2023

Telomeres: a Tool to Assess the Impacts of Mining Contaminants on Riparian Songbirds

Lillian Krach
lk149060@umconnect.umt.edu

Bridger Creel
bridger.creel@umconnect.umt.edu

Megan Fylling
megan.fylling@mso.umt.edu

Zac Cheviron
zac.cheviron@mso.umt.edu

Creagh Breuner
creagh.breuner@umontana.edu

Telomeres: a Tool to Assess the Impacts of Mining Contaminants on Riparian Songbirds

Lillian Krach

The University of Montana—The Breuner Lab

Abstract:

Mining has left massive environmental and physical scars across the landscape. Aquatic and riparian landscapes in particular have been significantly impacted by traditional mining practices. Waste products left over from hard-rock mining leech heavy metals onto the landscape and these metals spread from headwater streams to major waterways (Lottermoser 2010). Heavy metals have been shown to cause physiological stress and challenges to organisms depending on the metal and the concentration (Baos et al. 2019, Boyd & Rajakaruna 2013). While some mining-impaired areas have undergone restoration efforts, is it enough? Typical restoration methods replace the contaminated floodplain, but not the riverbed itself (Geum Environmental 2015). This raises the concern that heavy metal contaminants from the riverbed are still transferring to riparian organisms through the interconnected food webs. In an effort to assess the risk posed by mining contamination and the success of restoration in these mining-impaired areas, we have collected blood samples from six riparian songbird species to measure heavy metal concentration and relative telomere length (the size of the ends of the chromosomes that protect against replication damage) across riparian sites with different levels of contamination and remediation. Telomere length reflects long-term physiological stress and is considered a biomarker for life span in avian species (Powolny et al. 2020, Wilbourn et al. 2018). Riparian songbirds are an ideal group to assess the impacts of heavy metal contamination in riparian ecosystems because they consume both aquatic and terrestrial insects, putting them at significant risk of heavy metal contamination (Walters et al. 2008, Baxter et al. 2005) and they are considered indicators of ecosystem health. Thus, telomeres are informative of the integrated organismal impacts mining contamination has on songbirds. We found that songbirds at a non-contaminated reference site had significantly longer telomeres than songbirds at two remediated sites, indicating there may be some limitations to ecosystem recovery following remediation. In addition, while the site level pattern of change was consistent across species, we found consistent species differences in telomere length. We also found a relationship between a qualitative measure of fat content and relative telomere length. The results of this study provide

broad inference as to the efficacy of current riparian ecosystem restoration of mining contaminated areas and have implications for the adaptive management of songbirds, a group in overall decline.

Introduction:

Across the North American West, mining has left dramatic environmental impacts on the landscape, especially in riparian areas. Mining is deeply rooted in American history, and its environmental ramifications significantly impact ecosystems today. When valuable metals, such as gold and copper, are mined, massive volumes of waste rock are removed from the earth in the process. This waste rock is left on the landscape once the valuable ores are extracted, allowing contaminants to enter the landscape that would not otherwise. A major concern is these contaminants entering the headwaters of many major waterways at which point river flow and flood events can lead to high levels of contamination in riparian habitat (i.e. the river bed and floodplain) (Lottermoser 2010). Heavy metals and metalloids (hereafter referred to as "metals") are the primary contaminants of historic mining practices (Lottermoser 2010), and can include selenium, zinc, arsenic, lead, cadmium, copper, and mercury. These elevated metals can accumulate in the environment and significantly impact ecosystem health. While many of these metals are necessary for life to exist, high concentrations can lead to severe physiological consequences (Baos et al. 2019, Boyd & Rajakaruna 2013). Considering these wide-ranging impacts of metal contamination, it is vital to understand how metal exposure impacts riparian environments and their occupants.

Contamination of watersheds and riparian ecosystems has encouraged a wave of contemporary restoration in polluted areas. In many cases, the contaminated floodplain soils are replaced, but the riverbed itself is left untouched and unchanged. Despite these terrestrial restoration efforts, heavy metals can still bioaccumulate in the riparian food web from aquatic sources (Alberts et al. 2013) (Figure 1). Contaminants can transfer from the aquatic environment to insectivores in the riparian food web because the contaminated riverbed produces a large proportion of the food sources for terrestrial organisms (e.g. emergent macroinvertebrates). Thus, despite the restoration of the floodplain, terrestrial organisms may still be significantly impacted by metal exposure via trophic transfer from the aquatic food web. Riparian obligate songbirds are one of these groups of organisms at considerable risk for dietary metal exposure from their

environment through their primary food source—emergent aquatic macroinvertebrates (Baxter et al. 2005, Walters et al. 2008). Riparian songbirds are indicators of the health of their environment—and because of their exposure to heavy metals via ingestion of contaminated macroinvertebrates—songbirds are an ideal species to assess the physiological and greater ecosystem impacts of heavy metal mining contamination.

