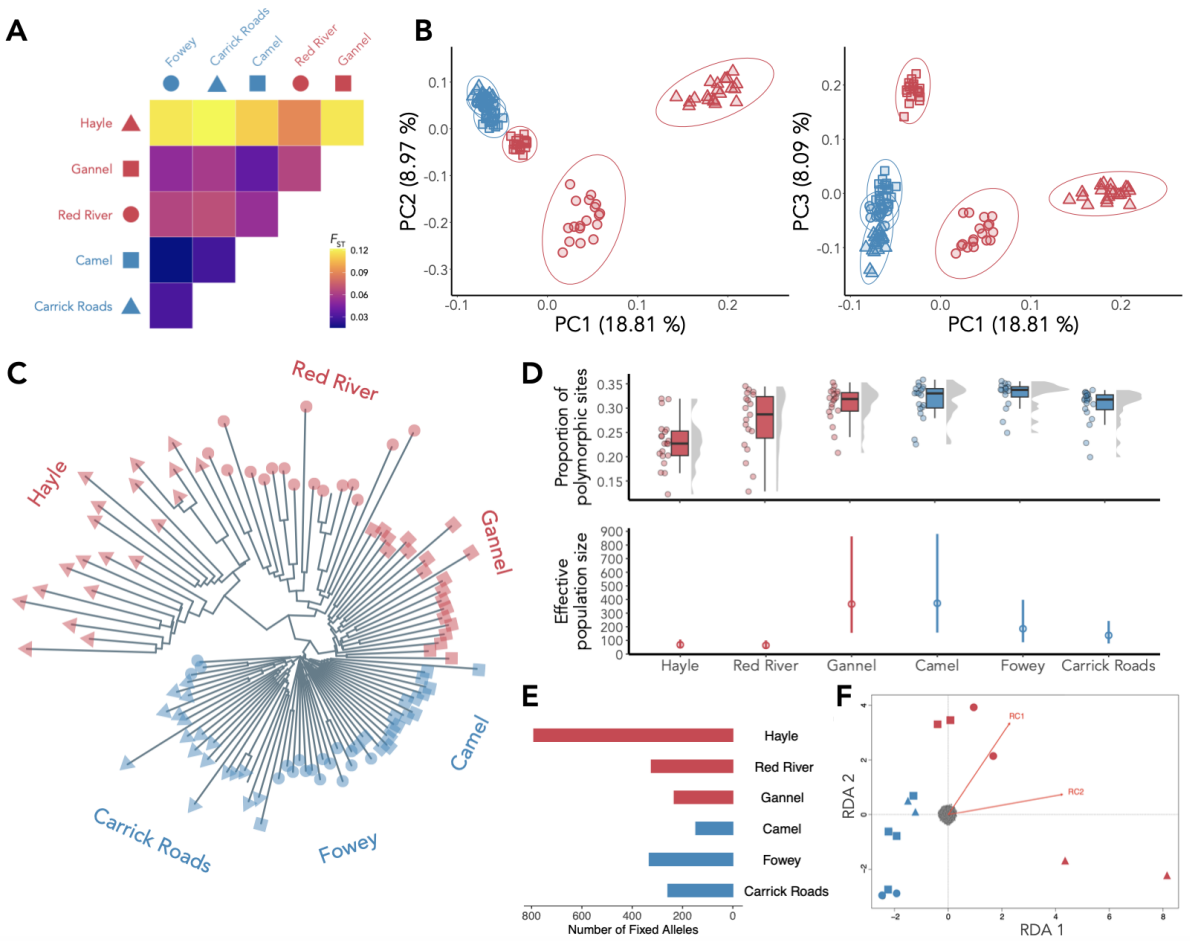

Figure 2. Population genomic data describing the metal-impacted and control trout. (**A**) Heatmap of pairwise F_{ST} calculated between all sampling sites. (**B**) Principal Component Analysis (PCA) showing PC1 (~19% of the variance) against PC2 (~9% of the variance) and PC1 against PC3 (~8% of the variance). Individuals are coded by colour and sampling site as in panel A. (**C**) Neighbour-joining tree showing the pairwise distances from aligned SNPs, computed using the K80 model. (**D**) Genetic diversity (calculated as the proportion of polymorphic sites) and effective population size (Ne) estimates calculated with currentNE. Genetic diversity was calculated at each individual sampled from each of the six sampling sites. (**E**) Quantification of fixed alleles calculated at each sampling site. (**F**) Globally significant Redundancy Analysis (RDA; p=0.007, R^2=0.2145) showing RDA1 (p=0.037) and RDA2 (p=0.1). Red vectors represent RC1 (water chemistry features; Table S7) and RC2 (Cd, Cu, Ni, Zn). In all panels, sampling sites are coloured by metal-impacted (red) or control (blue). For panels A, B, C, and F the sampling sites are coloured as: Hayle (red triangle); Red River (red circle); Gannel (red square); Camel (blue square); Fowey (blue circle) and Carrick Roads (blue triangle).

N_e estimates for the Hayle (N_e = 68; 90% CI: 43-106) and Red River (N_e = 64; 90% CI: 40-100) were low, but the Gannel (N_e = 366; 90% CI: 157-863) had a higher N_e than the Fowey (N_e = 186; 90% CI: 87-398) and Carrick Roads (N_e = 137; 90% CI: 77-243), and was comparable to that of the Camel (N_e = 372; 90% CI: 157-881). Overall, metal-impacted trout did not show a higher proportion of fixed alleles, except for the Hayle, which showed a much higher proportion compared to trout from all other sites (Figure 2E).

3.1.3 Identification of adaptive

Using four methods, we identified a total of 122 candidate loci potentially involved in metal adaptation (Table S6). Of the pcadapt candidates (n=40), the majority were associated with PC1 (n=18, which separates the Hayle). Remaining outliers were associated with PC2 (n=14; separates the Red River), and PC3 (n=8; separates the Gannel). OutFLANK identified eight candidates, which were primarily driven by divergent F_{ST} values observed in comparisons to the Hayle.

The RDA was globally significant (p=0.007; R^2=0.2145) and both axes were also significant (RDA axis 1: p=0.037; RDA axis 2: p=0.1, accepting a significance threshold ≤0.1). We identified a total of 30 loci which were significantly associated with these axes (two-tailed p-value = 0.012). RDA axis 1 (five loci) separated the Hayle, and to a lesser extent, the Red River and Gannel from the control group. RDA axis 2 (25 loci) separated the Red River and Gannel, suggesting this axis represents loci which are diverging in these populations, but not in the Hayle. RDA identified an overall significant association for two RCs (Figure 2F). Correlations between the loadings on the RCs and the RDA axes showed that RDA axis 1 was primarily associated with the environmental features of RC2: Cd, Cu, Ni and Zn (Table S7), which are the primary metal pollutants of the River Hayle. RDA axis 2 was primarily associated with the environmental features of RC1 (water chemistry features; Table S7). Using LFMM, we uncovered significant associations of 44 loci (RC1: 22 loci; RC2: 14 loci; RC3: 8 loci. We found no overlap amongst the loci identified using the four outlier approaches.

Of the 122 candidates, 60 loci showed good-quality alignments (MQ=60) to genes in the brown trout genome (Table S6). Twenty-eight of the variants were intronic, 21 variants were located upstream or downstream of genes, three were synonymous variants, and nine variants had functional gene-level predictor effects. Of the 1348 fixed variants identified in each of the metal-impacted populations, 42 aligned to genes in the brown trout genome. However, all variants were found to be intronic, except for a missense variant fixed in the Hayle.

3.2 Laboratory maintenance and fish morphometrics

In the laboratory, all fish were observed feeding; there were no mortalities and no negative health effects were observed. Stable water chemistry of the synthetic freshwater was maintained throughout the experiment (Table S8). Metal-impacted trout were significantly larger than control fish; however, there was no significant difference in condition factor (Table S9). The metal-impacted and control groups showed skewed sex ratios.

3.3 Tissue-metal burden analysis

In the river, gill metal concentrations were 7-, 6-, 1- and 2- fold higher in metal-impacted trout for Cd (p<0.0001), Cu (p<0.0001), Ni (p=0.01) and Zn (p<0.0001), respectively (Figure 3A). Cd, Cu and Ni decreased in concentration during the depuration period (Cd, p=0.0003; Cu, p<0.0001; Ni, p=0.01), but Zn did not (p=1). Although the concentration of Cd decreased during the depuration, it was still higher than the concentrations found in control trout after the

period of depuration ($p<0.0001$). Zn burdens also remained higher than control trout after the depuration period ($p<0.0001$).

In the gut, metal burdens at the point of sampling from the rivers were 14-, 11- and 2-fold higher in metal-impacted trout for Cd ($p<0.0001$), Cu ($p<0.0001$) and Zn ($p<0.0001$), respectively (Figure 3B). Ni burden was not higher ($p=0.99$), and did not decrease during the depuration period ($p=0.98$). Both Cu and Zn concentrations decreased after depuration (Cu, $p<0.0001$, Zn, $p<0.0001$), to concentrations comparable to those observed in control trout (Cu, $p=0.83$; Zn, $p=0.68$). Cd concentrations did not decrease (Cd, $p=0.56$), with 18-fold higher concentrations being measured in metal impacted fish compared to control trout after the period of depuration ($p<0.0001$).

In the kidney, metal burdens measured in fish immediately after sampling from the river were 27- and 23-fold higher in metal-impacted trout for Cd ($p<0.0001$) and Cu ($p<0.0001$) (Figure 3C). Although both metals showed a significant depuration (Cd, $p<0.0001$; Cu, $p=0.0002$), they remained 17- and 11-fold higher than control trout after depuration for 11 days (Cd, $p<0.0001$; Cu, $p=0.006$). Ni was higher in metal-impacted trout, but this was marginally non-significant in comparison to control trout ($p=0.09$). No statistical differences were observed for Zn.

