Impacts of heavy metal pollution on the ionomes and transcriptomes of Western mosquitofish (*Gambusia affinis*)

Running title: Responses to heavy metals in mosquitofish

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Abstract

Our understanding of the mechanisms mediating the resilience of organisms to environmental change remains lacking. Heavy metals negatively affect processes at all biological scales, yet organisms inhabiting contaminated environments must maintain homeostasis to survive. Tar Creek in Oklahoma, USA, contains high concentrations of heavy metals and an abundance of Western mosquitofish (*Gambusia affinis*), though several fish species persist at lower frequency. To test hypotheses about the mechanisms mediating the persistence and abundance of mosquitofish in Tar Creek, we integrated ionomic data from seven resident fish species and transcriptomic data from mosquitofish to test hypotheses about the mechanisms mediating the persistence of mosquitofish in Tar Creek. We predicted that mosquitofish minimize uptake of heavy metals more than other Tar Creek fish inhabitants and induce transcriptional responses to detoxify metals that enter the body, allowing them to persist in Tar Creek at higher density than species that may lack these responses. Tar Creek populations of all seven fish species accumulated heavy metals, suggesting mosquitofish cannot block uptake more efficiently than other species. We found population-level gene expression changes between mosquitofish in Tar Creek and nearby unpolluted sites. Gene expression differences primarily occurred in the gill, where we found upregulation of genes involved with lowering transfer of metal ions from the blood into cells and mitigating free radicals. However, many differentially expressed genes were not in known metal response pathways, suggesting multifarious selective regimes and/or previously undocumented pathways could impact tolerance in mosquitofish. Our systems-level study identified well characterized and putatively new mechanisms that enable mosquitofish to inhabit heavy metal-contaminated environments.

Keywords: ecological transcriptomics, environmental physiology, ionomics, local adaptation, Tar Creek, tolerance to heavy metals

Introduction

Anthropogenic alterations of the environment have caused the degradation and destruction of ecosystems worldwide (Chapin et al., 2000; Falkowski et al., 2000; Urabe, Naeem, Raubenheimer, & Elser, 2010). Pollution is a particularly widespread form of human impact and affects biological systems across all levels of organization. Populations living in disrupted ecosystems typically face three contrasting endpoints: migration toward more suitable habitats (Lenihan et al., 2001), adaptation to altered environmental conditions (Whitehead, Clark, Reid, Hahn, & Nacci, 2017), or extirpation (Wineland et al., 2019). There are numerous examples of population declines and extirpations caused by exposure to contaminants (e.g., Sánchez-Bayo, Goka, & Hayasaka, 2016; Silva et al., 2017), but some populations are able to persist even in heavily degraded ecosystems (see Oziolor & Matson, 2015). Organisms that persist in the presence of environmental contaminants must maintain physiological homeostasis to support critical biological processes, and there are four hypothetical mechanisms by which organisms can do so: exclusion, detoxification, resistance, and mitigation. In animals, exclusion is typically associated with alterations of the integument that minimize or eliminate uptake or maximize excretion of contaminants, thus maintaining low endogenous concentrations (see Goffredi, Childress, Desaulniers, & Lallier, 1997; Grieshaber & Völkel, 1998). In contrast, tolerance involves several coping strategies that minimize the pathological effects of contaminants when organisms are unable to exclude them from their bodies. Tolerance could be achieved through: enzymatic detoxification of contaminants into less harmful forms (Grieshaber & Völkel, 1998; Hildebrandt & Grieshaber, 2008), resistance to toxic effects by modification of direct toxicity targets (as in Pfenninger et al., 2014), or utilization of alternative physiological pathways that are not directly affected by the contaminants (i.e., mitigation; see Hildebrandt & Grieshaber, 2008). In most cases, we know little about the mechanisms allowing persistence in polluted environments. While there are clear predictions regarding the effects of individual contaminants on specific biological pathways (see below and Wood, Farrell, & Brauner, 2011a, 2011b) comparatively little is known about systems-level adjustments in response to multiple contaminants, particularly in a natural setting. As such, we used an agnostic approach to test how two organismal systems (i.e., the transcriptome, and the ionome) respond to differences in the concentration of heavy metals.

Heavy metals (e.g., lead [Pb], zinc [Zn], and cadmium [Cd]) sourced from mining activities are common contaminants in the biosphere (Pacyna & Pacyna, 2001). These elements have detrimental effects at all levels of biological organization, notably causing behavioral changes, reproductive damage, and increased mortality at an individual level, with cascading consequences for populations and ecosystems (Harmon, 2009; Neuberger, Hu, Drake, & Jim, 2009). In animals, the most toxic heavy metal ions are divalent cations that enter the body through branchial, cutaneous, and intestinal uptake (Gall, Boyd, & Rajakaruna, 2015; Wood et al., 2011a, 2011b). These ions then enter cells through calcium (Ca) channels by mimicking Ca^{2+} (see Wood, Farrell, & Brauner, 2011a, 2011b for reviews in fishes). Once inside the cell, the toxicity of metal ions is primarily caused by disruption of the redox balance (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012). Heavy metal ions are oxidized in Fenton-like reactions, which produce hydroxyl radicals from the reaction of a metal (traditionally iron [Fe^{3+}]) and hydrogen peroxide (Fenton & Jackson, 1899; Stohs & Bagchi, 1995). Oxygen-containing free radicals like hydroxyl are known as reactive oxygen species (ROS) and are highly reactive due to the presence of unpaired electrons (e.g., $^{\bullet}OH$) and/or catalytic activity (e.g., H_2O_2) (Craig, Hogstrand, Wood, & McClelland, 2009; Mager, 2011; McGeer, Niyogi, & Smith, 2011). ROS have naturally beneficial roles in animals by participating in signaling cascades (Fernandez-Marcos & Nóbrega-Pereira, 2016) and have even been shown to increase life span in *Drosophila melanogaster* in certain reaction pathways (see Scialò et al., 2016). However, when ROS concentrations exceed the antioxidant capacity to reduce or bind them, ROS oxidize cellular components, including nucleic acids (Cooke, Evans, Dizdaroglu, & Lunec, 2003), proteins (Davies, 2012), and lipids (Regoli & Giuliani, 2014), leading to oxidative damage. Heavy metal ions have also been shown to decrease ATP production by disrupting the electrochemical gradient across the inner mitochondrial membrane (Belyaeva, Sokolova, Emelyanova, & Zakharova, 2012; Kenderešová et al., 2012) and abstracting electrons bound for the electron transport chain (Meyer et al., 2013). Finally, heavy metal toxicity leads to a general suppression of the immune system via direct reduction of macrophage and lymphocyte activity (Sanchez-Dardon et al., 1999) and a decrease in antibody production (O'Neill, 1981; Viale & Calamari, 1984).

Metazoans have evolved strategies to counteract the toxic effects of heavy metals. Chief among these are antioxidant proteins (e.g., thiol-containing metallothionein and glutathione and radical-scavenging enzymes like superoxide dismutase and catalase) and nonenzymatic antioxidants like vitamins A, C, and E (Birben et al., 2012). There is also evidence of antioxidant regulation by hormones, such as melatonin, which can modulate the expression of antioxidant genes (Steinhilber et al., 1995) and directly scavenge free radicals with its reducing indole group (Poeggeler, Reiter, Hardeland, Tan, & Barlow-Walden, 1996). These antioxidants are crucial for metazoan life, as many of these proteins are highly conserved, with functional orthologs present in almost all aerobic organisms (Corona & Robinson, 2006). Despite these critical antioxidant defenses, heavy metal pollution often raises ROS levels beyond the binding and reductive capacity of natural defenses (Nemmiche, 2016). An important question is whether and how individuals use these existing antioxidant mechanisms to persist in polluted habitats.

