OF MICROPLASTICS AND MILKWEED: USING ‘OMICS TO ADDRESS BIODIVERSITY CHALLENGES IN THE ANTHROPOCENE

By

Miranda J. Wade

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ABSTRACT

As the Earth progresses through the Anthropocene Epoch, human-caused environmental changes continually impact the natural world. Some of the largest changes occur through land-use change or the development and improper disposal of pollutants. This dissertation aims to present the use of genome-wide molecular tools across two systems to investigate the potential implications and future consequences of these anthropogenic changes. The first of these investigations involves the use of population genomic tools to determine the effects of land-use change in the development of large, ground-mounted solar energy facilities in desert ecosystems. Sensitive arid land ecosystems are the second most common land-cover type for solar energy development globally, so it is necessary to understand existing diversity within desert plant populations to understand spatiotemporal effects of solar energy siting and design. I sampled Mojave milkweed (*Asclepias nyctaginifolia*) in and around the Ivanpah Solar Electric Generating Station (ISEGS) in the Mojave Desert of California to understand the species' population structure, standing genetic variation, and the intersection of this biodiversity with solar development. Using Restriction-site Associated Sequencing (RADseq), I found clear population structure over small spatial scales, suggesting each site sampled was a genetically distinct population of Mojave milkweed. This work suggests that the effects of land-cover change, especially those impacting core desert habitat, may impact long-term genetic diversity and persistence by increasing risks of genetic diversity loss or population extirpation. This highlights the need to consider the genetic diversity of species when predicting the impact and necessary conservation measures of large-scale land-cover changes.

My second investigation utilized genome-wide RNA and methylation analyses to understand the impacts of microplastics pollution on aquatic organisms. Microplastics

exposure correlates with evolutionary and ecological impacts across species, affecting organisms' development, reproduction, and behavior along with contributing to genotoxicity and stress. To gain a better understanding of organismal responses to microplastics, I performed an experiment using fathead minnows (*Pimephales promelas*) in different microplastic treatments. I tested two microplastic concentrations, reflecting both current and predicted future conditions, of pre-consumer, pristine plastic and environmentally exposed plastic gathered from Lake Ontario. I raised an F1 generation in the control treatment (no microplastic exposure) to determine intergenerational effects. I used directional mRNA and methylation sequencing to evaluate differences among treatments, sexes, and generations. I found evidence of metabolic stress-response changes in the fish exposed to microplastics compared to the controls. The effect of microplastics on gene expression was stronger in female minnows compared to males, but in epigenomic analyses the origin of the plastic had a larger effect in female minnows whereas the effect of concentration was stronger in males. Many of the differentially expressed or methylated genes interact with estrogenic chemicals associated with plastic. I observed intergenerational effects on gene methylation, highlighting a heritable mechanism in which parents can pass on the effects of microplastic exposure to their offspring. This study is among the first to highlight the persistent impacts of microplastic pollution on gene regulation in freshwater systems. As fathead minnows are an important toxicological model species, our hope is that the results of this study will have implications across aquatics species and ecosystems and highlights the importance of understanding the impact of microplastic exposure across levels of biological organization, from the cellular to population level. Altogether, my investigations in these two systems highlight the diverse ways in which anthropogenic change effects organisms across levels of molecular control and provides context for the ecological and evolutionary implications of these modifications.

ACKNOWLEDGEMENTS

If it takes a village to raise a child, it takes an entire collective to raise a scientist. I would first and foremost like to acknowledge the contributions, support, and sacrifices of my parents and family members throughout my life. Mom, Dad, I would neither be here on Earth or here in this position without you. Thank you. I also must acknowledge the many laughs, tears, and screams shared among my friends and lab mates through the years, especially through the stress monster that is finishing a dissertation. You know who you are. I can never express my gratitude to my advisor Dr. Mariah Meek for taking a chance on developing a hopeful undergrad into the scientist I am today. My committee members Gideon Bradburd, Sara Fitzpatrick, Chris Kozakiewicz, and Cheryl Murphy provided much wisdom and feedback during this journey. I would not have made it far without everyone.

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Finally, I want to express gratitude and appreciation to the Indigenous peoples across this continent who have been living and caring for this land from time immemorial, who are still here and will always continue to be present in this place. Michigan State

University occupies the ancestral, traditional, and contemporary Lands of the Anishinaabeg – Three Fires Confederacy of Ojibwe, Odawa, and Potawatomi peoples. In particular, the University resides on Land ceded in the 1819 Treaty of Saginaw. The Ivanpah Valley, the location of the organisms of my first chapter, is within the ancestral lands of the Nuwuvi (Southern Paiute), as well as the Newe (Western Shoshone) peoples. For thousands of years, the land on which the University of Toronto operates has been the traditional land of the Huron-Wendat, the Seneca, and the Mississaugas of the Credit. I acknowledge the truth that for more than five hundred years, Native nations have demonstrated resilience and resistance in the efforts to separate them from their land and ways of life. It is important for each of us to not only acknowledge but seek to understand the history that has brought us to reside on indigenous land and support the process of social justice and reconciliation. More action needs to be taken to reverse the long-standing practices to erase Indigenous people's history, language, culture, and existence. I recognize the sovereignty of these Indigenous nations and historic communities – both those who live here now and those who were forcibly removed from their Homelands.

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Introduction

Desert ecosystems are areas of high solar energy potential, making them the second largest recipient environment (by area) globally for large, ground-mounted solar energy development (hereafter solar energy, > 1 megawatts [MW_{DC}] (Prăvălie et al. 2019; Kruitwagen et al. 2021). While solar energy facilities provide low-carbon, efficient energy, they can have substantial ecological impacts on aridland ecosystems, which are often already threatened (Stoms et al. 2013; Hernandez et al. 2014; Grodsky & Hernandez 2020). Previous work illustrates that the effects of land-use and land-cover change on desert species are mostly negative (Sutherland et al. 2012) and include increased invasions by exotic annual grasses, higher fire risk, de-vegetation, extensive soil erosion, and reduced plant succession (Abella 2010; Abatzoglou & Kolden 2011; Cameron et al. 2012).

Land-use and land-cover changes associated with solar energy development may specifically alter patterns of wind, shade, hydrology (Armstrong et al. 2014; Suuronen et al. 2017; Tanner et al. 2020) plants (Grodsky & Hernandez 2020; Hernandez et al. 2020; Tanner et al. 2021), and patterns of herbivory and pollination (Lovich & Ennen 2011; Hernandez et al. 2014; Grodsky et al. 2021). Concentrating solar power (CSP) facilities, a type of solar energy, use large mirrors to reflect direct radiation to power towers and can

increase local albedo by 30-56%. The development of CSP may create localized drought conditions due to altered wind speeds, evapotranspiration, and excess heat (Lovich & Ennen 2011). As many desert species live near their physiological water and heat limits, even small changes in their habitat, such as changes in patterns of hydrological flows supporting washes, can have large consequences (Archer & Predick 2008; Grippo et al. 2015). Many desert species also struggle to recover from or adapt to rapid environmental changes, which can have long-term effects on population growth rates and individual fitness (Tanner et al. 2014; Moore-O'Leary et al. 2017).

Despite their economic importance and potential for large-scale habitat displacement, we have a limited understanding of the ecological impacts of solar energy development. Many desert plants have life history strategies that make them particularly vulnerable to impacts because they rely on conditions that only occur in a subset of climatic years for reproduction, dispersal, and geneflow (Shryock et al. 2014). Additionally, due to their relatively large footprints and land transformation compared to fossil fuels and other renewables, (Lovering et al. 2022) a single solar energy facility can encompass distributions of many plant species in a region. To increase the environmental sustainability of operating and planned solar energy facilities, an understanding of desert plant populations and their vulnerabilities to solar energy facility design is necessary. This need for an increased understanding of impacts of solar energy development on plant populations is particularly pressing as development of renewable energy increases in aridlands globally (Hernandez et al. 2015b, 2015a; Kruitwagen et al. 2021).

Information about the spatial structure and genetic health of desert plant populations is needed to inform solar energy development. However, few population genetics studies of North American desert plants, especially genome-wide studies, exist. Therefore, there is not

enough information currently to inform solar energy facility development, particularly siting and design, and to incorporate conservation actions that will protect geographically restricted plant species of conservation concern during development. Gaps in knowledge include a lack of understanding of how many unique populations exist in each area and how much genetic diversity may be lost from anthropogenic disturbances, such as solar energy development (Hoffmann et al. 2015; Allendorf 2017; Fraser 2017). Genetic information can fill the information gaps regarding population structure, the existence of local adaptation, and presence of unique genetic diversity within populations (Charlesworth et al. 2003; Nadeau & Jiggins 2010).

The southwestern United States is a hotspot for solar energy development (Lovich & Ennen 2011). Within this region is the Mojave Desert, covering over 13 million hectares, many of which are ecologically intact due to the sparseness of city centers and large swathes of public land (Cameron et al. 2012). This region is also a hotspot for endemic, environmentally sensitive, and evolutionarily diverse plant life that is foundational to desert ecosystems (Vandergast et al. 2013). In the Mojave, desert plants provide habitat and food resources to several charismatic invertebrates and vertebrates, including the federally listed desert tortoise (*Gopherus agassizii*, under the United States [U.S.] federal Endangered Species Act [ESA]), black-tailed jackrabbit (*Lepus californicus*), the state listed Mojave ground squirrel (*Xerospermophilus mohavensis*, under the California ESA, U.S.), the effectively listed monarch butterfly (*Danaus plexippus*, under the U.S. federal ESA), queen butterfly (*Danaus gilippus*), and sphinx moth (Sphingidae); (Esque et al. 2003; Grodsky et al. 2017, 2019, 2020; Moore-O'Leary et al. 2017; Saul-Gershenz et al. 2020). Increasingly, the Mojave Desert is stressed by anthropogenic change (Randall et al. 2010) and impacts to desert plants are widespread (Lovich & Bainbridge 1999; Agha et al. 2020; Tanner et al.

2021). As the Mojave Desert is progressively subjected to land-use and land-cover change, it is critical to understand and anticipate their impacts on plant species diversity (Smith et al. 2023).

The Ivanpah Solar Electric Generating System (ISEGS), a CSP facility located in the Mojave Desert of California, provides a model study system for determining how solar energy development affects rare plant populations. Located at the base of Clark Mountain, construction of ISEGS occurred between 2010 and 2014. At the time of its completion, ISEGS was the world's largest CSP facility, with an area of over 16 km^2 and a nameplate capacity of 392 megawatts (generating approximately 700,000 $MWhy^{-1}$ of electricity annually from 2014 - 2020). Incorporated into the facility design were mitigation strategies to reduce impacts on the desert community by (1) mowing vegetation below and in between the heliostats (in lieu of blading, which removes all aboveground biomass and all soil surface microtopography, including washes) in all areas beyond the innermost heliostat loops (i.e., closest to the power tower); and, (2) the creation of undisturbed 'halos' or micro-refugia where rare plants were identified prior to construction and left undisturbed (Grodsky and Hernandez 2020). Importantly, it was not clear at the time if such an approach would have a conservation benefit.

The construction plans of ISEGS specifically attempted to limit the impact of construction on endemic desert plants. One such plant is Mojave milkweed (*Ascelpias nyctaginifolia*, Apocynaceae), an iteroparous perennial plant common throughout Arizona, Nevada, and New Mexico, but considered seriously threatened in California (Schmid & Tibor 2001). In the Western Mojave, it is rare, found in the sandy soils of ephemeral and intermittent washes and slopes, and restricted to small microclimates in eastern California (Baldwin et al. 2002). Like many milkweed species, Mojave milkweed can propagate clonally and utilizes both wind and water for seed dispersal. This clonal propagation,

coupled with the ability of the milkweed to die back vegetatively and exist in the soil as rhizomes, may help preserve genetic diversity during times of non-ideal conditions and disturbance.

To avoid mowing sensitive desert plants, ISEGS biologists mapped 202 Mojave milkweed individuals across 59 sites in 2008 and protected them within halos. Most were found in small- to medium sizes washes with sandy to gravelly soil. No information on individual plant traits (e.g., size), location, and population structure of Mojave milkweed was made publicly available (CEC 2010). While the halos allowed milkweed to persist within ISEGS, the mowing and construction of concentric heliostats significantly altered the vegetation community within the ISEGS footprint (Grodsky and Hernandez 2020). Understanding the population structure of Mojave milkweed both within and around the solar installation will be imperative in determining the best strategy to conserve this rare plant. Here, we aimed to address key information gaps related to the conservation of Mojave milkweed by determining the population structure and diversity of Mojave milkweed in the Ivanpah Valley, and its overlay with ISEGS. Our questions included:

1. Is there distinct population structure within the Ivanpah Valley Mojave milkweed or is it a continuous, panmictic population?
2. How does the spatial layout of the ISEGS facility overlay with any potential population structure, i.e., how many unique populations does the ISEGS facility affect?
3. Is there unique genetic diversity found within ISEGS that could be lost due to disturbance?

Methods

Sample Collection

In 2015, we sampled leaf tissue of all vegetative Mojave milkweed plants at four locations: three sites undisturbed by facility construction and throughout ISEGS. The first of these sites ("Excelsior") is approximately 21 kilometers west of ISEGS. The other two locations are approximately five kilometers north ("Umberci)," and 60 kilometers south ("Bobcat") of the solar facility (Figure 1). We cut small sections of green leaf tissue from mature plants and stored them in individually labeled coin envelopes with desiccant packets to promote drying. When present, we collected seeds for subsequent growth in a greenhouse, where leaves were cut and similarly stored once the plants reached sufficient maturity. It is important to note that while we collected all the plants present at the time, there is the possibility that some plants died back prior to our ability to collect them or remained dormant as rhizomes that season.

We collected additional samples in the fall of 2018 from plants previously identified within the ISEGS halos (n= 8) as well as plants that emerged in halos after the 2015 sampling (n= 30). We also collected any previously unidentified plants that grew within the facility's footprint but outside of designated halo areas (n= 51). We acquired additional samples from the Umberci site of previously identified but unsampled plants (n= 32) and newly emerged plants (n= 16). We designated individuals (genets) based on the distance from other plants and sampled multiple ramets per genet if possible. We collected leaves from juvenile or adult individuals. For both years, we recorded the location of all plants present (Figure 1), even if they were too small to sample, to establish a census size (Table 1).

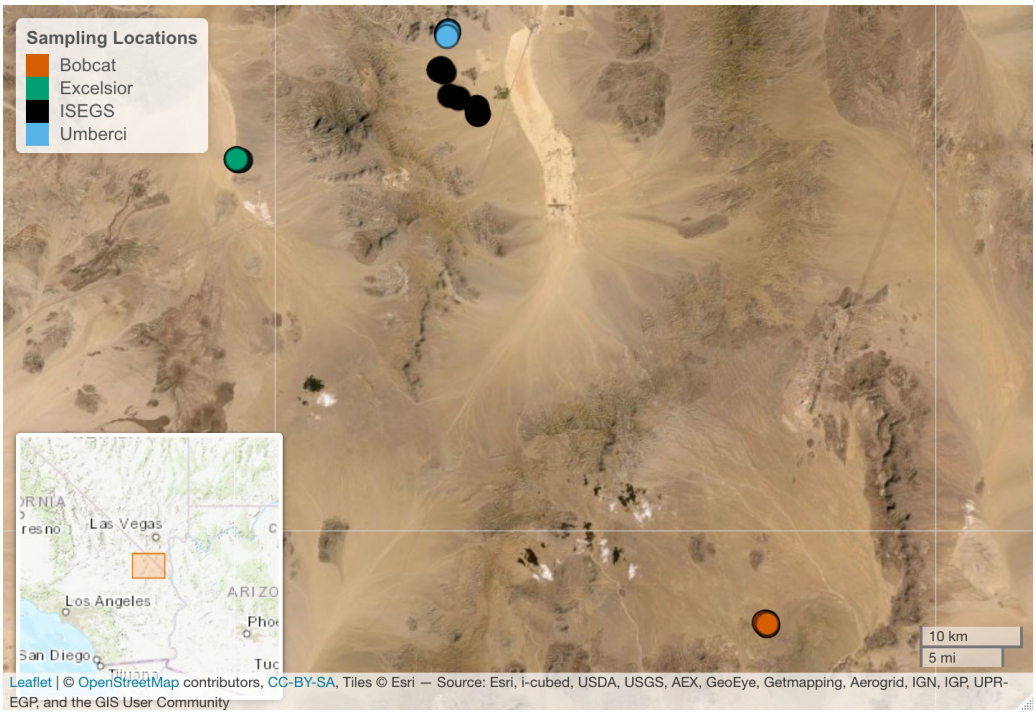

Figure 1. Map of the Mojave milkweed sampling sites. Note the spread of the sampling sites in the Ivanpah Solar Electricity Generating Station (ISEGS) facility compared to the natural populations. Samples from both 2015 and 2018 are included on the map.

Table 1. Measurements of diversity for Mojave milkweed populations of the Ivanpah Valley. Some samples were collected from the same plant across sampling years. All values were calculated using a dataset with clonal ramets removed. Ho = observed heterozygosity, He = Expected heterozygosity, Fis = inbreeding coefficient, Ne = effective population size, Av. Φ = within-population relatedness coefficient. There were no significant differences between observed and expected heterozygosity following Bartlett's test for the homogeneity of variances ($p << 0.001$) and a paired t-test (95% confidence intervals [CI]) in any of the populations except Umberci (denoted with an asterisk). Fis 95% CI were calculated by bootstrapping using boot.ppfis in hierfstat (significant intervals denoted with an asterisk). Reported CI for Ne were calculated using the pseudo-jackknife method outlined in (Jones et al. 2016).

Site Name	Samples collected 2015/2018	Samples analyzed 2015/combined years	Census size (total)	Allelic richness (rarefied)	Ho	He	Fis (95% CI)	Ne (95% CI)	Av. φ
Bobcat	27/0	20/20	30	1.47	0.138	0.138	0.001 (-0.013, 0.003)	33.0 (15.7, 212.0)	0.01
Excelsior	12/0	11/11	23	1.45	0.135	0.136	-0.001 (-0.008, 0.011)	71.5 (25.5, infinite)	0.001
ISEGS	23/90	17/84	226	1.5	0.142	0.144	0.007* (0.005, 0.018)	27.6 (12.2, 421.4)	0.003
Umberci	34/47	42/60	79	1.47	0.142*	0.140*	-0.004* (-0.016, -0.004)	23.8 (14.1, 46.5)	0.002

Sequencing

For both the 2015 and 2018 samples, we disrupted the dried plant tissue with steel beads using a bead mill prior to extracting DNA. We performed DNA extractions using the DNeasy Plant Mini kit (QIAGEN Inc., Valencia, CA, USA), and quantified the resulting concentrations of DNA using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). We diluted the purified DNA to a concentration of 10.0 ng/ul using low TE in preparation for Restriction site Associated DNA Sequencing (RADSeq) using the Best-RAD method (Ali et al. 2016). A modification to Ali et al. (2016) is that we used the restriction endonuclease *pstI* to digest the DNA due to the more favorable number of cut sites given the GC content and size of the *Asclepias syriaca* reference genome (Weitemier et al. 2019) (Genbank accession GFXT01000000). We sonicated samples to a fragment length of 200 base pairs for the 2015 samples and 300 base pairs for the 2018 samples using a Covaris m220 (Covaris, Woburn, MA, USA). Following library preparation with the NEBnext Ultra DNA kit for Illumina (New England Biolabs, Inc., Ipswich, MA), we performed library trace analysis using a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). We sequenced the 2015 samples on the Illumina HiSeq3000 platform at the University of California Davis DNA Technologies Core (PE-2x100bp). For the 2018 samples, we sequenced on the Illumina HiSeq X platform (PE-2x150bp) at the UC Davis Sequencing Center (Novogene Corporation Inc.). The longer read length for the 2018 samples was due to the technical specifications of the sequencing platform. Prior to analyzing data from both sampling years, we trimmed the 2018 samples to the same length as the 2015 samples (100bp) using trimmomatic v0.38 (Bolger et al. 2014).

SNP Discovery

Following sequencing, we demultiplexed data for all individuals from both sampling

years (n=233) using -process_radtags in STACKS v2.4 (Catchen et al. 2011, 2013) and the following tags: --bestrad, -c, -r, -D. We aligned the files to the *Asclepias syriaca* reference genome (Weitemier et al. 2019) (Genbank accession GFXT01000000) using the --very-sensitive-local wrapper in Bowtie2 v2.3.4 (Langmead & Salzberg 2012). We used the -ref_map.pl pipeline in STACKS v2.4 to call random SNPs (--write_random_snp) in the dataset. We retained loci that were present in at least 30% of individuals per population within a single population and proceeded with quality filtration on the resulting VCF file.

