NSF Graduate Research Proposal: Impacts of heavy metal contamination on the genetic diversity of Carolina wrens

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Introduction

Heavy metal contamination is one of the major environmental and public health concerns today, with a combined worldwide economic impact of more than US $10 billion annually (He et al. 2015). Heavy metals occur naturally in the Earth's crust, but most of the pollution is caused by anthropogenic sources such as mining, smelting, fossil fuel combustion and agricultural runoff (Ali et al. 2019; He et al. 2015). Once leached into the local waterways and soil, these metals are converted into their toxic forms which are highly persistent and capable of bioaccumulating, and sometimes biomagnifying, within the food chains (Duruibe et al. 2007). Some of the most toxic heavy metals include arsenic, cadmium, chromium, lead, and mercury which are known to have carcinogenic and neurotoxic effects on humans and the wildlife. These metal ions interact with cell components such as DNA and nuclear protein, and induce the formation of reactive oxygen species (ROS), causing DNA damage and conformational changes that initiate carcinogenic processes (Tchounwou et al. 2012). Due to these known physiological effects in certain individuals and species, studies have largely focused on the acute and short-term impacts of heavy metal pollution.

However, less is known about the evolutionary impacts of chronic metal exposure on population genetic diversity, despite 'genetic diversity' being one of three main pillars of biodiversity (Van Straalen and Timmermans 2002). Large populations with high levels of genetic variation can effectively cope with novel selection pressures (Frankham et al. 2010). But chronic exposure to pollution may decrease genetic diversity of populations which diminished their evolutionary potential (Van Straalen and Timmermans 2002). At a population level, metal contaminants can alter the genetic composition by increasing mutation rates, causing bottleneck events, enhancing directional selection on tolerant genotypes, and altering migrations (Bickham et al. 2000). Among the limited studies that have examined these effects, the results have rather been inconclusive and contrasting; species at historically contaminated sites have shown both an increase and a decrease in genetic diversity. For example, a study on tree sparrows (*Passer montanus*) near a historical metal smelting facility in China showed a decreased genetic diversity on its microsatellite loci (Yang et al. 2020) while a different study on great tit (*Parus major*) near a copper smelting site had a significantly higher nucleotide diversity in comparison to unpolluted sites (Eeva et al. 2006). In contrast, some studies have found no differences in the genetic diversity between populations at contaminated and uncontaminated sites, suggesting a possible influence of external factors like migration, gene flow and detoxification abilities on genetic diversity (Giska et al. 2005; Lagisz et al. 2010).

These results are also perhaps attributed to the varying response and effects of toxicants on neutral and selective markers (Fig 1). Selective markers that are closely related to protective biochemical mechanisms are directly responsive to the selective regime of the toxicant while neutral markers are indifferent to the selection pressure of a

specific toxic environment (Van Straalen and Timmermans 2002). Genetic variation may decrease if one of the variants of a selective marker is more tolerant to the toxicant and the selective pressure is directional and strong, causing homozygous genotypes that have a low tolerance to be eliminated. On the other hand, genetic variation may increase if the toxicants act on mutagenic neutral markers like non-coding DNA and increase its mutation rate (Giska et al. 2005). If mutations occur in coding regions, they may affect fitness, and selection can act on them. Furthermore, if the population size is small, then genetic variations in both neutral and selectable markers are susceptible to genetic drift ; drift effect may be reinforced by toxicants if these cause a long-term reduction or fragmentation of populations (Van Straalen and Timmermans 2002). Thus environmental toxicants can affect genetic variations through complex direct and indirect mechanisms, and an integrative perspective is necessary to understand these impacts. To date, only a handful of studies have utilized genome-wide polymorphism data to investigate the genetic and evolutionary impacts of metal pollution (Giska et al. 2015); most studies have only used small numbers of molecular markers which significantly limits the ability to detect population genetic processes that occur across the whole genome. In this study, I aim to provide a holistic insight into the genomic response of wild populations to metal contaminants by taking a genome-wide approach in the DNA sequencing.