The Tar Creek Superfund Site in Ottawa County, Oklahoma, USA is characterized by high levels of Cd and Zn stemming from flooded mines and runoff from tailings piles, relics of intensive mining operations in the area during the first half of the twentieth century (OWRB, 1983; United States Environmental Protection Agency, 2016). Runoff has caused increases in metal concentrations in the soil and water that pose significant risk to humans and wildlife inhabiting the area, and most tailings piles drain directly into Tar Creek (Datin & Cates, 2002; OWRB, 1983). Franssen (2009) demonstrated that the water in Tar Creek is extremely hard (i.e., high mineral content), which can buffer the toxic effects of metal cations (Hogstrand, 2011). However, even after correcting for the extreme hardness of the water, concentrations of both Cd and Zn in polluted reaches of Tar Creek exceed their respective national recommended water quality criteria for aquatic life from the United States Environmental Protection Agency (Franssen, 2009; United States Environmental Protection Agency, 2019). Despite ongoing remediation efforts (Cremeans, Devlin, Osorno, & Nairn, 2019), surface waters of Tar Creek are still polluted with potentially lethal concentrations of heavy metals (Neuberger et al., 2009; United States Environmental Protection Agency, 2016). In the fifty years since mining operations ceased, the Tar Creek watershed has exhibited altered fish community composition, decreased species richness, and impaired somatic and reproductive condition of the fishes that do persist (Franssen, 2009; Franssen, Brooks, Parham, Sutherland, & Matthews, 2006). While there are several fish species still inhabiting Tar Creek, Western mosquitofish (*Gambusia affinis*) are particularly abundant. Mosquitofish have been common in polluted sections of Tar Creek since at least the late 1960s (Branson, 1967), and they remain common during sampling today, representing over 80 percent of individuals in polluted reaches (Franssen et al., 2006; personal observations JLC, 2017-2019). There are numerous uncontaminated watersheds in close proximity to Tar Creek (Franssen, 2009; Franssen et al., 2006), allowing for comparative analyses that test hypotheses about how mosquitofish can persist in extreme environments like those found in Tar Creek.

Due to the extensive work on the physiological effects of heavy metals, we have a framework to form *a priori* hypotheses about the potential mechanisms that might mediate persistence of mosquitofish in Tar Creek. Here, we specifically asked whether mosquitofish in Tar Creek can actively maintain metal homeostasis through exclusion, and whether there is evidence for coping mechanisms associated with minimizing heavy metal toxicity (i.e., tolerance through detoxification, resistance, or mitigation). We hypothesized that mosquitofish have an increased ability to actively regulate their internal ion composition by blocking uptake of metals from the environment or eliminating metals from the body. Because no element operates in isolation (Baxter, 2015), we tested this hypothesis by comparing ionomes—concentrations of all mineral nutrients in an organism (Lahner et al., 2003)—across several fish species that inhabit both polluted and unpolluted watersheds and predicted that metal accumulation would be lower in mosquitofish than in other species that are less abundant in Tar Creek. We interpreted ionomic data using the framework of ecological stoichiometry that makes predictions about the mobility of elements (both essential and toxic) at multiple biological scales, from tissues to communities (Peace et al., 2021). We also hypothesized that mosquitofish could detoxify metals that enter the body by modulating the expression of genes in relevant pathways. We tested this hypothesis by comparing genome-wide gene expression patterns in mosquitofish from polluted and unpolluted habitats. We predicted transcriptional modifications to primarily occur in genes associated with known heavy metal response pathways.

Methods

Overview of study system

The Tar Creek Superfund site consists of approximately 100 square kilometers in Northeastern Oklahoma, centered around the impacted Tar Creek drainage. We selected sites in the Neosho River drainage with minimal environmental differences other than the presence or absence of heavy metals that were previously documented to contain mosquitofish populations that have been used for comparative analyses (Franssen, 2009; Franssen et al., 2006). These sites included a polluted section of Tar Creek (36.944° N, 94.854° W) as well as two sites in adjacent uncontaminated drainages, Coal Creek (36.850° N, 94.927° W) and Little Elm Creek (36.910° N, 94.806° W; see Figure 1). Coal Creek was used as a reference site for both ionomic and transcriptomic experiments, while Little Elm Creek was only used as a reference site for the transcriptomic experiment. Based on previous water chemistry monitoring in the area (Franssen, 2009; United States Environmental Protection Agency, 2016), we characterized levels of heavy metal pollution in each site as a binary variable (heavy metals present in Tar Creek or heavy metals absent in reference watersheds). All experimental protocols were approved by Kansas State University's Institutional Animal Care and Use Committee (protocol # 4379).

Do mosquitofish exhibit ionomic patterns indicative of active exclusion or increased elimination?

We tested whether mosquitofish had an increased ability to maintain low internal heavy metal concentrations compared to the other species inhabiting Tar Creek through exclusion or elimination. We predicted clear differences in elemental composition between fish from polluted and unpolluted sites across all species, and that mosquitofish would accumulate heavy metals to a lesser extent. Lower levels of heavy metal accumulation would suggest that mosquitofish can actively exclude or eliminate them from the body at a higher rate than other species.

Sampling and processing

To test our ionomic predictions, we sampled Tar and Coal Creeks (Table 1) and collected individuals of each inhabitant fish species using a 3-mm seine net. At each site, up to seven individuals per species were placed in aerated buckets and transported to a facility operated by the Grand River Dam Authority in Miami, OK for processing. All fish were individually sacrificed by cervical dislocation, and standard length (mm) was recorded for each individual. Visceral organs were removed and discarded using new stainless-steel dissection scissors and forceps. Viscera were removed because their ionomic signatures could be labile, depending on daily fluctuations of behavior or diet, whereas the ionomic signature arising from the remainder of the body is likely more robust to such changes. The remaining parts of the eviscerated carcass were dried in a food dehydrator (National Presto Industries, Eau Claire, Wisconsin, USA) at 55 °C for 48 hours to prevent sample decay. Samples were brought to Kansas State University for further desiccation in a drying oven at 60 °C for at least two weeks, weighed, and digested in a 2:1 mixture of trace metal grade HNO_3 and H_2O_2 for analysis using an inductively coupled plasma optical emission spectrometer (ICP-OES; Thermo Scientific iCAP 7400), following the methods of Rudman et al. (2019) to generate ionomes for each sample. After removing elements from our analysis that were below the limit of detection of the ICP-OES, our ionomic data consisted of mass-standardized concentrations of 24 biologically relevant elements (see Supplemental Table S1).

Statistical analyses

If species experienced elemental shifts (in metals or non-metals) in Tar Creek, we predicted that we would be able to identify differences between sites in the concentrations of individual elements and suites of covarying elements. To test these predictions, we first analyzed variation in our overall ionomic dataset using principal component analysis. We used the prcomp function with a correlation matrix in R (version 4.0.0; R Core Team, 2020) and retained six principal components with eigenvalues greater than 1, as in Kaiser (1960) (see Supplemental Table S1 for loadings for each element along each retained axis). The principal component scores for these six axes were used as dependent variables in subsequent analyses. We compared principal component scores between the contaminated and uncontaminated populations across all species to test for shared ionomic divergence across species between sites. Principal component scores were used as dependent variables in multivariate analysis of variance (MANOVA), and we included ‘site’ and ‘species’ as fixed factors. We found high covariance between species and standard length, which violated model assumptions. Hence, we calculated measures of relative standard length, and included this metric as a covariate to account for intraspecific allometry. All possible two-way interaction terms were included, and non-significant effects were removed from the final model. We used a type-III test to partition the sums of squares between the elements of our model using the Manova function from the car package in R (version 3.0-8; Fox & Weisberg, 2019) and Wilks' lambda to approximate F-statistics. Relative standard-length was not significant ($F_{6,57} = 0.788$, $P = 0.583$), so the main effect and all interactions including the relative standard-length term were removed from the final model. Thus, our final model contained the main effects of site and species and an interaction between site and species. Effect sizes were estimated with a multivariate analog of partial eta-squared using Wilks' lambda from the etasq function in the heplots package (version 1.3-5; Fox, Friendly, & Monette, 2018).

In the MANOVA, there was a significant interaction between site and species ($F_{36,288.2} = 5.338$, $P < 0.001$), indicating that the effect of site (polluted vs. unpolluted) varied among species (see results). The significant interaction term might indicate that some species do not exhibit differences across sites while others do, or that site differences are omnipresent but vary in nature and magnitude. To distinguish between these alternative explanations and assess the nature of convergent elemental differentiation between sites across species, we conducted a *post-hoc* canonical analysis of the site term in the MANOVA (as in Langerhans, 2009). We ran a PCA (covariance matrix) on the sums of squares and cross products matrix for the site term, which yielded an eigenvector (called a divergence vector) for PC1 that summarized the linear combination of variables in a single canonical axis that maximized ionomic differences between Tar and Coal Creek individuals across all species, while controlling for all other factors in our model. We then multiplied our matrix of dependent variables by the divergence vector, which yielded a vector of divergence vector scores, with one score for each individual. Total divergence in elemental composition is comprised of both convergent and non-convergent (i.e., unique to a single species) components of elemental differentiation, and divergence vector scores extract only the convergent component. We utilized Student's t-tests to compare divergence vector scores between individuals from polluted and unpolluted sites for each species. Our ionomic dataset is archived on Dryad (https://doi.org/10.5061dryad.q2bvq83kw), and all analytical scripts can be found on GitHub (https://github.com/michitobler/TarCreekGambusia).