We quality filtered the resulting file using VCFtools v0.1.15 (Danecek et al. 2011). Initially, we identified and removed individuals that were not genotyped at greater than 95% of loci and genotypes with a minimum read depth of less than 5. We then filtered out all genotypes with a gene quality score of less than 20. We subsequently removed loci with a minor allele count (MAC) of less than three [see (O'Leary et al. 2018)], followed by filtering out SNPs with a call rate of less than 90%. The final filtration step again identified and removed individuals with less than 85% of loci genotyped. We performed the SNP discovery on all samples combined to ensure the same loci were called across all samples and later separated out individual sampling years for downstream analysis. We removed any remaining monomorphic and uninformative loci using informloci in the R (R Core Team, 2020) package poppr (Kamvar et al. 2014, 2015) prior to proceeding with further analyses.

Genetic Diversity

We analyzed our SNP dataset to determine genetic diversity using allelic richness, effective population size (Ne), inbreeding coefficients (Fis), population differentiation, and observed/expected heterozygosity. We calculated heterozygosity, Fis, and allelic richness using the basic.stats and allelic.richness functions of hierfstat (Goudet 2005). Private alleles were determined using the private_alleles function in the R package poppr (Kamvar et al.

2014, 2015). In the private allele calculations, we used datasets from 2015 and a combined 2015 and 2018 dataset that excluded clones. We calculated the effective population size using NeEstimator v2.1 (Do et al. 2014) under the linkage disequilibrium model with an allele frequency of 0.05 using our clone-free dataset from 2015 (see below).

Population Structure

When determining population structure, we removed clonal ramets from the 2015 dataset according to their multilocus genotypes, using the mlg.filter function with a genetic distance threshold of 0.04 as calculated by the bitwise.dist function in poppr (Kamvar et al. 2014). We incorporated relatedness by calculating pairwise φ among all samples using the relatedness2 estimator in VCFtools (Manichaikul et al. 2010; Danecek et al. 2011). We removed individuals with pairwise φ values greater than 0.177, which corresponds with first-degree relatives such as full siblings and parent-offspring pairs, as clustering algorithms can be influenced by close relatives (Rodríguez-Ramilo & Wang 2012; Rodríguez-Ramilo et al. 2014). For each dataset, we evaluated the population structure of the plants using discriminant analysis of principle components via the R package adegenet (Jombart 2008; Jombart et al. 2010). We cross-referenced these results using the program *STRUCTURE* (Pritchard et al. 2000; Falush et al. 2003, 2007; Hubisz et al. 2009) and visualized the data using Structure Harvester (Earl & vonHoldt 2011). We also assessed population differentiation (Fst) using the method outlined by Weir and Cockerham (Weir & Cockerham 1984) using the package hierfstat (Goudet 2005). To further investigate the relationships between individuals, we calculated Minimum Spanning Networks (MSN) in poppr using the bitwise.dist and poppr.msn functions. Finally, we tested for isolation by distance in the samples using the R package conStruct (Bradburd et al. 2018).

Results

Sequencing and SNP discovery

We averaged 229,029,275 raw sequences per library, 4,306,523.6 mapped reads per individual, and an average coverage across loci of 30.5x. After filtering, we discovered 9,942 SNPs with <3% missingness in the combined dataset of 175 samples. For the dataset comprised of individuals identified in 2015, we discovered 9,503 SNPs across 90 individuals with an average missingness of 3.23%. For the dataset comprised of individuals identified in 2018, we found 9,643 polymorphic SNPs across 113 individuals with an average missingness of 2.46%. The discrepancy in the number of SNPs between the dataset is due to the removal of monomorphic loci from the dataset following the separation by sampling year.

Genetic Diversity

Overall, genetic diversity was similar across sites (Table 1). All sites had similar values for allelic richness, with ISEGS having the highest allelic richness (1.5) and Excelsior the lowest (1.45; Table 1). The values of observed heterozygosity (Ho) were also similar across sites, with ISEGS and Umberci have the highest observed value (Ho= 1.42) and Excelsior the lowest (Ho= 1.35, Table 1). Umberci and ISEGS were also the only sites with a significant difference in expected versus observed heterozygosity. However, in ISEGS the observed heterozygosity was less than expected and in Umberci we saw the opposite trend, where observed heterozygosity was greater than expected. Both ISEGS and Umberci had significant inbreeding coefficients, with ISEGS having a slightly positive value (0.007) and Umberci a slightly negative measurement (-0.004, Table 1). Bobcat had the highest average coefficient of relatedness (φ) of 0.01. Clonal ramets were confirmed in all sites but Excelsior. The effective population sizes (Ne) of each population ranged from 24-72 (Table

1). The calculated Ne is smaller than the census size in both Umberci and ISEGS. The Ne estimate of Bobcat is closest to the census number, and the estimate for Excelsior is roughly five times greater than the census size, even though it had the smallest values for diversity metrics such as observed heterozygosity and allelic richness.

We found alleles private to each population, with more unique diversity discovered in ISEGS in the additional year of sampling (Table 1, Figure 2). For the 2015 dataset, 1,896 out of the 9,503 loci had alleles private to a single site, with a portion of the private alleles present in only a single individual (Table 2, Figure 2). The number of private alleles per site ranged from 186 in Excelsior to 841 in Umberci, representing 1-5% of total allelic diversity per site (Table 2, Figure 2). The site with the largest proportion of private alleles in 2015 was Umberci (0.050) and the site with the lowest was Excelsior (0.013). For the dataset comprising both 2015 and 2018 samples, 1,059 out of the 9,942 alleles were private to a single sampling site, and all private alleles were present in at least two individuals in each population. Excelsior again contained the fewest private alleles (15) and ISEGS contained the greatest (673), representing between <1-4% of the allelic diversity (Table 2). Like the original dataset, the combined-year samples had the lowest proportion of private alleles in Excelsior (0.001), but the population with the largest proportion of private alleles was ISEGS (0.035, Table 2).

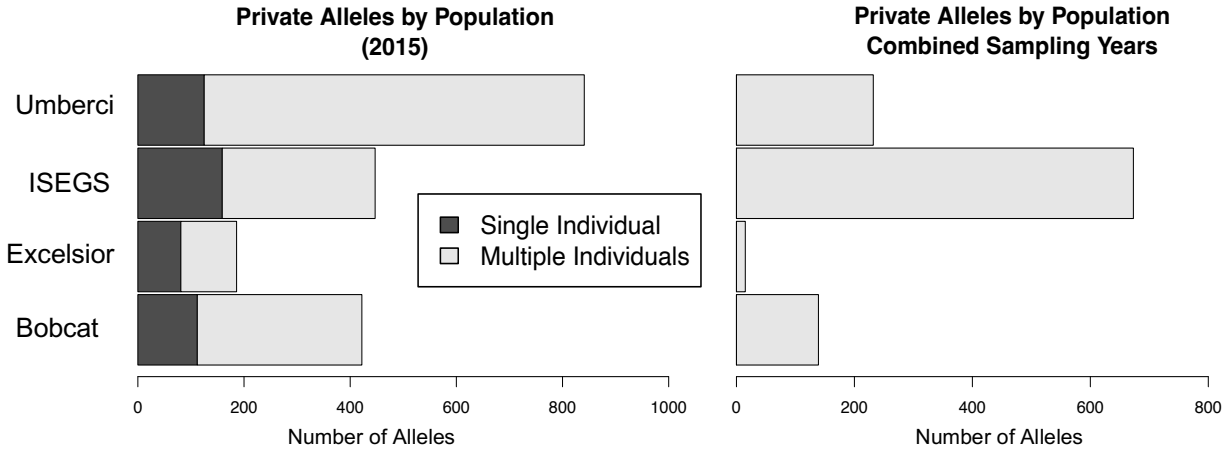

Figure 2. Private alleles of Mojave milkweed from the 2015 sampling year and a combination of individuals from sampling years. As the additional sampling year included many individuals from both ISEGS and Umberci, an overall decrease in private alleles is not unexpected as the populations are geographically close to one another and would reasonably share many alleles. No private alleles were found in a single genet in the combined years, while there were many cases of a single genet containing alleles in 2015.

Table 2. Proportion of private alleles in Mojave milkweed populations across sampling years. Allele counts were calculated using the mk.counts function in the R package PopGenReport.

Population	2015			Combined Years		
	Total alleles in population	Total private alleles	Relative proportion of alleles	Total alleles in population	Total private alleles	Relative proportion of alleles
Bobcat	15860	422	0.027	16304	139	0.009
Excelsior	14749	186	0.013	15182	15	0.001
Ivanpah Solar Electric Generating Station (ISEGS)	16050	447	0.028	19212	673	0.035
Umberci	16785	841	0.050	17861	232	0.013

Population Structure

Using the samples from 2015, we confirmed distinct populations of Mojave milkweed across the Ivanpah Valley. Principal components analysis (PCA) and K-means clustering prior to discriminant analysis of principal components (DAPC) indicated that two to five groups had the best fit (Appendix, Figures 13, 14, 15, 17), with the Umberci site consistently separating out first, followed by ISEGS, Bobcat, and Excelsior (Figure 3). At K=5, the Umberci population began subdividing (Appendix, Figure 16). During cross-validation, STRUCTURE analysis indicated four populations (Appendix, Figure 18). The MSN analysis revealed a similar pattern of genetic distances that corresponded with this population structure, placing ISEGS as the center of the network with a few individuals from the surrounding sites clustering with the ISEGS samples (Figure 4). As the ISEGS Mojave milkweed individuals are located centrally in the network, this suggests that they contain variation that is ancestral to the other populations. ISEGS also had the lowest genetic differentiation (Fst) from the surrounding populations (0.036 [pairwise with Bobcat], 0.038 [pairwise with Excelsior], and 0.040 [pairwise with Umberci], Table 3), however all Fst values were statistically significant, indicating that each population is well-differentiated (Table 3). The isolation by distance model was not significant, indicating that isolation by distance does not appear to drive the population structure in our system.

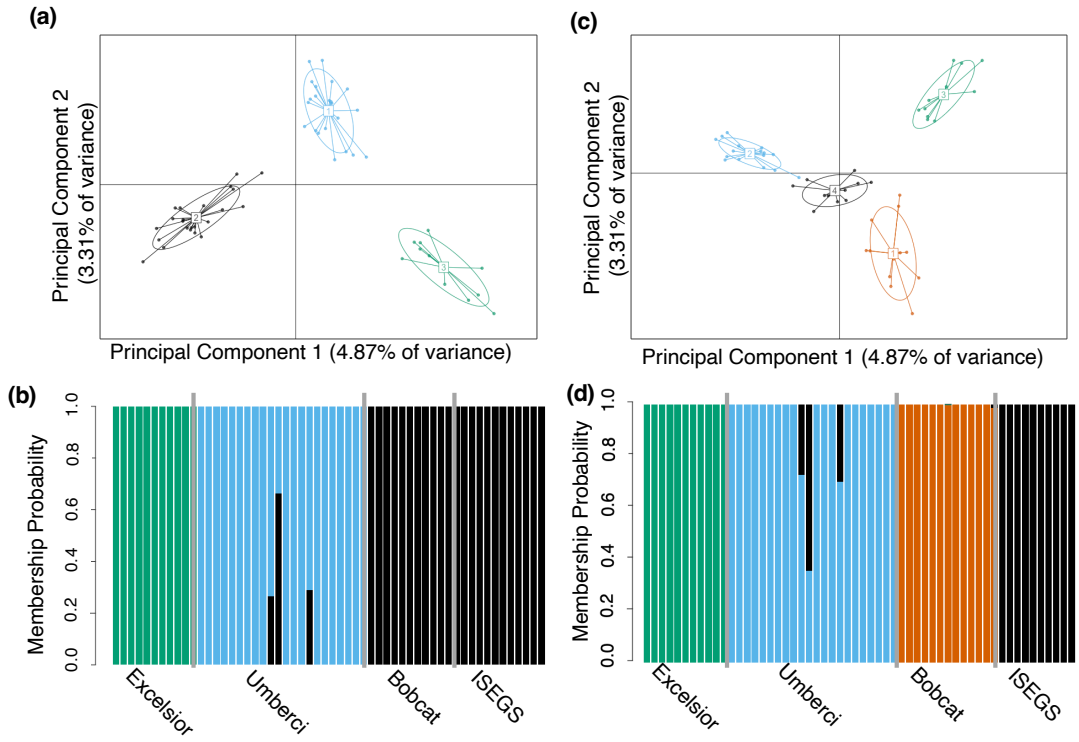

Figure 3. Discriminant analysis of principal components (DAPC) and membership probability plots of the Mojave milkweed individuals identified in 2015. There is clear population structure present in the Mojave milkweed populations of the Ivanpah Valley. Shown are scatterplots of individuals under K=3 (a) and K=4 (c) genetic groups and their corresponding membership probabilities (b and d, respectively) from the DAPC analysis. The analysis also suggests past gene flow between ISEGS and Umberci, as there are individuals clustered in Umberci that have membership probability in ISEGS.

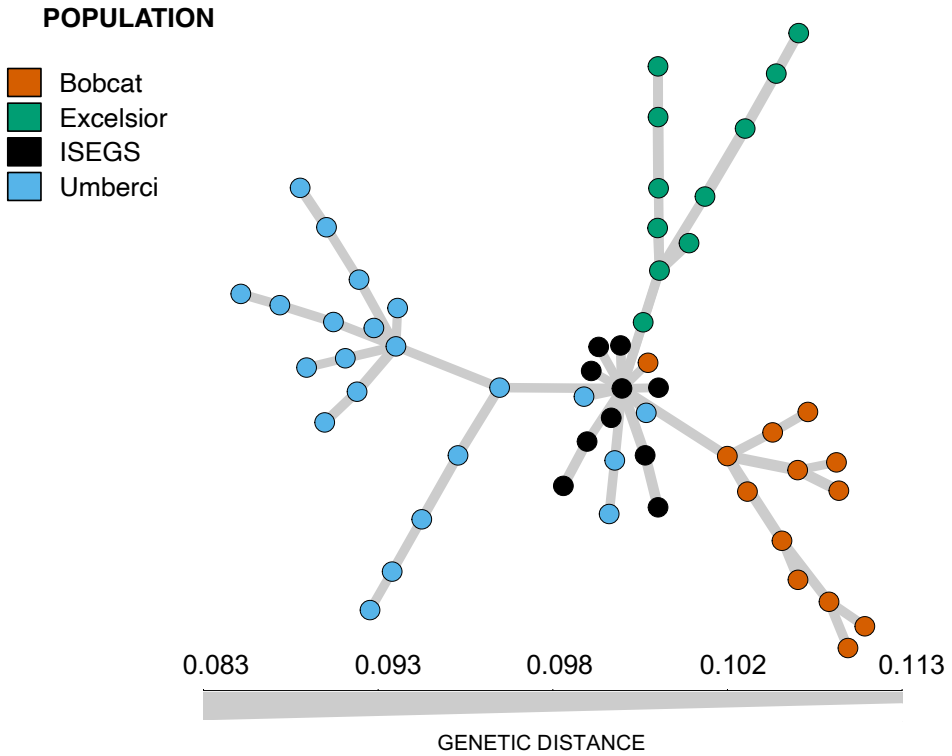

Figure 4. Minimum Spanning Network of the 2015 samples. Horizontal axis indicates pairwise Euclidean distance of each sample, which has no underlying biological assumptions. The ISEGS Mojave milkweed individuals are located centrally in the network, suggesting that they contain variation that is ancestral to the other populations. The populations' ancestral position is further supported by some individuals that were sampled from other populations clustering closer, based on genetic distance, to ISEGS samples than their putative populations.

Table 3. Pairwise differentiation among the Mojave milkweed populations. Upper diagonal is Fst calculated according to Weir and Cockerham (1984). Lower diagonal shows 95% confidence intervals following 999 permutations.

	Bobcat	Excelsior	ISEGS	Umberci
Bobcat		0.045	0.036	0.053
Excelsior	(0.042, 0.049)		0.038	0.056
Ivanpah Solar Electric Generating Station (ISEGS)	(0.034, 0.038)	(0.035, 0.040)		0.040
Umberci	(0.050, 0.055)	(0.053, 0.060)	(0.038, 0.042)	

Discussion

Our study shows that Mojave milkweed is highly structured throughout the Ivanpah Valley, and the footprint of the ISEGS facility supports an entire, genetically distinct population of this rare plant species. We found that each sampled population of Mojave milkweed contained unique genetic variation. The DAPC analysis shows that populations throughout the Ivanpah Valley are highly structured; however, there is evidence of some recent gene flow between the ISEGS and Umberci populations (Figure 3). This is most likely due to the relatively closer proximity between these populations compared to others and facilitated by the ability of the milkweed seeds to be dispersed by wind (Wyatt & Broyles 1994). Interestingly, the MSN based on genetic distance placed some individuals from other sites within the ISEGS cluster (Figure 4). This finding, coupled with the central location of the ISEGS cluster in the network, the number of private alleles, and the lowest pairwise Fst values associated with ISEGS, strengthens the idea that ISEGS plants serve as a source of rare genetic diversity for Mojave milkweed in the Ivanpah Valley. This finding coincides with other studies of endemic plants of narrow geographic range (Surina et al. 2014; Radosavljević et al. 2015) where the central populations tend to have higher genetic diversity than other populations across the range.

The distinct population structure of Mojave milkweed in the Ivanpah Valley, coupled with the small effective population size of each individual population, may lead to eventual genetic erosion (Aguilar et al. 2008). This is especially true given the small Ne values (Table 1), leaving these populations susceptible to reduced population viability (Frankham et al. 2014). The calculated Ne values as well as most of the confidence intervals of the Mojave milkweed populations were less than the 50/500 rule, where in the short term an $Ne \geq 50$ reduces inbreeding depression and a long-term $Ne \geq 500$ maintains evolutionary potential

(Jamieson & Allendorf 2012). For one of the populations, Excelsior, the pseudo-jackknifed upper-bound confidence interval returned a value of ‘infinite’ (Table 1). This finding may be interpreted as an insignificant interval as the genetic results may be explained entirely by the sample size being too small rather than the effects of genetic drift (Waples & Do 2010). As Excelsior was the population with the smallest census and sampling sizes, this is not altogether unsurprising. However, given that the Excelsior population had the smallest observed and expected heterozygosity values, the least allelic richness, and the lowest proportion of private alleles, there are likely impacts to the population’s long-term evolutionary capacity regardless of the insignificant Ne upper bound (Allendorf 1986; Lesica & Allendorf 1992). Finally, while the sampled populations of Mojave milkweed have similar overall genetic diversity, the extremely small size of each population and high proportion of private alleles within each population means the loss of a single individual could result in the loss of a significant amount of the genetic diversity within that population.

Genetic diversity is essential for the persistence of populations of rare species. When populations have extremely small numbers of individuals, they are at increased risk of inbreeding depression, loss of genetic diversity, and fixation of maladaptive traits (Lande 1998). These are important considerations in mitigation and management strategies (Clarke et al. 2012; Coates et al. 2018), especially in plants (Oostermeijer et al. 2003). Overall genetic diversity, one of the pillars of biodiversity (DeWoody et al. 2021), is critical in maintaining population longevity because increased diversity is linked to increased fitness (Willis 1993; Frankham 1995) and adaptive potential (Fernandez-Fournier et al. 2021). This is especially prescient in an age of unprecedented anthropogenic change (Foley et al. 2013).

The distinct population structure of Mojave milkweed in the Ivanpah Valley, coupled with the small effective population size of each individual population, may lead to eventual

genetic erosion (Aguilar et al. 2008). This is especially true given the small Ne values (Table 1), leaving these populations susceptible to reduced population viability (Frankham et al. 2014). The observed Ne values of each of the Mojave milkweed populations were much less than the 50/500 rule, where in the short term an Ne of 50 reduces inbreeding depression and a long-term Ne of 500 maintains evolutionary potential (Jamieson & Allendorf 2012). Coupled with these consequences, populations with consistently small Ne values are at a greater risk for the loss of important genetic variation due to the process of genetic drift (Ellstrand & Elam 1993).

Another concern for these populations of rare plants lies in one of its life history strategies, clonality, as excessive clonal propagation increases allelic diversity and heterozygosity at the expense of increased inbreeding (Balloux et al. 2003; Meloni et al. 2013). Additionally, as Mojave milkweed is self-incompatible, it is overall more susceptible to the loss of genetic variation following disturbance and habitat fragmentation (Honnay & Jacquemyn 2007), which may impact the ISEGS population due to the presence of the solar energy facility. The combined effects of clonality and self-incompatibility appear to reduce genetic diversity, potentially due to reduced mate availability (Honnay & Jacquemyn 2008). Following disturbance in the form of urbanization, clonal, self-incompatible plants had decreased clonal diversity and reproductive success (Bartlewicz et al. 2015), which would further affect population viability, especially in populations with already low numbers (Honnay & Bossuyt 2005). As our dataset included putative clones in all sites except for Excelsior, the potential effects of clonality on the reproductive strategy of Mojave milkweed and its population longevity should be investigated (Witte & Stöcklin 2010).