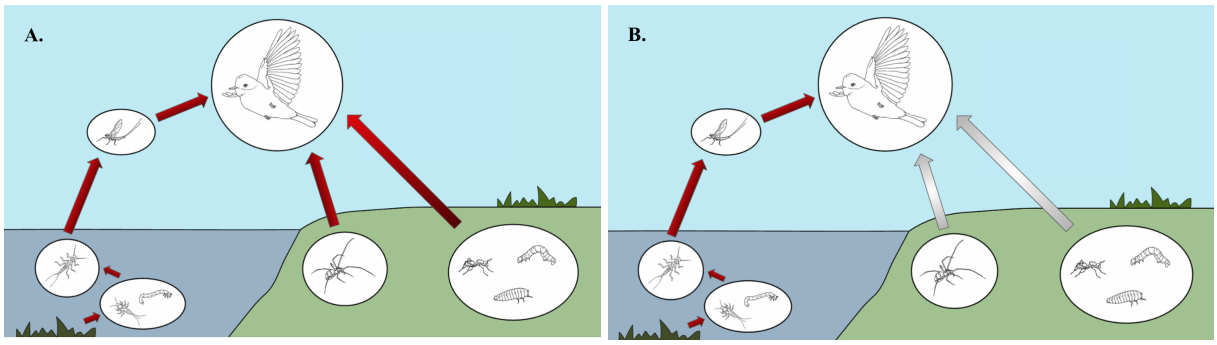

Figure 1: Riparian food web diagrams in which red arrows represent metal transfer alongside energy transfer and gray arrows represent energy transfer without metal transfer. A) Metal transfer from the aquatic and terrestrial ecosystem to songbirds via their diet in a riparian ecosystem with a metal-contaminated riverbed and floodplain. B) Metal transfer from the aquatic ecosystem to songbirds via their diet in a riparian ecosystem with a metal-contaminated riverbed and restored floodplain.

Metal exposure has been well evidenced to cause organismal stress (Baos et al. 2019, Boyd & Rajakaruna 2013). While an organismal stress response is a vital factor in immediate organismal survival, chronic stress, can have negative impacts on an organism's overall fitness. Chronic stress can lead to a decrease in growth, reproduction, and overall fitness by allocating an organism's resources to biological processes that promote immediate survival, instead of long-term fitness (Breuner et al. 2008, Herborn et al. 2014). Heavy metal accumulation on riparian landscapes is thus of primary concern for causing chronic stress to organisms in the riparian ecosystem.

Chronic stress can have negative effects across bodily systems and the signature of stress can be measured by using various physiological biomarkers. One such biomarker is relative telomere length. Telomeres are repeating genetic elements at the end of chromosomes that protect against degradation during the replication process. Telomeres naturally shorten due to the

cellular “end replication problem” (Wilbourn et al. 2018). However, telomeres can also shorten prematurely due to both external (e.g., habitat quality; Grunst et al. 2020) and internal (e.g., elevated corticosterone; Pegan et al. 2019) stressors. In addition, telomere length is directly linked to lifespan, especially in mammals and birds (Powolny et al. 2020, Wilbourn et al. 2018). Within a species, shorter relative telomere length is predictive of a shorter life span and lower overall organismal health (Herborn et al. 2014). Thus, telomeres are considered to be biomarkers of an organism’s overall fitness, but it is unclear at this point if they are a symptom of age or play a causal role in the aging process. Despite this unknown, telomeres are a strong predictor of lifespan (Haussmann et al. 2005, Wilbourn et al. 2018), and are the strongest predictor of avian life span during early development (Haussmann et al. 2005, Powolny et al. 2020). While metals are shown to cause stress to organisms (Baos et al. 2019, Boyd & Rajakaruna 2013), and many stressors have been shown to shorten relative telomere length (Herborn et al. 2014, Powolny et al. 2020), to our knowledge, there has been no research showing how metals impact songbird telomere length.

We compared the relative telomere lengths of insectivorous riparian songbirds across three different habitats, with relatively consistent riverbed heavy metal contamination and varying floodplain heavy metal contamination in comparison to an uncontaminated reference habitat to assess the physiological impacts of metals and the mining-impaired environment. By measuring relative telomere length in songbirds facing varying challenges from mining contamination, this work ties the impacts of metal exposure to a measure of overall organismal health (Haussmann et al. 2005, Wilbourn et al. 2018). Specifically, this study addresses the following question:

Question) How does metal exposure from mine wastes impact songbird relative telomere length?

Hypothesis: Metal accumulation acts as a physiological stressor, causing negative impacts on overall organismal health.

P: The negative physiological impacts from metal accumulation will be seen as a decrease in relative telomere length as telomere degradation is a common result of

physiological stress. We expect an inverse relationship between metal accumulation and relative telomere length across sites.

Null Hypothesis: Songbirds are resilient to metal contamination and accumulation of metals does not cause telomere-measured physiological stress.

Pa: Telomere length will be unchanged across sites. There will be no significant difference in relative telomere length of songbirds within sites or across sites.