Are there differences in metal-responsive gene expression patterns?

We tested for gene expression differences between mosquitofish from polluted and unpolluted habitats, focusing on multiple organs in the body. In addition to differences between sites in heavy metal content, there could be other unmeasured environmental differences, so we predicted that among the various environmentally related transcriptional changes, genes involved with heavy metal binding and redox reactions would be upregulated in all organs of individuals from Tar Creek, compared to uncontaminated populations.

Sampling and processing

To quantify gene expression, we captured six adult female *G. affinis* from Tar Creek, Coal Creek, and Little Elm Creek (total *N* = 18 individuals; see Figure 1 and Supplemental Table S2 for sampling information). Two reference watersheds were utilized to better capture potential spurious, site-specific expression variation that could lead to false inferences when comparing to the contaminated site. Specimens were immediately euthanized by cervical dislocation upon capture, and standard length was measured. Gill, liver, and brain tissues were dissected and placed in separate tubes with 1 ml of RNAlater (Ambion), resulting in a total of 54 tissue samples. Samples were stored at 4 °C for approximately two weeks, at which point they were stored at -80 °C until further processing.

RNA extraction, library preparation, and sequencing

We followed RNA extraction (Macherey-Nagel Nucleospin RNA kit) and library preparation (New England Biolabs NEBNext Ultra Directional RNA Library Prep Kit with NEBNext Poly(A) mRNA

Magnetic Isolation Module) protocols previously established and utilized for other members of the family Poeciliidae (Passow et al. 2017), with the following minor modifications. During RNA extraction, the tissue sample input was smaller (between 3 and 25 mg of RNAlater-preserved tissue) because some samples (especially brains) of *G. affinis* were smaller than those of the species studied by Passow et al. (2017). Furthermore, libraries were enriched with twelve cycles of PCR amplification of adaptor ligated DNA. All library samples were stored at 4 °C to minimize freeze-thaw cycles. After assessing library quality on an Agilent Bioanalyzer High Sensitivity DNA chip, libraries were pooled into two groups of 27 samples that had equal proportions of samples from each site and tissue to minimize lane effects during sequencing. Paired-end, 100-bp sequencing was completed on two lanes of an Illumina HiSeq 2500 (Admera Health Biopharma Services, South Plainfield, NJ, USA) with one pool of 27 samples per lane.

Trimming, mapping, and assembly

Of the 54 samples sequenced, two Tar Creek gill samples and one Tar Creek brain sample were removed from the analysis due to improper indexing or low sequencing output. Thus, we analyzed gene expression in 16 gill samples, 18 liver samples, and 17 brain samples, for a total of 51 raw transcriptomes. Low quality reads (Phred score < 24) and adapter sequences were trimmed from the raw RNA-seq reads using TrimGalore! (version 0.4.1; Krueger, 2014). Only paired reads longer than 50 bp were retained. Across all tissues and sites, each sample had an average of 25,106,758 ± 9,999,182 reads. To reduce confounding effects of library size, we randomly subsampled 26 million reads (13 million paired-end reads) from 24 libraries that had significantly higher read counts than the other libraries using seqtk (Li, 2013b). After subsampling, the samples had an average of 21,502,509 ± 5,314,437 reads. The remaining high quality reads were mapped to the southern platyfish (*Xiphophorus maculatus*) reference genome (Schartl et al., 2013) using BWA-MEM (version 0.7.17; Li, 2013a) with default parameters. Reads mapped to a genome assembly of *G. affinis* (Hoffberg et al., 2018) yielded similar results, but the better assembly and annotation of the *X. maculatus* genome led to improved functional interpretation of gene expression results.

Using fixmate from samtools (version 1.2; Li et al., 2009), we added read groups to each alignment, removed soft-clipped bases, sorted aligned reads in coordinate order, and ensured all paired read alignments contained correct mate information. We used Stringtie (version 1.3.2d; Pertea et al., 2015) to extract expressed transcripts for each sample, and then used a python script (prepDE.py, provided with Stringtie) to generate a count matrix with all 27,266 genes for each sample. Supplemental Table S2 contains read counts and mapping efficiency throughout our analytical pipeline.

Quantification of differential expression

We removed genes with low expression (fewer than 2 counts per million [cpm], and present in fewer than 15 of the 51 transcriptomes), which removed 8,573 genes and brought the total number of genes in the dataset to 18,693. We then compared expression between Tar Creek and both uncontaminated creeks in each tissue separately. Using the edgeR package from Bioconductor (version 3.30.0; Robinson, McCarthy, & Smyth, 2010), we created a DGEList and calculated normalized factors based on library size for each sample. We then separated the data by tissue and created a design matrix (using the model.matrix function) for each tissue type separately, which consisted of an intercept of zero and site as the independent variable. For each tissue, we used the design matrix to calculate common and tag-wise dispersion across all three sites using estimateDisp. Then we fit a negative binomial generalized linear model to the normalized counts for each tag, using the glmFit function. We generated contrast matrices using the makeContrasts function in the limma R package (version 3.44.1; Ritchie et al., 2015) to compare expression in each tissue between all sites in a pairwise manner. Using each contrast matrix of comparisons and the fitted negative binomial models for each tag, we tested for statistically significant differences in gene expression using likelihood ratio tests (with the glmLRT function), while accounting for error due to multiple testing with a false discovery rate <0.05 using the Benjamini-Hochberg correction (Benjamini & Hochberg, 1995). To eliminate spurious gene expression differences due to inherent site-specific differences other than the factor of interest in our study (i.e., the presence of heavy metals), we used only genes that were significantly differentially expressed in the same direction in both unpolluted sites for our analyses (Supplemental Tables S3 and S4). To visualize expression variation among sites in shared differentially expressed genes, we performed a discriminant analysis of principal components (DAPC) using the adegenet package in R (version 2.1.4; Jombart, 2008). We separated the differentially expressed genes by tissue and used the dapc function to linearly transform the count data using PCA and perform a linear discriminant analysis on a chosen subset of the principal components. For all tissues, we retained eight principal components and two discriminant functions.

Functional annotation of differentially expressed genes

To understand the functions of differentially expressed genes between polluted and unpolluted sites, we annotated the genes from the *X. maculatus* reference set with functional information from human orthologs in the SwissProt database (http://ca.expasy.org/sprot/), following Passow et al. (2017). For our analyses, we used SwissProt annotations from two lists of genes in our dataset—consistently differentially expressed candidate genes in mosquitofish (the target set) and genes from the *X. maculatus* reference genome that were retained after filtering out lowly expressed genes (the background set). Each list of candidate gene annotations was uploaded as an unranked list to GOrilla (Eden, Navon, Steinfeld, Lipson, & Yakhini, 2009), which identifies the Gene Ontology (GO; The Gene Ontology Consortium, 2004) terms associated with each SwissProt entry provided and tests for enrichment of

GO terms in the target set relative to the background set. We restricted our analysis to GO terms related to biological processes. There were 11,186 genes in the background set that were uniquely associated with at least one of these GO terms. Using GOrilla, we tested for enrichment of GO terms with FDR <0.05. Of these enriched GO terms, we retained those with an enrichment score >2, and those that were associated with at least 5 genes in our target gene set, resulting in 621 total enriched GO terms (see Supplemental Table S5 for enriched terms in each tissue). Up- and downregulated genes in each tissue were analyzed separately.

Results

Mosquitofish exhibit a different shift in ionomic composition than other Tar Creek inhabitants

We generated ionomes of 4 to 7 individuals in each population of 7 species that inhabit Tar Creek (see Table 1). If mosquitofish can exclude metals, we predicted that all species except mosquitofish would experience a significant ionomic shift. To determine whether species exhibited ionomic shifts and which elements drove divergence between habitats, we analyzed ionomes between polluted and unpolluted populations across all species simultaneously. After principal component analysis, we retained 6 axes that cumulatively explained 81.8 % of the variance in elemental composition (Supplemental Table S1). The first principal component explained 38.1 % of the variation in overall elemental composition (Figure 2), and principal component scores along this axis were most positively correlated with concentrations of Be, Bi, Ca, Co, Cr, S, Tl, Zn, though all elements had a positive loading along this axis. The second principal component explained 18.4 % of the variance in elemental composition. Positive scores along PC2 were associated with higher concentrations of B, Cd, Fe, and Si and lower concentrations of Ba, Mn, Ni, and Sr. Principal component 3, which explained 9.0 % of the overall variance, was positively correlated with As, Ca, S, and Zn, and negatively correlated with Co, Se, Sr, and V. There was significant segregation of samples from different sites along each of the first three principal components (Student's *t*-tests; PC1: $t_{89} = 4.574$, $P < 0.001$; PC2: $t_{89} = -7.415$, $P < 0.001$; PC3: $t_{89} = -4.583$, $P < 0.001$), and Tar Creek samples tended to have higher scores along PC1 and lower scores along PC2 and PC3. Segregation along these three axes indicated that Tar Creek individuals were enriched in As, B, Ca, Cd, Cr, Fe, S, and Zn, relative to Coal Creek individuals.