In the liver, metal burdens were 21-, 30-, 2-, and 2-fold higher in metal-impacted trout measured after sampling from the river for Cd ($p<0.0001$), Cu ($p<0.0001$), Ni ($p=0.001$) and Zn ($p<0.0001$), respectively (Figure 3D). Metal-impacted trout showed a depuration of all metals (Cd, $p<0.0001$, Cu, $p<0.0001$, Ni, $p=0.002$; Zn, $p<0.0001$). However, concentrations of Cd and Cu remained 7- and 17-fold higher than control trout after depuration (Cd, $p=0.003$; Cu, $p<0.0001$).

In the muscle, metal-impacted trout showed 6-fold higher Cd burden concentrations compared to control trout (Cd, $p<0.0001$) when measured after sampling from the river, and Cd did not depurate (Cd, $p=0.05$) (Figure 3E). Although slightly higher burdens of Cu were observed in metal-impacted trout, this was non-significant ($p=0.41$); Cu did however depurate ($p=0.03$). Ni and Zn did not show any significant differences.

Sex differences did not alter the significance of the burden data. Full statistical results for the metal-burden data can be found in Table S10).

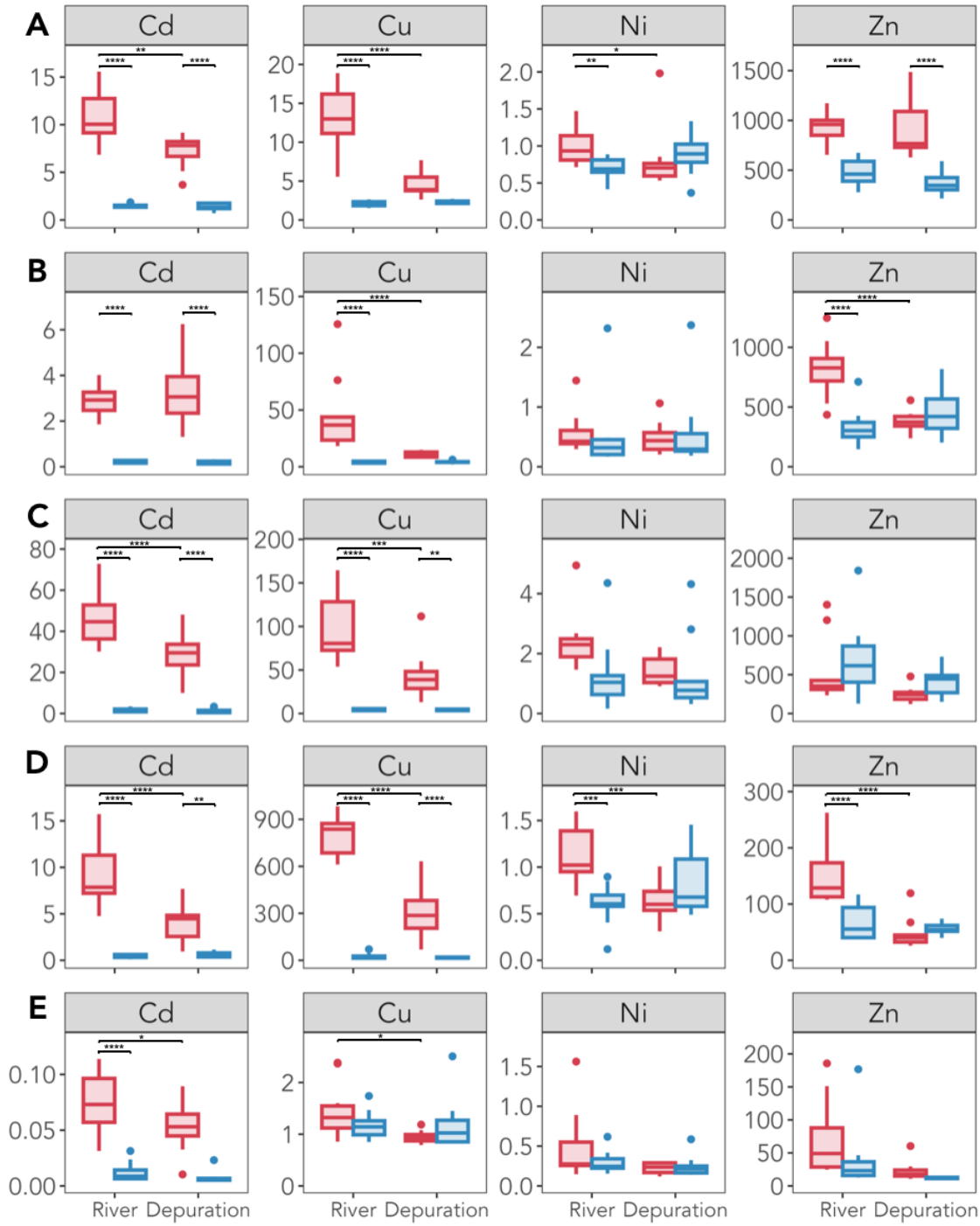

Figure 3. Tissue-metal burden data of cadmium (Cd), copper (Cu), nickel (Ni), and zinc (Zn) measured in metal-impacted (in red) and control (in blue) trout measured at Timepoint 1 (River) and Timepoint 2 (Depuration). Metal burden is measured as ng/mg of dry weight tissue for the (**A**) Gill; (**B**) Gut; (**C**) Kidney; (**D**) Liver; and (**E**) Muscle. Significance was measured using a Two-way ANOVA (full details in Table S10). Only significant results from pairwise Tukey tests are included where **** indicates p≤0.0001, *** indicates p≤0.001, ** indicates p≤0.01, and * indicates p≤0.05.

3.3 Comparison of the transcriptional landscapes between metal-impacted and control trout following a period of depuration

3.3.1 RNAseq libraries and gene expression profiles

Across the six samples per group, we generated an average of 16,549,620 (SEM ± 1,341,005) reads for the gill, and 11,588,849 (SEM ± 1,329,081) reads for the liver (Table S11). Evaluation of the ordination of the samples via PCA revealed a clear separation between metal-impacted and control trout in their gene expression profiles. In the gill, replicates were separated along PCA axis 1 (49% of the variation; Figure 4A). Liver samples were also differentiated, but to a lesser extent, showing a separation of the samples along PCA axis 2 (21% of the variation; Figure 4B).

3.3.2 Identification of differentially expressed genes

After depuration, analysis of the differentially expressed genes (DEGs) revealed 2,042 DEGs (FDR ≤ 0.05) in the gill, of which 1,180 were over-expressed (logFC > 1) and 862 were under-expressed (logFC <-1) in metal-impacted trout compared to control trout (Figure 4C; see Table S12 for the full list of significant DEGs). We identified several metal-binding and antioxidant genes that remained over-expressed in the gill tissue of metal-impacted trout, including metallothionein 2 (*mt2*), metal response element binding transcription factor 2 (*mtf2*), metalloreductase (*steap4*), methionine sulfoxide reductase B2 (*msrb2*), thioredoxin-related transmembrane protein 2 (*tmx2a*), three glutathiones (*tstd1*, *mgst3*, *gstz1*), and lysyl oxidase (*loxl2a*, *loxl2b*). We also identified genes with well-known roles in oxidative stress response and response to xenobiotics, including two cytochrome p450 genes, five cytochrome c oxidase subunits, a cytochrome b-245 chaperone (*cybc1*), both aryl hydrocarbon receptors (*ahr1a*, *ahr1b*), and fourteen heat-shock proteins. Related specifically to the known activity of the gill in response to metals, we also identified ATP-binding cassette (ABC) transporters (*abca4a*, *abcg2a*, *abcc5*), and two copies of carbonic anhydrase 4 (*ca4*). Amongst the under-expressed genes, we observed a high number of immune-system related genes, including interferon-induced proteins, interleukins, and E3 ubiquitin protein ligases.

In the liver, 311 DEGs (FDR ≤ 0.05) were identified, of which 200 were over-expressed (logFC > 1), and 111 were under-expressed (logFC <-1) in metal-impacted trout compared to control trout after the depuration period (Figure 4D; see Table S13 for the full list of DEGs). We did not observe the same level of metal-induced transcriptional changes in the liver, with very few over-expressed metal-binding, oxidative stress response, or xenobiotic handling genes. However, we did identify a gene annotated as *heavy metal-binding protein HIP-like*, and the well-known xenobiotic metabolism gene, cytochrome P450 1A1 (*cyp1a1*).

Intersecting the gill and liver DEGs, we identified 25 shared over-expressed genes, and eight shared under-expressed genes (Table S14). Of note, *DENN domain-containing protein 5B-like* was over-expressed in both tissues, and in both cases, was the most strongly over-expressed gene.