Pb: There will be no significant relationship between metal accumulation and relative telomere length within sites, but there will be differences in relative telomere length across sites, due to differences in site quality.

Methods:

One-hundred and fourteen riparian songbird blood samples were collected between the months of May 2022 and August 2022 by Ph.D. candidate Bridger Creel and others to measure metal concentration and relative telomere length. A total of four different sites were sampled. Three of these sites are in the upper Clark Fork River Superfund Complex: a fully contaminated and pre-remediation site (i.e. Forcella Ranch), a restoration in progress site (i.e. Grant Kohrs National Park; referred to as "Remediated" below), and a completely restored site (i.e. Warm Springs; referred to as "Restored" below). We compared samples from these sites to a reference site on the Bitterroot River (i.e. MPG Ranch), that is comparatively not affected by mining contamination (referred to as "Reference" below). See Figure 2 for a complete map of all sites. Each site is at different stages of restoration; however, all sites (excluding the reference) have been polluted with heavy metals and metalloids, including zinc (Zn), copper (Cu), selenium (Se), arsenic (As), lead (Pd), cadmium (Cd), and mercury (Hg). We sampled six species of obligate riparian songbirds for this experiment: *Melospiza melodia* (song sparrow), *Dumetella carolinensis* (gray catbird), *Setophaga petechia* (yellow warbler), *Empidonax traillii* (willow flycatcher), *Molothrus ater* (brown-headed cowbird), and *Agelaius phoeniceus* (red-winged blackbird). Although these songbirds have important similarities, such as insectivory and location, we chose these species to identify the impacts of heavy metal contaminants on songbirds with differing diets to infer general patterns across the local riparian songbird community assemblage.

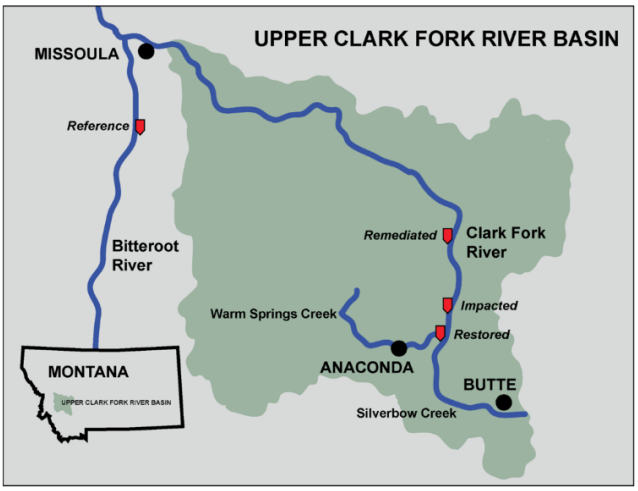

Figure 2: map of sampled sites in the Clark Fork water shed and the Bitterroot River in Montana. Red points indicate site locations. Reference refers to MPG Ranch, impacted refers to Forcella Ranch, remediated refers to Grant Kohrs National Park, and restored refers to Warm Springs.

Blood samples were collected from nestling songbirds. Sites were searched throughout the breeding season for nests. Once a nest was identified, they were tracked until success (i.e. fledge) or failure. Once nestlings were approximately 6-7 days old, a 1% volume/body mass blood sample was collected from the alar vein using 26-gauge needles and capillary tubes. The volume of the blood collected was at least 80 microliters, if possible, as 70 microliters were needed for metals analysis and 10 microliters were needed for telomere analysis. Blood samples for telomere and heavy metal concentration analysis were collected simultaneously in the field. Once blood samples were collected, they were frozen on ice within an hour and stored at -80℃.

For the metal analysis component of this study, 70 microliters of the blood samples are digested and analyzed via ICP-MS for Zn, Cu, Se, As, Pd, Cd, and Hg concentrations in the Environmental Biogeochemistry Lab. Ph.D. Candidate Bridger Creel will be conducting the metal analysis component for this study, but I will have access to the data to assess the relationship between relative telomere length and heavy metal concentration.

For relative telomere analysis, 10 microliters of whole blood were analyzed for relative telomere length. Once blood metal concentration analysis is completed, these samples (i.e. metals and relative telomere length) will be paired from the same individual. Red blood cells provide adequate tissue to assess variation in relative telomere length (Aubert & Lansdorp 2008, Herborn et al. 2014). To measure relative telomere length from whole blood samples, DNA was

extracted using Qaigan DNeasy kits. We then conducted qPCR following an adapted method from Heidinger et al. 2012 in Dr. Zac Chevriron's lab at the University of Montana.

Quantitative polymerase chain reaction (qPCR) measures the average telomere length in the genome by measuring the qPCR product of a single gene copy (S) and relating it to the telomere length (T). For this experiment, we tested the primers amplified a control gene (GAPDH) in all six focal species using PCR (Figure 3). Given that the primers successfully amplified the GAPDH gene, it was used as S. The ratio of telomere gene repeats per single gene copy (T/S ratio) produced then represents the average telomere length (Lin et al. 2019). This ratio is normalized to a "golden sample" (more information below) on each qPCR plate, facilitating cross-plate comparisons. Each plate was checked for contamination using both the "golden sample" and negative control. There was no meaningful correlation between DNA quality of DNA concentration and relative telomere length.