Analyses of principal component scores in the MANOVA (Table 2) identified a significant effect of site ($F_{6,65} = 6.978$, $P < 0.001$, $\eta_P^2 = 0.392$), indicating that—irrespective of species—Tar Creek and Coal Creek individuals differed in ionomic composition, and that site explained approximately 39 % of the non-error variance in ionomic composition after controlling for other factors in the model. This variance can be thought of as the shared ionomic divergence between sites among all species. We also found that ionomic composition varied by species ($F_{36,288.2} = 6.785$, $P < 0.001$, $\eta_P^2 = 0.362$), and there was a significant interaction effect between site and species ($F_{36,288.2} = 5.338$, $P < 0.001$, $\eta_P^2 = 0.312$), demonstrating that there were differences in how each population pair responded to heavy metal stress. *Post-hoc* comparisons of the divergence vector scores from the canonical axis from the site term of our model showed significant differentiation in divergence vector scores between sites in every species except mosquitofish (for *Gambusia affinis*: $t_{10} = 1.891$, $P = 0.088$; for other species, see Figure 3), suggesting the presence of non-convergent ionomic changes in mosquitofish and that all other species exhibit significant shared ionomic variation.

Significant differential gene expression in each tissue

Due to the inherent complexity of environmental differences between our sites, we analyzed genes that were differentially expressed in the same direction in both uncontaminated sites compared to Tar Creek. We found 2,388 shared differentially expressed genes in gill samples, 499 in liver samples, and only 18 in brain samples (FDR < 0.05; Figure 4A). A separate ionomic analysis of gill, liver, and brain tissues solely in mosquitofish closely mirrored these results (see Supplemental Figure S1), showing divergence in elemental composition in gills and livers, but not in brains. The expression patterns of each mapped gene can be found in Supplemental Table S3, and visualization of divergence in gene expression across sites in each tissue can be found in plots of linear discriminant scores in Figure 4B-D.

In addition to understanding how expression differed at each gene as a function of metal exposure, we were also interested in understanding whether any genes had shared expression responses to metal exposure. Co-expressed genes may be regulated by the same transcriptional machinery or be related functionally, as they likely belong to the same biological pathway (Langfelder & Horvath, 2007). We generated weighted gene co-expression networks using weighted gene co-expression network analysis (i.e., WGCNA; see online supplement for methods) and obtained modules of genes that showed similar expression profiles and were correlated with the presence of environmental heavy metals. We found that our network-based analyses of gene expression largely matched our differential expression analyses. We found nine modules of co-expressed genes across all three tissues sampled that were associated with the presence of environmental heavy metals (Supplemental Figure S2), but functional analysis of genes within these modules did not identify any genes with known functions related to heavy metal homeostasis (see online supplement for details).

Antioxidant genes are upregulated in the gill in response to heavy metal stress

To understand the functional implications of the observed gene expression patterns, we annotated the differentially expressed genes with human SwissProt accessions and biological process GO terms. We found that several known antioxidant genes were upregulated in gill tissues, including metallothionein, glutaredoxin, nucleoredoxin, glutathione peroxidase, and glutathione S-transferase (see Supplemental Table S4 for SwissProt annotations of differentially expressed genes). Products of these genes have been shown to guard against oxidative stress by binding metal ions prior to the production of ROS or rendering free radicals inert by donating spare electrons (Hogstrand, 2011; Mager, 2011).

Using a broader systems-level approach to understand patterns of differential expression, we expected to see enriched GO terms and modules of co-expressed genes (see online supplement) related to heavy metal exposure. We found enrichment of eight GO terms in the upregulated genes in the gill, 383 terms in downregulated genes in the gill, and 230 terms in downregulated genes in the liver of fish from the polluted site (Supplemental Table S5). There was no significant enrichment for upregulated genes in the liver or for up- or down-regulated genes in the brain. To search the enriched GO terms for biological processes related to our *a priori* hypotheses regarding heavy metals, we queried the Gene Ontology database using AmiGO2 and searched for "metal" (http://amigo.geneontology.org/amigo; accessed 16 September 2021), which yielded a list of 66 biological process GO terms broadly related to metals (see Supplemental Table S6). We intersected this list with the 621 enriched GO terms and found enrichment of three biological process GO terms related to metals. There were 39 genes that were downregulated in gill samples that were associated with the "regulation of metal ion transport" GO biological process, and 57 such genes that were associated with "cellular metal ion homeostasis". The "cellular metal ion homeostasis" GO term was also enriched in downregulated genes in the liver, which was driven by downregulation of 24 genes in the liver. Finally, there were 25 genes that were downregulated in the liver that were associated with the "metal ion homeostasis" GO term.

The majority of the enriched GO terms were related to diverse biological processes not typically invoked in responses to heavy metals. The top five biological processes over-represented in upregulated genes in the gill were angiogenesis, regulation of angiogenesis, regulation of nuclear cell cycle DNA replication, regulation of vasculature development, and neuron projection guidance. In downregulated genes in the gill, the top five biological processes that were enriched in our dataset were immune system process, immune response, cell activation, defense response, and leukocyte activation, which was largely mirrored by the top five GO terms in downregulated genes in the liver—immune system process, immune response, defense response, signal transduction, and positive regulation of immune system process. Annotation and enrichment results for each tissue are summarized in Supplemental Table S5.

Discussion

Environmental perturbations represent a significant challenge to the maintenance of homeostasis. While it is clear that organisms residing in polluted environments must maintain homeostasis despite the suboptimal conditions, we have a limited understanding of the mechanisms by which they are able to do so. We utilized an integrative approach to understand whether mosquitofish are able to actively maintain homeostasis by blocking metal uptake from the environment or tolerate internal metal accumulation through transcriptional changes. We compared whole body elemental composition and transcriptome changes between populations of *G. affinis* that inhabit a heavy metal-polluted watershed and nearby unpolluted habitats to understand the mechanisms behind the persistence of these fish in the polluted watershed. We found evidence for the accumulation of multiple elements in all species in Tar Creek, but mosquitofish exhibited a pattern of ionomic divergence distinct from that of other species. We also identified significant upregulation of several antioxidant genes that are known to minimize cellular intake of metal ions and maximize detoxification of ROS. Overall, this study uncovered putative molecular mechanisms underlying the persistence of mosquitofish in Tar Creek.

Maintenance of homeostasis through exclusion of metals from the body

We found evidence for multivariate ionomic shifts in the fishes of Tar Creek, where Tar Creek individuals across all species were generally enriched in As, B, Ca, Cd, Cr, Fe, S, and Zn, and relatively depleted in Al, Ba, Co, Mn, Ni, Se, Sr, and V (Figure 2). This suggests that the water chemistry in Tar Creek is associated with changes in tightly regulated suites of elements, which has implications for how fishes build and maintain their bodies in heavy metal-contaminated water. Each species, including mosquitofish, experienced a shift in ionomic composition between populations, but the shift in mosquitofish was unique. Mosquitofish were the only species that did not show a significant difference in the site-specific divergence vector scores (i.e., the convergent differences in elemental composition across species); all other species showed evidence of significant convergence in ionomic shifts between sites (Figure 3). To better understand the unique nature of divergence in mosquitofish compared to the other species, we plotted the average principal component scores for each population along the first three principal component axes (Figure 5). Along each PC axis, the non-convergent nature of ionomic shifts in mosquitofish becomes apparent, as both populations of mosquitofish appear substantially different from the other species in their respective habitats. Furthermore, despite showing significant differences in divergence vector scores between sites (see Figure 3), *Pimephales notatus* also appeared to show large non-convergent differences in multivariate space, which warrants further study. These findings suggest that there are unique ionomic shifts in mosquitofish, but that metals are nonetheless accumulated, demonstrating that mosquitofish—as all other species in this study—lack the ability to actively maintain metal homeostasis by blocking uptake from the environment.

Other animals also show negligible evidence for exclusion of heavy metals, though several studies have found evidence of such mechanisms in the metal-chelating properties of algal cell walls (e.g., Pinto et al., 2003). Originally, we considered the possibility that mosquitofish may possess novel adaptations to limit uptake of environmental contaminants, and that mosquitofish would be the only species to not exhibit elemental differences, thus explaining the higher frequency of mosquitofish in Tar Creek compared to other resident species. However, mosquitofish still differed in ionomic composition between sites (see Figure 2 and Supplemental Table S7). The bioaccumulation of heavy metals in mosquitofish suggests that exclusion is not used as a mechanism to maintain physiological homeostasis.