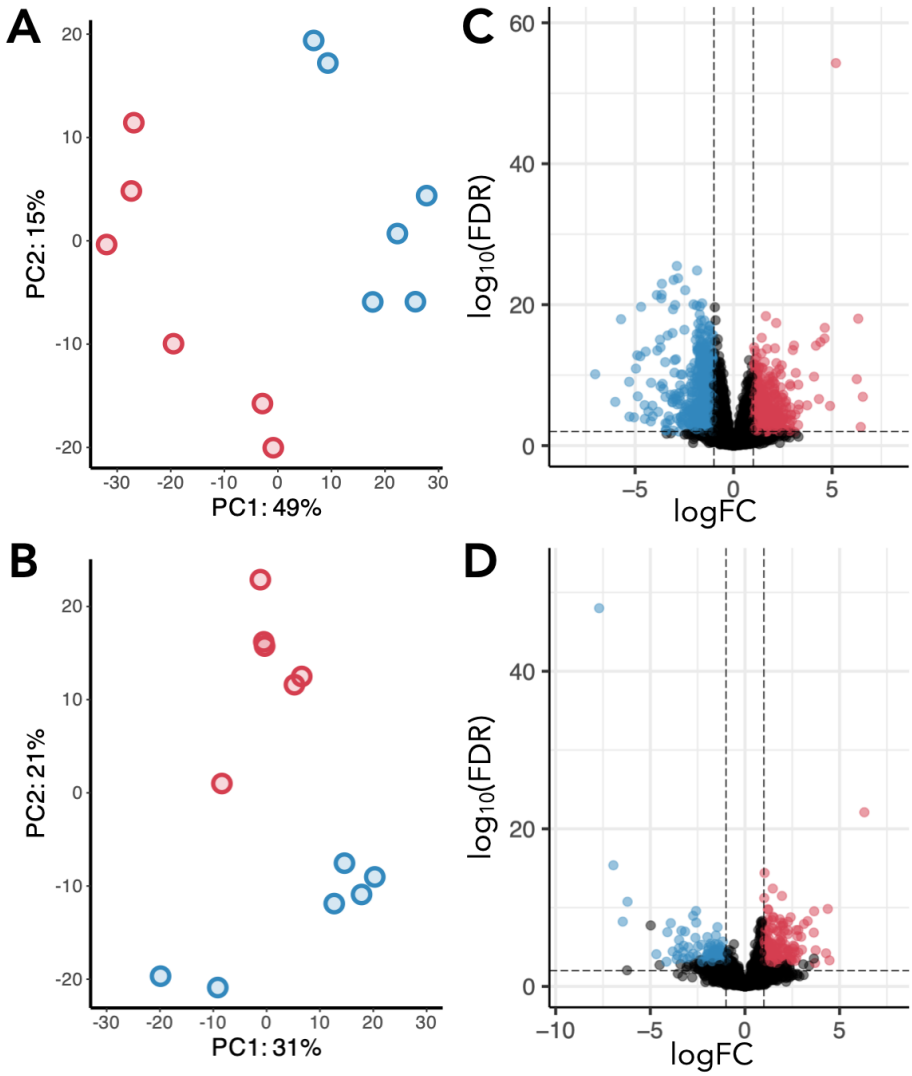

Figure 4. Landscapes of differential gene expression between metal-impacted (in red) and control (in blue) trout. (**A**) Principal Components Analysis (PCA) of the vst normalised counts in the gill (PC1: 49% of the variance; PC2: 15% of the variance). (**B**) Principal Components Analysis (PCA) of the vst normalised counts in the liver (PC1: 31% of the variance; PC2: 21% of the variance). (**C**) Volcano plot of the 28,261 genes in the gill. (**D**) Volcano plot of the 21,820 genes in the liver. Coloured dots correspond to differentially expressed genes (DGEs) with FDR ≤ 0.05 and a log2foldchange of 1 (red) or -1 (blue), meaning genes are over-expressed, or under-expressed in metal-impacted trout, respectively.

3.3.3 GO enrichment

To further investigate the transcriptomic landscapes of metal-impacted trout following depuration, we explored activated and suppressed GO terms via GSEA. In the gill, we identified several activated biological processes indicative of changes in ionoregulation: chloride, calcium, sodium, and potassium ion transport; and oxidative stress: regulation of small GTPase mediated signal transduction, and WnT signalling. Suppressed biological processes included microtubule movement; DNA repair and replication; immune responses, and protein folding, catabolism, and ubiquitination. Amongst the activated molecular function

pathways, we identified ion channel activity, including voltage-gated potassium and calcium channel activity, metallocarboxypeptidase activity, cytochrome-c oxidase activity, and copper ion binding and iron sulphur cluster binding. Suppressed pathways were related to protein folding and ubiquitination. Gill GSEA terms can be found in Table S15.

In the liver, we found a weak concerted biological signal related to metal tolerance. We found no significantly activated or suppressed biological pathways, but cytochrome-c oxidase activity was a significantly activated molecular function. Liver GSEA terms can be found in Table S16.

Discussion

We applied an integrative approach, using genomics, transcriptomics, and tissue-metal burdens, to investigate the signatures of potential long-term adaptation to metal pollutants in wild brown trout. Population genomics analysis showed that metal-impacted trout are highly differentiated from control trout, and in some cases, show significantly lower genetic diversity, highlighting the effects of metal pollutants on the genetic make-up of these populations. Using a series of outlier tests, we identified selection signatures for loci associated with oxidative stress and metal homeostasis. Measured from the river, metal-impacted trout showed considerably higher concentrations of metals across multiple tissues indicating that they are indeed absorbing metals, and thus supporting the requirement for mechanisms to cope with increased metal burden. After 11 days in control water, patterns of depuration were tissue- and metal-specific, pointing towards a complex network of biological responses involved in metal handling. Analysis of gill and liver RNAseq data revealed marked differences in the transcriptomic profiles between metal-impacted and control trout. In the liver, we did not observe a strong concerted signature. However, in the gill, we identified over-expressed genes and activated biological processes involved in ionoregulation, metal homeostasis and oxidative stress.

4.1 High concentrations of metals in metal-impacted trout demonstrate that metals are absorbed and necessitates processes of metal handling

Metal burden data quantified from individuals sampled at their river of origin showed significantly higher concentrations of metals in the tissues of metal-impacted trout. The varying amounts of metal concentrations across the different tissues points to the diverse roles of these organs in terms of metal uptake, storage, and detoxification. The gill, being in intimate contact with the water, and the gut, via ingestion of metal-contaminated prey, are well-characterised as the main routes of metal uptake in fish (Wood, 2011b). We found that the gill had elevated concentrations of all metals measured in this study (Cd, Cu, Ni and Zn), pointing to this tissue as the main target of metal entry. Meanwhile, elevated concentrations of Cd, Cu and Zn in the gut suggests brown trout are ingesting metals from food resources in the river. However, Ni was not elevated in the gut; previous research has shown that pre-exposure to Ni reduces gastrointestinal uptake of Ni, suggesting a homeostatic interaction might exist for this metal (Chowdhury et al., 2008).

The liver and kidney provide key functions in the detoxification and excretion of metals (Wood, 2011a). All metals measured (Cd, Cu, Ni and Zn) were significantly elevated in the liver, supporting its role as the main tissue f

or detoxification in vertebrates, including metals. In contrast, only Cd and Cu were elevated in the kidney. As found here, the kidney has been previously found to accumulate the highest concentrations of Cd (Andres et al., 2000; De Smet et al., 2001). Muscle generally shows a weak accumulating potential for metals (Jia et al., 2017), and we found that only Cd was significantly elevated.

Overall, the observation of elevated tissue burdens confirms that trout living in metal-impacted rivers are affected by metal pollution and supports the hypothesis that genomic and/or transcriptomic regulatory mechanisms must be involved in the dynamics of metal storage and detoxification, and in preventing the harmful effects associated with such high metal loads.

4.2 Impacts of metal pollutants on the genetic structure and diversity of metal-impacted trout

We observed strong genetic sub-structuring between metal-impacted and control trout across all our analyses. Such patterns have been observed previously using both neutral microsatellites (Paris et al., 2015) and SNPs (Osmond et al., 2024). Despite their geographic proximity, metal-impacted trout showed strong genetic separation, from one another, and also from control trout. In contrast, differentiation was low among metal-sensitive trout occupying clean rivers, which are geographically much more distant from one another. For example, the mouths of the River Hayle and Red River are separated by just ~9 km, whereas the River Camel and the Carrick Roads are separated by ~270 km of coastline.

Population genetic studies on salmonids often report a high incidence of population structure (Bekkevold et al., 2020; King et al., 2020), and this has been frequently attributed to processes of local adaptation (Fraser et al., 2011; Primmer, 2011; Taylor, 1991). However, identifying what proportion of the genetic structure is explained solely by local adaptation is confounded by neutral demographic processes. Indeed, our analysis of fixed alleles in the metal-impacted populations showed that only one variant was located within an exon, suggesting that strong genetic drift is acting in these small populations. It can also be argued that if populations are geographically close, but experience substantially different environmental pressures, the expected differentiation due to non-selective factors is small (Lotterhos & Whitlock, 2015; Wadgymar et al., 2022). There are no known physical barriers separating the mouths of the metal-impacted rivers, and thus the strong population structure could also indicate that non-adapted individuals which stray into metal-impacted rivers suffer adverse toxic effects (Sergeant et al., 2022). Over time, a reduction in gene flow would be expected to further increase genetic drift and strengthen genetic differentiation.

The effect (neutral or non-neutral) of metal pollutants was particularly marked in the River Hayle, where we observed micrographic structuring based on metal contamination levels,

with trout sampled upstream and downstream of an extremely toxic middle section of the river (~1.5km), showing strong genetic differentiation (Paris et al., 2015). As well as indicating that metal toxicity may act as a chemical barrier to migration within the river (Hecht et al., 2007; Williams & Gallagher, 2013), these microgeographic differences could also indicate finer-scale adaptive responses. Indeed, gill cell cultures showed different metallothionein gene expression patterns when exposed to upstream and downstream Hayle River water (Minghetti et al., 2014).

Metal-impacted populations do not appear to be experiencing the same genetic impact in response to metal pollutants. The River Hayle, the Red River, and the River Gannel, are all impacted by different 'cocktails' of metal pollutants and also differ in their water chemistry features (which affect metal bioavailability). This result could stem from a number of factors: that metal pollutants are behaving at different toxicity thresholds within each river; disparate adaptive responses in each of the metal-impacted populations; differences in the population size and demographic history of these populations. Unfortunately, our attempts to analyse demographic history were unsuccessful due to poorly constructed site-frequency spectra, but previous analysis using approximate bayesian computation (ABC) showed that bottlenecks occurred in all metal-impacted populations (Paris et al., 2015). It is therefore very probable that the observed patterns of population structure relate to the impacts of both historical (population bottlenecks) and contemporary (potentially adaptive) metal pollution.

The River Hayle and Red River trout appeared to be most affected by metal pollution, with these populations showing the strongest population structure and particularly long branches in the neighbour-joining tree. The latter observation indicates higher substitution rates in these populations, which can be the result of multifarious biological processes, including drift, episodes of heightened adaptive evolution, but also higher mutation rates, or differences in life-history traits (Mendes & Hahn, 2016). Increased mutation rates have been evidenced in metal-impacted sunfish (*Lepomis auritus*) (Theodorakis et al., 2006), and metal-impacted yellow perch (*Perca flavescens*) died younger than control conspecifics (Couture & Pyle, 2008). Genetic diversity and N_e were also lower in the River Hayle and Red River populations, but particularly so in the former, which also had a higher inbreeding coefficient. Such observations support the hypothesis that environmental pollution can cause significant genetic erosion (van Straalen & Timmermans, 2002). Nevertheless, the fact that trout remain in these rivers, and show evidence of extremely high concentrations of metals in their tissues, suggests that an adaptive component to metal tolerance is probable.