The ISEGS facility clearly overlays an entire population of Mojave milkweed that contains considerable genetic distinctiveness, so local extirpation of the milkweed could result in the loss of crucial genetic diversity for the persistence and adaptive potential of the

species in this region (Ricklefs 1987). This highlights the importance of protecting this population within ISEGS. It is also important to note that construction of the facility likely resulted in mortality of some individuals prior to this study (as Mojave milkweed can remain dormant belowground for greater than one year), thus resulting in the undetected loss of their genetic diversity. While mowing instead of blading within ISEGS preserved some plants, a recent study found that the mowed areas in ISEGS had less plant cover and structure of cacti and Mojave yucca (*Yucca schidigera*) than undeveloped areas (Grodsky & Hernandez 2020), indicating an overall loss of biodiversity. The question of how the construction of USSE impacts long-term diversity is ripe for investigation. Of special interest would be if the construction and operation of the ISEGS facility significantly alters (1) hydrological patterns supporting intermittent and ephemeral washes that are preferred substrates for Mojave milkweed germination and establishment (Grippo et al. 2015) (2) patterns of herbivory owing to fencing that may impact animal movement within and outside the facility's footprint (Grodsky et al. 2020; Sawyer et al. 2022) and (3) habitat fragmentation. All effects individually or combined may lead to reduced genetic variation and loss of local genetic structure in plant populations (Young et al. 1996).

Our findings demonstrate the importance of understanding population structure and genetic composition of rare and imperiled plants when designing large, ground-mounted solar energy facilities. The insights gained from this study are useful for siting and designing future solar energy facilities sustainably, including the importance of multi-year species monitoring in deserts prior to construction. Our work shows that creating the halos was beneficial to the genetic health of the Mojave milkweed in the area, as it preserved an entire, genetically unique population. However, the impact of solar energy infrastructure on patterns of hydrological flow that create and sustain desert washes—washes that serve as critical substrates for Mojave milkweed—remains a critical research gap. Loss of the unique

genetic diversity found in this population could be detrimental to the long-term persistence and adaptive capacity of this important plant. In the future, the siting of large, ground-mounted solar energy should consider the population structure of rare and imperiled species in the area as to ensure sites do not entirely overlay single populations. Understanding the population structure of species impacted by solar energy development can serve to align goals for a rapid, renewable energy transition and biological conservation.

CHAPTER 2: CONTEMPORARY CONCENTRATIONS OF MICROPLASTICS IN AQUATIC ECOSYSTEMS CAUSES MOLECULAR STRESS RESPONSES IN FISH, WITH LARGEST IMPACTS ON FEMALES

Introduction

Humans have produced over 6.3 billion metric tons of plastic waste since the mid 20th century (Geyer et al. 2017). Anywhere between 92 and 236 thousand metric tons of this waste is microplastics (Sebille et al. 2015). Due to its ubiquitous nature, microplastics (plastic particles that are less than five millimeters in length) are increasingly recognized as a critical and pervasive pollutant worldwide (Browne et al. 2007; Saal et al. 2008; Frias et al. 2014). Microplastics were first recognized in the marine environment in the 1960s (Ryan 2015) and are now globally distributed and found in most systems, including the air (Dris et al. 2016), terrestrial ecosystems (Costa et al. 2018; Machado et al. 2018), and aquatic environments (Reid et al. 2018). The aquatic ecosystems impacted by microplastics pollution are as diverse as the deep sea (Cauwenberghe et al. 2013; Woodall et al. 2014), the Antarctic and Southern Oceans (Cunningham et al. 2020), the arctic (Lusher et al. 2015), and freshwater environments such as the Laurentian Great Lakes (Rochman et al. 2013b). In 2012, the average abundance of microplastics in the Great Lakes was approximately 43,000 particles/km^2 (Eriksen et al. 2013), resulting in the designation of microplastics as an emerging environmental threat.

Given their pervasiveness, it is important that we understand the effects of microplastic exposure on aquatic organisms. Observations of marine microalgae have shown the formation of heteroaggregates with microplastics (Long et al. 2017) as well as alterations in growth, gene expression, and photosynthesis (Yokota et al. 2017). Organisms at various trophic levels mistake microplastics for food, especially non-discriminatory or filter-feeding

species (Lusher 2015; Lusher et al. 2016). In habitat-forming aquatic organisms such as corals (genus *Corallium*), microplastics exposure is associated with feeding impairment and tissue abrasion (Corinaldesi et al. 2021). Other non-discriminatory feeders such as crustaceans can accumulate microplastics through both feeding and burrowing (Setälä et al. 2016; Welden & Cowie 2016), highlighting the complexity of organismal impacts resulting from microplastics exposure. Consuming microplastics correlates with negative effects in bivalves such as digestive tract blockage and translocation to the cardiac system (Browne et al. 2008; Moos et al. 2012). Bivalves may have delayed egestion of microplastics due to the formation of aggregates in the digestive system, increasing the chance of trophic transfer (Egbeocha et al. 2018).

Microplastics can move throughout the aquatic food web through trophic transfer (Farrell & Nelson 2013; Nelms et al. 2018; Costa et al. 2020; Hasegawa & Nakaoka 2021). Diverse aquatic organisms such as fish, turtles, and whales can ingest microplastics by either mistaking microplastics for food or by consuming prey contaminated by microplastics (Rezania et al. 2018). In common goby (*Pomatoschistus microps*) juveniles, microplastics exposure inhibited acetylcholinesterase (ACHe) activity, which may affect neuromuscular function (Oliveira et al. 2013). A similar reduction in ACHe was observed in adult zebrafish (*Danio rerio*) (Limonta et al. 2021) exposed to microplastics. Following 60 days of exposure to polystyrene microplastics, marine medaka (*Oryzias melastigma*) had microplastics accumulation in the gills, intestines, and liver as well as reduced fecundity in females (Wang et al. 2019). Microplastics have also been documented in commercially and culturally important fish species such as Chinook salmon (*Oncorhynchus tshawytscha*), European seabass (*Dicentrachus labrax*), Atlantic horse mackerel (*Trachurus trachurus*), Atlantic chub mackerel (*Scomber colias*), Brazilian mojarra (*Eugerres brasilianus*), and mullets (*Mugil spp.*) (Collicutt et al. 2019; Barboza et al. 2020; Nunes et al. 2021). The ubiquity of species

exposed to microplastics is concerning, as microplastics can act as stressors in multiple ways.

An additional consequence of microplastics is the ability to sorb persistent organic pollutants (POPs) from the environment, thereby transmitting potentially harmful molecules to organisms that consume them (Egbeocha et al. 2018). This vector property of microplastics has been observed under laboratory conditions (Avio et al. 2015; Paul-Pont et al. 2016; Santos et al. 2021a). POPs documented in microplastics include organochlorine pesticides, metals, endocrine disrupting compounds, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) (Ashton et al. 2010; Rochman et al. 2013b; LI et al. 2016). In some systems, concentrations of PCBs in microplastics were found to be a millionfold higher than in the surrounding water (Betts 2008). This poses a further challenge as the sorbed chemicals could have additional effects on organisms interacting with microplastics, increasing the difficulty of determining responses due to the physical presence of the microplastic itself versus the consequences of the additional exposure to POPs. The longer a plastic is in the environment, the more chemicals it has the chance to sorb, possibly creating an additive effect on organisms that encounter microplastics. Additionally, the weathering process of plastics can create roughened edges, which may further increase their ability to negatively affect organisms associating with them (Rubin et al. 2021). The ingestion of both microplastics directly from the manufacturer and those collected in the environment (where they sorbed other contaminants) has been linked to hepatic stress and increased transcription of genes related to abnormal germ cell proliferation in adult Japanese medaka, *Oryzias latipes* (Rochman et al. 2013a). In a study using zebrafish, microplastics exposure increased cadmium accumulation in the livers, guts, and gills (Lu et al. 2018). This further highlights the complicated interaction of microplastics, the environment, and the potential impacts on aquatic organisms.

Differential gene expression analysis provides insight into the molecular response to ecological variables and changing environments (Alvarez et al. 2015). Studying differential gene expression along physiological timescales such as in exposure scenarios helps shape hypotheses along evolutionary timescales such as population effects (Reid & Whitehead 2016), which is necessary in understanding the effects of persistent pollutants such as microplastics. Thus, we investigated the genome-wide expression changes associated with microplastics exposure. Previous gene expression studies of microplastics exposure have documented myriad responses across aquatic organisms. In red corals, microplastics exposure resulted in the upregulation of various cytochrome and heat shock protein genes (Corinaldesi et al. 2021). Marine mussels exposed to microplastics had transcription effects in genes related to antioxidant effects and detoxification (Avio et al. 2015). Zebrafish exposed to polystyrene microplastics had altered expression of antioxidant genes as well as genes related to apoptosis (Umamaheswari et al. 2021). A separate study of gene expression in zebrafish head kidneys found upregulation of genes involved in xenobiotic metabolism and adaptive immunity (Limonta et al. 2021). In marine medaka, microplastics exposure correlated with gene transcription changes along the hypothalamic-pituitary-gonadal (HPG) axis in both male and female fish (Wang et al. 2019).

As many major freshwater ecosystems are experiencing declines in biodiversity (Reid et al. 2018), it is essential to understand how microplastics impact aquatic environments and examine the varied effects of microplastics on aquatic organisms. However, current studies are limited largely by sample size or the scope of exposure scenarios and the implications of microplastic pollution on freshwater biodiversity remain unclear (Eerkes-Medrano et al. 2015; Reid et al. 2018). To date, it has proven difficult to comprehensively assess the effects of microplastics on aquatic organisms (Burns & Boxall 2018), and many knowledge gaps remain in the scientific literature regarding the effects of microplastics at the

organismal level, especially along the interactions of plastic sizes, shape, exposure duration, concentration, and polymer type. (Bucci et al. 2020). We addressed some of these knowledge gaps by examining the molecular effects of polyethylene microplastic ingestion on fathead minnows (*Pimephales promelas*), a model toxicological organism and prey fish native to the Great Lakes region of the North America. We investigated the effects of various concentrations as well as origins of plastics to elucidate some of the more complicated aspects of microplastics exposure. Our questions included:

1) Is there an effect of microplastics exposure on gene expression in fathead minnows, and does this effect vary by sex?
2) Does the magnitude of the effect of microplastics on gene expression vary with the relative concentration of microplastics?
3) How does the effect vary between exposure to pristine microplastic versus microplastic collected from the environment, which may have sorbed additional pollutants?

Evaluating the impacts of microplastic exposure across the genome as well as over multiple plastic sources and concentrations provides a better understanding of the effects of microplastic pollution on aquatic communities and may clarify the consequences of living in an environment polluted with microplastics. Deducing molecular responses to microplastics exposure in a model organism will provide further insight to the mechanistic consequences of organisms interacting with this new pollutant, which are especially prescient concerns for species of conservation concern or in already-threatened ecosystems. Furthermore, taking a genome-wide look at the response to microplastics may reveal unexpected effects on molecular functioning and provide insights on the outcomes of exposure to this complex pollutant.

Methods

Experimental Design

We used fathead minnows, a toxicological model organism, throughout this experiment to determine the effects of microplastics across life stages. We adapted rearing methods from a previously published fathead minnow life cycle experiment (Parrot 2005). We obtained fathead minnows from the breeding stock of the Ontario Ministry of the Environment, Climate, and Parks (MECP) and raised these fish in the MECP facilities under experimental conditions with four treatments and a negative control (N). The treatments included a low, environmentally relevant concentration (240 particles/L) of virgin polyethylene (PL) microplastic (Grbić et al. 2020), as well as a 10-fold increase in concentration representative of a future scenario (2400 particles/L) of virgin polyethylene (PH), low concentration of polyethylene gathered from Lake Ontario (EL), or a high concentration of polyethylene microplastic originating in Lake Ontario (EH) for a total of 5 treatments. We used 5 replicates per treatment for a total of 25 aquaria. The experimental design is summarized in Figure 5. We reduced microplastic size to 100-500μm using a burr mill grinder.

Each treatment received twice daily feedings. The initial diet consisted of newly hatched brine shrimp at a concentration of 15 nauphii/μL until 30 days post hatch (dph), followed by a gradual introduction of frozen brine shrimp into the diet until 50 dph, after which point the diet consisted solely of thawed brine shrimp. Throughout the experiment, we maintained the water bath at 25˚C and lights turned on each day for a total of 16 hours. We completed 30% water changes three times a week. During cleaning, we scraped the aquaria to remove algae buildup and tested a random tank from each treatment for dissolved oxygen, pH, and conductivity to determine water quality. Additionally, we checked ammonia levels in a

sequential manner for each treatment during cleaning. The experiment concluded at six months, or 180 dph. We euthanized fish using a lethal bath of buffered tricaine methanesulphonate (MS-222) at a concentration ≥ 250 mg/L. We left fish in the bath until two minutes after respiration ceased. For molecular analysis, we dissected liver tissue from one male and two females from each tank (n=75) and placed the tissue in RNAlater. Between fish, we cleaned dissection tools with 10% bleach, molecular-grade deionized water, and 90% ethanol.

Differential gene expression analysis

To evaluate gene expression patterns among different treatments, we extracted RNA from the samples using the Quick-RNA Miniprep Kit (Zymo Research, Irvine, CA, USA) and quantified the resulting genomic material using Qubit RNA assay kits (Invitrogen, Carlsbad, CA, USA). Following quantification, we prepared the samples for sequencing using the QuantSeq 3' mRNA-seq FWD library kit (Lexogen, New Hampshire, MA, USA). Samples were normalized to 50ng prior to undergoing reverse transcription. For the second strand synthesis, we used the UMI module for QuantSeq FWD (Lexogen, New Hampshire, MA, USA) to identify PCR duplicates. We pooled all 75 samples prior to sequencing at the RTSF Genomics Core at Michigan State University. We used the high-output, 75 base-pair SE format on the NextSeq 500. We produced additional technical replicates by resequencing the entire library twice for a total of three runs.

Following sequencing, we used bbduk v35.92 to trim poly-A tails and low-quality nucleotides (commands k=13 ktrim=r useshortkmers=t mink=5 qtrim=r trimq=10 minlength=20m, see http://jgi.doe.gov/data-and-tools/bb-tools/). We aligned the reads to the fathead minnow genome (GenBank accession GCA_016745375.1) using STAR (Dobin et al. 2013) with modified Encode settings --outFilterType BySJout --outFilterMultimapNmax

20 --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --outFilterMismatchNmax 999 --outFilterMismatchNoverLmax 0.6 --alignIntronMin 20 --alignIntronMax 1000000 --alignMatesGapMax 1000000 --outSAMattributes NH HI NM MD as recommended by Lexogen. Identical reads were collapsed using the dedup function of the umi-tools package in Python 3.6.6 (Smith et al. 2017). We used HTSeq v 0.11.2 for counting of sorted, indexed, and deduplicated bam files for all three libraries prior to exporting the data to R.

We combined the resulting count data from the three sequencing runs into a single DESeq dataset using the DESeq2 package (Anders & Huber 2010). We used explanatory variables such as sex, treatment type (control, pristine low, pristine high, environmental low, environmental high), plastic concentration (high vs low), and plastic type (pristine vs environmental) to categorize our data for pairwise comparison (Figure 5). We further filtered this dataset by removing samples with a total count number of less than 100,000 followed by removing tags with less than 10 counts across 15 samples. We used DESeq2 to identify differential expression between treatments, concentration levels, plastic types, and sex as well as to investigate the interaction of sex on the treatments, concentrations, and plastic types. For variables with greater than 20 genes with significant expression changes, we performed functional enrichment analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang et al. 2009; Sherman et al. 2022) to determine any enrichment in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis or along the gene ontology (GO) terms of biological process, molecular function, and cellular component. For this analysis, we used lists of the associated locus tags and compared to the *Pimephales promelas* database within DAVID. Significance was assessed at the Benjamini-Hochberg adjusted $p \leq 0.05$.

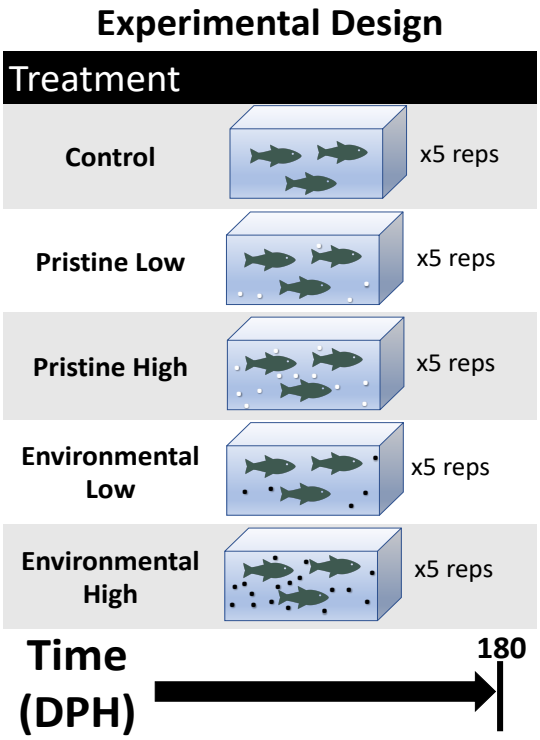

Figure 5. Graphical representation of the experimental design. Briefly, we raised five replications of a single generation of fish from eggs through 180 days post hatch (DPH) in either a control or one of four treatment types. The treatments varied by concentration (low or high) and plastic source (pristine [directly from manufacturer] or environmental [collected from the beach of Lake Ontario]). Pristine plastics are depicted with open circles in the experimental tanks, environmental plastics are depicted with filled circles.

Weighted gene co-expression network analysis

To assess the genome-wide effects of microplastics exposure, we performed weighted gene co-expression network analysis (WGCNA) to identify modules of co-expressed genes with coordinated variation (Langfelder & Horvath 2008; Rose et al. 2015). This analysis allowed us to test for significant interactions between expression levels in gene modules and our experimental variables. For this analysis we used three initial datasets which included all samples that passed filtration (n=58), only male samples (n=21), and only female samples (n=37). We analyzed the datasets according to suggestions from the developers. These data underwent variance stabilized transformation prior to continuing

the analysis. For the analysis, we used a power of 6, 7, and 8 for all, female, and male datasets, respectively. For adjacency and TOMsimilarity we specified the hclust method “average”. We used a deepSplit of 2 and minClusterSize of 30 for cutreeDynamic. We merged similar modules at a cutHeight of 0.25 based on the distance between their eigengenes. We then examined the Pearson correlations between module eigengenes and traits for the following indicator variables: control (treated or control), sex (female or male), treatment type (control, pristine low, pristine high, environmental low, environmental high), plastic concentration (high vs low), and plastic type (pristine vs environmental). As with the differentially expressed gene lists from our DESeq analysis, we performed functional enrichment analysis on significantly correlated modules ($p \leq 0.05$) using DAVID across the levels of biological process, molecular process, and cellular component and within the KEGG pathway. Again, significance was assessed at the Benjamini-Hochberg adjusted $p \leq 0.05$ and we used lists of the associated locus tags and compared to the *Pimephales promelas* database within DAVID.

Results

Differential expression between treatments

Following sequencing, we averaged 12,806,340 reads and 1,642,706 alignments per individual after deduplication across the three technical replicates. Our initial dataset included 23,143 locus tags with at least a single count. Following filtration, the final dataset for analysis featured 7471 locus tags across 58 samples. The final dataset included 21 male and 37 female individuals. We found differentially expressed genes among all treatments and the controls (see Table 4). For the initial differential expression (DE) analysis (n=58) comparing the treated fish and the control, 20 genes were differentially expressed at $p<0.05$, with the most positive log fold change (LFC) in hemoglobin subunit beta-2 [*LOC120489274* (LFC= 0.914, p=0.003)] and the most negative in the protein-coding gene *Heat Shock Protein 90 Beta Family Member 1 (HSP90B1)*, a molecular chaperone involved in protein folding (LFC = -1.199, p=0.003) [Figure 5, Table 4]. Exposure to estrogenic chemicals common to plastics is linked to decreased expression of *HSP90B1* in liver metabolic pathways in mice (Diamante et al. 2020) and expression changes in zebrafish (Villeneuve et al. 2012).The treatment of high concentration of pristine plastic compared to the control had DE in 129 genes, with *HSP90B1* again having the most negative (LFC=-1.1, p=0.01, Figure 6) and *Ribosomal Protein S11* (*RPS11*) the most positive (LFC= 0.953, p=0.04) fold changes. DAVID analysis indicated enrichment for the ribosomal KEGG pathway. 29 genes had DE between the environmental low treatment and the control, with the most negative change (LFC=-2.359, p=0.002) occurring in *ADP-ribosylation factor-like 4aa* (*ARL4AA*) and the most positive in diamine acetyltransferase 1-like [*LOC120471604* (LFC=0.634, p=0.023)]. The only KEGG pathway enriched was for protein processing in endoplasmic reticulum. For the comparison of the environmental high treatment and the

control, the only differentially expressed genes were *Adenosylmethionine Decarboxylase 1* [*AMD1* (LFC= 1.167, p=0.012)] and *Period circadian clock 1b* [*PER1B* (LFC= 2.027, p=0.001)], which are both known to interact with estrogenic chemicals common in plastics (Davis et al. 2022). The only gene differentially expressed between the pristine low treatment and the control was *Eukaryotic translation initiation factor 4E family member 1c* [*EIF4E1C* (LFC=-1.715, p=0.029)], also known to interact with estrogenic chemicals common in plastics in both fathead minnows and zebrafish (Villeneuve et al. 2012).