Protocol from the Qaigan DNeasy kit for extraction of "nucleated blood" was followed to extract DNA. After DNA extraction, DNA was quantified using a Thermoscientific nanodrop to determine the quality and approximate concentration of the DNA. The DNA was then stored in a -80°C freezer until DNA could be diluted. DNA was then thawed and diluted to 3.33 ng/uL. Stock concentrations of DNA were placed back into the -80°C freezer and the diluted DNA was stored in the refrigerator.

Primer sequences for GAPDH and telomere genes from Heidinger et al. (2012) were used for PCR and qPCR reactions. Prior to qPCR, PCR reactions were completed with all six species to ensure that the GAPDH and telomere sequences were correct and that no unwanted products were being produced. Thermocycles used in PCR and qPCR were identical. Refer to Tables 1, 2, 3, and 4 for explicit cycles and recipes.

Before a qPCR plate was constructed, the qPCR machine was turned on to begin warming up the lamp. A qPCR plate was then made using diluted DNA (i.e. 3.33ng/uL), Quantabio sybr green, PCR water, and GAPDH/telomere primers diluted to 1um solution. Refer to Table 2 for the recipe used.

PCR Recipe

Reagent	*volume (uL)*
Dream Taq	25
PCR water	15
forward primer	2.5
reverse primer	2.5
DNA template	5

Table 1: Recipe used for both telomere and GAPDH PCR. The volumes in this table represent the amount needed for a single individual. PCR water was used in place of the DNA template for a negative control.

qPCR Recipe

Reagent	*volume (uL)*
Sybr green	12.5
PCR water	6
forward primer	0.5
reverse primer	0.5
DNA template	6

Table 2: Recipe used for both telomere and GAPDH qPCR. The volumes in this table represent the amount needed for a single individual (i.e. represent a single well in the 96 well plate). PCR water was used in place of the DNA template for a negative control.

PCR/qPCR thermocycles for GAPDH

Cycles	*Temperature/Time*
1	95 for 10m
40	95 for 30s, 60 for 30s, 72 for 60s
1	dissociation curve

Table 3: Thermocycles used for GAPDH in PCR and qPCR. Temperature is in celsius. “S” represents seconds and “M” represents minutes.

PCR/qPCR thermocycles for Telomeres

Cycles	*Temperature/Time*
1	95 for 10 m
27	95 for 15s, 58 for 30s, 72 for 30s
1	dissociation curve

Table 4: Thermocycles used for telomeres in PCR and qPCR. Temperature is in celsius. “S” represents seconds and “M” represents minutes.

Each plate contained a "golden sample"—a sample with high quality DNA that was in triplicate on all plates—to calculate repeatability across plates and allow for comparison across plates. Each sample was run in triplicate, so 30 unique individuals were run on a single plate with a "golden sample" and a negative control that contained PCR water. Figure 3 displays the plate map used for all qPCR runs. Sybr green was thawed and protected from light as much as possible while making a master mix (MM) (i.e. sybr green, GAPDH/telomere primers, and PCR water). The MM was made according to Table 2 with 10% extra for any pipette error. Once a MM was made, it was distributed into 8-series PCR tubes to then pipette 19uL of MM into each of the 96-wells on the qPCR plate using a multichannel Eppendorf pipette. During plate construction, the plate was kept on ice by inserting the plate into a frozen 96-well gel mold. After MM was added to all 96 wells, 6uL of the individual sample was pipetted into the plate according to the plate diagram in Figure 3. When the DNA sample was added to the well, the well was mixed by using a pipette to draw up and expel the solution 6-8 times.

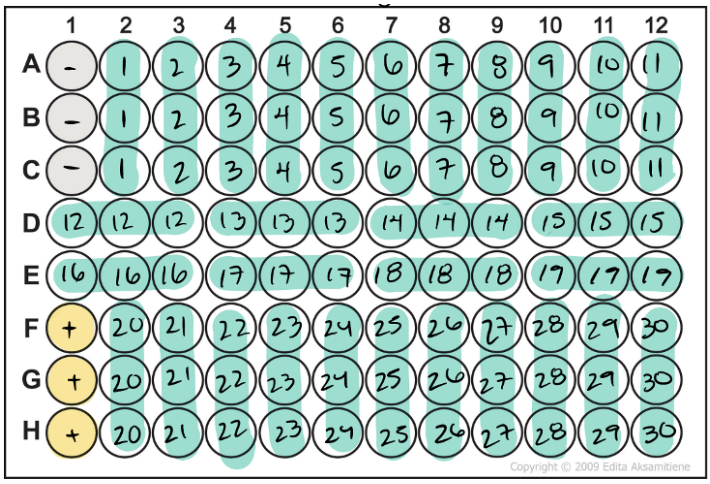

Figure 3: 96-well diagram that was used for qPCR. Blue wells represent individual samples run in triplicate. Yellow wells represent the golden sample that was used across plates. Grey wells represent a negative control that contains PCR water.