4.3 Assessment of candidate loci involved in local adaptation to metal pollution

To detect candidate loci underpinning potential metal tolerance we applied several statistical tests, each with different assumptions, using a particularly rigorously set of filtered loci and stringent cut-off criteria for significance. This was to reduce spurious correlations between loci and environmental variables, and the strong population structure we observed (Ahrens

et al., 2018; Hoban et al., 2016). Amongst the 122 outlier loci, we identified 12 variants with potential gene-level effects, several of which have functional annotations with potential relevance to metal adaptation. We identified a splice-gene variant in *map35k*, which is activated by various stresses including oxidative stress and calcium overload (Ichijo et al., 1997) and is regulated in response to copper (O'Doherty et al., 2020); a synonymous variant in *scara3* which is an oxidative stress gene involved in scavenging oxidative molecules (Han et al., 1998; Sone et al., 2010); a missense variant in *ferritin, heavy subunit-like* which is involved in the storage of iron in a nontoxic state (Munro & Linder, 1978); a stop-loss mutation in *cyp7a1*, one of the cytochrome P450 enzymes involved in mono-oxygenation reactions in a wide range of endogenous and exogenous compounds (Uno et al., 2012); and which is regulated in response to toxic metals (Chi et al., 2019); a 3'-UTR variant in ZNF239 and a missense variant in PCGF3, both of which regulate transcription; a splice donor variant in *hmmr*, which regulates cell growth (He et al., 2020) and is modified in exposure to arsenic (Dodmane et al., 2013); and missense variants in *mfhas1* and *inavaa* which both function in innate immunity: *mfhas1* is a regulator of *tlr2* and *tlr4* (Shi et al., 2017; Zhong et al., 2015), and *inavaa* is stimulated by pattern recognition receptors including *tlr2* and *tlr4* (Yan et al., 2017). Whilst these genes represent promising candidates underpinning local adaptation to metals, we recognise that we have not surveyed the full variation potentially responsible for metal tolerance due to, for example, limitations in the RADseq approach (Lowry et al., 2017, but see Catchen et al., 2017), and neglecting the role of structural variants (SVs; Mérot et al., 2020), especially duplications arising from the salmonid whole-genome duplication event (Moriyama & Koshiba-Takeuchi, 2018). Ongoing research using whole-genome sequencing methods are aimed at increasing our understanding of potential candidates involved in adaptation to metals in these trout populations.

4.4 Limited metal depuration indicates the importance of molecular mechanisms for metal handling

We observed metal- and tissue- specific patterns of depuration pointing to a contrasting affinity of each of the measured metals to different tissues for deposition and excretion. The patterns of depuration in the gill, liver and kidney are of particular interest. In the gill, Cd, Cu and Ni depurated, but Zn did not, and although Cd depurated from the gill, it did not reduce to concentrations observed in the control fish. Toxic concentrations of all metals can have deleterious effects on the gill, including oxidative stress (Sevcikova et al., 2011), impaired ionoregulation (Atli & Canli, 2013), damage and mucus accumulation (Playle, 1998), necessitating their rapid detoxification and removal (Saibu et al., 2018). Rapid clearance (within 16h) of Cd and Cu from the gill has been reported previously in rainbow trout (*Oncorhynchus mykiss*), with accompanied increases in the liver (Handy, 1992). Indeed, metal-impacted trout continued to show high burdens of all metals in the liver, but especially Cd and Cu, both of which showed evidence of depuration, but not to comparable concentrations observed in the control fish. We observed similar patterns for Cd and Cu in the kidney, suggesting overall that the gill is essential in detoxifying these metals, and that they are subsequently transported to the liver and kidney for excretion (Garai et al., 2021).

We also observed a high retention of Cd across all tissues, which has been observed previously, and over much longer time periods than our experiment allowed for (Defo et al., 2015; Kraemer et al., 2005).

As others have shown, these results indicate that tissue metal concentrations can be high, but the metals within the tissue are likely non-toxic (*e.g.*, Campbell et al., 2008; Leonard et al., 2014; Shekh et al., 2021; Wang & Rainbow, 2006). The physiological processes governing this are attributed to subcellular partitioning, whereby metals are compartmentalised into either a metal-sensitive fraction (MSF), which is considered the target of attack of metals in cells, or the biologically detoxified metal portions (BDM), which is considered to alleviate toxicity (see review by (Wang, 2013). Notably, there is evidence that over time, metals are moved from the MSF to the BDM, and that fish surviving long-term in chronically metal-impacted environments have partitioned the majority of their tissue metal burdens into the BDM, although the MSF may also be important (Wood, 2011b). Overall, the observation of tissue-specific shifts in metal burdens, as well as the retention of high metal burdens among particular tissues, emphasises the importance of detoxification and excretion in metal-impacted brown trout, necessitating molecular mechanisms underpinning these processes.

In our experimental design, we attempted to adhere to the Three R's (replacement, reduction, and refinement) (Díaz et al., 2020). Attention to these ethical considerations means our study design was limited by the number of sampling timepoints. For example, the use of only two timepoints for the tissue-metal burdens limits our ability to quantify depuration over both i) additional timepoints, which would have allowed a more detailed insight into the journey of metal depuration, and ii) a longer depuration period, permitting a more confident determination of "true" depuration (including our insights into constitutive gene expression). However, we highlight the challenges of maintaining wild fish under laboratory conditions (Huntingford et al., 2006), including acclimation to captivity (Baillon et al., 2016), and perhaps, more importantly, uncertainty regarding if metal-impacted trout would ever completely depurate to reach the background tissue-metal burdens observed in the control population.

4.5 Constitutive gene expression as a potential mechanism of adaptation to metal pollution

We observed different transcriptional profiles between metal-impacted and control trout, indicative of the role of constitutive gene regulation in mitigating metal toxicity. In particular, the gill had a higher number of DEGs (2,042), 58% of which were over-expressed in comparison to the control trout, including over-expressed genes known to be involved in metal detoxification, oxidative stress and response to xenobiotics. This included the well-known metal-binding gene metallothionein (*mt2*), a key protein involved in the homeostasis and detoxification of Cd, Cu and Zn (Hamilton & Mehrle, 1986). In the previous study quantifying transcriptional differences measured from trout sampled directly from the river, *mt2* was over-expressed in both the gill (logFC: 8.2) and liver (logFC: 5.6) (Uren Webster et al., 2013), whereas after depuration we found that it was over-expressed (albeit to a lesser extent), and only in the gill (logFC: 1.6). We also identified genes with well-known roles in

stress and xenobiotic response, including heat-shock proteins (Gupta et al., 2010). Furthermore, we identified the overexpression of both isoforms of the aryl hydrocarbon receptor (*ahr1a*, *ahr1b*), which mediate the biochemical response to toxic compounds including dioxins and polyaromatic hydrocarbons (Hahn, 2002), and have been implicated in toxicant adaptation in killifish (*Fundulus heteroclius*) (Aluru et al., 2015; Whitehead et al., 2017). Related specifically to the activity of the gill in response to metals, we also identified the overexpression of several ABC transporters, which are known to be involved in the uptake, transfer and elimination of xenobiotics (Kropf et al., 2020). We observed little overlap in the DEGs identified in the gill and liver, pointing to the distinctive roles of these tissues in metal homeostasis. However, the most highly over-expressed gene in both tissues was *DENN domain-containing protein 5B-like*. The DENN domain is an evolutionarily conserved module found in all eukaryotes (Zhang et al., 2012). DENN domain-containing proteins directly interact with Rab GTPases (Marat et al., 2011), which act as molecular switches in the regulation of multiple intracellular trafficking processes (Homma et al., 2021), which respond to stress (Ganotra et al., 2023) and interact with metallothionein (Knipp et al., 2005; Wolff et al., 2008).

Overall, despite the high number of identified DEGs, we identified a low number of significant GO terms. This could indicate that processes regulating metal tolerance become more random over time, indicating that there is low concordance in the function of the genes which govern the recovery process. Although we sampled individuals of the same age, this result could also indicate the stochasticity in using wild fish in our experiment, and moreover, that wild trout might not experience metal toxicity in the same way, nor bear the same metal tolerance phenotype. Finally, a different affinity to acclimation in the laboratory could also confound our identity of genes related to the recovery and continued detoxification to metals. Related to this, although we identified some GO terms related specifically to metal homeostasis in the gill, we identified fewer than anticipated, and the liver showed very little signal related to metal homeostasis. Moreover, a recent study of mosquitofish (*Gambusia affinis*) inhabiting metal-polluted environments, also found that many of the DEGs were not associated with known metal response pathways, highlighting the multifariousness of metal toxicity and indicating that undocumented pathways could be responsible (Coffin et al., 2022). Generally, these results highlight the complexity of understanding metal tolerance, and call attention to our lack of knowledge surrounding adaptation and acclimation to metals in wild vertebrates.

Nevertheless, in the gill, we did identify a convincing signal of pathways involved in ion transport. Metals mainly cause toxicity by disrupting ion transport pathways in the gill, which eventually causes disruptions of ionic balance in the body (Evans 1987; Wood, 1992). In particular, Cd and Zn are known to share uptake routes in the gill (Ca transport pathways), where acute toxicity occurs as a result of the disruption of Ca homeostasis (Hogstrand et al., 1996; Niyogi & Wood, 2004). On the other hand, Cu uptake in the gill is known to occur mainly via Na transport pathways, and acute toxicity manifests via the disruption of Na homeostasis (Grosell & Wood, 2002). We identified both Ca ion transmembrane transport,

voltage-gated calcium channel activity, and Na ion transport as significantly activated in the gill. Additionally, metals can cause the inhibition of the Na+/K+-ATPase pump (Grosell et al., 2002; Haverinen & Vornanen, 2023), and Cu can increase Cl- loss (Mazon et al., 2002). Both potassium and chloride ion transport were activated, as well as voltage-gated potassium channel activity. Together, this suggests that compensatory increases in ion transport activity may underpin responses to limit the effects of metals in the gill of metal-impacted trout.