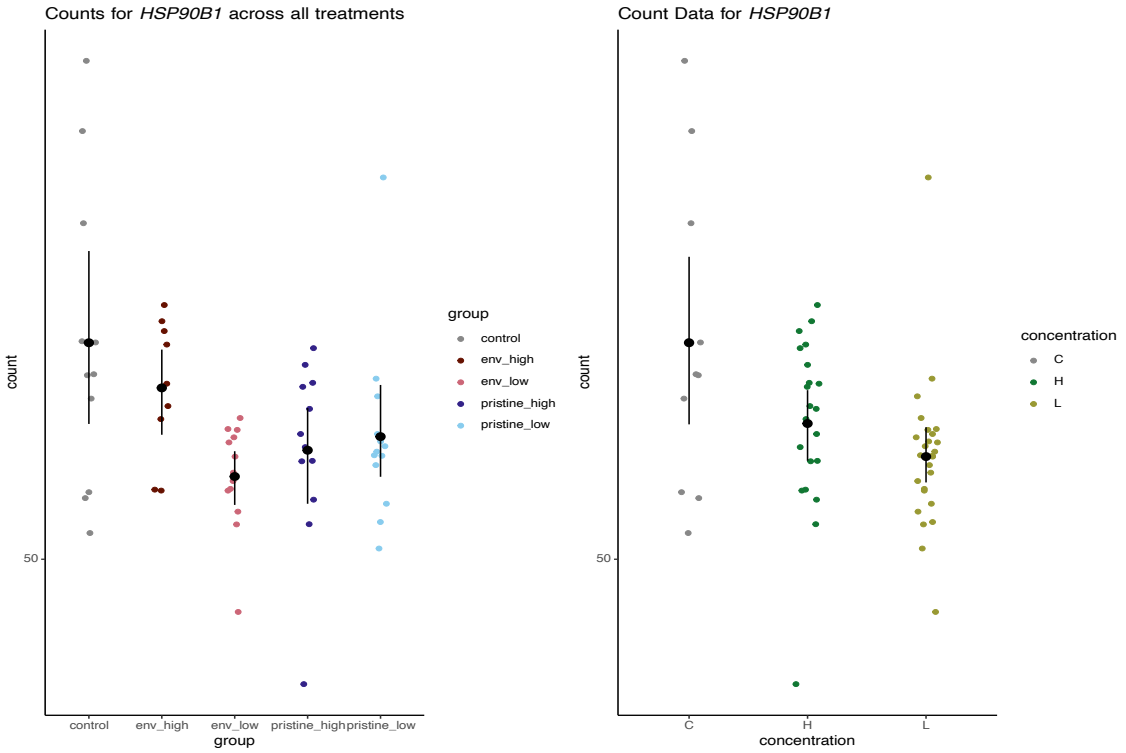

Figure 6. Counts for the gene *Heat Shock Protein 90 Beta Family Member 1 (HSP90B1)*. Differential expression from the control is designated with an asterisk. This gene had decreased expression across all treated samples regardless of concentration or plastic origin compared to the control minnows [Table 1]. However, this correlation was likely driven by the female individuals as this gene did not have significant DE in the analysis of male minnows. This trend was also seen in the female treated samples (LFC=-1.435, p=0.005), pristine samples (LFC=-1.426, p=0.017), environmental treatment (LFC= -1.447, p=0.01), and the pristine high treatment (LFC= -1.63, p=0.005), high concentration (LFC=-1.335, p=0.022) compared to the control. There was also DE in the female pristine high vs pristine low (LFC=-1.878, p=0.005) treatments.

We also found genes with DE across the concentrations (low, high, control) and plastic types (environmental, pristine, control) comparisons (see Table 4). There were 92 DE genes between the high concentration of plastics compared to the controls, and DAVID analysis indicated enrichment for the ribosomal KEGG pathway. 23 genes had DE between the low concentration and control treatments, with the most negative LFC in hemoglobin subunit beta-2 (LFC=-0.952, p=0.01) and the most positive in *Activating transcription factor 3* [*ATF3,* (LFC=2.252, p=0.032)], which plays roles in modulating immunity, metabolism, and oncogenesis (Ku & Cheng 2020). 69 genes had differential expression between the pristine plastic and the control, and DAVID analysis again indicated enrichment for the ribosomal KEGG pathway. For the comparison between environmental plastic and the control, there were 16 differentially expressed genes, with the most negative LFC in hemoglobin subunit beta-2 (LFC= -0.852, p=0.05) and the most positive LFC in *ATP binding cassette subfamily F member 1* [*ABCF1* (LFC= 1.846, p=0.034)] which is orthologous to the human gene of the same name and may play a role in enhancement of protein synthesis and the inflammation process [(Consortium et al. 2022), release 5.4.0].

Table 4. Differential gene expression results from DESeq analysis. Only significantly differentially expressed ($p \leq 0.05$) genes or functional annotations reported. Functional annotations are Benjamini-corrected.

Dataset	Comparison	Number of genes	Functional annotation (official gene symbols if n≤25)
All	All male (M) vs female (F) minnows	1923	Ribosome (KP, p=2.6E-42) Metabolic pathways (KP, p=2.2E-5) PPAR signaling pathway (KP, p=3.6E-5) Fatty acid metabolism (KP, p=1.0E-4) Fatty acid degradation (KP, p=3.8E-4) Ferroptosis (KP, p=0.002) Pyruvate metabolism (KP, p=0.002) Protein processing in endoplasmic reticulum (KP, p=0.006) Cysteine and methionine metabolism (KP, p= 0.006) Carbon metabolism (KP, p=0.01) Valine, leucine, and isoleucine degradation (KP, p=0.01) Peroxisome (KP, p=0.02) Biosynthesis of amino acids (KP, p=0.02) Tyrosine metabolism (KP, p=0.05) Biosynthesis of unsaturated fatty acids (KP, p=0.05)
	Treated M vs treated F	1421	Ribosome (KP, p=1.7E-49) Fatty acid metabolism (KP, p=1.2E-4) Metabolic pathways (KP, p=1.2E-4) PPAR signaling pathway (KP, p=1.8E-4) Ferroptosis (KP, p=0.001) Fatty acid degradation (KP, p= 0.003) Pyruvate metabolism (KP, p=0.02) Biosynthesis of unsaturated fatty acids (KP, p=0.02) Pantothenate and CoA biosynthesis (KP, p=0.03) Cysteine and methionine metabolism (KP, p=0.04)

Table 4 (cont'd)

	Untreated M vs untreated F	294	Ribosome (KP, p= 9.9E-26) Protein processing in endoplasmic reticulum (KP, p=0.003) Fatty acid metabolism (KP, p=0.005) Aminoacyl-tRNA biosynthesis (KP, p=0.05)
	Treated vs control	20	*LOC120471604, LOC120489274, HSP90B1, USO1, WU:FJ16A03, SRPRA, GPX3, BHMT, EIF3JB, AMD1, CDKN1BA, RTN4A, PRDX2, CIDEB, HDAC5, LOC120476048, DIABLOA, RPS28, SERPINC1, LOC120465073*
	High concentration plastic vs control	92	Ribosome (KP p=2.8E-57)
	Pristine plastic, high concentration (PH) vs control	129	Ribosome (KP p=4.5E-92)
	Environmental plastic, low concentration (EL) vs control	29	Protein processing in endoplasmic reticulum (KP, p=0.001)
	Low concentration plastic vs control	23	*HSP90B1, EIF4E1C, DNAJC3A, LOC120471604, LOC120489274, H6PD, SRPRA, CLTB, ZGC:162964, SLC17A9B, CRELD2, CALR, ATF3, GPX3, USO1, CARS1, CDKN1BA, MANF, WU:FJ16A03, HNF4A, FICD, PRDX2, BHMT*
	Environmental plastic vs control	16	*AMD1, ELP3, SI:CH211-132B12.7, LOC120471604, PER1B, HSP90B1, DNAJC3A, SEC61A1L, SRPRA, ABCF1, MRTO4, SRP72, LOC120489274, CUNH1ORF131, PER3, TEFA*
	Pristine plastic vs control	69	Ribosome (KP p=1.3E-30)
	Environmental plastic, high concentration (EH) vs control	2	*PER1B, AMD1*
	Pristine plastic, low concentration (PL) vs control	1	*EIF4E1C*
Females	Treated vs control	76	Protein export (KP, p=0.007)
	Pristine plastic vs control	79	Ribosome (KP, p=2.3E-43)

Table 4 (cont'd)

	Environmental plastic vs control	90	Protein processing in endoplasmic reticulum (KP, p=2.4E-4) Protein export (KP, p=0.009) Aminoacyl-tRNA biosynthesis (KP, p=0.05)
	PH vs control	157	Ribosome (KP, p=3.3E-91)
	PH vs PL	59	Protein processing in endoplasmic reticulum (KP, p=1.7E-5)
	EL vs control	19	*PNPLA6, ADIPOR2, TNPO3, TRAK1A, CGNL1, LOC120460994, LOC120473388, RNF130, ANKRD11, SOX6, CNSTA, ETNPPL, LOC120483477, SETD1A, RND1A, RWDD1, NIP7, LYRM7, ZMP:0000001301*
	EH vs control	20	*CUNH1ORF131, AMD1, RAB27A, SLC49A4, DDX27, TOMM70A, SRPRA, ZGC:154046, COPB2, NPM1A, HMGN3, SRP72, SI:CH211-132B12.7, CLOCKA, SLC38A3A, PER1B, BRF1A, NOP58, LGMN, EIF2B4*
	EH vs PL	1	*PER1B*
	EL vs PH	1	*ZGC:112148*
	EH vs PH	1	*SLC38A3A*
	EH vs EL	6	*RPS6KA1, IFT81, C4, ARL4AA, SLC38A3A, LOC120486762*
	PL vs control	3	*EIF4E1C, NPM1, SLC49A4*
	High concentration of plastic vs control	102	Ribosome (KP, p=1.2E-48)
Males	Pristine vs environmental plastic	1	*ELOVL5*
	Treated vs control	2	*PLAAT1L, LOC120477409*
	EH vs EL plastic	1	*LOC120495555*
	EH vs PL	2	*DHX58, RNF213A*

Differential expression between the sexes

We found a stark difference in how the minnows responded to microplastics between the sexes (Table 4). The comparison between all male and female individuals (regardless of plastic exposure) contained 1923 differentially expressed genes and DAVID analysis of the genes revealed changes in several KEGG Pathways (KP) related to metabolism and inflammation (Table 4). When comparing control male and female minnows, only 294 genes had differential expression. DAVID analysis revealed enrichment in genes related to metabolism but not inflammation, as expected given that these individuals were not exposed to microplastics. However, when the treated males and females were compared, we again saw differential expression in 1423 genes and functional enrichment in both metabolism and inflammation, indicating a difference in response to microplastics between the sexes.

In female individuals (n=37), when compared to the female controls, there were 76 differentially expressed genes and DAVID analysis indicated enrichment in the KEGG pathway of protein export (p=0.007). For the individual treatments in females, 19 genes had DE between the environmental low samples and the control, with the most negative LFC in piezo-type mechanosensitive ion channel component 2 [*LOC120460994* (LFC= -2.164, p=0.034)] and the most positive change in *NIP7 nucleolar pre-rRNA processing protein* [*NIP7* (LFC= 1.627, p=0.046)], which is orthologous to a human gene involved in ribosomal large subunit biogenesis [(Consortium et al. 2022) release 5.4.0]. 157 genes differed between the pristine high treatment and control and were enriched for the ribosome KEGG pathway (KP, p=3.3E-91). 20 genes differed between the environmental high treatment and control in females, with the most negative LFC in *cunh1orf131* (LFC= -2.6, p=3.8E-4), which is orthologous to human chromosome 1 open reading frame 131, which enables RNA

binding activity [(Consortium et al. 2022), release 5.4.0], and the most positive LFC in *solute carrier family 49 member 4* (*SLC49A4*), predicted to enable transmembrane transporter activity [(Consortium et al. 2022), release 5.4.0]. Exposure to estrogenic chemicals associated with plastics has correlated with increased transcription of *SLC49A4* in zebrafish (Wu et al. 2021). Transcription changes in *SLC49A4* are also associated with liver injury, hepatomegaly, and necrosis. The genes *EIF4E1C*, *nucleophosmin 1a* (*NPM1A*), which acts upstream of hemopoiesis and the regulation of apoptotic processes [(Consortium et al. 2022), release 5.4.0], and *SLC49A4* differed between the pristine low treatment and control. 59 genes differed between the pristine high and low treatments and were enriched for protein processing in the endoplasmic reticulum (KP, p=1.7E-5).

Some comparisons of treatments in the females yielded only a single gene with differential expression. These included the environmental high and pristine low treatments [*PER1B* (LFC=0.445, p=0.001)], the environmental low and pristine high treatments [*ZGC:112148* (LFC=0.364, p=0.04)], predicted to be involved in protein secretion and vesicle-mediated transport, (Consortium et al. 2022), release 5.4.0], and the high concentrations of environmental and pristine plastics, [*SLC38A3A* (LFC=0.426, p=0.031), Figure 2]. Finally, 6 genes differed between the environmental high and low treatments (Table 4). The female samples also had differential expression between the plastic types and concentrations. Compared to controls, there were 79 DE genes in the female pristine plastic treatments, enriched for the KEGG pathway for ribosomes (p=2.3E-43) and 90 DE genes in the female treatments with environmental plastics, enriched for the KEGG pathways of protein export (p=0.009), protein processing in the endoplasmic reticulum (p=2.4E-4), and Aminoacyl-tRNA biosynthesis (p=0.05). The females exposed to high concentrations, regardless of plastic type, had 102 differentially expressed genes, which again corresponded to a functional increase in the KP for ribosomes (p=1.2E-48).

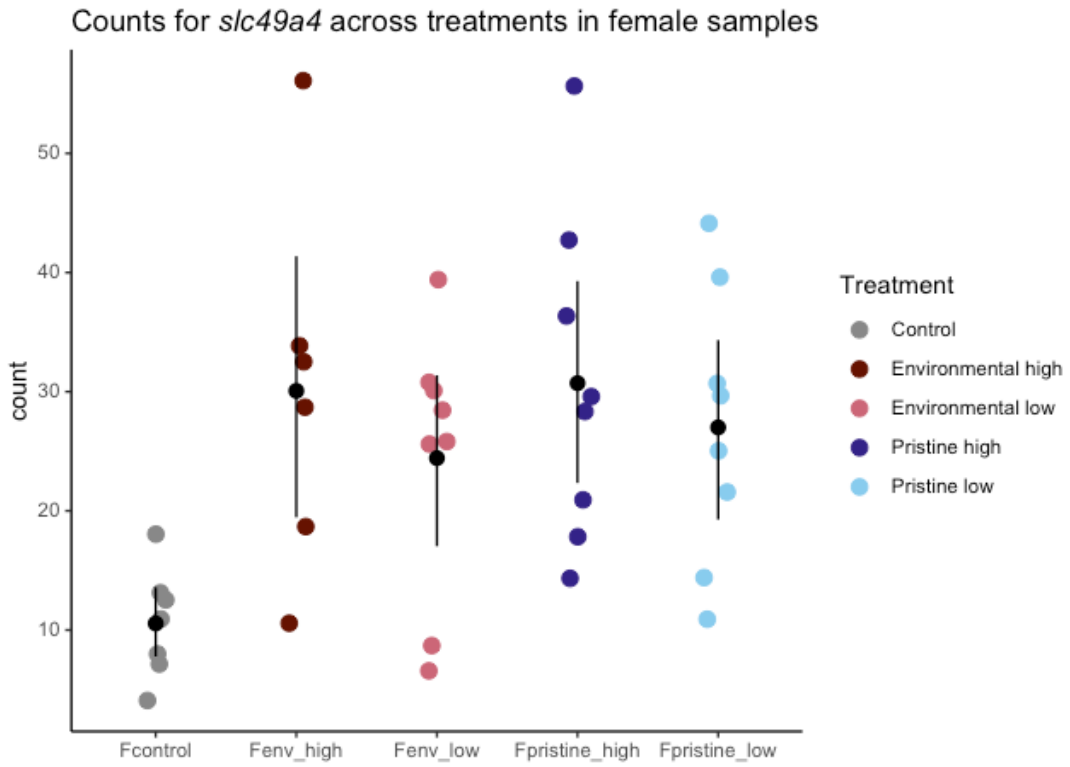

Figure 7. Counts for *SLC49a4* across treatments of female samples. There was significant difference in log fold change between the treatment and control samples LFC=1.403 (p=0.001), pristine high and controls LFC=1.63 (p=0.005), environmental high and control LFC=1.521 (p=0.015), pristine low and control LFC=1.354 (p=0.34), pristine high compared to pristine low LFC=1.213 (p=0.04), pristine plastic and control LFC=1.445 (p=0.002), and environmental plastic and control LFC=1.354 (p=0.004).

Contrastingly, in the males (n=21), only two genes--*phospholipase A and acyltransferase 1-like* [*PLAAT1L* (LFC=-2.09, p=0.007)], predicted to be involved in N-acylphosphatidylethanolamine metabolism [(Consortium et al. 2022), release 5.4.0] and the uncharacterized locus *LOC120477409* (LFC=7.076, p=0.007) were differentially expressed between the treated and control samples. The gene *ELOVL5* (LFC=1.323, p=0.007) differed between the pristine and environmental individuals in the males. Among the males, cytochrome P450 2F2-like [*LOC120495555* (LFC=-2.5, p -0.023)] expression differed between the environmental high and low treatments. The genes DEXH (Asp-Glu-X-His) box polypeptide 58 [*DHX58* (LFC=2.568, p=0.028)], predicted to be involved in the innate immune response, and ring finger protein 213a [*RNF213A* (LFC=2.31, p=0.030)], predicted

to act upstream of or within blood circulation, differed between the environmental high and pristine low treatments in males [(Consortium et al. 2022), release 5.4.0]. There were no other comparisons with significantly differentiated gene expression in males.

Table 5. Gene modules from weighted correlated network analysis. Only significantly differentially expressed (p≤0.05) genes or functional annotations reported. Functional annotations are Benjamini-corrected.

Dataset	Module (number of genes)	Association (p≤0.05)	Functional annotation
All	A1 (1047)	Sex (-0.95)	Metabolic pathways (KP p=8.5E-7) Peroxisome (KP p=0.05) Pantothenate and CoA biosynthesis (KP p=0.05)
	A2 (438)	Sex (-0.42) Low concentration of plastic (0.31)	Sequence-specific DNA binding (Molecular function, GO:0043565) Aminoacyl-tRNA biosynthesis (KP p=2.0E-10) Fatty acid degradation (KP p=0.002) Metabolic pathways (KP p=0.02)
	A3 (2709)	Sex (-0.65) Pristine plastic (-0.27) Pristine plastic, high concentration (PH) (-0.31) Treatment vs control (0.28)	Ribosome (KP p=1.4E-30, entry map03010) mRNA surveillance pathway (KP p=0.001, entry hsa03015) nucleocytoplasmic transport (KP p=0.03, entry ko03013)
	A4 (179)	Treatment vs control (0.4)	Ribosome biogenesis in eukaryotes (KP p=1.1E-19, entry ko03008)
	A5 (104)	Treatment vs control (0.32)	Proteasome (KP p=8.7E-5, entry hsa03050) Ferroptosis (KP p=0.02, entry hsa04216)
	A6 (340)	Low concentration of plastic (-0.27) Environmental plastic, low concentration (EL) (-0.26) Treatment vs control (0.34)	Protein processing in the endoplasmic reticulum (KP p=3.2E-14) N-Glycan biosynthesis (KP p=5.7E-4, entry map00510) Various types of N-glycan biosynthesis (KP p=0.001) Protein export (KP p=0.001)

Table 5 (cont'd)

Females	F1 (1497)	High concentration plastic (-0.41) Pristine plastic (-0.38) PH (-0.58) Treatments vs control (0.46)	Ribosome (KP p=2.4E-49)
	F2 (271)	High concentration (-0.33)	--
	F3 (707)	High concentration (-0.33) PH (-0.49) Pristine plastic, low concentration (PL) (0.32)	--
	F4 (424)	Low concentration plastic (-0.36)	--
	F5 (974)	Low concentration plastic (-0.36)	Aminoacyl-tRNA biosynthesis (KP p=5.5E-5) Fatty acid degradation (KP p=0.005) Metabolic pathways (KP p=0.01) PPAR signaling pathway (KP p=0.01)
	F6 (486)	Environmental plastic (-0.32) Treatments vs control (0.39)	Protein processing in endoplasmic reticulum (KP p=8.8E-7) Proteasome (KP p=7.1E-4) N-Glycan biosynthesis (KP p=0.001) Various types of N-glycan biosynthesis (KP p=0.01) Metabolic pathways (KP p=0.04)
	F7 (424)	Treatments vs control (0.38)	Ribosome biogenesis in eukaryotes (KP p=4.7e-11)
	F8 (308)	Treatments vs control (0.4)	--
Males	M1 (119)	PL (-0.49)	--
	M2 (104)	PL (-0.45)	Proteasome (KP p=8.7E-5) Ferroptosis (KP p=0.02)
	M3 (80)	Environmental plastic, high concentration (EH) (-0.71)	--
	M4 (889)	Treatments vs control (-0.54)	Ribosome (KP p=4.9E-53)
	M5 (160)	PL (-0.45)	--

Table 5 (cont'd)

	M6 (135)	High concentration plastic (-0.52) PH (-0.66)	--

Weighted gene co-expression networks between treatments

The weighted gene co-expression network analysis (WGCNA) yielded groups of genes with highly correlated expression. In the dataset of all samples (n=58), three modules were significantly ($p \leq 0.05$) associated with sex (between 438-2709 genes per module, Figure 8, Table 5). These modules included significant associations with metabolic pathways, the peroxisome, and pantothenate and CoA biosynthesis. The A3 module, also significantly associated with treated samples, featured functional enrichment for ribosome, the mRNA surveillance pathway (KP p=0.001), and nucleocytoplasmic transport (KP p=0.03). The A4 (n=179), A5 (n=104), and A6 (n=340) modules significantly associated with the treated samples and were functionally enriched for ribosome biogenesis in eukaryotes, proteasome, ferroptosis, protein processing in the endoplasmic reticulum, N-Glycan biosynthesis, and protein export. The A6 module was also associated with the low concentration of environmental plastic and the low concentration of plastic regardless of plastic type. The A2 module was also significantly associated with the low concentration of plastic and associated with the molecular function of sequence-specific DNA binding and the KEGG Pathways of aminoacyl-tRNA biosynthesis, fatty acid degradation, and metabolic pathways.