After plate construction was complete, the plate was centrifuged for 15 seconds to pull all reagents into the solution. The plate was then placed into a MX3005P qPCR machine and the respective cycle was run (Table 3, Table 4). The qPCR lamp was always allowed to warm for at least 30 minutes prior to running the first plate of the day, plates were always run 0-30 min after construction, and plates were always made separately (i.e. GAPDH and telomere plates were not made at the same time because only one plate was able to be run at a single time).

Statistical analysis:

We used general linear models (GLMs) to assess the influence of site, species, fat score (a discrete visual score from 0-3), and age (days since hatch) on T/S. We ran a model selection procedure testing for GLM fit with different family and link functions, assessed by AIC. As we expected, given T/S data was bounded by 0 and right-tailed, our best-fit model was with a Gamma family and log link.

We then used a backward elimination process to exclude variables with p-value > 0.05 (assessed via ANOVA of included variables) to produce minimum adequate models. Our initial model included the variables site, species, fat score, and age. Our final GLM contained site, species, and fat score, with model selection only removing the age variable.

There is some grouping of T/S values among individuals from the same nest, however, we did not have the statistical power to include a random effect for nest of origin given small sample sizes, so samples are assumed to be independent within site/species combinations in our models.

Models could not include individuals from Forcella Ranch (the fully contaminated site, or un-remediated site) and two species of riparian songbirds: willow flycatchers and song sparrows. These data were unusable in models because there is expected variation in T/S across species and the only nestlings we were able to sample at Forcella Ranch were willow flycatchers and song sparrows, which we were not able to sample at other sites. As a result, we cannot resolve the influence of site versus species in these samples and they cannot be included in any statistical analyses.

Blood samples for heavy metal concentration have been collected but have not been analyzed. In the near future, we will use generalized linear mixed-effects models to assess the relationship between metal concentrations and T/S. This will allow for further analysis of the relationship between songbird relative telomere length and metal concentration.

Results:

PCR results.

PCR was used to ensure that primer sequences amplified in each sampled species, and to confirm that there was no contamination in the primer sequences or the diluted songbird DNA.

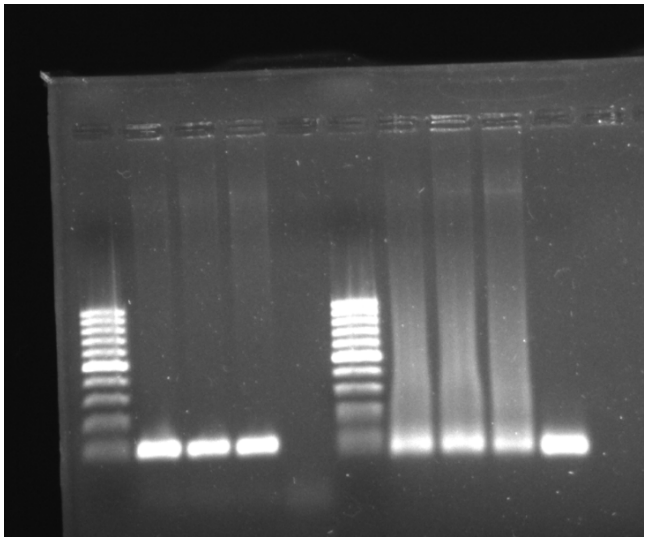

Figure 4: PCR results for GAPDH (i.e. left grouping of columns) and telomere (i.e. right grouping of columns) primers in three riparian songbird species: yellow warblers, brown-headed cowbirds, and red-wing blackbirds. From left to right, the columns represent 100 base pair ladder, yellow warbler, brown-headed cowbird, red-wing blackbird, and negative control using PCR water.

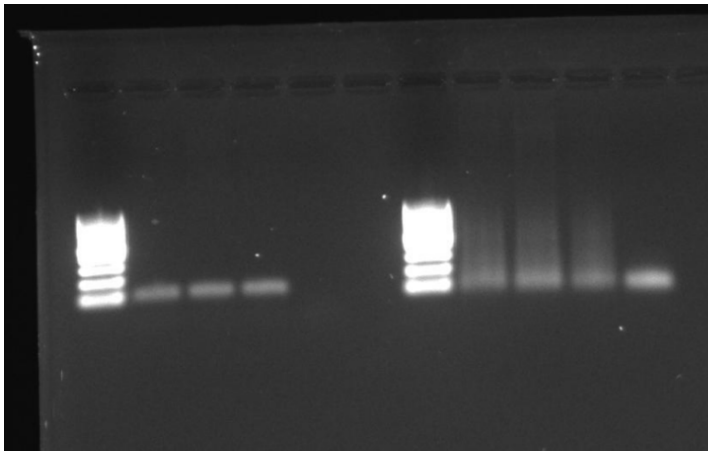

Figure 5: PCR results for GAPDH (i.e. left grouping of columns) and telomere (i.e. right grouping of columns) primers in three riparian songbird species: song sparrows, willow flycatchers, and grey catbirds. From left to right, the columns represent 100 base pair ladder, song sparrow, willow flycatcher, grey catbird, and negative control using PCR water.