Our results suggest that constitutive gene expression could be an important adaptive mechanism permitting metal tolerance. Plasticity theory predicts that when populations experience consistent inducing conditions over multiple generations, a plastic response can eventually become constitutively expressed, even in the absence of the original cues that induced the change (*i.e.* genetic assimilation; Pigliucci et al., 2006; Waddington, 1961). It has also been suggested that constitutive expression can provide higher fitness than short-term responsive expression (Evans, 2015; Geisel, 2011; Snell-Rood et al., 2010). We note that we are limited in fully deciphering what proportion of the DEGs are constitutively expressed solely due to metal handling, as the DEG signatures could also be indicative of other environmental factors. However, the clear separation of the gene expression profiles, the identification of relevant genes and pathways in the gill, accompanied by the observed differences in tissue-metal concentrations, and their depuration, does implicate an important role of constitutive gene expression in metal tolerance.

5 Conclusions and considerations

Using the findings from our genomic and transcriptomic analysis of populations of brown trout from a region of southwest England heavily impacted by metal pollution, we provide valuable new insights into the evolution of metal tolerance in this species. The observation of elevated tissue-metal burdens confirms that metal pollution affects the trout residing within these rivers and implicates the evolution of adaptive strategies to cope with the insult of elevated metals. Across our genomic and transcriptomic analyses, we identified a shared biological functionality of genes and pathways involved in oxidative stress and response to chemical exposure. Several RADseq outlier loci were identified as genes with known roles in oxidative stress and we also identified transcriptomic pathways which respond to oxidative stress, including the regulation of small GTPases (Ferro et al., 2012), Wnt signalling (Funato et al., 2006), heat shock proteins (Kalmar & Greensmith, 2009), AhR pathway activation (Kou et al., 2024) and cytochrome-c oxidase activity (Srinivasan & Avadhani, 2012).

The high number of constitutively expressed genes in the gill, accompanied by the strong patterns of metal accumulation and depuration, highlights this organ as functionally important for metal tolerance. As a respiratory organ, gills are essential for survival. The gill also acts as the main route of metal uptake, and the known disruptive impact of metals on ionoregulation (and osmoregulation), likely results in compensatory mechanisms to balance the necessity to breathe and ionoregulate, whilst limiting metal toxicity. The observation of activated biological pathways involved in ionoregulation in the gill highlights the multifaceted impacts of metals

on these processes. However, increased ionoregulation to compensate for ion loss likely results in an overall increased uptake in metals. Such increased metal uptake would necessitate the evolution of modified mechanisms and routes of internal metal handling. The tissue-metal data supports this hypothesis, with the gill showing a high uptake of all metals, but significant depuration of these metals over time. Crucially, such increased metal load would be expected to drive the evolution of adaptive mechanisms to mitigate metal-stress, and our identification of genomic and transcriptomic candidates involved in oxidative stress and response to chemicals emphasises these processes as being particularly important. Continuing research involving laboratory experiments related to the physiology of metal tolerance in these trout, and whole-genome sequencing of trout surviving in metal-impacted rivers in the southwest of England, and elsewhere, should help to further illuminate these processes.

Data Availability

RADseq data for the following samples (cam01, cam02, fal01, gan01, gan02, hay06-hay13) were previously published in Paris *et al.* 2017, (https://doi.org/10.1111/2041-210X.12775) and are available at the NCBI Sequence Read Archive, BioProject PRJNA379215, accession numbers SRR5344602–SRR5344617. De-multiplexed RADseq data for remaining samples is available in the European Nucleotide Archive (ENA) under the project accession number (XXX). RNAseq data is available from ArrayExpress accession E-MTAB-10209.

Author contributions

JRP and JRS conceived the study. JRP designed and performed the research, analysed the data and wrote the paper with contributions from co-authors. RAK, SS, AL, VB and PBH performed research and analysed data. LVL designed research. KAM and AF contributed analytical tools. MAU, RWW, NRB, and JMC designed and performed research. EMS and JRS designed and performed research and co-wrote the paper. All authors read the manuscript and provided comments.

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References

Ahrens, C. W., Rymer, P. D., Stow, A., Bragg, J., Dillon, S., Umbers, K. D. L., & Dudaniec, R. Y. (2018). The search for loci under selection: trends, biases and progress. *Molecular Ecology*, *27*(6), 1342–1356.

Aluru, N., Karchner, S. I., Franks, D. G., Nacci, D., Champlin, D., & Hahn, M. E. (2015). Targeted mutagenesis of aryl hydrocarbon receptor 2a and 2b genes in Atlantic killifish (Fundulus heteroclitus). *Aquatic Toxicology* , *158*, 192–201.

Andres, S., Ribeyre, F., Tourencq, J.-N., & Boudou, A. (2000). Interspecific comparison of cadmium and zinc contamination in the organs of four fish species along a polymetallic pollution gradient (Lot River, France). *The Science of the Total Environment*, *248*(1), 11–25.

Atli, G., & Canli, M. (2013). Metals (Ag(+) , Cd(2+) , Cr(6+)) affect ATPase activity in the gill, kidney, and muscle of freshwater fish Oreochromis niloticus following acute and chronic exposures. *Environmental Toxicology*, *28*(12), 707–717.

Badyaev, A. V. (2005). Stress-induced variation in evolution: from behavioural plasticity to genetic assimilation. *Proceedings. Biological Sciences / The Royal Society*, *272*(1566), 877–886.

Baillon, L., Pierron, F., Pannetier, P., Normandeau, E., Couture, P., Labadie, P., Budzinski, H., Lambert, P., Bernatchez, L., & Baudrimont, M. (2016). Gene transcription profiling in wild and laboratory-exposed eels: Effect of captivity and in situ chronic exposure to pollution. *The Science of the Total Environment*, *571*, 92–102.

Bekkevold, D., Höjesjö, J., Nielsen, E. E., Aldvén, D., Als, T. D., Sodeland, M., Kent, M. P., Lien, S., & Hansen, M. M. (2020). Northern European Salmo trutta (L.) populations are genetically divergent across geographical regions and environmental gradients. *Evolutionary Applications*, *13*(2), 400–416.

Bélanger-Deschênes, S., Couture, P., Campbell, P. G. C., & Bernatchez, L. (2013). Evolutionary

change driven by metal exposure as revealed by coding SNP genome scan in wild yellow perch (Perca flavescens). *Ecotoxicology* , *22*(5), 938–957.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* , *30*(15), 2114–2120.

Bourret, V., Couture, P., Campbell, P. G. C., & Bernatchez, L. (2008). Evolutionary ecotoxicology of wild yellow perch (Perca flavescens) populations chronically exposed to a polymetallic gradient. *Aquatic Toxicology* , *86*(1), 76–90.

Bourret, V., Dionne, M., & Bernatchez, L. (2014). Detecting genotypic changes associated with selective mortality at sea in Atlantic salmon: polygenic multilocus analysis surpasses genome scan. *Molecular Ecology*, *23*(18), 4444–4457.

Bradley, R. W., & Morris, J. R. (1986). Heavy metals in fish from a series of metal-contaminated lakes near Sudbury, Ontario. *Water, Air, and Soil Pollution*, *27*(3), 341–354.

Brown, B. E. (1977). Effects of mine drainage on the River Hayle, Cornwall a) factors affecting concentrations of copper, zinc and iron in water, sediments and dominant invertebrate fauna. *Hydrobiologia*, *52*(2), 221–233.

Bryan, G. W., Gibbs, P. E., Hummerstone, L. G., & Burt, G. R. (1987). Copper, zinc, and organotin as long-term factors governing the distribution of organisms in the Fal estuary in Southwest England. *Estuaries*, *10*(3), 208–219.

Burt, R., Burnley, R., Gill, M., & Neill, A. (2014). *Mining in Cornwall and Devon: Mines and Men*. University of Exeter Press.

Campbell, P. G. C., Kraemer, L. D., Giguère, A., Hare, L., & Hontela, A. (2008). Subcellular Distribution of Cadmium and Nickel in Chronically Exposed Wild Fish: Inferences Regarding Metal Detoxification Strategies and Implications for Setting Water Quality Guidelines for Dissolved Metals. *Human and Ecological Risk Assessment: An International Journal*, *14*(2), 290–316.

Capblancq, T., Luu, K., Blum, M. G. B., & Bazin, E. (2018). Evaluation of redundancy analysis to identify signatures of local adaptation. *Molecular Ecology Resources*, *18*(6), 1223–1233.

Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., & Cresko, W. A. (2013). Stacks: an analysis tool set for population genomics. *Molecular Ecology*, *22*(11), 3124–3140.

Catchen, J. M., Hohenlohe, P. A., Bernatchez, L., Funk, W. C., Andrews, K. R., & Allendorf, F. W. (2017). Unbroken: RADseq remains a powerful tool for understanding the genetics of adaptation in natural populations [Review of *Unbroken: RADseq remains a powerful tool*

for understanding the genetics of adaptation in natural populations]. *Molecular Ecology Resources*, *17*(3), 362–365.

Chandrapalan, T., & Kwong, R. W. M. (2021). Functional significance and physiological regulation of essential trace metals in fish. *The Journal of Experimental Biology*, *224*(24). https://doi.org/10.1242/jeb.238790

Chi, L., Lai, Y., Tu, P., Liu, C.-W., Xue, J., Ru, H., & Lu, K. (2019). Lipid and cholesterol homeostasis after arsenic exposure and antibiotic treatment in mice: Potential role of the Microbiota. *Environmental Health Perspectives*, *127*(9), 97002.

Chowdhury, M. J., Bucking, C., & Wood, C. M. (2008). Pre-exposure to waterborne nickel downregulates gastrointestinal nickel uptake in rainbow trout: indirect evidence for nickel essentiality. *Environmental Science & Technology*, *42*(4), 1359–1364.