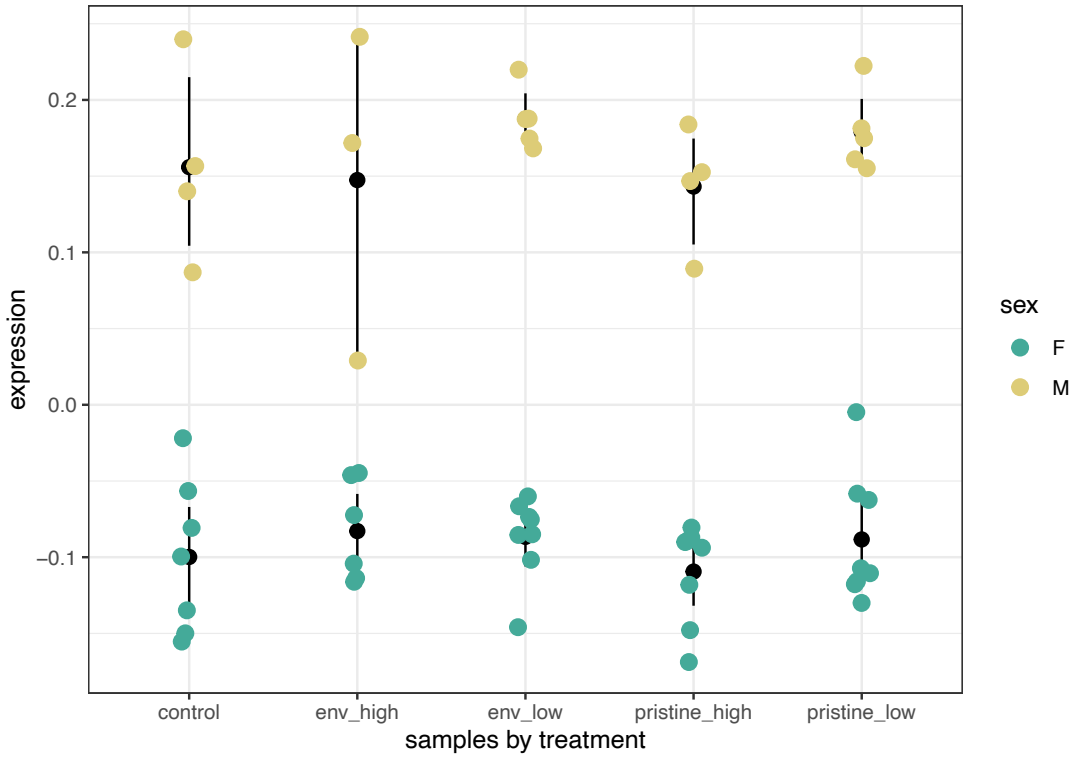

Figure 8. Eigengene expression for the A1 module from weighted gene co-expression analysis (WGCNA) across treatments [see Table 2]. This module significantly correlated with sex (-0.95, p=1E-29) and illustrates broad differences in expression between the male and female minnows independent of their response to the treatments.

Weighted gene co-expression networks by sex

When the analysis was separated by sex, there were several modules associated with overall plastic exposure in females and were enriched for various metabolic processes, including protein processing in endoplasmic reticulum, proteasome, and metabolic pathways (F6) as well as ribosome biogenesis (F7) [Table 5]. Three modules significantly correlated with high concentrations of plastic in the females. One module (F1) was significantly enriched for ribosomes (KP p=2.4E-49) and associated with pristine plastic. The F4 (n=424) and F5 (n=974) modules associated significantly with the low concentration of plastic. While the F4 module was not functionally enriched, the F5 module was enriched for aminoacyl-tRNA biosynthesis, fatty acid degradation, metabolic pathways, and the

PPAR signaling pathway. For the WGCNA analysis of male minnows, the M4 (n=889) module was the only module significantly associated with the individuals exposed to plastic and enriched for ribosome. Several modules were associated with the low concentration of pristine plastic (M1, M2, M5), but only the M2 module had any significant functional enrichment, in proteasome and ferroptosis (Table 1). The remaining modules, while associated with environmental plastic at high concentration (M3) and both the high concentration of plastic and pristine plastic (M6) had no significant functional enrichment.

Discussion

Our results indicate many transcriptional changes in the livers of individuals exposed to any kind of microplastics. Many of the differentially expressed genes are associated with chemical and drug induced liver injury (Davis et al. 2022). Interestingly, there were markedly more genes differentially expressed among the sexes in the control (N=1923) compared across sexes in the treatments (N=1421) [Table 4]. Furthermore, the plastic exposed individuals had additional functional enrichment in the KEGG pathways, most notably in the peroxisome proliferator-activated receptor (PPAR) signaling pathway, demonstrating greater metabolic disruption between males and females exposed to microplastics compared to the unexposed individuals. This indicates that female gene expression is more affected by microplastic exposure than in the males. This finding complements previous work demonstrating evidence of sex-dependent effects in other species. Female mice appear more susceptible to microplastics than males (Wei et al. 2022; Yang et al. 2022). A study in Japanese medaka (*Oryzias latipes*) found dose-dependent decreases in female fecundity associated with microplastics, but no effects on fecundity in males (Zhu et al. 2020). However, other work suggests this affect varies with life stage, as juvenile male guppies (*Poecilia reticulata*) are adversely affected by microplastics exposure (Rahman et al. 2022). However, this work did not include female guppies so is an incomplete comparison. Collectively, our results, along with this previous work, highlights the importance of determining the sex-specific responses to microplastic exposure to fully understand how environmental microplastics may impact population dynamics and individual fitness.

We also found significant effects of microplastic exposure on the expression of multi-gene modules. Several gene modules featured enrichment for a suite of KEGG pathways

related to stress responses, including the detection and degradation of abnormal mRNAs (mRNA surveillance), vital cellular processes and cellular metabolism (e.g., nucleocytoplasmic transport, ribosome biogenesis, proteosome, N-Glycan biosynthesis, protein export), and regulated cell death (proteasome, ferroptosis). The modules enriched for mRNA surveillance, ferroptosis, and proteasome activity are especially notable, as these pathways are known to activate under pathological conditions, oxidative damage, or stress (Dixon et al. 2012; Baumann 2014; Jamar et al. 2017). There is also evidence of metabolic disruption associated with microplastics exposure, which is a finding consistent with several other studies in aquatic organisms (Wan et al. 2019; Medriano & Bae 2022; Shin & Jeong 2022; Wang et al. 2022). Finally, the changes in pathways related to regulated cell death are consistent with previous work finding that microplastics exposure reduces cell viability (Palaniappan et al. 2022). Taken together, the changes in these pathways are consistent with and related to pathologies of the liver and demonstrate the potential for microplastics exposure to negatively affect organisms.

Across the dataset featuring all individuals, we found more differentially expressed genes associated with exposure to the high concentration of microplastics (N=92) compared to the low concentration (N=23). In the females, we found 102 genes associated with the high concentration of plastic with associated functional enrichment for the ribosome KEGG pathway, but no significant DE in the low concentration. Contrastingly, there was no significant difference in either concentration in the male minnows. Additionally, our WGCNA analysis across all minnows found modules significantly associated with both the low and high concentrations of microplastics, showing evidence for small perturbations in expression across many genes leading to pathway changes (Table 2). In the low concentration treatments, we saw changes in sequence-specific DNA binding as well as pathways involved in metabolism and the stress response, specifically the PPAR signaling pathway,

which can increase antioxidant gene expression during oxidative stress (Muzio et al. 2021). This serves as additional support for the idea that microplastic exposure leads to perturbations in metabolism and links to stress pathways. For the modules associated with the high concentration treatments, the only pathway significantly enriched was that of the ribosome. This result is somewhat counter to work that found an association between higher concentration of plastics and differential expression in inflammatory and stress genes in hybrid snakehead (*Channa*) (Zhang et al. 2022). However, work in zebrafish also showed slightly more genes differentially expressed in low concentration treatments compared to the high concentration (Limonta et al. 2019). If we observed a larger response to higher concentrations of microplastics across all stages when compared to the lower concentration, this would imply that there would be more severe changes in organisms as microplastic levels rise. However, our results show that microplastics at low and high concentrations correlate with gene expression changes, but the magnitude of the changes in expression do not generally correlate with concentration.

If we observed a marked difference in the relative expression levels between the pristine and environmental treatments, this may indicate that environmental plastic that may contain sorbed contaminants has a different effect on organisms. Interestingly, across all samples we found the greatest number of differentially expressed genes, when compared to the controls, in the minnows exposed to the pristine plastic compared to the those exposed to the environmental plastic. Despite this, there were no significant expression changes when comparing the pristine and environmental treatments to each other. As both of our plastic origin types correlated with similar gene modules, our work suggests that microplastics themselves are a more-impactful stressor on gene expression than the contaminants that sorb to plastics during environmental exposure. Several gene modules associated with the different plastic treatments contained functional enrichment in pathways linked to

metabolism and the stress response. These results are complementary to work done in grass carp (*Ctenopharyngodon idella*) that found subsequent oxidative stress and metabolic perturbations across the transcriptome after microplastics exposure (Liu et al. 2022) and findings from marine medaka (*Oryzias melastigmas*) associating microplastics exposure with liver metabolism perturbation (Ye et al. 2021).

In this study, the responses to the origin of the plastic differed between the sexes. we found the opposite trend in the female-only dataset, where the environmental plastic treatments had a higher overall number of DE genes compared to the genes in the pristine treatments. Again, there was no significant difference in gene expression when comparing the treatments to each other, only the individual treatments compared to the control. In the dataset containing only male minnows, there were no significant differences between the respective treatments and controls, however a gene involved in fatty acid biosynthesis was differentially expressed between the environmental and pristine treatments. These findings further highlight the complexity of the organismal response to microplastics exposure. On the cellular level, the weathering process of microplastics exposed to environmental conditions induces higher cytotoxicity, even though the pristine microplastics were associated with higher inflammatory potential (Völkl et al. 2022), further emphasizing the complexity of microplastics as a pollutant. Additional work with other types of plastics (polystyrene, high-density polyethylene, plastics treated with plasticizers of various environmental risk, etc) as well as aged plastics from other locations and aquatic environments will help elucidate further if and how microplastics act as multiple stressors.

Here, we show microplastics act as a stressor on aquatic organisms at the molecular level via changes in genome-wide gene expression patterns. This builds our mechanistic understanding of the many ways microplastics negatively impact species and compliments previous work showing evidence for microplastic associated oxidative stress and changes in

neurological, endocrine, and immunity (Franzellitti et al. 2019; Mao et al. 2021), decreased survival, increased inflammation, and necrosis (Kögel et al. 2020). Due the myriad of recorded organismal responses to microplastics, microplastics exposure poses a threat to biodiversity that should not be understated. As prior work has suggested a greater effect of exposure to contaminated or environmental plastics on developing fish (Rainieri et al. 2018; Bucci et al. 2022; Tarasco et al. 2022), the potential for long-term negative population consequences of microplastics exposure should not be overlooked. This study sought to offer a broader genomic understanding for the molecular effects of microplastics exposure across the liver transcriptome of an important model organism. To this end, this study successfully increases our knowledge of the molecular response of aquatic species to this anthropogenic pollutant and emphasizes the necessity for better monitoring and management of the negative impacts of this ubiquitous and long-lasting contaminant.

CHAPTER 3: THE EPIGENOMIC AND INTERGENERATIONAL EFFECTS OF MICROPLASTICS EXPOSURE ON THE MODEL TOXICOLOGICAL ORGANISM *PIMEPHALES PROMELAS* (FATHEAD MINNOW)

Introduction

Microplastics (plastic particles <5mm in length) are increasingly recognized as a threat to aquatic biodiversity due to its ubiquity, potential impacts across organisms, and environmental longevity (Reid et al. 2018; Corinaldesi et al. 2021). Microplastics pollution is a unique challenge of the Anthropocene, as large-scale plastic production began in the early 20th century and has since risen to over 350 million metric tons (Mt) annually (Geyer et al. 2017; Napper & Thompson 2020). Of this plastic, an estimated 20 Mt of plastic waste is generated annually (Borrelle et al. 2020), most of it entering aquatic environments (Chen et al. 2020). To date, microplastics have been recorded in marine (Coyle et al. 2020), freshwater (Wagner et al. 2014; Li et al. 2018), atmospheric (Zhang et al. 2020a), and terrestrial (Dissanayake et al. 2022) ecosystems and interacting with the biota therein (Anbumani & Kakkar 2018). Given this pervasiveness, it is important we understand the various effects of microplastic exposure on organisms.

Exposure to microplastics is linked to many physiological and ecological effects in aquatic biota. For example, microplastic ingestion negatively affects feeding activity in lugworms (*Arenicola marina*), leading to weight loss (Besseling et al. 2013). Increased oxidative stress following microplastics exposure has been recorded in the marine copepod *Tigriopus japonicus* (Choi et al. 2020). Multiple instances of uptake, translocation, and genotoxic effects of microplastics have been reported in the marine mussel *Mytilus edulis* (Browne et al. 2008; Moos et al. 2012; Avio et al. 2015). In juvenile Chinese mitten crabs (*Eriocheir sinensis*), exposure to polystyrene microplastics resulted in tissue accumulation

and reduced growth (Yu et al. 2018). Many studies using zebrafish (*Danio rerio*) as a model found myriad changes related to microplastics exposure, from developmental toxicity through negative reproductive effects (Bhagat et al. 2020). The important commercial fish species European seabass (*Dicentrachus labrax*), Atlantic horse mackerel (*Trachurus trachurus*), and Atlantic chub mackerel (*Scomber colias*) not only had microplastics translocated to their gills, dorsal muscle, and gut, but also higher levels of lipid peroxidation consistent with oxidative damage (Barboza et al. 2020). This evidence across a gamut of species illustrates the many ways in which microplastics pollution affects aquatic organisms.

One concerning feature of microplastics is the ability to sorb persistent organic pollutants (POPs) from the environment (Ziccardi et al. 2016; Sleight et al. 2017; Anbumani & Kakkar 2018; Amelia et al. 2021; Santos et al. 2021b), and thereby transmit additional contaminants to organisms interacting with them (Avio et al. 2015; Paul-Pont et al. 2016; Hal et al. 2020). Additionally, prior work has suggested microplastics aggravate the effects of other contaminants when co-exposed (Zhang et al. 2020b). Exposure to microplastics with sorbed chemicals is linked to increased mortality and the reduction of important ecosystem services in lugworms (*Arenicola marina*) (Browne et al. 2013). In marine medaka (*Oryzias melastigma*), exposure to microplastics with sorbed phenanthrene increased bioaccumulation and reproductive toxicity (Li et al. 2022). Zebrafish exposed to microplastics spiked with Benzo[α]pyrene experienced increases in histopathological signs of intestinal inflammation and reduced fecundity (Tarasco et al. 2022). The additional complication of sorbed chemicals highlights the complexity of microplastics interacting with the environment and organisms therein.

Many previous studies focused on physiological and ecological effects, but microplastics also have the power to affect molecular level processes, such as gene expression (Wade *et al.*, in prep) and gene regulation. Looking across levels of molecular

control, such as DNA methylation, will allow the elucidation of the implications of microplastics exposure across levels of biological organization and help understand the mechanism behind phenotypic and population-level effects of microplastics exposure (Vandegehuchte & Janssen 2014). Very few studies to date have investigated the epigenetic effects of microplastics exposure. In male rats, polyethylene microplastic exposure increased DNA methylation in a dose-dependent manner (Farag et al. 2023). Exposure to polyethylene microplastics had no significant effect on global DNA methylation levels in *Daphnia magna* (Song et al. 2022), however individual genes were not analyzed. Chemicals commonly associated with plastics such as Bisphenol-A are associated with decreased global methylation in zebrafish (*Danio rerio*) (Laing et al. 2016), and phthalates have well-documented epigenetic effects (Dutta et al. 2020). Additionally, epigenetic modification not only affects molecular processes in the exposed animal but can also persist through generations (Vandegehuchte et al. 2010b). Studying responses to microplastic exposure in aquatic environments will provide a better understanding of the potential for long-lasting ecotoxicological implications of microplastic exposure.

Some prior work investigated the trans- and intergenerational effects of microplastics exposure in various species. In *Daphnia magna,* parental microplastics exposure was associated with transgenerational effects in growth and reproduction (Song et al. 2022). A cross-generational study in oriental river prawn (*Macrobrachium nipponense*) found paternal exposure to microplastics correlated with decreased survival and reduced immunity-related enzymatic activity in the F1 larvae (Sun et al. 2022). For the acorn barnacle (*Amphibalanus amphitrite*), parental exposure to microplastics increased larval mortality and delayed development in the F1 generation (Yu & Chan 2020). In Pacific oysters (*Crassostrea gigas*), the offspring of parents exposed to microplastics had reduced locomotor activity (Bringer et al. 2022). A 60-day exposure to polystyrene microplastics in a single generation of marine

medaka (*Oryzias melastigma*) led to decreased hatching, heartrate changes, and body length in offspring (Wang et al. 2019). Another study in marine medaka again found heartrate changes as well as slowed growth and premature hatching in the F1 generation following parental exposure to polystyrene microplastics for 150 days (Wang et al. 2021). As these previous studies helped elucidate the presence of important generational impacts due to microplastics exposure, there is a need to investigate the mechanisms behind these alterations. One such method for exploring these mechanisms is through determining heritable changes in DNA methylation levels due to microplastics exposure, an area ripe for investigation. We addressed these knowledge gaps by studying the molecular effects of microplastic ingestion on fathead minnows (*Pimephales promelas*), a fish native to the Laurentian Great Lakes region of North America. Our questions were:

1) Does microplastic exposure cause changes in methylation patterns in adult fathead minnows?
2) Does the effect of microplastic exposure vary between different concentrations and types of microplastic exposure?
3) Are there intergenerational effects of microplastic exposure on methylation patterns?

To address these questions, we raised fish under four treatments: two microplastic concentrations (present and predicted future concentrations) and two types of microplastic [polyethelene sourced from the manufacturer ('pristine') and that collected from the beach of Lake Ontario ('environmental')]. We raised the offspring of minnows exposed to microplastics in control (no microplastic exposure) conditions to determine if there are any persistent, transgenerational effects of microplastic exposure. This approach of evaluating the impacts of microplastic exposure from multiple plastic origins and concentrations and across generations provides a provides crucial insight into the intergenerational effects of

microplastics on freshwater organisms, allows us to tease out the effect of the microplastic itself from sorbed chemicals, and the results from this study help clarify the consequences of developing, living, and reproducing in an environment polluted with microplastics.

Methods

Experimental Design

We used fathead minnows, a toxicological model organism, throughout this experiment to determine the effects of microplastics across life stages. We adapted rearing methods from a previously published fathead minnow life cycle experiment (Parrot 2005). We obtained fathead minnows from the breeding stock of the Ontario Ministry of the Environment, Climate, and Parks (MECP). From eggs through 180 DPH, we raised fish under experimental conditions with four treatments and a negative control (CTRL). The treatments were: 1) a low, environmentally relevant concentration (240 particles/L) of virgin polyethylene (PL) microplastic, 2) high, future scenario concentration (2400 particles/L) of virgin polyethylene (PH), 3) low concentration of polyethylene gathered from Lake Ontario (EL), and 4) a high concentration of polyethylene microplastic originating in Lake Ontario (EH). We used 5 replicates per treatment for a total of 25 aquaria. We reduced microplastic size to a standardized 100-500μm using a burr mill coffee grinder. The experimental design is summarized in Figure 9.