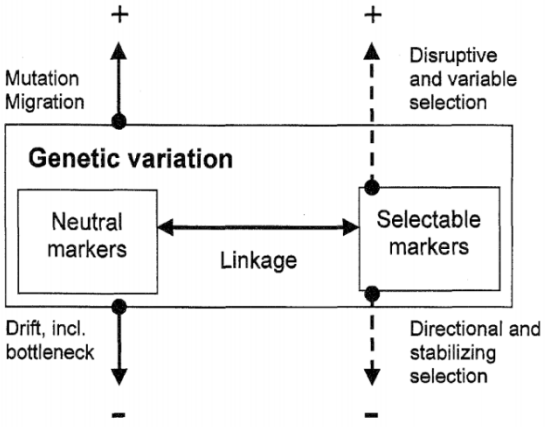

Fig 1. A conceptual framework for effects of toxicants on genetic variation in natural populations. A distinction is made between neutral and selectable markers. On both types of markers, factors operate that may increase (+) or decrease (-) genetic variation. Factors related to population size (drift and bottlenecks) will affect neutral and selectable markers indiscriminately. Mutation and immigration will also affect both types of markers. Selection acts only on selectable markers and different types of selection may increase or decrease genetic variation. Neutral markers may still respond to selection if they are linked to selectable markers.

(Van Straalen and Timmermans 2002)

Objective and Hypothesis:

My overall goal is to investigate whether heavy metal pollution affects the genetic diversity of terrestrial wildlife, and if so, whether it increases or decreases. In order to examine this, I will compare the genetic variability of Carolina wrens (*Thryothorus ludovicianus*) between historically contaminated sites and uncontaminated sites. Carolina wrens is a non-migratory terrestrial songbird commonly found in the eastern United States. They primarily feed on soil-dwelling insects which exposes them to elevated levels of metal accumulation. Both the male and female remain on their territories year-round as breeding pairs, making them ideal candidates for indicators of contaminants on a small geographical scale. Further, Jackson et al. (2011) reported a 34% reduction in the nesting success of Carolina wrens near a forested floodplain of mercury-contaminated rivers in Virginia, USA, suggesting that heavy metal pollution has caused great environmental stress on resident populations which may alter their population genetic characteristics.

I hypothesize that the genetic diversity of Carolina wrens at contaminated sites and uncontaminated sites will be *different* i.e. population genetic responses to metal exposure will affect genetic diversity in terrestrial songbirds. Since I assume minimal gene flow from migration due to their year-long residency, I expect that the direct impacts of heavy metals on gene alteration will overshadow the effects of other external factors that may exchange genotypes between different environments. The population at contaminated sites may respond with *increased genetic diversity* as a result of new mutation directly induced by metal toxicants on non-coding DNA sequences that tend to have higher mutation rates (Van Straalen and Timmermans 2002). In contrast, we may observe a *decreased genetic variation* (genetic erosion) due to population bottleneck or directional selection as a result of strong selection for tolerant genotypes. In either case, it is highly likely that changes in the genetic makeup result as a consequence of adaptation to metal contaminants.

Research Plan

My research is a combination of field collections and laboratory analysis. For my fieldwork, I will select multiple historically contaminated Superfund sites listed under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) environmental law and locate corresponding uncontaminated reference sites (>50 km away from contaminated sites). If I observe a consistent pattern in genetic diversity between all contaminated sites and reference sites, it is more likely that the metal contamination is causing the observed patterns as opposed to stochastic natural processes like migration and outbreak of diseases (Bickham et al. 2000). Multiple populations will be studied from each site to investigate a gradient effect such as downstream from a contaminant source. At each site, I will collect feather samples of Carolina wrens using feeder trap and mist-net using acoustic lures for heavy metal and genetic analysis. Feathers are the most commonly used non-invasive sampling matrix and are highly reflective of their dietary metal intake and chronic body burden (Condon and Cristol 2009). Feather metal levels from each site will be correlated with the observed genetic effects. I will obtain all relevant permits from the state and the USGS Bird Banding Lab where each bird will be equipped with a unique U.S Fish and Wildlife Service band and released unharmed.

The laboratory component will comprise two parts:

(i) Measurement of heavy metal levels: Cleaned feathers will be oven-dried and acid digested for measuring arsenic, cadmium, lead, zinc and chromium using ICP-MS while mercury will be measured using the Tekran 2600 Mercury Analyzer following EPA Method 1631. The data will be used to observe the correlation between metal levels and genetic diversity. These analyses will be conducted at the W.M. Keck Collaboratory for Plasma Spectrometry at Oregon State University.