Coffin, J. L., Kelley, J. L., Jeyasingh, P. D., & Tobler, M. (2022). Impacts of heavy metal pollution on the ionomes and transcriptomes of Western mosquitofish (Gambusia affinis). *Molecular Ecology*, *31*(5), 1527–1542.

Couture, P., & Pyle, G. (2008). Live Fast and Die Young: Metal Effects on Condition and Physiology of Wild Yellow Perch from along Two Metal Contamination Gradients. *Human and Ecological Risk Assessment: An International Journal*, *14*(1), 73–96.

Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., Handsaker, R. E., Lunter, G., Marth, G. T., Sherry, S. T., McVean, G., Durbin, R., & 1000 Genomes Project Analysis Group. (2011). The variant call format and VCFtools. *Bioinformatics* , *27*(15), 2156–2158.

Defo, M. A., Bernatchez, L., Campbell, P. G. C., & Couture, P. (2015). Transcriptional and biochemical markers in transplanted Perca flavescens to characterize cadmium- and copper-induced oxidative stress in the field. *Aquatic Toxicology* , *162*, 39–53.

De Smet, H., De Wachter, B., Lobinski, R., & Blust, R. (2001). Dynamics of (Cd,Zn)-metallothioneins in gills, liver and kidney of common carp Cyprinus carpio during cadmium exposure. *Aquatic Toxicology* , *52*(3-4), 269–281.

Díaz, L., Zambrano, E., Flores, M. E., Contreras, M., Crispín, J. C., Alemán, G., Bravo, C., Armenta, A., Valdés, V. J., Tovar, A., Gamba, G., Barrios-Payán, J., & Bobadilla, N. A. (2020). Ethical Considerations in Animal Research: The Principle of 3R's. *Revista de Investigacion Clinica; Organo Del Hospital de Enfermedades de La Nutricion*, *73*(4), 199–209.

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2012). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* , *29*(1), 15–21.

Dodmane, P. R., Arnold, L. L., Kakiuchi-Kiyota, S., Qiu, F., Liu, X., Rennard, S. I., & Cohen, S. M. (2013). Cytotoxicity and gene expression changes induced by inorganic and organic trivalent arsenicals in human cells. *Toxicology*, *312*, 18–29.

Eastwood, S., & Couture, P. (2002). Seasonal variations in condition and liver metal concentrations of yellow perch (Perca flavescens) from a metal-contaminated environment. *Aquatic Toxicology* , *58*(1-2), 43–56.

Etter, P. D., Bassham, S., Hohenlohe, P. A., Johnson, E. A., & Cresko, W. A. (2011). SNP discovery and genotyping for evolutionary genetics using RAD sequencing. *Methods in Molecular Biology* , *772*, 157–178.

Evans, D. H. (1987). The fish gill: site of action and model for toxic effects of environmental pollutants. *Environmental Health Perspectives*, *71*, 47–58.

Evans, T. G. (2015). Considerations for the use of transcriptomics in identifying the “genes that matter” for environmental adaptation. *The Journal of Experimental Biology*, *218*(Pt 12), 1925–1935.

Ferro, E., Goitre, L., Retta, S. F., & Trabalzini, L. (2012). The Interplay between ROS and Ras GTPases: Physiological and Pathological Implications. *Journal of Signal Transduction*, *2012*, 365769.

Forester, B. R., Lasky, J. R., Wagner, H. H., & Urban, D. L. (2018). Comparing methods for detecting multilocus adaptation with multivariate genotype-environment associations. *Molecular Ecology*, *27*(9), 2215–2233.

Fraser, D. J., Weir, L. K., Bernatchez, L., Hansen, M. M., & Taylor, E. B. (2011). Extent and scale of local adaptation in salmonid fishes: review and meta-analysis. *Heredity*, *106*(3), 404–420.

Frichot, E., Schoville, S. D., Bouchard, G., & François, O. (2013). Testing for associations between loci and environmental gradients using latent factor mixed models. *Molecular Biology and Evolution*, *30*(7), 1687–1699.

Funato, Y., Michiue, T., Asashima, M., & Miki, H. (2006). The thioredoxin-related redox-regulating protein nucleoredoxin inhibits Wnt–β-catenin signalling through Dishevelled. *Nature Cell Biology*, *8*(5), 501–508.

Ganotra, J., Sharma, B., Biswal, B., Bhardwaj, D., & Tuteja, N. (2023). Emerging role of small GTPases and their interactome in plants to combat abiotic and biotic stress. *Protoplasma*, *260*(4), 1007–1029.

Garai, P., Banerjee, P., Mondal, P., Ch, N., & Saha, R. (2021). Effect of heavy metals on fishes: Toxicity and bioaccumulation. *Journal of Clinical Toxicology*, 1–10.

Geisel, N. (2011). Constitutive versus responsive gene expression strategies for growth in changing environments. *PloS One*, *6*(11), e27033.

Ghalambor, C. K., McKAY, J. K., Carroll, S. P., & Reznick, D. N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*, *21*(3), 394–407.

Goudet, J. (2005). hierfstat, a package for r to compute and test hierarchical F-statistics. *Molecular Ecology Notes*, *5*(1), 184–186.

Grosell, M., Nielsen, C., & Bianchini, A. (2002). Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, *133*(1-2), 287–303.

Grosell, M., & Wood, C. M. (2002). Copper uptake across rainbow trout gills: mechanisms of apical entry. *The Journal of Experimental Biology*, *205*(Pt 8), 1179–1188.

Gupta, S. C., Sharma, A., Mishra, M., Mishra, R. K., & Chowdhuri, D. K. (2010). Heat shock proteins in toxicology: how close and how far? *Life Sciences*, *86*(11-12), 377–384.

Hahn, M. E. (2002). Aryl hydrocarbon receptors: diversity and evolution. *Chemico-Biological Interactions*, *141*(1-2), 131–160.

Hamilton, P. B., Rolshausen, G., Uren Webster, T. M., & Tyler, C. R. (2017). Adaptive capabilities and fitness consequences associated with pollution exposure in fish. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *372*(1712). https://doi.org/10.1098/rstb.2016.0042

Hamilton, S. J., & Mehrle, P. M. (1986). Metallothionein in Fish: Review of Its Importance in Assessing Stress from Metal Contaminants. *Transactions of the American Fisheries Society*, *115*(4), 596–609.

Handy, R. D. (1992). The assessment of episodic metal pollution. I. Uses and limitations of tissue contaminant analysis in rainbow trout (Oncorhynchus mykiss) after short waterborne exposure to cadmium or copper. *Archives of Environmental Contamination and Toxicology*, *22*(1), 74–81.

Han, H. J., Tokino, T., & Nakamura, Y. (1998). CSR, a scavenger receptor-like protein with a protective role against cellular damage causedby UV irradiation and oxidative stress. *Human Molecular Genetics*, *7*(6), 1039–1046.

Hansen, M. M., Olivieri, I., Waller, D. M., Nielsen, E. E., & GeM Working Group. (2012). Monitoring adaptive genetic responses to environmental change. *Molecular Ecology*, *21*(6), 1311–1329.

Hart, M. B., Molina, G. S., & Smart, C. W. (2020). Estuarine foraminifera from South West England: impact of metal pollution in a mining heritage area. *Journal of Sedimentary Environments*, *5*(1), 1–16.

Hartwig, A., Asmuss, M., Ehleben, I., Herzer, U., Kostelac, D., Pelzer, A., Schwerdtle, T., & Bürkle, A. (2002). Interference by toxic metal ions with DNA repair processes and cell cycle control: molecular mechanisms. *Environmental Health Perspectives*, *110 Suppl 5*, 797–799.

Haverinen, J., & Vornanen, M. (2023). Dual effect of metals on branchial and renal Na,K-ATPase activity in thermally acclimated crucian carp (Carassius carassius) and rainbow trout (Oncorhynchus mykiss). *Aquatic Toxicology* , *254*, 106374.

Hecht, S. A., Baldwin, D. H., Mebane, C. A., Hawkes, T., Gross, S. J., & Scholz, N. L. (2007). *An overview of sensory effects on juvenile salmonids exposed to dissolved copper: Applying a benchmark concentration approach to evaluate sublethal neurobehavioral toxicity*. https://repository.library.noaa.gov/view/noaa/3524

Hendry, A. P. (2016). Key Questions on the Role of Phenotypic Plasticity in Eco-Evolutionary Dynamics. *The Journal of Heredity*, *107*(1), 25–41.

Hendry, A. P., Gotanda, K. M., & Svensson, E. I. (2017). Human influences on evolution, and the ecological and societal consequences. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *372*(1712), 20160028.

He, Z., Mei, L., Connell, M., & Maxwell, C. A. (2020). Hyaluronan Mediated Motility Receptor (HMMR) Encodes an Evolutionarily Conserved Homeostasis, Mitosis, and Meiosis Regulator Rather than a Hyaluronan Receptor. *Cells* , *9*(4). https://doi.org/10.3390/cells9040819

Hoban, S., Kelley, J. L., Lotterhos, K. E., Antolin, M. F., Bradburd, G., Lowry, D. B., Poss, M. L., Reed, L. K., Storfer, A., & Whitlock, M. C. (2016). Finding the Genomic Basis of Local Adaptation: Pitfalls, Practical Solutions, and Future Directions. *The American Naturalist*,

188(4), 379–397.

Hogstrand, C., Verbost, P. M., Bonga, S. E., & Wood, C. M. (1996). Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. *The American Journal of Physiology*, *270*(5 Pt 2), R1141–R1147.

Homma, Y., Hiragi, S., & Fukuda, M. (2021). Rab family of small GTPases: an updated view on their regulation and functions. *The FEBS Journal*, *288*(1), 36–55.

Horn, J. L. (1965). A RATIONALE AND TEST FOR THE NUMBER OF FACTORS IN FACTOR ANALYSIS. *Psychometrika*, *30*, 179–185.