Maintenance of homeostasis through detoxification

Persistence of mosquitofish in Tar Creek could also be mediated by modulating gene expression—and ultimately physiological function—to mitigate the adverse effects of heavy metal exposure and increased internal concentrations, allowing mosquitofish to maintain homeostasis through detoxification. In each tissue, we identified genes that were significantly differentially expressed in the same direction in both unpolluted sites, with the majority of these shared differentially expressed genes found in gill tissues.

In gills, where the highest accumulation of metals occurred, we found a coordinated downregulation of the *ZIP3* gene, which codes for a zinc transport protein that allows zinc to enter the cytoplasm, and upregulation of several antioxidant proteins (Supplemental Tables S3 and S4). We also found upregulation of enzymes that are able to reduce ROS into less harmful intermediates (e.g., glutathione peroxidase and several isoforms of glutathione S-transferase), as well as metal-binding proteins such as metallothionein (see Supplemental Tables S3 and S4), which matches with established patterns of gene expression and protein activity following heavy metal exposure in fishes (Basha & Rani, 2003; Ransberry, Morash, Blewett, Wood, & McClelland, 2015; Uren Webster, Bury, van Aerle, & Santos, 2013; Woo, Yum, Park, Lee, & Ryu, 2009). Counterintuitively, peroxiredoxin-6, which is important in the reduction of hydrogen peroxide with glutathione (Chen, Dodia, Feinstein, Jain, & Fisher, 2000), was downregulated in the gill, suggesting that some canonical antioxidant proteins may be triggered or repressed upon metal exposure, depending on the context of exposure. Consistent with our data, previous studies have found evidence for upregulation of some, but not all, antioxidant genes following heavy metal exposure, and the antioxidants identified varied by study. There are few studies that found evidence for differential expression of all antioxidants known to play a role in heavy metal homeostasis, suggesting that there are likely distinct responses to different mixtures of metals and that there could be functional redundancy in antioxidant responses. Alternatively, species-specific evolutionary histories could produce different genomic architectures, leading to historical contingencies in terms of which genes are responsive to heavy metal exposure in different species.

While the whole-body ionomic data showed that mosquitofish experience an influx of metal from the environment into the body, the gene expression results identified downregulation of genes coding for proteins related to transport of ions from the blood into cells and upregulation of several genes that code for antioxidant proteins. If the gene expression patterns noted here lead to proteomic differences, then the amount of heavy metals that enter cells from the bloodstream may be limited, thus lessening the adverse effects of the heavy metals once they are in the cell.

Liver and brain tissues did not exhibit the transcriptional responses to heavy metal exposure that we hypothesized or observed in the gills. While there was significant accumulation of metals and many genes were differentially expressed in the liver, the vast majority of the gene-by-gene and systems-level (GO enrichment and WGCNA) expression patterns we identified revealed downregulation of genes related to Ca^{2+} homeostasis (see Supplemental Tables S3, S5, and S8) and a general suppression of the immune system, which is a common response when organisms are stressed, by heavy metals or otherwise (Sanchez-Dardon et al., 1999). In contrast to our findings in the gill, however, we did not find significant differential expression of any genes known to play a role in heavy metal responses. This discrepancy in responses between tissues could be caused by gills being in direct contact with heavy metals in the environment.

The majority of transcriptional differences in the liver did not appear to be related to heavy metal homeostasis, but we found evidence of differential expression of at least one gene in the liver related to metabolism with known ties to heavy metal stress. There is ample evidence of disruption of mitochondrial function upon exposure to heavy metals (e.g., Belyaeva et al., 2012; Kenderešová et al., 2012; Meyer et al., 2013), and we found significant upregulation of cytochrome c, which is a protein associated with the inner mitochondrial membrane that shuttles electrons from complex III to complex IV of the electron transport chain (Hüttemann et al., 2011). In addition to furthering aerobic respiration through the oxidative phosphorylation pathway, the upregulation of cytochrome c could also represent an antioxidant response, as cytochrome c has been shown to reduce superoxide radical, thus diminishing total cellular ROS (Andreyev, Kushnareva, & Starkov, 2005; Skulachev, 1998). We hypothesize that the upregulation of cytochrome c could be an adaptive mechanism by which mosquitofish are able to maintain aerobic respiration despite the disruptions to mitochondrial function caused by heavy metals. However, further studies specifically focusing on mitochondrial physiology are required to further explore and validate this hypothesis.

Addressing alternative hypotheses

In addition to the documented upregulation of key antioxidant genes in the gill, we also saw modulation of hundreds of genes across tissues that did not appear to be related to heavy metal stress and homeostasis. There are two non-mutually exclusive hypotheses that could explain these patterns. First, mosquitofish in Tar Creek are likely under stress, which has cascading effects genome-wide (Aluru & Vijayan, 2009). Likewise, the presence of heavy metals may not be the only strong source of selection between polluted and unpolluted habitats. The Tar Creek watershed is not a static system with constant input of a few major pollutants; it is a dynamic patchwork of fluctuating heavy metal concentrations, pH, oxygen and food availability, ecological interactions, and human impact. While the unpolluted sites were selected carefully to minimize environmental differences other than heavy metal pollution, there may be unmeasured ecological differences between sites that could cause physiological responses in inhabitants. There could be numerous stressors other than heavy metals that differentially impact the organisms inhabiting Tar Creek compared to the unpolluted sites, which could lead to diverse gene expression responses that do not belong to a single pathway. This pattern has been seen in other poeciliid species inhabiting extreme environments (Passow et al., 2017; Tobler, Kelley, Plath, & Riesch, 2018). Some abiotic stressors are environmentally correlated (e.g., hypoxia and toxic hydrogen sulfide are positively correlated in freshwater: Chen & Morris, 1972; Cline & Richards, 1969); individuals coping with toxicity from one physiochemical stressor must sometimes also survive in suboptimal conditions, leading to broad gene expression responses. In addition to selection from physiochemical stressors, we likewise see reduced species richness in many extreme environments (Greenway, Arias-Rodriguez, Diaz, & Tobler, 2014), which has been shown to impact behaviors (Plath et al., 2007; Tobler, Riesch, Tobler, & Plath, 2009) and ecological interactions (Laske, Rosenberger, Wipfli, & Zimmerman, 2018; Tobler & Plath, 2011; Tobler et al., 2015). We posit that similar mechanisms could be at play in Tar Creek, leading to broad stress-related gene expression patterns. Future work should focus on the interplay of these multifarious interactions and include controlled common garden laboratory experiments.

In addition to the transcriptional modifications that could enable survival of *G. affinis* in Tar Creek, mosquitofish may also be able to persist in heavy metal-contaminated habitats due to species-specific ecological attributes that went unmeasured in our experiments. Mosquitofish have a high reproductive potential with long reproductive seasons (Pyke, 2005), representing an opportunistic life history strategy that is particularly well suited for repopulation of disturbed habitats (Winemiller & Rose, 1992). Furthermore, as the only livebearing fish species in Tar Creek (Franssen et al., 2006), internal gestation could enable persistence of mosquitofish by providing a buffer between the developing embryos and the toxic environment (Greenway et al., 2014), though this remains to be tested experimentally. Finally, because of the life history traits noted above and the high connectivity between Tar Creek and neighboring uncontaminated sites, mosquitofish may not be adapted (generally or locally) for life in Tar Creek at all; instead, they may be a sink population that merely persists due to continuous migration from populations in unpolluted habitats. Indeed, an examination of population genetic variation in our transcriptome data (see online supplement for details) indicated that there was only one genetic cluster represented in our data, suggesting there is no genetic differentiation or population structure in the genomic regions captured by the transcriptome. Future work to address the mechanistic basis of pollution tolerance should utilize common garden laboratory exposure experiments to identify local adaptation through plastic and genetic responses to pollution and population genomic data to understand patterns of gene flow genome-wide. An open question that remains is whether gene expression variation in this system is purely driven by environmental differences among populations or whether genetic and/or epigenetic differences in gene regulation across these subpopulations also play a role.