(ii) Genetic diversity analysis: Using a purification kit, I will extract genomic DNA from each feather sample. Library preparation and restricted site Associated DNA (RAD) sequencing will be performed on Illumina HiSeq 2000, as suggested by other studies (Fischer et al. 2017; Giska et al. 2015). As for the mitochondrial ATP synthase 6 sequencing, PCR reactions will be performed and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit. I will then perform statistical analysis using Stacks for RAD sequencing and DnaSP and Arlequin 3.5 for mitochondrial mtATP6 which will

help me compare the number of private alleles, number of haplotypes, haplotype diversity, nucleotide diversity, number of polymorphic sites and measure of population differentiation (Giska et al. 2015) between contaminated and uncontaminated sites. The Mantel test will reveal whether the genetic differentiation between populations is correlated with geographical distance (isolation by distance hypothesis), and the extent of influence from the contaminants. Lastly, I will use the Bayes factor to describe the effect of metal pollution on the allele frequency of each SNP. These analyses will be performed at the Centre for Genome Research and Biocomputing at Oregon State University under the mentorship of Prof. William Baird, Prof. Susanne Brander and her team at the Brander Lab who have extensively worked on evolutionary ecotoxicology. Below is the anticipated cost for the project:

Research Expense	**Cost (USD)**
Field expense (travel, food, birding equipment)	8,000
Heavy metal laboratory analysis	500
Genome sequencing and genetic analysis	6,000
Research stipend and mentorship program	28,000
Grand total	42,500

Table 1: Expected budget for research

Anticipated results

If I observe a *significant difference between genetic diversity* in all contaminated and uncontaminated sites, in addition to a strong correlation between metal levels and genetic diversity, then the results would suggest that the toxicants are impacting the genetic variability in Carolina wrens. If the Mantel's test shows that the genetic differentiation between populations is significantly correlated with geographical distance, then we can further infer that heavy metals have a strong influence on the genetic differentiation of Carolina wrens population. *Lower genetic diversity* in contaminated sites would support the 'genetic erosion' hypothesis while a *higher genetic diversity* would likely be an indication of mutations from oxidative stress. The consequences of increased mutation are often deleterious as it decreases fitness especially in a small population where mutation rates are accelerated. This case was observed in barn swallow *Hirundo rustica* following the nuclear Chernobyl accident where their mutation rate in microsatellite loci significantly increased by a factor greater than 2-3 per locus (Ellegren et al. 1997). Ellegren et al (1997) also showed that the average fitness was lower and the breeding population size smaller after the accident than in the control regions. Unless compensated for by higher reproduction or immigration (Bickham et al. 2000), the increased mutation rate in combination with population bottleneck and subsequent fixation of deleterious alleles could threaten fitness and eventually lead to extinction. Thus, understanding the impacts of chronic metal exposure on wild populations should be of important concern in biodiversity conservation.

In contrast, my hypothesis on decreased genetic diversity from chronic metal exposure may not be supported if I observe no difference in genetic variability between contaminated and uncontaminated sites. Although I have attempted to reduce the effects of migration, gene flow between local populations may still counteract the effects of metal pollution on genetic diversity. A study by Costa et al. 2013 on soil invertebrates with low dispersal capacity still showed that gene flow masked the genotoxic effect of heavy metals. Furthermore, if other factors such as habitat fragmentation or small population size do not strongly co-occur with metal pollution to cause genetic isolation, then we may not observe a strong genetic reaction to environmental toxicants. Thus, many ecological factors influence the effects of chronic metal exposure on population genetic diversity, which makes it harder to detect the exact impacts of such pollutants. However, it is still important to strengthen pollution regulations and protect populations from the possible loss of genetic diversity.

Broader Impacts

The research will shed light on the evolutionary response of terrestrial wildlife to human-mediated environmental contamination, bridging the gaps between evolutionary biology and ecotoxicology. This interdisciplinary approach of the project will be beneficial to different members of the scientific community including evolutionary and molecular biologists, ecotoxicologists and chemists as well as the general public. Results from this study will be aimed for a publication in both a peer-reviewed scientific journal and as well as scientific articles which will be accessible to a wide range of audiences. This information will help local communities be aware of the environmental issues around them. Furthermore, the data will not only benefit wildlife conservationists and ecotoxicologists but also the public health sectors for risk assessment in human health. It will also serve as useful data for policy-makers in framing laws surrounding biodiversity conservation and environmental pollution.

As an advocate for diversity, equity and inclusion in the field of STEM, my research team will incorporate undergraduate and graduate students from underrepresented groups. I will provide opportunities to engage in both laboratory and fieldwork to minority students and to those who may have had limited access to advanced scientific resources. The proposed budget includes summer stipend and mentorship programs allocated for undergraduate and graduate research interns to help develop their passion for evolutionary biology and ecotoxicology. I will use my previous laboratory experiences in mentoring my teammates. During the recruitment phase, I will contact the STEM Leaders Program (Centre for Diversity and Inclusion) at Oregon State University and advertise the opportunities among underrepresented communities on campus. At the end of the project, I plan to present our findings at the 2025 SACNAS-The National Diversity in STEM conference.

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