Huntingford, F. A., Adams, C., Braithwaite, V. A., Kadri, S., Pottinger, T. G., Sandøe, P., & Turnbull, J. F. (2006). Current issues in fish welfare. *Journal of Fish Biology*, *68*(2), 332–372.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., & Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science*, *275*(5296), 90–94.

Jia, Y., Wang, L., Qu, Z., Wang, C., & Yang, Z. (2017). Effects on heavy metal accumulation in freshwater fishes: species, tissues, and sizes. *Environmental Science and Pollution Research International*, *24*(10), 9379–9386.

Kalmar, B., & Greensmith, L. (2009). Induction of heat shock proteins for protection against oxidative stress. *Advanced Drug Delivery Reviews*, *61*(4), 310–318.

King, R. A., & Stevens, J. R. (2020). An improved genetic sex test for Atlantic salmon (Salmo salar L.). *Conservation Genetics Resources*, *12*(2), 191–193.

King, R. A., Stockley, B., & Stevens, J. R. (2020). Small coastal streams-Critical reservoirs of genetic diversity for trout (Salmo trutta L.) in the face of increasing anthropogenic stressors. *Ecology and Evolution*, *10*(12), 5651–5669.

Knipp, M., Meloni, G., Roschitzki, B., & Vasák, M. (2005). Zn7metallothionein-3 and the synaptic vesicle cycle: interaction of metallothionein-3 with the small GTPase Rab3A. *Biochemistry*, *44*(9), 3159–3165.

Kou, Z., Tran, F., & Dai, W. (2024). Heavy metals, oxidative stress, and the role of AhR signaling. *Toxicology and Applied Pharmacology*, *482*, 116769.

Kraemer, L. D., Campbell, P. G. C., & Hare, L. (2005). A field study examining metal elimination kinetics in juvenile yellow perch (Perca flavescens). *Aquatic Toxicology* , *75*(2), 108–126.

Kropf, C., Fent, K., Fischer, S., Casanova, A., & Segner, H. (2020). ABC transporters in gills of

rainbow trout (Oncorhynchus mykiss). *The Journal of Experimental Biology*, *223*(Pt 15). https://doi.org/10.1242/jeb.221069

Kumar, V., Parihar, R. D., Sharma, A., Bakshi, P., Singh Sidhu, G. P., Bali, A. S., Karaouzas, I., Bhardwaj, R., Thukral, A. K., Gyasi-Agyei, Y., & Rodrigo-Comino, J. (2019). Global evaluation of heavy metal content in surface water bodies: A meta-analysis using heavy metal pollution indices and multivariate statistical analyses. *Chemosphere*, *236*, 124364.

Laporte, M., Pavey, S. A., Rougeux, C., Pierron, F., Lauzent, M., Budzinski, H., Labadie, P., Geneste, E., Couture, P., Baudrimont, M., & Others. (2016). RAD sequencing reveals within-generation polygenic selection in response to anthropogenic organic and metal contamination in North Atlantic Eels. *Molecular Ecology*, *25*(1), 219–237.

Leonard, E. M., Banerjee, U., D'Silva, J. J., & Wood, C. M. (2014). Chronic nickel bioaccumulation and sub-cellular fractionation in two freshwater teleosts, the round goby and the rainbow trout, exposed simultaneously to waterborne and dietborne nickel. *Aquatic Toxicology* , *154*, 141–153.

Liao, Y., Smyth, G. K., & Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* , *30*(7), 923–930.

López-Maury, L., Marguerat, S., & Bähler, J. (2008). Tuning gene expression to changing environments: from rapid responses to evolutionary adaptation. *Nature Reviews. Genetics*, *9*(8), 583–593.

Lotterhos, K. E., & Whitlock, M. C. (2015). The relative power of genome scans to detect local adaptation depends on sampling design and statistical method. *Molecular Ecology*, *24*(5), 1031–1046.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550.

Lowry, D. B., Hoban, S., Kelley, J. L., Lotterhos, K. E., Reed, L. K., Antolin, M. F., & Storfer, A. (2017). Breaking RAD: an evaluation of the utility of restriction site-associated DNA sequencing for genome scans of adaptation. *Molecular Ecology Resources*, *17*(2), 142–152.

Luu, K., Bazin, E., & Blum, M. G. B. (2017). pcadapt: an R package to perform genome scans for selection based on principal component analysis. *Molecular Ecology Resources*, *17*(1), 67–77.

Macnair, M. R. (1987). Heavy metal tolerance in plants: A model evolutionary system. *Trends in*

Ecology & Evolution, *2*(12), 354–359.

Maes, G. E., Raeymaekers, J. A. M., Pampoulie, C., Seynaeve, A., Goemans, G., Belpaire, C., & Volckaert, F. A. M. (2005). The catadromous European eel Anguilla anguilla (L.) as a model for freshwater evolutionary ecotoxicology: Relationship between heavy metal bioaccumulation, condition and genetic variability. *Aquatic Toxicology* , *73*(1), 99–114.

Marandel, F., Charrier, G., Lamy, J.-B., Le Cam, S., Lorance, P., & Trenkel, V. M. (2020). Estimating effective population size using RADseq: Effects of SNP selection and sample size. *Ecology and Evolution*, *10*(4), 1929–1937.

Marat, A. L., Dokainish, H., & McPherson, P. S. (2011). DENN domain proteins: regulators of Rab GTPases. *The Journal of Biological Chemistry*, *286*(16), 13791–13800.

Mazon, A. F., Cerqueira, C. C. C., & Fernandes, M. N. (2002). Gill cellular changes induced by copper exposure in the South American tropical freshwater fish Prochilodus scrofa. *Environmental Research*, *88*(1), 52–63.

McLaren, W., Gil, L., Hunt, S. E., Riat, H. S., Ritchie, G. R. S., Thormann, A., Flicek, P., & Cunningham, F. (2016). The Ensembl Variant Effect Predictor. *Genome Biology*, *17*(1), 122.

Meirmans, P. G. (2020). genodive version 3.0: Easy-to-use software for the analysis of genetic data of diploids and polyploids. *Molecular Ecology Resources*, *20*(4), 1126–1131.

Mendes, F. K., & Hahn, M. W. (2016). Gene Tree Discordance Causes Apparent Substitution Rate Variation. *Systematic Biology*, *65*(4), 711–721.

Mérot, C., Oomen, R. A., Tigano, A., & Wellenreuther, M. (2020). A Roadmap for Understanding the Evolutionary Significance of Structural Genomic Variation. *Trends in Ecology & Evolution*, *35*(7), 561–572.

Minghetti, M., Schnell, S., Chadwick, M. A., Hogstrand, C., & Bury, N. R. (2014). A primary FIsh Gill Cell System (FIGCS) for environmental monitoring of river waters. *Aquatic Toxicology* , *154*, 184–192.

Moriyama, Y., & Koshiba-Takeuchi, K. (2018). Significance of whole-genome duplications on the emergence of evolutionary novelties. *Briefings in Functional Genomics*, *17*(5), 329–338.

Munro, H. N., & Linder, M. C. (1978). Ferritin: structure, biosynthesis, and role in iron metabolism. *Physiological Reviews*, *58*(2), 317–396.

Niyogi, S., & Wood, C. M. (2004). Kinetic analyses of waterborne Ca and Cd transport and their interactions in the gills of rainbow trout (Oncorhynchus mykiss) and yellow perch

(Perca flavescens), two species differing greatly in acute waterborne Cd sensitivity. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology*, *174*(3), 243–253.

O'Doherty, C., Keenan, J., O'Neill, F., Clynes, M., Sinkunaite, I., Horgan, K., Murphy, R., & O'Sullivan, F. (2020). Gene expression profiling of copper-resistant Caco-2 clones. *Metallomics: Integrated Biometal Science*, *12*(10), 1521–1529.

Ortiz, E. M. (2019). vcf2phylip v2. 0: convert a VCF matrix into several matrix formats for phylogenetic analysis. *URL Https://doi org/105281/zenodo*, *2540861*.

Osmond, D. R., King, R. A., Russo, I.-R. M., Bruford, M. W., & Stevens, J. R. (2024). Living in a post-industrial landscape: repeated patterns of genetic divergence in brown trout (Salmo trutta L.) across the British Isles. *Diversity and Distributions*, *Early View Online*.

Paradis, E., & Schliep, K. (2019). ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* , *35*(3), 526–528.

Paris, J. R., King, R. A., & Stevens, J. R. (2015). Human mining activity across the ages determines the genetic structure of modern brown trout (Salmo trutta L.) populations. *Evolutionary Applications*, *8*(6), 573–585.

Paris, J. R., Stevens, J. R., & Catchen, J. M. (2017). Lost in parameter space: a road map for stacks. *Methods in Ecology and Evolution / British Ecological Society*, *8*(10), 1360–1373.

Pierron, F., Bourret, V., St-Cyr, J., Campbell, P. G. C., Bernatchez, L., & Couture, P. (2009). Transcriptional responses to environmental metal exposure in wild yellow perch (Perca flavescens) collected in lakes with differing environmental metal concentrations (Cd, Cu, Ni). *Ecotoxicology* , *18*(5), 620–631.

Pigliucci, M., Murren, C. J., & Schlichting, C. D. (2006). Phenotypic plasticity and evolution by genetic assimilation. *The Journal of Experimental Biology*, *209*(Pt 12), 2362–2367.

Playle, R. C. (1998). Modelling metal interactions at fish gills. *The Science of the Total Environment*, *219*(2), 147–163.

Primmer, C. R. (2011). Genetics of local adaptation in salmonid fishes. *Heredity*, *106*(3), 401–403.

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A. R., Bender, D., Maller, J., Sklar, P., de Bakker, P. I. W., Daly, M. J., & Sham, P. C. (2007). PLINK: a tool set for whole-genome association and population-based linkage analyses. *American Journal of Human Genetics*, *81*(3), 559–575.

Rainbow, P. S. (2020). Mining-contaminated estuaries of Cornwall – field research laboratories for trace metal ecotoxicology. *Journal of the Marine Biological Association of the United Kingdom. Marine Biological Association of the United Kingdom*, *100*(2), 195–210.