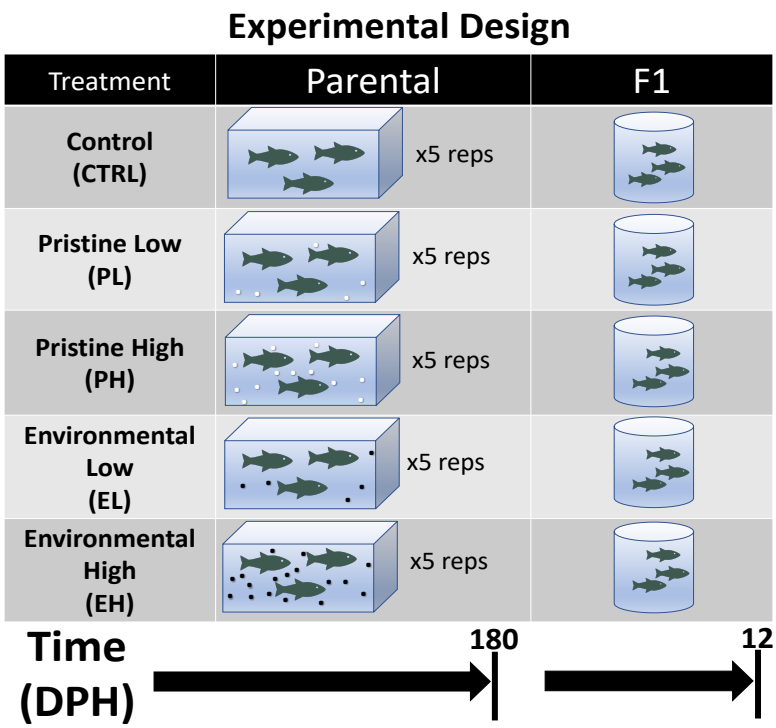

Figure 9. Experimental design. Sampling time points are given below the figure. DPH=days post hatch. Pristine plastics are depicted with open icons in the experimental tanks, environmental plastics collected from the environment are depicted with black dots.

Each treatment received twice daily feedings. The initial diet consisted of newly hatched brine shrimp at a concentration of 15 nauphii/µL until 30 days post hatch (dph), followed by a gradual introduction of frozen brine shrimp into the diet until 50 dph, after which point the diet consisted solely of thawed brine shrimp. Throughout the experiment, we maintained the water bath at 25˚C and lights turned on each day for a total of 16 hours. We completed 30% water changes three times a week and cleaned the aquaria to remove algae buildup, we also tested a random tank from each treatment for dissolved oxygen, pH, and conductivity to determine water quality. Additionally, we checked ammonia levels in a sequential manner for each treatment during cleaning.

We encouraged mating by provided egg tiles in each tank. We obtained clutches of offspring from 23 of the 25 tanks. After the minnows laid eggs on the clay tiles located in

each tank, we removed and counted the eggs. If more than 100 eggs were laid, we set 50 aside for the intergenerational portion of the study where we raised a second generation of minnows to 12 dph under negative control conditions (no microplastics) to isolate the intergenerational effect of parental exposure. We fed newly hatched brine shrimp to the larvae once daily starting on the second day following the hatching of the first individual. A complete water change was done every other day. At the completion of the experiment, we euthanized larvae using MS-222, rinsed them with deionized water, and flash froze all samples. The experiment concluded at 180 dph. For final processing, we euthanized fish using a lethal bath of buffered MS-222 at a concentration ≥ 250 mg/L. We left fish in the bath until two minutes after respiration ceased. We dissected liver tissue from one male and two females from each tank (n=75) and placed the tissue in 95% ethanol. Between fish, we cleaned dissection tools with 10% bleach, molecular-grade deionized water, and 90% ethanol.

Library preparation and quality filtration

We homogenized the liver tissue of adults (n=75) and several larvae from familial clutches (n=21) using a bead mill with steel beads prior to extraction. We used the bead-based method outlined in Ali et al. (2016) to extract and purify genomic DNA. To determine methylation patterns in our samples, we used the NEBNext Enzymatic Methyl-seq kit (#E7120L) for enzymatic conversion of methylated nucleotides, individual barcoding, and library preparation (New England Biolabs, Inc, Ipswich, MA). The samples (n=96) were pooled into a single library and sequenced twice on the Novaseq6000 on one lane of a SP flowcell (PE 2x150) at the RTSF Genomics Core at Michigan State University.

Following sequencing, reads from both sequencing runs were trimmed using *TrimGalore!* (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to remove

adapter-contaminated sequences and the first ten base pairs from the 5' and 3' ends of the fragments. We converted the fathead minnow reference genome (GenBank accession GCA_016745375.1) (Burns et al., 2016 & Saari et al., 2017) using *Bismark* (Krueger & Andrews 2011) and aligned reads to the converted genome using Bowtie2 (Langmead & Salzberg 2012) within *Bismark* with a modified stringency setting of --score_min L,0,-0.6. Alignments were deduplicated from each library using deduplicate_bismark and samples from each sequencing run merged into a single file using SAMtools version 1.16.1 (Danecek et al. 2021). Prior to further analysis, we removed samples from the dataset with less than one million reads. We subsequently extracted methylation calls using bismark_methylation_extractor.

Relatedness determination

To determine relatedness among individuals, we used the merged and sorted bam files to extract SNPs using *BCFtools* mpileup (Danecek et al. 2021) piped to *BCFtools* call (Li 2011). We quality filtered the resulting file using *VCFtools* v0.1.15 (Danecek et al. 2011), initially removing genotypes not represented in at least 50% of individuals and any SNPs with a count less than five. The resulting data was then further filtered to remove genotypes with fewer than three reads, genotypes with a call rate of less than 90% and filtered out SNPs with a minor allele frequency less than 0.05. We removed individuals with a missingness greater than 15% and then calculated pairwise φ among all samples using the relatedness2 estimator. Samples with pairwise φ ranges [0.177, 0.354], were denoted as first-degree relatives, which includes full siblings and parent-offspring pairs. Kinship coefficient ranges [0.0884, 0.177] and [0.0442, 0.0884] were considered 2nd-degree and 3rd-degree relationships, respectively (see https://www.kingrelatedness.com/manual.shtml).

Differential methylation analysis

We used the R packages bsseq (Hansen et al. 2012), dmrseq (Korthauer et al. 2017), DSS (Wu et al. 2013, 2015; Feng et al. 2014; Park & Wu 2016), and MethylSig (Park et al. 2014) to additionally quality filter and determine regions of differential methylation. Briefly, we read in the coverage files from *Bismark* to a BS-seq object, first filtering out any loci with coverage of less than 10 and then filtering out any individuals with an average read count of less than 0.5. Our final filtration step involved filtering out any loci that did not occur in at least 90% of individuals from the parental generation (n= 58) and the F1 generation (n=21). We tested for differentially methylated loci (DMLs) and regions (DMRs) across the variables of sex (female, male), exposure (treated, control), concentration (high, low, control), plastic type (environmental, pristine, control), individual treatments (environmental high, environmental low, pristine high, pristine low, control), and generation (parental, F1). We also investigated the interactions of sex across treatments. The stringency values for a locus/region to be called differentially methylated were a delta of 0.1 and false discovery rate (FDR) of 0.05. We used the NCBI Genome Data Viewer for the *Pimephales promelas* annotation release 100 to search for genes within the DMRs (or DMLs if there were not statistically significant DMRs).

Results

Combined dataset differential methylation analysis

Our libraries averaged >60% genome alignment and >98% cytosine conversion. The average number of merged, aligned, and deduplicated reads per sample was 8,032,241. We ultimately removed 16 individuals from our final dataset due to poor sequencing (<1 million reads). Following quality filtration, the final dataset used in differential methylation analysis consisted of 19,338 methylated loci across 79 individuals. Of these, 21 individuals were from the F1 generation and 58 from the parental. Overall, there were few instances of significant differential methylation across the treatments (four comparisons), plastic types (environmental vs pristine plastic), or plastic concentrations when analyzing both generations of minnows together in a single dataset. The only variable with many differentially methylated loci (DMLs) or regions (DMRs) was between the generations themselves, which is expected as methylation levels differ across developmental stages (Goll & Halpern 2011). Within the parental generation, we found DMLs and DMRs found between the male and female minnows as well as between the environmental and pristine plastics, the high concentration plastic and control, and in five comparisons between treatments (Table 6). Altogether, the total number of DMLs and DMRs were 339 and 19, respectively. Using the NCBI genome browser, we found that many of the loci/regions with differential methylation were in transfer RNAs, uncategorized genes, or regions with no known genes. This was consistent throughout all analyzed datasets. Genes found within DMRs among the parental analyses include basic proline-rich proline like (LOC120473533), 5s ribosomal RNA, (LOC120465807), and fap1 adhesin-like (see Table 6).

Table 6. Summary of differentially methylated loci (DMLs) and differentially methylated regions (DMRs) from DSS analysis with a false discovery rate (FDR) <0.05 and a minimum delta of 0.1.

Dataset	Comparison	DMLs	DMRs	Genes (if known)
All (n=79)	Parental vs F1	17615	892	*Not investigated*
Parental (n=55)	Male vs Female	65	3	*LOC120473533, TRNAG-GCC*
	Environmental vs Pristine plastic	4	--	--
	High vs low concentration	11	1	*TRNAG-GCC*
	Environmental Low (EL) vs Control	4	--	--
	EL vs Pristine high (PH)	158	11	*TRNAA-AGC, LOC120467080, TRNAG-GCC, TRNAP-CGG, LOC120491149, LOC120465807*
	PH vs pristine low (PL)	4	--	--
	EL vs PL	43	1	--
	EL vs environmental high (EH)	50	3	--
Female parental (n=34)	Environmental vs Pristine plastic	1539	73	*LOC120489314, TRNAQ-UUG, TRNAA-AGC, TRNAP-AGG, TRNAQ-CUG, TRNAL-CAG, TRNAM-CAU, TRNAL-UAG, ANKRD3, LOC120475989, TRNAR-ACG, TRNAP-CGG , TRNAT-UGU, GRIA4B, LOC120491149, LOC102465807, NPFFL, TRNAG-UCC, LOC120473156, LOC120473586, LOC120473585, LOC120473590, LOC120473532, LOC120473533, LOC120473555, LOC120475309, LOC120475315, ADARB2, ZMP:0000000936, LOC120481455, LOC120486186*
	Environmental plastic vs control	11	--	*ADARB2, LOC120475832*
	Pristine plastic vs control	84	4	*TRNAL-UAG*

Table 6 (cont'd)

	Low vs control	9	1	--
	EL vs control	538	13	*LOC120489314, ANKRD31, LOC120473589, LOC120473532, LOC120473590, LOC120473554*
	PL vs control	10	2	*TRNAG-GCC, LOC120473156*
	PH vs control	173	15	*TRNAA-AGC, LOC120467080, TRNAG-GCC, LOC120491149*
	EH vs PL	147	13	*LOC120475989, TRNAT-UGU, LOC120473156*
	EH vs PH	473	32	*LOC120472127, TRNAP-CGG, TRNAT-UGU, LOC120491149, LOC120465807, NPFFL, TRNAG-UCC, GPM6BB, ZMP:0000000936, LOC120486186*
	EL vs PH	3020	147	*LOC120486679, LOC120489314, ARHGAP17B, APOL, TRNAA-AGC, TRNAQ-UUG, TRNAP-AGG, TRNAQ-CUG, TRNAL-AAG, TRNAL-UAG, TRNAP-UGG, TRNAP-CGG, LOC120467080, TRNAF-GAA, ARHGEF12B, ANKRD31, PKD1A, TBX4, LOC120480528, SI:CH211-232B12.5, ACSL2, TRNAR-ACG, TRNAE-UUC ,TRNAG-GCC, TRNAE-CUC, TRNAG-CCC, TRNAD-GUC, TRNAG-CCC, TRNAR-CCG, TRNAP-CGG, TRNAP-UGG, TRNAP-AGG, CAVIN4B, TRNAT-UGU, LOC120491149, INO80DB, ZGC:165604, LOC120460519, LOC120461512, MMP14A, LOC120465807, NPFFL, TRNAG-UCC, LOC120473156, LOC120473588, LOC120473533, LOC120473584, LOC120473586, LOC120473589, LOC120473554, LOC120473585, LOC120473590, LOC120473555, LOC120473533, LOC120475315, LOC120475309, LOC120475320, GJB7, ADARB2, GPM6BB, LOC120484212, LOC120484214, LOC120486186*
	EL vs PL	2046	96	*LOC120486679, TPP1, LOC120489314, ARHGAP17B, TRNAQ-UUG, TRNAP-AGG, TRNAA-AGC, TRNAQ-CUG, TRNAL-AAG, TRNAL-UAG, TRNAP-UGG, TRNAP-CGG, LOC120467080, ARHGEF12B, ANKRD31, LOC120469624, LOC120475989, TRNAR-AGC, TRNAG-GCC, TRNAR-CCG, TRNAP-UGG, TRNAP-CGG, TRNAP-AGG, TRNAT-UGU, TRNAA-UGC, ZGC:165604, LOC120461512, TRNAG-UCC, LOC120473156, LOC120473586, LOC120473532, LOC120473554, LOC120473533, LOC120473588, LOC120473585, LOC120473590, LOC120473555, ADARB2, LOC120481455, LOC120486186*

Table 6 (cont'd)

	EL vs EH	231	13	*TRNAA-AGC, ANKRD31, LOC120473554*
Male parental (n=24)	Control vs treated	56	6	*LOC120472127*
	Environmental vs pristine plastic	11	1	--
	Environmental plastic vs control	1	--	--
	Control vs pristine plastic	126	11	*LOC120472127, si:ch211-232b12.5, LOC120465808, LOC120465806, LOC120484212*
	High vs low concentration	415	27	*Trnap-agg, Trnal-uag, Trnaf-gaa, Trnar-ucg, Trnag-gcc, LOC120491149, LOC120465807, LOC120465806, LOC120465808, LOC120473156, LOC120473555*
	Control vs low concentration	395	28	*Trnap-agg, Trnar-ucg, LOC120472127, si:ch211-232b12:5, Trnag-gcc, LOC120488227, LOC120491149, LOC120465806, LOC120465808, LOC120465807, LOC120473533, LOC120473665, LOC120484212*
	EL vs control	397	22	*Trnaf-gaa, LOC120472127, Trnar-ucg, si:ch211-232b12.5, LOC120491149, LOC120465806, LOC120465808, LOC120473533, LOC120473665*
	EH vs control	1	--	--
	PL vs control	354	25	*Trnap-agg, LOC120472127. si:ch211-232b12.5, Trnag-gcc, LOC120488227, LOC120465806, LOC120465808, LOC120465807, LOC120473533, LOC120484212*
	PH vs control	14	1	--
	PH vs PL	67	3	*Trnag-gcc, LOC120465807, LOC120465806,*

Table 6 (cont'd)

	EH vs PL	2644	139	*LOC120489314, LOC120489314, TSPAN2A, LOC120463284, trnaa-agc, trnaq-uug, trnap-agg, trnaq-cug, trnal-aag, trnal-uag, trnap-ugg, trnap-cgg, trnal-cag, trnam-cau, trnal-uag, trnaf-gaa, trnar-ucg, SFTPBB, ARHGEF12B, PKD1A, LOC120459336, LOC120471030, LOC120475989, LOC120481110, trnar-acg, trnae-uuc, trnag-gcc, trnae-cuc, trnag-ccc, trnad-guc, trnap-cgg, trnap-ugg, trnar-ccg, trnap-agg, LOC120488227, LOC120491149, LOC120491149, INO80DB, SHANK2A, LOC120459923, LOC120461512, MMP14A, LOC120465807, LOC120465806, LOC120465808, LOC120468055, trnag-ucc, LOC120473156, LOC120473589, LOC120473532, LOC120473555, LOC120473585, LOC120473590, LOC120473533, LOC120473554, trnar-ccu, LOC120475309, LOC120475315, GPM6BB, LOC120484212, LOC120484213, LOC120484212, LOC120484214*
	EH vs PH	1215	62	LOC120489314, LOC120489968, LOC120463284, trnaq-uug, trnap-agg, trnal-aag, trnal-uag, trnap-ugg, trnap-cgg, trnap-agg, SOUL5L, trnar-ucg, PKD1A, LOC120459336, LOC120475989, LOC120481110, trnar-acg, LOC120486626, SI:CH211-57I17.5, MMP14A, SI:DKEY-172J4.3, NPFFL, trnag-ucc, LOC120473264, LOC120473589, LOC120473532, LOC120473588, LOC120473585, LOC120473590, LOC120473555, LOC120473533, LOC120475309, LOC120475315, GPM6BB, LOC120484212, LOC120484214,
	EL vs PH	14	--	--
	EL vs PL	2	--	--

Table 6 (cont'd)

	EL vs EH	3786	210	LOC120489314, LC120489968, tspan2a, LOC120463284, Trnaq-uug, trnap-agg, trnaa-agc, trnaq-cug, Trnal-aag, trnal-uag, trnap-ugg, trnap-cgg, Trnal-uag, trnap-ugg, trnap-cgg, trnaa-agc, trnal-aag, Trnaf-gaa, soul5l, Trnar-ucg, sftpbb, LOC120459336, LOC120471010, trnas-gcu, LOC120471019, tbx4, acsl2, LOC120481110, Trnar-acg, Trnae-uuc, LOC120486626, Trnag-gcc, Trnar-ccg, trnap-cgg, trnap-ugg, trnap-agg, Trnat-ugu, LOC120488227, LOC120491149, Trnaa-ugc, trnaa-cgc, SI:CH211-57I17.5, MMP14A, TMEM145, LOC120465807, LOC120465806, LOC120465808, LOC120468055, Trnag-ucc, LOC120473264, LOC120473586, LOC120473533, LOC120473588, LOC120473532, LOC120473589, LOC120473554, LOC120473532, LOC120473555, LOC120473585, LOC120473665, Trnar-ccu, LOC120475315, LOC120475309, GJB7, GPM6BB, ZMP:0000000936, LOC120484212, LOC120484213, LOC120484212, LOC120484214, LOC120485500,
F1 juveniles (n=21)	Environmental vs. pristine plastic	12	--	TNFRSF19 (TNF receptor superfamily member 19)
	Environmental plastic vs control	17	--	TNFRSF19 (TNF receptor superfamily member 19)
	Pristine plastic vs control	33	3	trnat-ugu
	High vs low concentration	24	2	--
	High concentration vs control	14	1	--
	Low concentration vs control	59	4	LOC120469624
	EL vs control	109	9	trnaf-gaa, LOC120475658
	EH vs control	39	2	--
	PL vs control	97	6	LOC120481110
	PH vs control	24	2	--

Table 6 (cont'd)

	PH vs PL	96	6	TPP1, LOC120481110, NPFFL
	EH vs PL	93	8	TPP1, TNFRSF19, LOC120468725, LOC120481455
	EH vs PH	24	1	--
	EL vs PH	88	6	PPP1R12A
	EL vs PL	119	9	Tpp1, trnaf-gaa, LOC120481110
	EL vs EH	119	9	trnal-aag, trnal-uag, trnap-ugg, trnap-cgg, trnaf-gaa, LOC120481110, LOC120475658

Parental generation methylation analysis

Within the parental generation, we consistently found DMRs containing genes related to transfer RNAs and ribosomal subunit formation (Table 6). When we separated the parental minnows by sex, there were a total of 8281 DMLs and 409 DMRs found among the females. The variables with the greatest levels of differential methylation in the females revolved around whether the plastic was pristine from the manufacturer or the environmental plastic from Lake Ontario. While the concentration variables did not yield any significant differential methylation in the females, the interaction of the plastic origin and concentration yielded differential methylation between several treatments (Table 6). The comparisons with the greatest number of DMRs were the environmental plastic compared to the pristine plastic (n=73), the environmental low treatment compared to the pristine high treatment (n=147), and the environmental low compared to the pristine low treatment (n=96). These three comparisons shared regions within several genes. One of these is the ankyrin repeat domain 31 (ankrd31) gene, which featured greater methylation in each treatment associated with pristine plastic. Differential expression in ankrd31 is also associated with chemical and drug induced liver injury (Davis et al. 2022) and known to interact with estrogenic-like chemicals associated with plastics (Ali et al. 2014; Lei et al. 2021). Another gene of interest with differential methylation between these comparisons is adenosine deaminase RNA specific B2 (adarb2), which also showed increased methylation in the pristine plastic treatments (average methylation of 0.338, 0.532, and 0.52 for the environmental, pristine and control treatments, respectively; see Figure 10). Adarb2 is known to have DNA methylation changes associated with exposure to estrogenic compounds (Jadhav et al. 2017; Awada et al. 2019). We did not find these two genes, however, to be significantly differentially methylated in the male minnows.