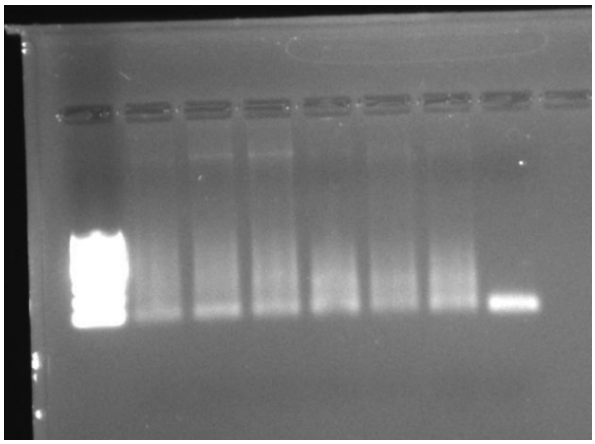

Figure 6: PCR results for telomere primers across six riparian songbird species: yellow warblers, brown-headed cowbirds, red-wing blackbirds, song sparrows, willow flycatchers, and grey catbirds. From left to right, the columns represent 100 base pair ladder, yellow warbler, brown-headed cowbird, red-wing blackbird, song sparrow, willow flycatcher, grey catbird, and negative control using PCR water.

No amplification was observed in the negative control for the GAPDH gene for any of the sampled species (Figure 4, Figure 5). However, there was amplification of the negative control in all runs of telomere PCR for all sampled species (Figure 4, Figure 5, Figure 6), indicating there was some kind of contamination. However, quality controls were run on all qPCR data to ensure there was no contamination of collected data, and all qPCR data passed the quality control tests. The source of contamination in the PCR is still unknown.

Relative telomere length results.

The final GLM revealed three significant predictors of relative telomere length: site, species, and fat score. Figure 7 displays the marginal relationship between fat score and T/S within the final GLM. The data revealed strong evidence that relative telomere length (as represented by T/S) and fat score are positively correlated (Figure 7, ß = 0.23, p-value = 0.02).

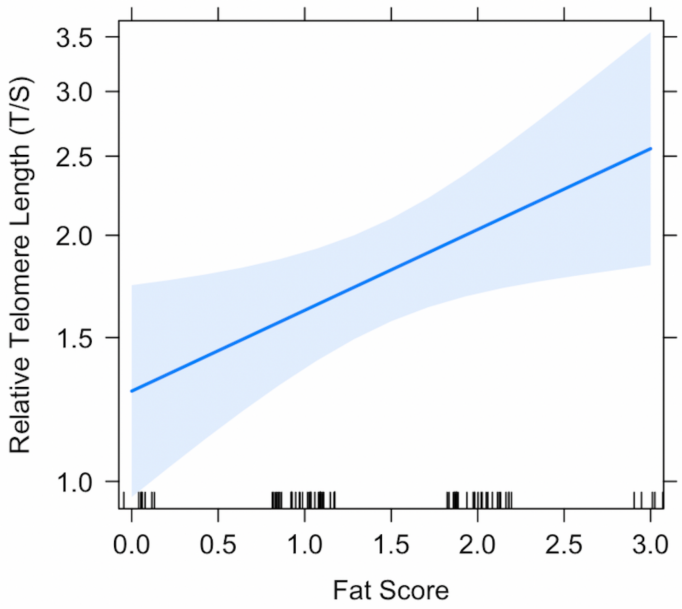

Figure 7: The marginal relationship between relative telomere length (T/S) and fat score from a general linear model with covariates of site and species (ß = 0.23, SE = 0.09, p = 0.02). The light blue area represents a 95% confidence interval.

We found strong evidence of site-level differences in relative telomere length (Figure 8). The marginal effect of site within the final GLM suggests that songbirds sampled at the reference site had the longest T/S compared to the remediated and restored site. While there is no evidence that the difference in relative telomere length between the restored and remediated site is meaningful (Figure 8, p-value >0.05), there is strong evidence that the difference between the

reference site and both the restored and remediated sites is meaningful (Figure 8, p-value <0.001).