Conclusions and other considerations

Our analyses of elemental differentiation across multiple species inhabiting Tar and Coal Creeks demonstrated that all species experienced an ionomic shift that included accumulation of heavy metals; however, the axis of ionomic divergence was different in mosquitofish than other species, suggesting mosquitofish cannot limit uptake of metals from the environment. We also found evidence of differential gene expression in gill, liver, and brain tissues between individuals from polluted and unpolluted sites, providing a first look into transcriptional mechanisms that could mediate tolerance of mosquitofish to life in heavy metal-contaminated environments. The gills exhibited the largest transcriptomic response and appeared to be involved in mediating heavy metal tolerance by upregulating genes involved with limiting uptake of heavy metal ions from the blood and binding and reducing free radicals. However, several known antioxidant proteins (e.g., superoxide dismutase, catalase, and glutathione) were not differentially expressed in our study, and we also found differential expression of hundreds of genes with diverse functions other than metal homeostasis. This indicates the inherent complexity of organismal responses to diverse mixtures of pollutants like those found in Tar Creek and highlights the need for further research to understand the intricacies of responses to contaminant exposure. Despite the lack of data on local adaptation in this system, the ionomic and transcriptomic shifts noted in the present study likely play a major role in pollution tolerance in mosquitofish. Building on our existing systems framework by integrating genomic data would vastly improve our understanding of how mosquitofish maintain homeostasis and survive in Tar Creek.

Pollution tolerance is a complex phenotype involving the interplay of atoms, genes, RNA and protein molecules, tissues, organisms, and ultimately larger ecological entities. Systems-level approaches have been instrumental to our understanding of these responses by integrating multiple lower-level components to elucidate phenomena at higher levels of biological organization (Pinu et al., 2019). By analyzing multiple elements simultaneously, our study improves the resolution over modular studies of individual contaminants, which may lack biological significance, since contaminants rarely exist in isolation (Escher, Stapleton, & Schymanski, 2020; Wood, 2011). The results of this study—and particularly the complex changes in gene expression—demonstrate the need for future studies to test for effects of multiple concomitant stressors simultaneously.

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Data Accessibility

All transcriptomes have been deposited in GenBank (BioProject accession: PRJNA707024). The *Xiphophorus maculatus* assembly used as a reference genome for our analyses was also obtained from Genbank (BioProject accession: PRJNA72525). Raw ionomic and transcriptomic datasets are archived on Dryad (https://doi.org/10.5061/dryad.q2bvq83kw), and scripts used for all analyses are available on GitHub (https://github.com/michitobler/TarCreekGambusia).

Author Contributions

JLC and MT designed the experiments and collected samples in the field. Reagents were provided by JLK, PDJ, and MT. Computing resources were provided by JLK and MT. JLC, JLK, and PDJ conducted laboratory work. JLC and MT conducted the analyses and wrote the manuscript. All authors made substantial contributions to revisions of the manuscript.

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Table 1. Sample sizes for ionomic data and taxonomic information for each species in both Tar and Coal Creeks.

Family	**Species**	$\mathbf{N_{Tar}}$	$\mathbf{N_{Coal}}$
Poeciliidae	*Gambusia affinis*	6	6
Fundulidae	*Fundulus notatus*	7	6
Cyprinidae	*Pimephales notatus*	6	6
Centrarchidae	*Lepomis cyanellus*	6	4
Centrarchidae	*Lepomis gulosus*	4	6
Centrarchidae	*Lepomis macrochirus*	7	6
Centrarchidae	*Lepomis megalotis*	7	7

Table 2. Type-III multivariate analysis of variance summary table comparing principal component scores of ionomic differentiation between sites and species. We used the Wilks' lambda test statistic and estimated effect sizes with a multivariate analog of η_p^2, as calculated with the Wilks' lambda test statistic (Λ): $\eta_p^2 = 1 - \Lambda^{1/s}$, where s is the number of levels of each factor minus 1, or the number of dependent variables, whichever is smaller.

	df	**Wilks' Λ**	**approx F**	**num df**	**den df**	**Pr(>F)**	**η_p^2**
Intercept	1	0.64	6.083	6	65	<0.001	0.360
Site	1	0.608	6.978	6	65	<0.001	0.392
Species	6	0.068	6.785	36	288.2	<0.001	0.362
Site × Species	6	0.106	5.338	36	288.2	<0.001	0.312

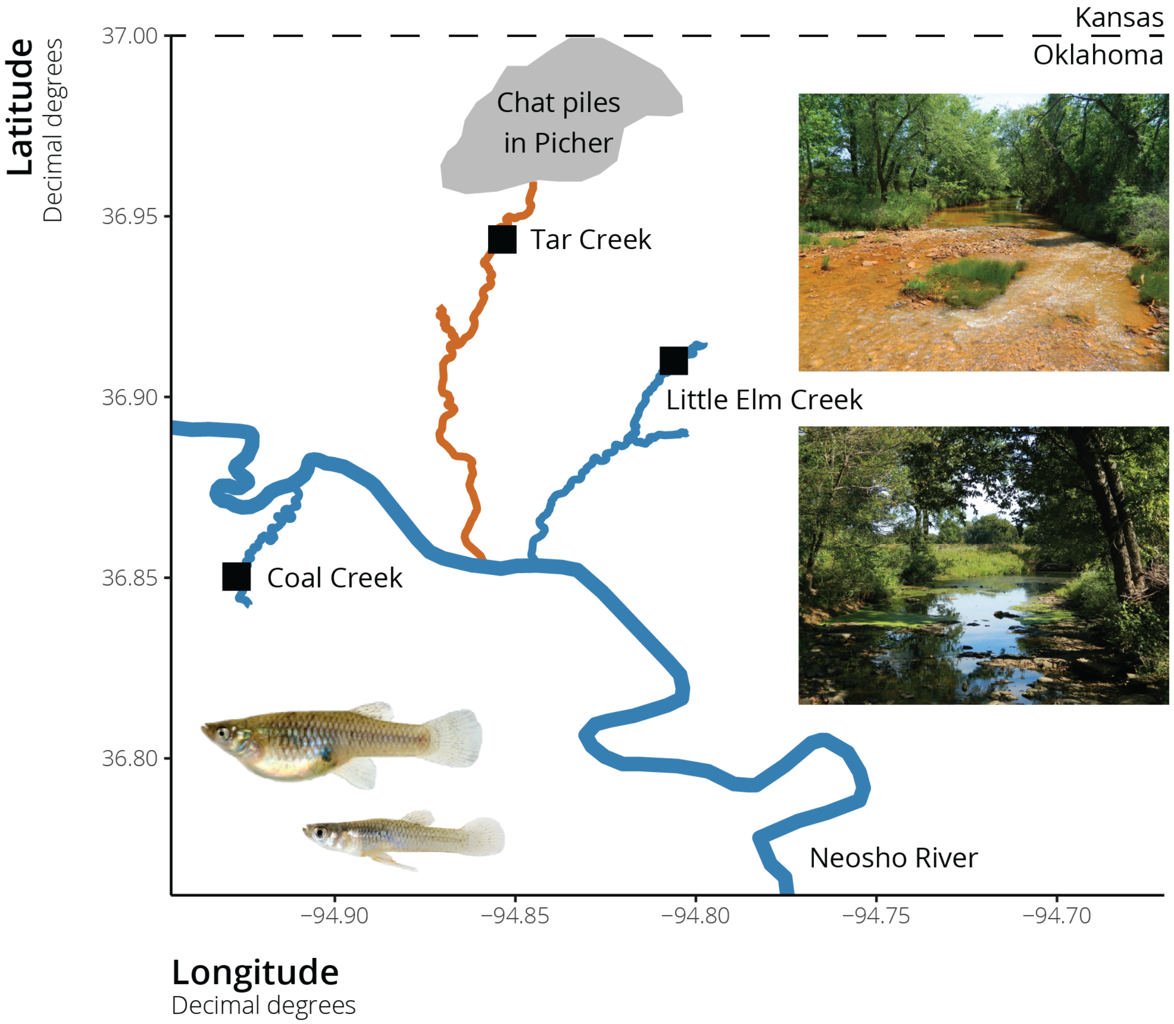

Figure 1. Map of the Tar Creek Superfund Site. Acid mine drainage directly enters Tar Creek through two routes: i) runoff from surface piles of mining waste known as chat piles in the area of Picher, OK, and ii) through boreholes to inundated mines throughout the area. The Neosho River and tributaries sampled in this study (Tar, Coal, and Little Elm Creeks) are represented by latitude and longitude digitized from Google Earth. Black squares represent sampling locations on each tributary. A male and female *G. affinis* are pictured in the bottom left, and Tar Creek (top-right) and Coal Creek (bottom-right) are pictured for reference.