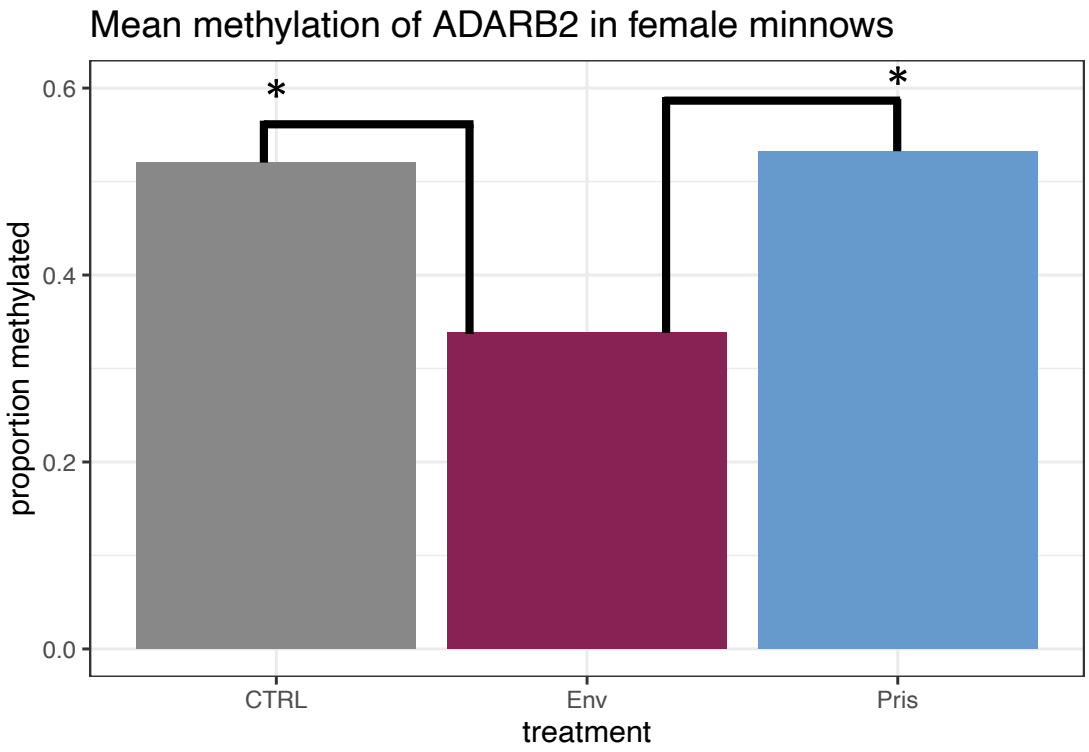

Figure 10. Average methylation levels of ADARB2, a gene involved in RNA editing, among the Pristine (Pris), Environmental (Env) and Control (CTRL) treatments. The female minnows exposed to environmental microplastics had reduced methylation in across this gene. Significantly different methylation donated with asterisks.

Within the male minnows, we found a total of 9498 significant DMLs and 528 significant DMRs. Overall, the male minnows exhibited a greater quantity of differentially methylated loci and regions than the females. Like the females, there was differential methylation attributed to whether the plastic was pristine or environmental, but unlike the females the concentration of the plastic, irrespective of the type, also elicited a difference in the methylation levels (high compared to low concentration, control compared to low concentration; Table 6). The comparisons with the largest number of DMRs were between the environmental high and pristine low (n=139), environmental high and pristine high (n=62) and the environmental low and environmental high (n=210) treatments. There were many DMLs and DMRs spanning genes for transfer RNAs, ribosomal RNAs, uncategorized,

or regions with no known genes. Another gene of interest found within multiple DMRs of analyses is ras-related protein Rab-19 (LOC120472127), which is orthologous to RAB19, a member of the RAS oncogene family and known to interact with estrogenic chemicals associated with plastics (Awada et al. 2019) and is a key component of many cellular processes (Jewett et al. 2021). This gene was found in analyses between the treated (average methylation = 0.489) pristine plastic (average methylation = 0.462), low concentration (average methylation = 0.45), and control (average methylation = 0.719) groups of male minnows as well as in one comparison in the females (Table 6, Figure 11).

Figure 11. Average methylation levels of the ras-related protein Rab-19 gene in the male parental minnows exposed to the control (CTRL), low concentration (Low), pristine plastic (Pris), and across all males exposed to microplastics (treat) treatments. This gene is a key component in intracellular protein transport and autophagosome assembly. All treatments were significantly differentially methylated compared to the control individuals.

Intergenerational methylation effects

Across the F1 generation juveniles, we found significant differentiation in 68 DMRs and 967 DMLs. Outside of transfer RNAs, the only genes with differential methylation shared among the parental minnows were glucocorticoid-induced transcript 1 protein-like (LOC120481455, Figure 12) and src-like-adapter 2 (LOC120469624), both of which were found in comparisons across plastic origin in the females and across the interaction of parental exposure to differing plastic origins and concentrations in the F1 generation. In both generations, there was reduced methylation in the treatments related to environmental plastic compared to the pristine, pre-consumer plastic. Orthologs of both glucocorticoid-induced transcript 1 protein-like and src-like-adapter 2 have shown methylation changes when exposed to chemicals associated with plastics (Jorgensen et al. 2016; Jadhav et al. 2017). Within the F1 analysis, there were also genes with multiple instances of differential methylation across the comparisons. Notably, the genes TNF receptor superfamily member 19 (TNFRSF19) and tripeptidyl peptidase 1 (TPP1) were differentially methylated in several comparisons. Loci within TNFRSR19 were differentially methylated in the environmental plastic treatments compared to both the controls and pristine treatments (average methylation values of 0.241, 0.039, and 0.062, respectively, Figure 13), as well as between the environmental high and pristine low treatments. This gene is related to development and is known to have differential methylation when exposed to estrogenic chemicals (Jadhav et al. 2017). TPP1 was differentially methylated in treatments with interacting concentrations and plastic origins (i.e., the high and low concentrations of pristine, plastic, the low concentrations of both plastic origins, and the environmental high treated parents compared to the pristine low), and again is a known gene that interacts with estrogenic chemicals (Jorgensen et al. 2016; Awada et al. 2019).

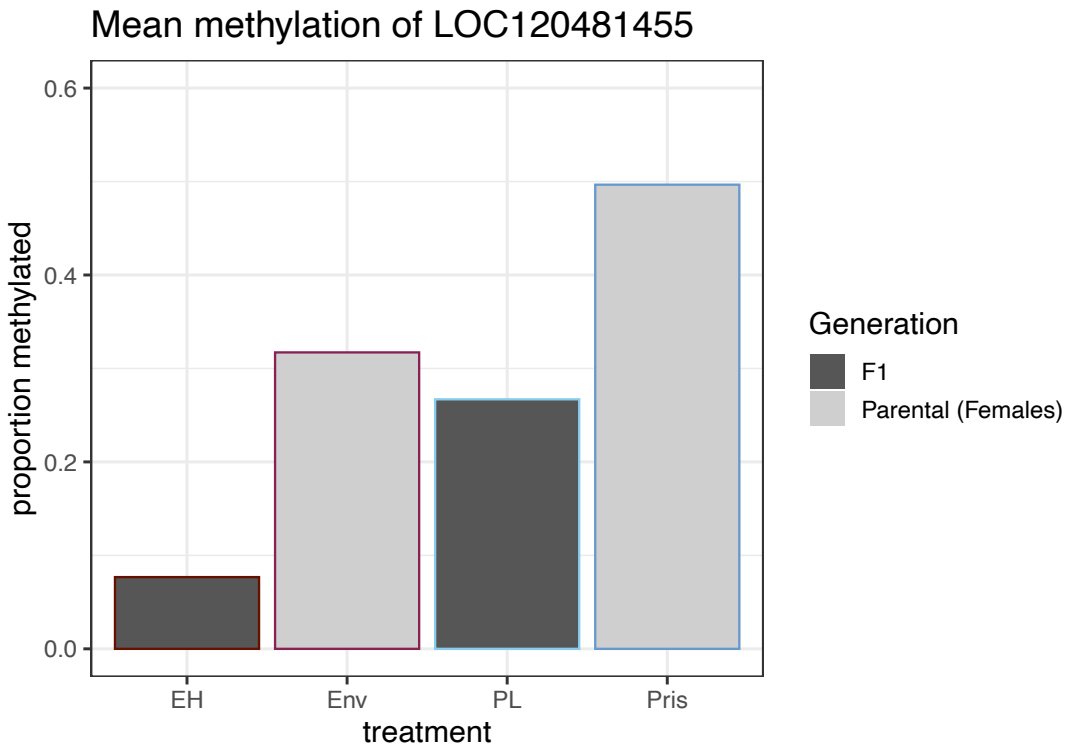

Figure 12. Average methylation levels of glucocorticoid-induced transcript 1 protein-like gene, which may be an early marker for glucocorticoid-induced apoptosis, in the Pristine (Pris) and Environmental (Env) treatments in the parental female minnows and the Pristine Low (PL) and Environmental (EH) treatments of the F1 minnows. Both analyses were significantly differentially methylated. This gene was not within differentially methylated regions in any of the other comparisons in the F1 minnows.

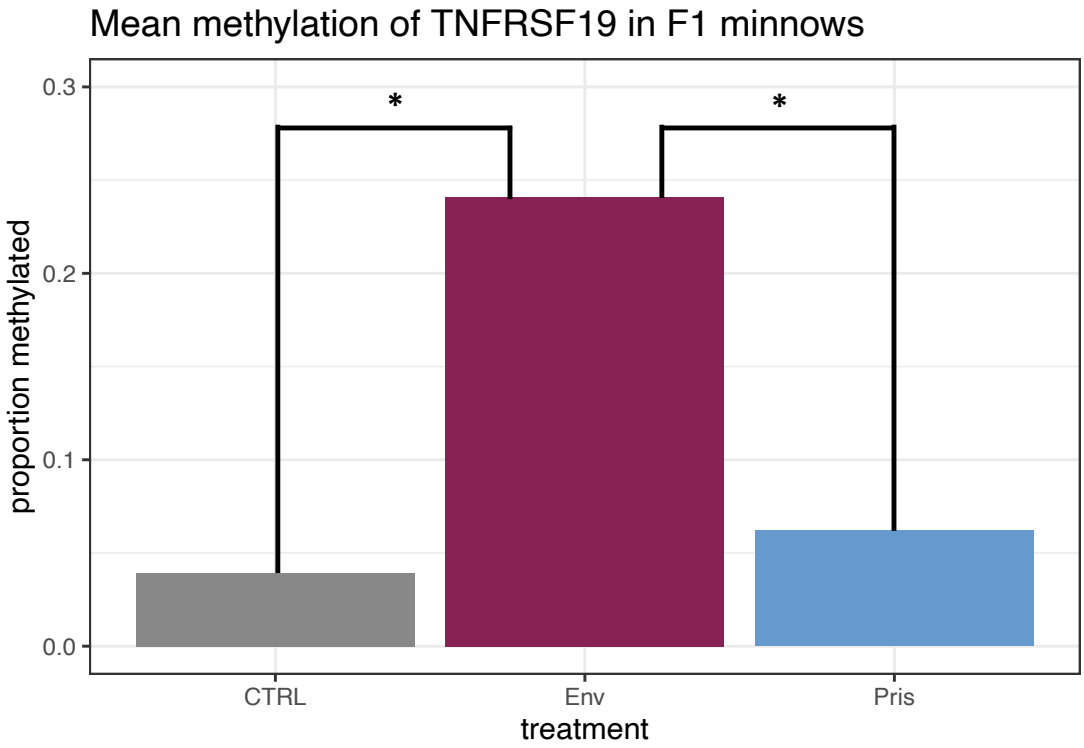

Figure 13. Average methylation levels of the TNF receptor superfamily member 19 (TNFRSF19) gene in the offspring of parental minnows exposed to the control (CTRL), environmental plastic (Env), and pristine plastic (Pris) treatments. This gene plays a role in embryonic development and can induce apoptosis. Significantly differential methylation levels denoted by asterisks.

Relatedness analysis

Following quality filtration, the dataset used to analyze relatedness consisted of 26,463 SNPs across 72 samples. The average missingness among the samples was less than 5%. Unsurprisingly for a lab-bred lineage, all minnows analyzed were highly related to one another. Most of the minnows had pairwise relatedness values consistent with first-degree relatives. The average relatedness among all minnows from both generations was 0.368. We found no evidence of family effects contributing to gene methylation responses.

Discussion

The goal of this study was to increase our understanding of the molecular response to plastic pollution in an important model fish species. Here, we provide evidence for a correlation between microplastics exposure and methylation changes across the sexes, exposure scenarios, and generations of fathead minnows. Overall, we found more differential methylation in the males of the parental generation compared to both the females and F1 generation. Many genes within the differentially methylated regions of both the parental and F1 minnows are related to cellular components and processes (i.e., the suite of ribosome-related genes and transfer RNAs), indicating differences in cellular metabolism related to the exposure to microplastics. The presence of transfer RNAs (tRNA) found differentially methylated in our analysis should not be discounted, however, as there is evidence of methylation-induced tRNA dysregulation in complex diseases such as cancer (Rosselló-Tortella et al. 2022), so the tRNA methylation changes found throughout this study may be related to a negative response to microplastics exposure. It is unclear, however, what kind of gene-regulatory effect the differential methylation of ribosome-related genes such as 5s ribosomal RNA has since methylation may not affect transcription in these genes (Besser et al. 1990).

In the second generation of minnows, the effects across microplastics treatment were largely in regions not shared with the parental minnows, many of which most likely differ due to tissue type and developmental stage. There was, however, a relatively consistent number of DMLs/DMRs across plastic origins and concentrations, however many of these were in areas of the genome with no known genes. However, as the fathead genome continues to be updated and improved upon, these loci and regions may be eventually described. The greater amount of F1 DMLs/DMRs between each of the parental treatments,

however, provides evidence toward a transgenerational additive effect, at least affecting younger life stages. Our study confirming changes in gene methylation provides additional molecular context that complements evidence for changes in enzymatic activity, RNA expression, and histopathology correlated with microplastics exposure (Patra et al. 2022). If the gene methylation levels in the minnows responded differently between the pristine and environmental treatments, this could indicate plastic with sorbed contaminants effects organisms differently than microplastics without additional contaminants. Similarly, a larger response to higher concentrations of microplastics implies greater impacts in organisms as microplastic levels rise. Interestingly, we observed subtle differences in gene methylation levels attributed to changes in plastic concentration or plastic origin (pre-consumer or environmental).

Female minnows experienced the largest magnitude of differentially methylated regions and loci between plastic origin comparisons. We found over a thousand DMLs regardless of plastic concentration, over two thousand DMLs between the environmental and pristine plastics at low concentration, and over 100 DMRs between the environmental low and pristine high treatments in females (Table 6). This finding is complementary to work in developing zebrafish (Tarasco et al. 2022), larval fathead minnows (Bucci et al. 2022), and adult Japanese medaka (*Oryzias latipes*) (Rochman et al. 2013a) where environmental or chemical-spiked microplastics elicit a greater response than pristine plastics. As many of the genes within these differentially methylated loci/regions in the females are known to interact with estrogenic chemicals that plastics are known to leach (Yang et al. 2011; Bittner et al. 2014), the differences in the concentrations of these chemicals between pre-consumer, pristine microplastics and those exposed to the environment likely attribute to this observed difference in relative methylation.

Contrastingly, we found a greater number of DMRs across the plastic concentrations in

the male minnows, indicating males may have a higher sensitivity to microplastics concentration than females, or perhaps that females have a similar response to microplastics regardless of concentration. This result coincides with previous work suggesting a dose-dependent effects of microplastics on rainbow trout *Oncorhynchus mykiss* (Roch et al. 2022), dose-dependent reduction in fecundity following microplastics exposure in Japanese medaka (Zhu et al. 2020), and research suggesting male Chinese mitten crabs (*Eriocheir sinensis*) are increasingly sensitive to microplastics exposure as concentration increases (Sun et al. 2022). However, the latter work was in the testis specifically, whereas our findings are liver-specific, so future work should investigate the methylation changes in gonads and other tissues/organs to fully characterize the differences in sensitivity between males and females. Overall, our findings provide more evidence that microplastics act as multiple stressors, including the response to the physical presence of microplastics themselves and the additive effect of the sorbed chemicals.

As we raised the F1 generation under the same conditions as the control, seeing similar methylation patterns in the F1 minnows across the parental treatment types may implicate chronic effects of microplastics that can persist through generations. Inter- and transgenerational inheritance of methylation patterns may impact gene transcription and thus disrupt many molecular processes (Vandegehuchte et al. 2010a). For example, if these epigenetic methylation changes are in genes involved in the stress response, as we have shown here, this may lead to inherited compensation or tolerance to microplastics with yet-unrecorded physiological and population consequences (Vandegehuchte & Janssen 2014). For the most part, we observed a lesser magnitude of differential methylation in the offspring compared to the parental generation (Table 6). Overall lesser methylation in juveniles is not an unexpected result given previously recorded variations in methylation across development (Goll & Halpern 2011).

Interestingly, the generations shared few genes within DMRs/DMLs. As we compared whole larvae from the F1 generation to liver samples from the parental generation, there are likely more methylation changes found throughout other tissue types in the adult minnows, especially in the gonads, that we were unable to capture or confirm transgenerational effects of with this experimental design. Genes with shared differential methylation throughout the F1 juveniles included transfer RNAs, transmembrane proteins (i.e., LOC120481110) and genes related to inflammation and necrosis (i.e., LOC120469624, TNFRSF19, TPP1), several of which are known to interact with plastic-related chemicals. We found a greater magnitude of differential methylation in the F1 juveniles from the interactions of plastic origins and concentration than from either variable independently. Our gene-level methylation findings expand upon findings of inter- and transgenerational reproductive toxicity but not global methylation changes in *Daphnia magna* following microplastics exposure (Song et al. 2022) and serve as some of the first evidence of DNA methylation changes attributed to microplastics, as most epigenetic work to date has featured nanoplastics (López de las Hazas et al. 2022). Given that fish species such as salmonids develop methylation changes to shifts in their environment associated with domestication (Barreto et al. 2019; Koch et al. 2023), and exposure to toxic hydrogen sulfide results in inherited methylation changes in *Poecilia mexicana* (Kelley et al. 2021), our findings provide evidence that similar epigenetic adaptation attributed to plastic pollution may occur in fish.

Our study shows that exposing even a single generation of fish to microplastics can have long-term and multi-generational methylation effects, even once the microplastic exposure is removed. As altered methylation patterns can indicate rapid, intergenerational adaptation to environmental changes (Vandegehuchte & Janssen 2011, 2014), our findings help anchor the molecular response to microplastics to the potential ecological and

evolutionary implications of DNA methylation. Additionally, this is some of the first epigenetics work outside of nanoplastics and in a fish species native to the Laurentian Great Lakes. In an ecotoxicological context, there remains a knowledge gap in the transmittance of pollutant-induced epigenetic changes and the subsequent ability to induce small evolutionary changes (Morgan et al. 2007). As previous work reveals a correlation between patterns of DNA methylation and genome-wide adaptive divergence (Herrera & Bazaga 2016), studying mechanisms of differential gene expression along physiological timescales such as exposure analyses helps shape hypotheses along evolutionary timescales such as predicting population changes, physiological compensation/tolerance, emigration, or ultimately extirpation (Chevin et al. 2010; Reid & Whitehead 2016; Shama et al. 2016; Garant 2020a), which is necessary in understanding the effects of persistent, chronic pollutants such as microplastics. As the rapid, repeated evolution of tolerance to toxins has been reported in killifish (Reid et al. 2016), linking methylation data with evidence of plastic pollution adaptation or tolerance is a logical direction for future work. This study provides information on the persistent impacts of microplastic pollution that could impact freshwater environments even beyond future restoration efforts, increasing our knowledge of the molecular responses to anthropogenic pollution. Predicting future effects of microplastics pollution using a model organism is an ideal method of identifying the negative, and potentially enduring, effects of plastic pollution and informing plans to mediate the effects of plastic pollution.

CHAPTER 4: ‘OMICS IN THE ANTHROPOCENE: A SMALL SYNTHESIS

Introduction

It is inarguable that humans have had unprecedented impacts on the environment, increasing with the continued industrialization of societies (Crutzen 2002). The joint press conference of the Anthropocene Working Group/Max Planck Society announced the location of the testing for the validity of the Anthropocene hypothesis, wherein we collectively entered a new epoch in the 1950s with the testing of nuclear bombs (see the Anthropocene Working Group, http://quaternary.stratigraphy.org/working-groups/anthropocene/). One of the largest anthropogenic changes have been the creation of greenhouse gasses leading to global climate change (IPCC, 2023), but other changes include land-use changes such as deforestation and large-scale development (see Chapter 1 for an example of land-use change impacting a rare species), or the development of and subsequent release of toxic or damaging chemicals into the environment (Chapters 2 and 3 discuss the effects of microplastics pollution). These changes led to the development of new fields of study, such as conservation biology, a crisis discipline addressing the preservation of biodiversity (Soulé 1985). While still in a scientific age where much of the biodiversity of this planet is still relatively unknown or understudied, conservation biologists are tasked with addressing the effects of anthropogenic change across species, often making recommendations based on incomplete knowledge (due to technology and/or time constraints) and grappling with the fear of failure (Meek et al. 2015).