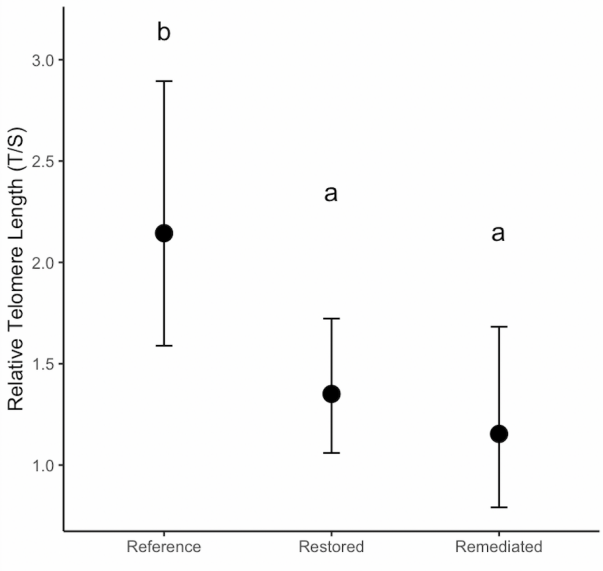

Figure 8: Mean relative telomere length (T/S) in reference, restored, and remediated sites. Points are marginal means from a general linear model with covariates of species and fat score. Error bars are 95% confidence intervals. Compact letter display represents statistical differences of site marginal means below an alpha value of 0.05.

We found strong evidence of interspecific differences in relative telomere length among the songbird species sampled (Figure 9). In assessing the marginal effect of species in our final GLM, we found that brown-headed cowbirds (BHCO), red-winged blackbirds (RWBL), and yellow warblers (YEWA) had similar relative telomere lengths (Figure 9, p-value >0.05). Gray catbirds (GRCA) had the longest relative telomere length, and there is strong evidence that there is a meaningful difference between yellow warbler and gray catbird relative telomere length (Figure 9, p-value <0.01). However, there was no evidence that there is a meaningful difference in relative telomere length among red-wing blackbirds, brown-headed cowbirds, and gray catbirds (Figure 9, p-value >0.05).

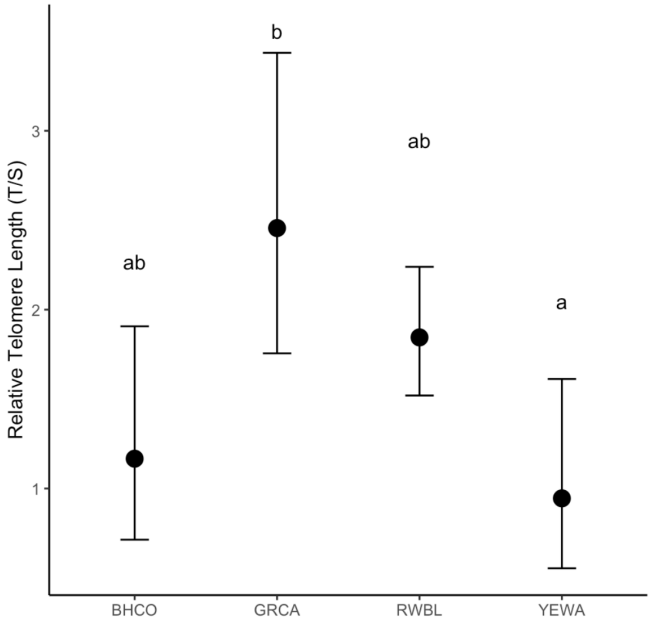

Figure 9: Mean relative telomere length (T/S) in brown-headed cowbirds (BHCO), grey catbirds (GRCA), red-wing blackbirds (RWBL), and yellow warblers (YEWA). Points are marginal means from a general linear model with covariates of site and fat score. Error bars are 95% confidence intervals. Compact letter display represents statistical differences of site marginal means below an alpha value of 0.05.

Discussion:

Heavy metals are prominent on the environmental landscape as a result of traditional mining practices (Lottermoser 2010). While restoration is taking place, and in some instances completed, it may not be sufficient to reverse the damage done. Current restoration efforts in the Clark Fork watershed remove the contaminated floodplain but leave the contaminated riverbed intact. Removal of the floodplain may reduce the magnitude of heavy metals songbirds are subjected to; however, songbirds are likely still being exposed to heavy metals via their ingestion of aquatic macroinvertebrates (Figure 1). It was unclear what impact removal of the floodplain would have on reducing heavy metal exposure for songbirds, but considering our results, riparian restoration may have limitations as represented by impacts on organismal health.

Although some important comparisons of this data are currently missing (i.e. comparison of relative telomere length at Forcella Ranch and comparison of heavy metal concentration and relative telomere length), we have strong evidence to show that there is a meaningful difference in relative telomere length between non-contaminated (i.e. reference) and contaminated riverbed sites (i.e. remediated and restored) (Figure 8, p-value <0.01). While we are unable to directly assess the relationship between relative telomere length and heavy metal concentration at this point, our results followed our prediction that telomeres from nestlings at sites with metal

contamination are shorter. This result is in line with the prediction that there will be a relationship between metals and relative telomere length

With current data, the mechanism underlying the relatively shorter telomere lengths at contaminated sites remains unresolved. Likely, even with more data (i.e. metal concentration data, more representation of samples from Forcella Ranch, and more samples of willow flycatchers and song sparrows at other sites) would still not yield an exact mechanism. However, even with this limitation that is common to field experiments, there is strong evidence showing songbird relative telomere length is shorter at contaminated sites and is still biologically meaningful. We would argue that regardless of the mechanism, the correlation of shorter telomere length is enough to warrant further investigation into assessing riparian restoration and the negative physiological impacts on riparian songbirds.