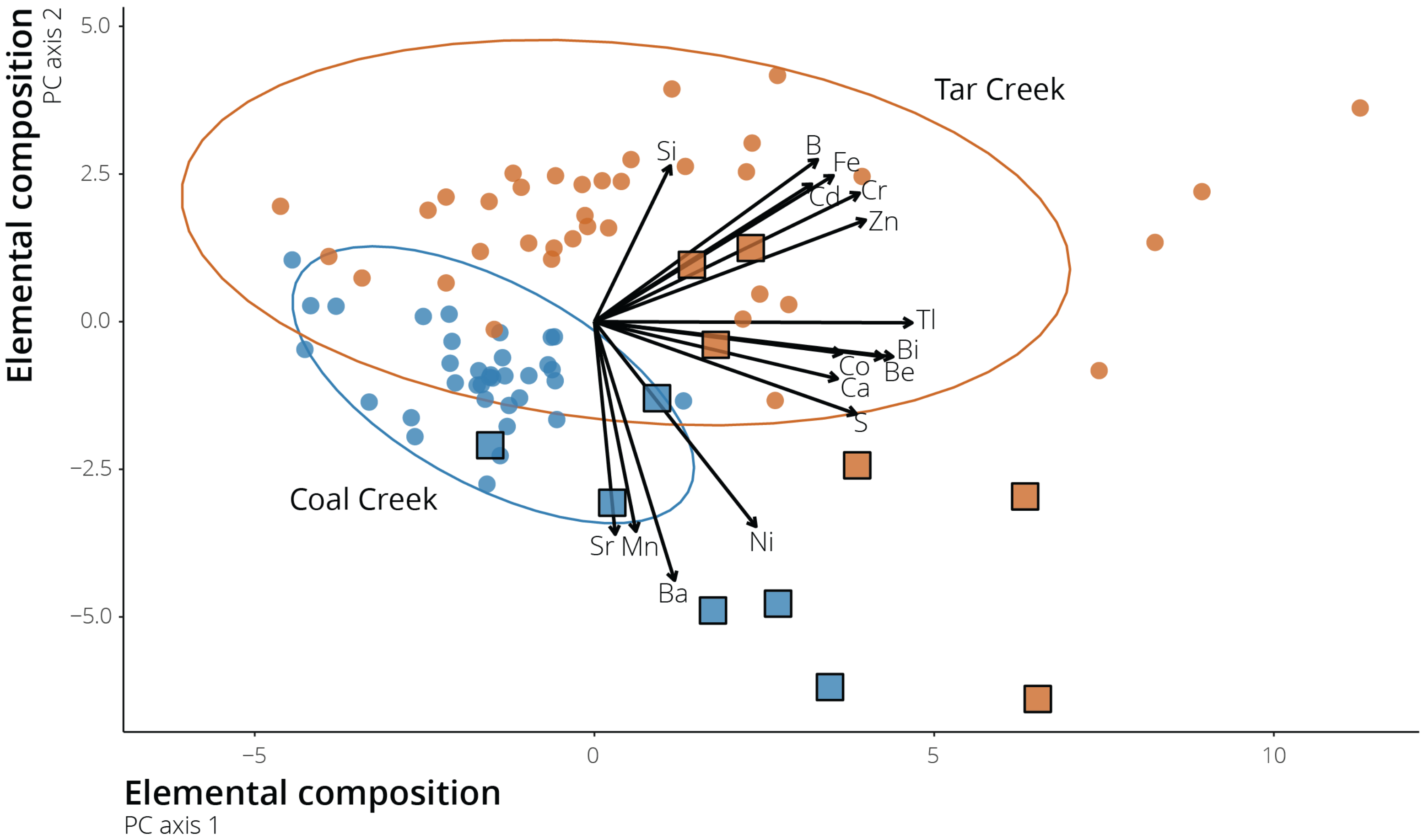

Figure 2. Plot of individual elemental principal component scores along the first two principal component axes. Observations in orange are from Tar Creek, and observations in blue are from Coal Creek. Mosquitofish from both sites are represented by squares, while all other species are shown as circles. Ellipses show 95 % confidence intervals in both dimensions for each site. Loadings are shown in black for the eight elements with the largest absolute loadings along each axis. Loadings were calculated as the eigenvector multiplied by the square root of the eigenvalue for that axis. The magnitude of each loadings arrow was magnified by a factor of five for ease of viewing.

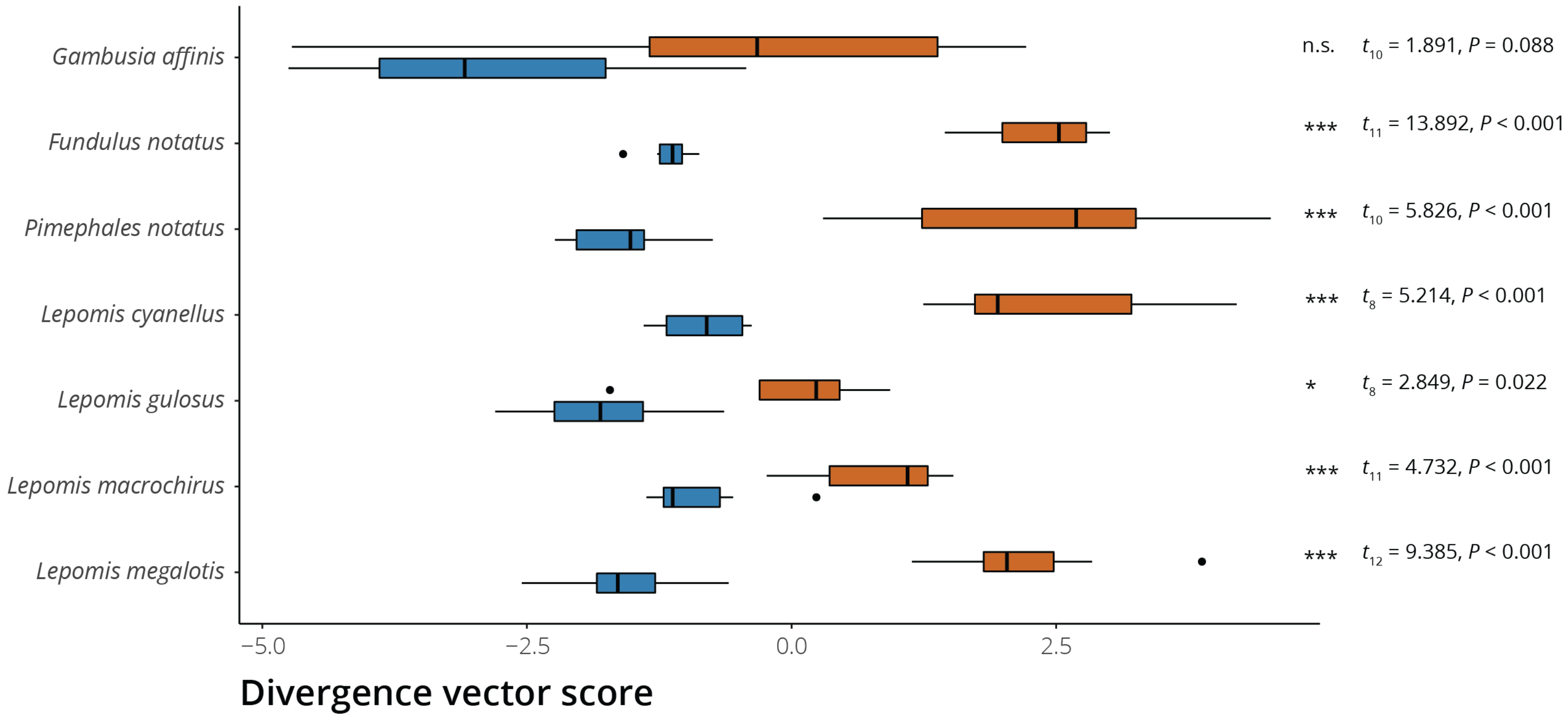

Figure 3. Boxplots of divergence vector scores comparing convergent elemental differentiation between sites across all species. To obtain divergence vector scores, we used multivariate analysis of variance to test for the main effects of site and species and the interaction between site and species on the principal component scores from the first six axes. We ran a PCA on the sums of squares and cross products matrix for the 'Site' term from this MANOVA and multiplied the resulting eigenvector for PC1 by the matrix of principal component scores for the first six axes to obtain divergence vector scores for each individual. See the Methods section and Table 2 for more details. Scores were plotted for species (shown on the y-axis) and sites (blue corresponding to Coal Creek and orange corresponding to Tar Creek individuals) separately to show differences between sites in each species. Separate *t*-tests were conducted on each species, and statistics and *P*-values are shown at right. *Gambusia affinis* is the only species that did not have significantly different divergence vector scores between sites.