Rivera, H. E., Aichelman, H. E., Fifer, J. E., Kriefall, N. G., Wuitchik, D. M., Wuitchik, S. J. S., & Davies, S. W. (2021). A framework for understanding gene expression plasticity and its influence on stress tolerance. *Molecular Ecology*, *30*(6), 1381–1397.

Saibu, Y., Kumar, S., Jamwal, A., Peak, D., & Niyogi, S. (2018). A FTIRM study of the interactive effects of metals (zinc, copper and cadmium) in binary mixtures on the biochemical constituents of the gills in rainbow trout (Oncorhynchus mykiss). *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, *211*, 48–56.

Santiago, E., Caballero, A., Köpke, C., & Novo, I. (2024). Estimation of the contemporary effective population size from SNP data while accounting for mating structure. *Molecular Ecology Resources*, *24*(1), e13890.

Schwarzenbach, R. P., Egli, T., Hofstetter, T. B., von Gunten, U., & Wehrli, B. (2010). Global Water Pollution and Human Health. *Annual Review of Environment and Resources*, *35*(1), 109–136.

Sergeant, C. J., Sexton, E. K., Moore, J. W., Westwood, A. R., Nagorski, S. A., Ebersole, J. L., Chambers, D. M., O'Neal, S. L., Malison, R. L., Hauer, F. R., Whited, D. C., Weitz, J., Caldwell, J., Capito, M., Connor, M., Frissell, C. A., Knox, G., Lowery, E. D., Macnair, R., … Skuce, N. (2022). Risks of mining to salmonid-bearing watersheds. *Science Advances*, *8*(26), eabn0929.

Sevcikova, M., Modra, H., Slaninova, A., Svobodova, Z., & Others. (2011). Metals as a cause of oxidative stress in fish: a review. *Veterinary Medicine*, *56*(11), 537–546.

Shahjahan, M., Taslima, K., Rahman, M. S., Al-Emran, M., Alam, S. I., & Faggio, C. (2022). Effects of heavy metals on fish physiology - A review. *Chemosphere*, *300*(134519), 134519.

Shekh, K., Saeed, H., Kodzhahinchev, V., Brinkmann, M., Hecker, M., & Niyogi, S. (2021). Differences in the subcellular distribution of cadmium and copper in the gills and liver of white sturgeon (Acipenser transmontanus) and rainbow trout (Oncorhynchus mykiss). *Chemosphere*, *265*, 129142.

Shi, Q., Xiong, B., Zhong, J., Wang, H., Ma, D., & Miao, C. (2017). MFHAS1 suppresses TLR4 signaling pathway via induction of PP2A C subunit cytoplasm translocation and inhibition of c-Jun dephosphorylation at Thr239. *Molecular Immunology*, *88*, 79–88.

Smedley, D., Haider, S., Ballester, B., Holland, R., London, D., Thorisson, G., & Kasprzyk, A. (2009). BioMart--biological queries made easy. *BMC Genomics*, *10*(1), 1–12.

Smith, T. B., & Bernatchez, L. (2008). Evolutionary change in human-altered environments. *Molecular Ecology*, *17*(1), 1–8.

Snell-Rood, E. C., Van Dyken, J. D., Cruickshank, T., Wade, M. J., & Moczek, A. P. (2010). Toward a population genetic framework of developmental evolution: the costs, limits, and consequences of phenotypic plasticity. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*, *32*(1), 71–81.

Sone, H., Akanuma, H., & Fukuda, T. (2010). Oxygenomics in environmental stress. *Redox Report: Communications in Free Radical Research*, *15*(3), 98–114.

Srikanth, K., Pereira, E., Duarte, A. C., & Ahmad, I. (2013). Glutathione and its dependent enzymes' modulatory responses to toxic metals and metalloids in fish--a review. *Environmental Science and Pollution Research International*, *20*(4), 2133–2149.

Srinivasan, S., & Avadhani, N. G. (2012). Cytochrome c oxidase dysfunction in oxidative stress. *Free Radical Biology & Medicine*, *53*(6), 1252–1263.

Taylor, E. B. (1991). A review of local adaptation in Salmonidac, with particular reference to Pacific and Atlantic salmon. *Aquaculture* , *98*(1), 185–207.

Theodorakis, C. W., Lee, K.-L., Adams, S. M., & Law, C. B. (2006). Evidence of altered gene flow, mutation rate, and genetic diversity in redbreast sunfish from a pulp-mill-contaminated river. *Environmental Science & Technology*, *40*(1), 377–386.

Timberlake, S. (2017). New ideas on the exploitation of copper, tin, gold, and lead ores in Bronze Age Britain: The mining, smelting, and movement of metal. *Materials and Manufacturing Processes*, *32*(7-8), 709–727.

Travis, C. C., & Hester, S. T. (1991). Global chemical pollution. *Environmental Science & Technology*, *25*(5), 814–819.

Uno, T., Ishizuka, M., & Itakura, T. (2012). Cytochrome P450 (CYP) in fish. *Environmental Toxicology and Pharmacology*, *34*(1), 1–13.

Uren Webster, T. M., Bury, N., van Aerle, R., & Santos, E. M. (2013). Global transcriptome profiling reveals molecular mechanisms of metal tolerance in a chronically exposed wild population of brown trout. *Environmental Science & Technology*, *47*(15), 8869–8877.

Valko, M., Morris, H., & Cronin, M. T. D. (2005). Metals, toxicity and oxidative stress. *Current Medicinal Chemistry*, *12*(10), 1161–1208.

Vangronsveld, J., & Clijsters, H. (1994). Toxic effects of metals. *Plants and the Chemical Elements: Biochemistry, Uptake, Tolerance and Toxicity*, 149–177.

van Straalen, N. M., & Timmermans, M. J. T. N. (2002). Genetic Variation in Toxicant-Stressed Populations: An Evaluation of the “Genetic Erosion” Hypothesis. *Human and Ecological Risk Assessment: An International Journal*, *8*(5), 983–1002.

Vareda, J. P., Valente, A. J. M., & Durães, L. (2019). Assessment of heavy metal pollution from anthropogenic activities and remediation strategies: A review. *Journal of Environmental Management*, *246*, 101–118.

Waddington, C. H. (1961). Genetic assimilation. *Advances in Genetics*, *10*, 257–293.

Wadgymar, S. M., DeMarche, M. L., Josephs, E. B., Sheth, S. N., & Anderson, J. T. (2022). Local Adaptation: Causal Agents of Selection and Adaptive Trait Divergence. *Annual Review of Ecology, Evolution, and Systematics*, *53*(1), 87–111.

Wang, W.-X. (2013). Prediction of metal toxicity in aquatic organisms. *Chinese Science Bulletin = Kexue Tongbao*, *58*(2), 194–202.

Wang, W.-X., & Rainbow, P. S. (2006). Subcellular Partitioning and the Prediction of Cadmium Toxicity to Aquatic Organisms. *Environmental Chemistry* , *3*(6), 395–399.

Waples, R. S. (2024). Practical application of the linkage disequilibrium method for estimating contemporary effective population size: A review. *Molecular Ecology Resources*, *24*(1), e13879.

Whitehead, A. (2014). Evolutionary genomics of environmental pollution. *Advances in Experimental Medicine and Biology*, *781*, 321–337.

Whitehead, A., Clark, B. W., Reid, N. M., Hahn, M. E., & Nacci, D. (2017). When evolution is the solution to pollution: Key principles, and lessons from rapid repeated adaptation of killifish (Fundulus heteroclitus) populations. *Evolutionary Applications*, *10*(8), 762–783.

Whitlock, M. C., & Lotterhos, K. E. (2015). Reliable Detection of Loci Responsible for Local Adaptation: Inference of a Null Model through Trimming the Distribution of F(ST). *The American Naturalist*, *186 Suppl 1*, S24–S36.

Williams, C. R., & Gallagher, E. P. (2013). Effects of cadmium on olfactory mediated behaviors and molecular biomarkers in coho salmon (Oncorhynchus kisutch). *Aquatic Toxicology* , *140-141*, 295–302.

Wolff, N. A., Lee, W.-K., Abouhamed, M., & Thévenod, F. (2008). Role of ARF6 in internalization of metal-binding proteins, metallothionein and transferrin, and cadmium-metallothionein

toxicity in kidney proximal tubule cells. *Toxicology and Applied Pharmacology*, *230*(1), 78–85.

Wood, C. M. (1992). Flux measurements as indices of H+ and metal effects on freshwater fish. *Aquatic Toxicology* , *22*(4), 239–263.

Wood, C. M. (2011a). 1 - An introduction to metals in fish physiology and toxicology: basic principles. In C. M. Wood, A. P. Farrell, & C. J. Brauner (Eds.), *Fish Physiology* (Vol. 31, pp. 1–51). Academic Press.

Wood, C. M. (2011b). An Introduction to Metals in Fish Physiology and Toxicology: Basic Principles. In Wood, C.M., Farrell, A.P., Brauner, C.J. (Ed.), *Homeostasis and Toxicology of Non-Essential Metals* (Vol. 31B, pp. 2–40). Elsevier.

Yan, J., Hedl, M., & Abraham, C. (2017). An inflammatory bowel disease-risk variant in INAVA decreases pattern recognition receptor-induced outcomes. *The Journal of Clinical Investigation*, *127*(6), 2192–2205.

Yu, G., Wang, L.-G., Han, Y., & He, Q.-Y. (2012). clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. *Omics: A Journal of Integrative Biology*, *16*(5), 284–287.

Zhang, D., Iyer, L. M., He, F., & Aravind, L. (2012). Discovery of Novel DENN Proteins: Implications for the Evolution of Eukaryotic Intracellular Membrane Structures and Human Disease. *Frontiers in Genetics*, *3*, 283.

Zhong, J., Shi, Q.-Q., Zhu, M.-M., Shen, J., Wang, H.-H., Ma, D., & Miao, C.-H. (2015). MFHAS1 Is Associated with Sepsis and Stimulates TLR2/NF-κB Signaling Pathway Following Negative Regulation. *PloS One*, *10*(11), e0143662.