With industrialization came many technological advances in both computation and biology. One of the most notable advances in biology was in the field of genomics, beginning with the Molecular Clock concept in the 1960s (Zuckerkandl & Pauling 1965), followed by the complete genome sequencing of bacteria (Fleischmann et al. 1995; Fraser

et al. 1995) in the 1990s and the (almost) complete sequencing of worm, fly, and human genomes rounding out the century (Consortium* 1998; Adams et al. 2000; Venter et al. 2001). Since this time, the number of molecular tools available to investigate biodiversity exponentially increased. These new technologies opened the doors for the inference of genomic population structure, calculation of genomic diversity, investigation of the molecular response of organisms to stimuli, and even a new understanding of heritability outside of the nucleotides within a genetic sequence. Altogether, these technological advances ushered in the transition from conservation genetics, where only a few genes or selectively neutral regions are used to resolve questions, to conservation genomics that span the genome of organisms and include insight into responses to selective pressures (Primmer 2009; Frankham 2009).

Incorporating genomic molecular tools into conservation biology allows for the investigation of questions along evolutionary timescales, such as if a population contains the genetic diversity necessary for potential adaptation to new or changing environmental variables (Garant 2020b). Genomics facilitates a better understanding of contemporary population sizes through the estimation of the effective population size (Ne) as well as a view of the past demography of a population, such as history of bottlenecks, selective events, or even the relatedness of individuals. Understanding this genomic history and contemporary standing of a population allows for stronger prediction of its persistence through a selection event or if the negative effects of lack of gene flow, inbreeding depression, or genetic drift may lead to maladaptation or eventual extirpation. This can largely be inferred through measurements of allelic richness, the proportion of alleles private to a single population, relatedness and/or inbreeding, heterozygosity, and Ne.

Synthesis

The first chapter of this dissertation illustrates an example of these metrics being used for inferences in rare species conservation in the face of land-use change. Using these and other metrics, we can infer the necessity of evolutionary or genetic rescue or delineate key populations to serve as conservation units for the preservation of biodiversity (Funk et al. 2012; Whiteley et al. 2015). For example, my work demonstrated that the population of Mojave milkweed within the solar facility contained greater diversity that the surrounding populations. This means the extirpation of that population would have a more detrimental impact on the biodiversity of the Ivanpah valley Mojave milkweed compared to the loss of the other populations found in the study. This population genomic knowledge of the milkweed shows that the construction and ongoing presence of the facility could have long-lasting consequences on the desert ecosystem if the biodiversity is not properly considered. As anthropogenic change persists, the reality of many populations of species is that they will be exposed to new environments and selective pressures at unprecedented, rapid timescales.

Molecular tools are also useful in determining the responses of individuals and/or populations to contemporary stressors along physiological timescales. A popular and often-used method for this is transcriptomics, which can now be applied more easily to wild as well as laboratory populations (Alvarez et al. 2015). Transcriptomics are especially useful in linking a particular phenotype to its molecular mechanism as we can measure the changes in expression an organism experiences at a given point in time. Importantly, the broad range of data developed from the transcriptomic approach reveals unpredicted responses, which expands our knowledge of stressors' effects across organisms (Reid & Whitehead 2016). The second chapter of this dissertation exemplifies transcriptomics revealing as-yet

unrecorded responses to microplastics pollution, and that these changes differ in organisms with different sexes. As fields such as predictive ecotoxicology continue to expand, it becomes easier to link transcriptomic responses to their phenotypic results, which will help elucidate both the phenotype and mechanism undergoing selective pressure. We are increasingly able to make these inferences through the development of gene ontologies, where groups of enriched genes are strongly correlated with differences in molecular functions, cellular components, or even biological processes. This allows for a more functional assessment of molecular data and allows the scaling of the phenotypic consequences of transcriptomic findings upwards through the levels of biological organization. Thus, I was able to take suites of individual genes with differential expression and relate them to physiological processes that may have ecological impacts.

An additional, notable physiological response is DNA methylation. Genome-wide methylation changes in response to stressors alter gene function, although there are many other effects that are largely unknown or understudied, especially across non-model species. In my work in Chapter 3, I show that microplastics exposure has a marked effect on gene methylation levels at both the level of the individual nucleotide and across genomic ranges. Importantly, observations of heritable DNA methylation changes contribute to the study of epigenomics, heritable changes in gene function that do not rely on DNA sequence alterations (Vandegehuchte & Janssen 2011). In my work, I illustrated that some DNA methylation changes attributed to microplastics exposure affect the next generation of organisms, even when that generation was not directly exposed to microplastics. It is by this feature that genome-wide DNA methylation is both a response at physiological timescales and may result in population-wide methylation changes that affect species along evolutionary timescales. However, as methylation reversal is possible, this mechanism can complicate predications in conservation.

Discussion

As the scientific field of conservation biology is often under experience, time, study viability, and monetary constraints, the method of ‘omics used in addressing conservation challenges requires consideration. Out of genomics, transcriptomics, and epigenomics, the most widely accessible method is genomics, especially when studying entire populations. The largest number of samples can be reliably sequenced at one time under genomic protocols, especially those that feature reduced representation. Genomics methods are also easily done under non-destructive sampling schemes, which is especially important when considering that the loss of individuals, in small populations especially, can have negative consequences. As stated above, genomics methods are ideal in analyses concerning the current diversity of a population. Another key feature of genomics studies is that are equally possible for organisms that do not have a reference genome available as they are for organisms with more developed genomic resources. Transcriptomic and epigenomic methods are more resource-intensive on all fronts so are not applied as widely in conservation questions. For most transcriptomic questions, scientifically rigorous experimental design requires multiple samples as well as replicates, which can be challenging to replicate outside of laboratory conditions. The complexity of isolating a response specific to a certain variable cannot be understated and requires careful consideration. Furthermore, a high-quality genome, or ideally a transcriptome, for the species is necessary for the best data analysis in these studies. DNA methylation research requires more complicated laboratory preparation and is also most useful when used with excellent genome resources. However, one of the largest caveats of DNA methylation is the lack of information of the regulatory effects in most species. Determining the full scale of the gene regulation triggered by methylation is an active field of study and should provide a

more complete picture within another decade.

Perhaps the most exciting feature of incorporating these various ‘omics into conservation questions is the potential for the comprehensive view. For example, after understanding the existing variation within populations using genomics, transcriptomics of those populations along altitudinal gradients could reveal differential gene expression in consistent genes or suites of genes. Following that, running a DNA methylation assay may reveal the actual mechanism behind the expression changes in the form of differential methylation along gene or regulatory regions. Forward-thinking questions are also easily developed in this framework, as linking expression changes to heritable methylation allows for hypotheses along the evolutionary trajectory of a population, whether by epigenomic changes or the more traditional changes in the genome itself. Humans have come far in both altering our environment as well as developing the technology to understand and mitigate these changes. As the complexity of the effects of anthropogenic change increase, the questions and hypotheses correspondingly increase in intricacy. ‘Omics remains along the frontier in biodiversity conservation. It will be exciting to see what new technologies develop or become accessible as technology continues to advance and provides a bright spot in an otherwise murky future on Earth.

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APPENDIX

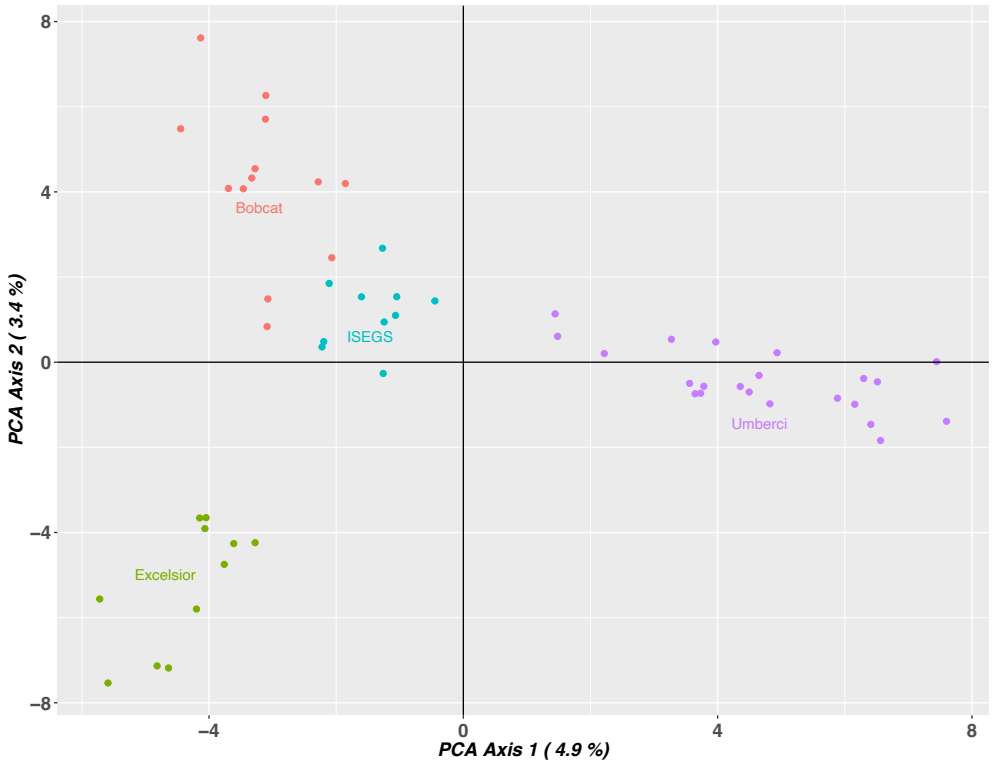

Figure 14. Principal components analysis of the Mojave Milkweed samples collected in 2015.

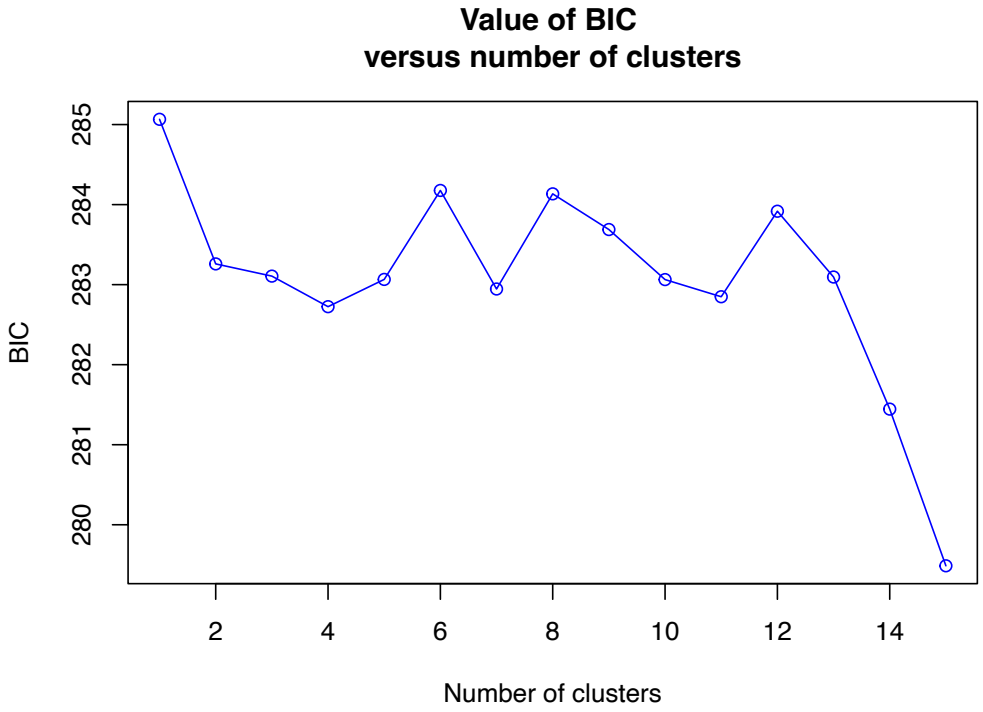

Figure 15. Value of Bayesian information criterion (BIC) as a function of k-means clustering in adegenet. Data shows similar values for 2 to 5 clusters.

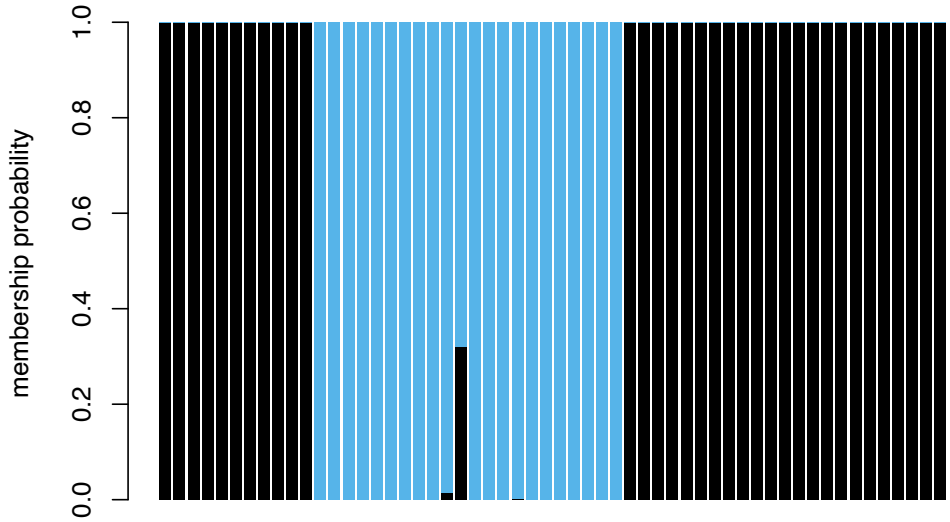

Figure 16. Structure-like plot from the discriminant analysis of principal components (DAPC) showing the membership probabilities at k=2.

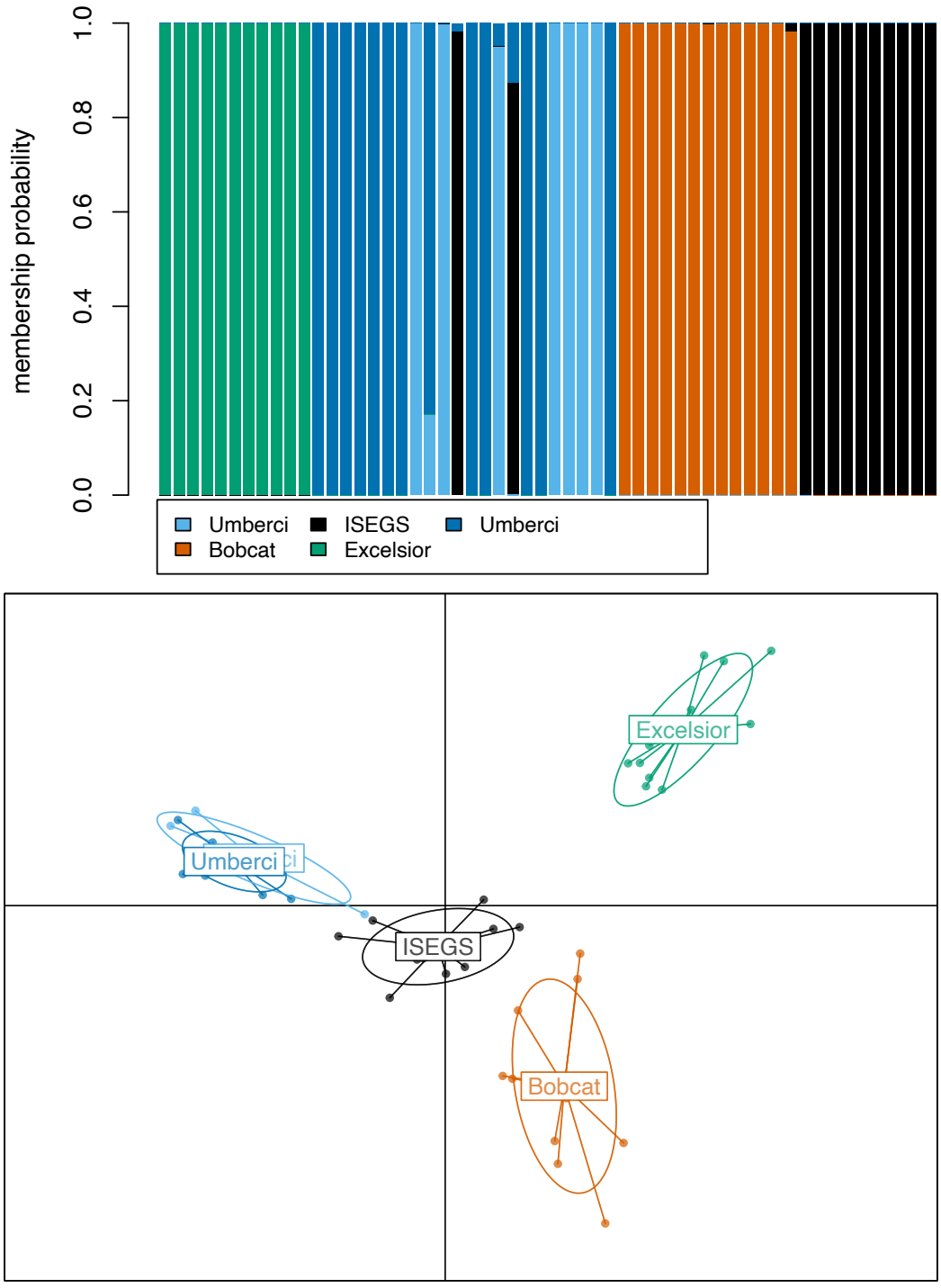

Figure 17. Structure-like (upper plot) and scatter plot (lower plot) from discriminant analysis of principal components (DAPC) analysis of k=5.

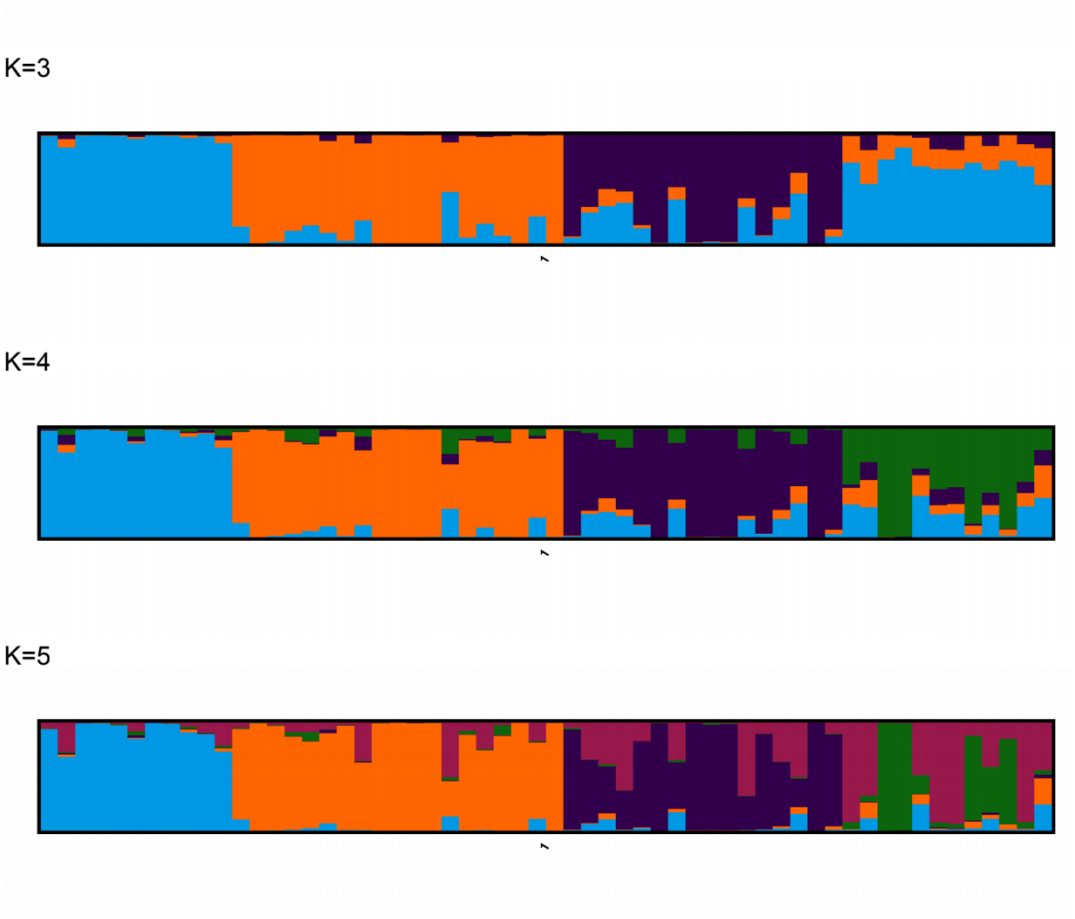

Figure 18. Output from STRUCTURE Harvester showing K=3-5.