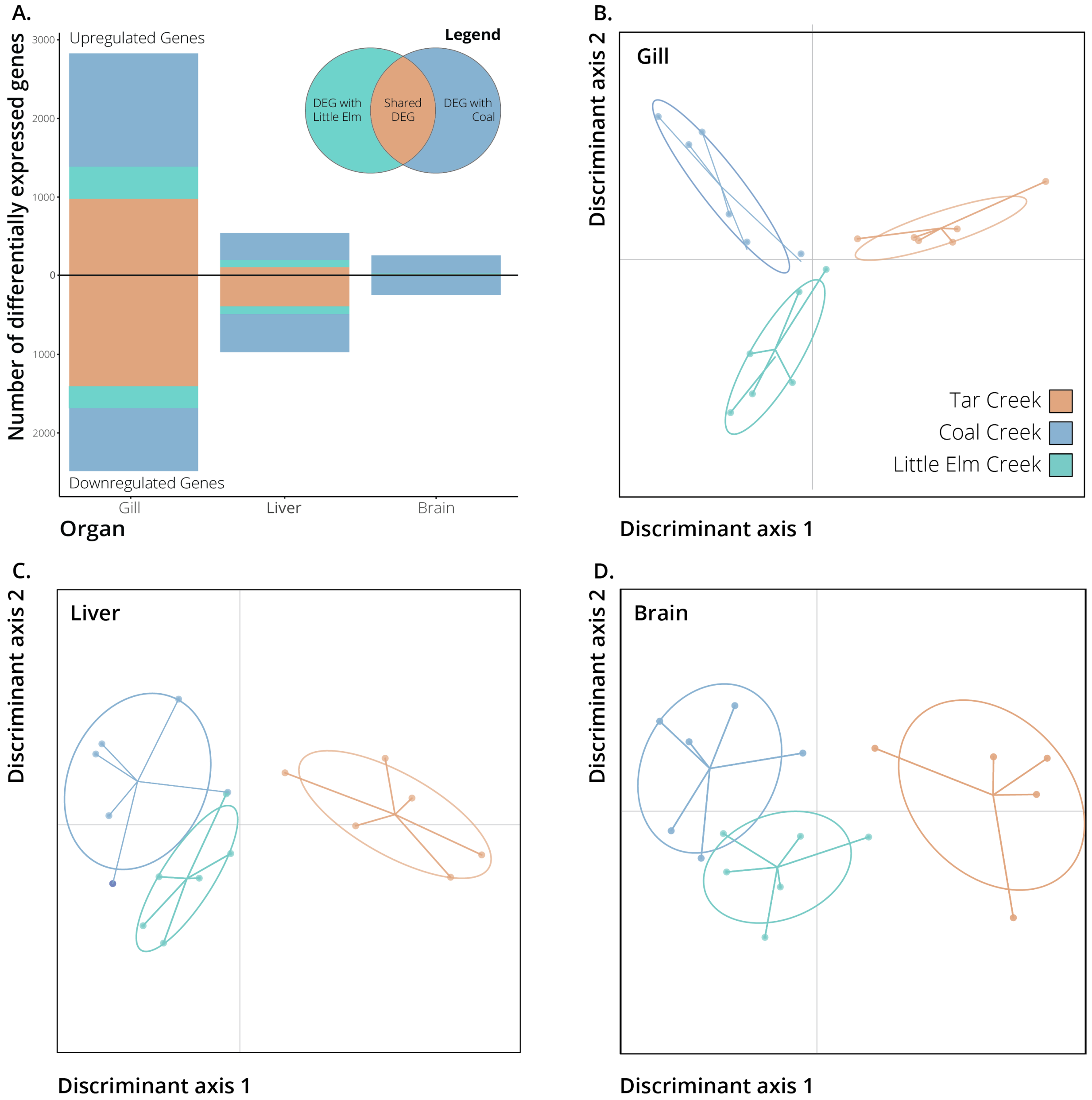

Figure 4. (A) Stacked bar plot of the number of differentially expressed genes (DEG) in each tissue. Genes above zero on the y-axis were upregulated in Tar Creek individuals, and genes below zero were downregulated in Tar Creek individuals. The orange portion of each bar shows the number of genes that were differentially expressed in both Coal and Little Elm Creeks in the same direction, compared to Tar Creek. The blue portion of bars signifies the number of genes differentially expressed only in Coal Creek individuals compared to Tar Creek individuals. The turquoise portion of each barplot represents genes that were only differentially expressed in Little Elm Creek compared to Tar Creek. Plots of discriminant scores from a discriminant analysis of principal components (DAPC) to visualize multivariate divergence in gene expression in (B) gill, (C) liver, and (D) brain samples.

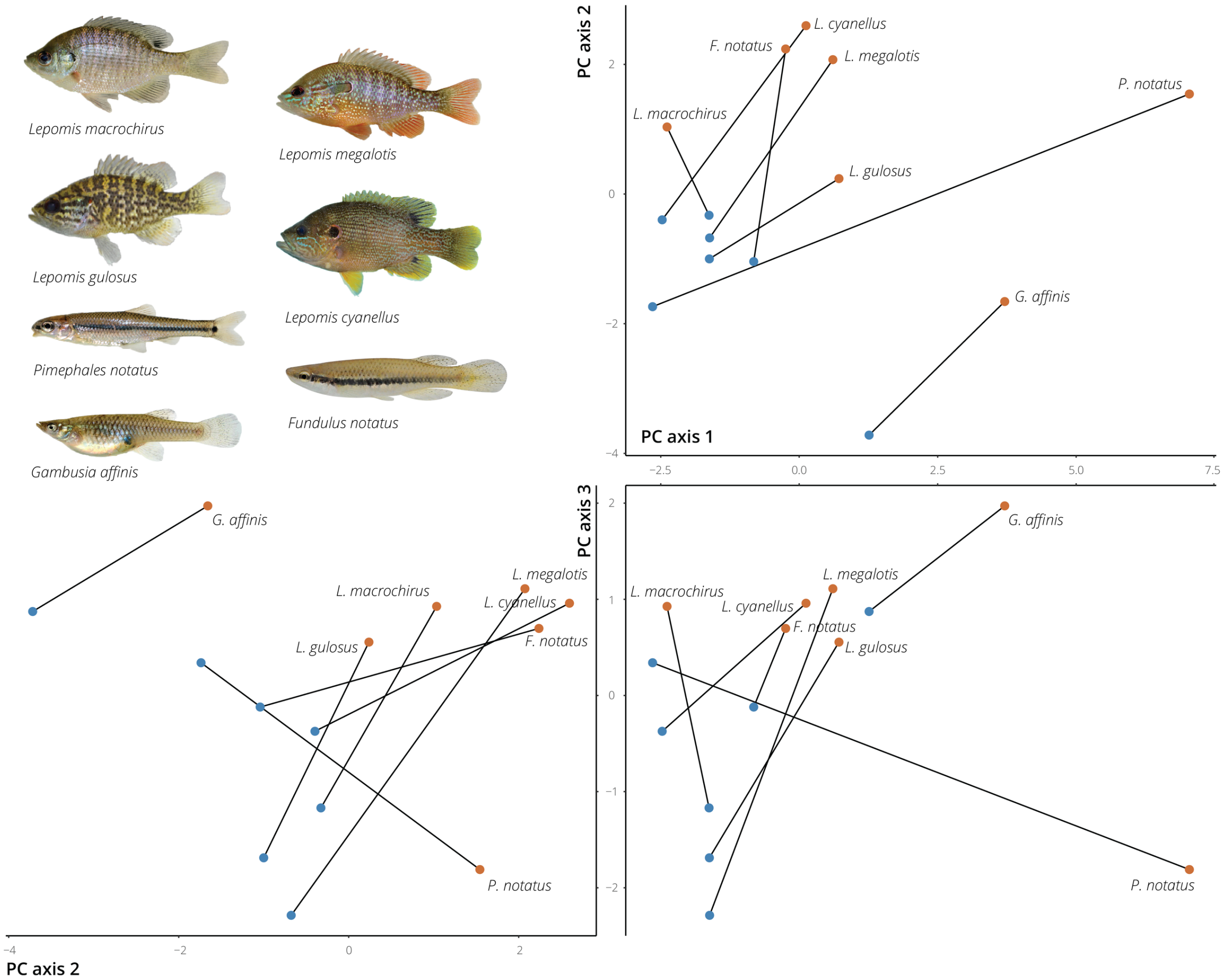

Figure 5. Images of each sampled species are included in the top left. Plot of average principal component scores generated from whole organism ionomes for each population in each of the first three principal component axes. Populations of the same species are connected with a line to demonstrate species-specific differences. Populations from Tar Creek are colored orange, while Coal Creek populations are colored blue.