Interestingly, in comparing relative telomere length to nestling fat score, there was strong evidence to show that birds with a higher fat score had longer telomeres (Figure 7, p-value < 0.01). Fat score is a commonly used measure of nestling condition, with a higher fat score indicating better condition (Labocha & Hayes 2012). A positive correlation of relative telomere length with fat score indicates that songbirds sampled with a longer relative telomere length were also in better condition. This is the expected relationship, as better condition as represented by fat score and longer relative telomere length would both represent a “healthier” organism (Haussmann et al. 2005, Herborn et al. 2014, Wilbourn et al. 2018). However, we are not aware of any previous evidence that has linked fat score to relative telomere length. As a result, while this relationship validates our use of relative telomere length to measure songbird organismal health, it also demonstrates an incredibly interesting relationship within itself. Whether the positive correlation between relative telomere length and fat score is mediated by heavy metals, site quality, or something else, the relationship suggests that within this study we are seeing real, biologically important differences in organismal health among nestlings across these sites.

Habitat quality varies dramatically across sites, with Warm Springs (i.e. restored) and MPG Ranch (i.e. reference) having the highest quality habitat, Grant Kohrs National Park (i.e. remediated) having only moderate habitat quality, and Forcella Ranch (i.e. impacted or pre-remediation) having the poorest habitat quality. Regardless of the mechanism, our data present strong evidence that there is something mediating differences in songbird relative telomere length across sites (Figure 8, p-value < 0.001). However, considering previous literature

and these results together, relative telomere length is likely impacted due to habitat quality, heavy metal exposure, or even more likely, an interaction between the two. If habitat was the only driver of relative telomere length, we would expect Warm Springs and MPG Ranch to have the longest relative telomere lengths with Grant Kohrs National Park and Forcella Ranch to have the shortest. However, we found Warm Springs and Grant Kohrs National Park to have very similar relative telomere lengths (Figure 8).

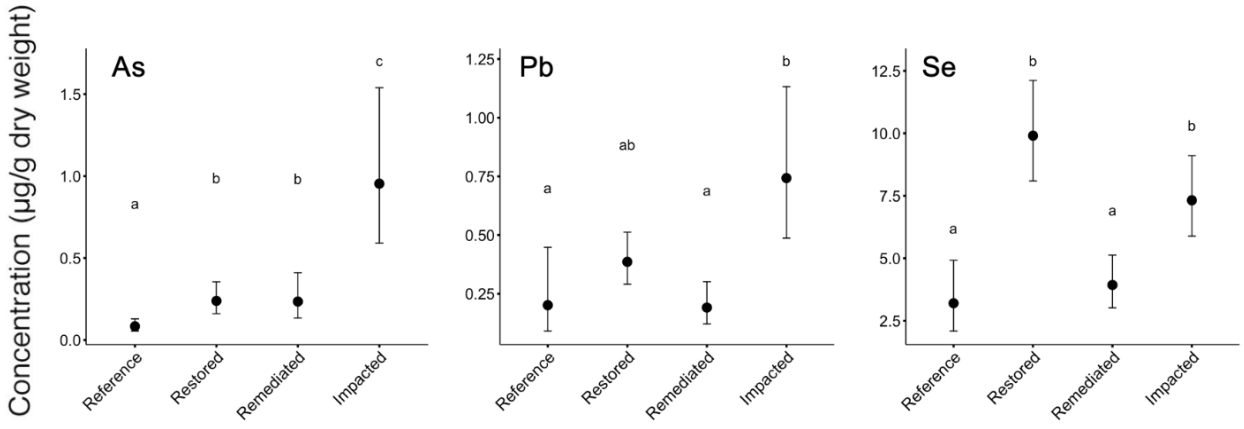

Figure 10: Heavy metal concentration in blood sampled from nestling riparian songbird species from May-August 2020 at reference, remediated, restored, and impacted sites. From left to right, the heavy metals represented are selenium (Se), lead (Pb), and arsenic (As). Points are marginal means extracted from a GLM with a gamma distribution and log link and covariates species and nestling age. Error bars represent a 95% confidence interval. Compact letter display represents statistical differences of site marginal means below an alpha value of 0.05

Metal analysis has been performed on songbird samples from 2020 collected at the same sites. Copper, lead, and zinc were not elevated in songbird blood and showed no strong differences across sites. However, arsenic, lead, and selenium concentrations were beyond levels of concern at some sites and revealed site-level differences. Arsenic and lead concentrations across reference, restored, and remediated sites have very similar concentrations, but blood concentrations are elevated at the impacted site. Selenium shows a different site-level pattern, with blood concentration decreasing from upstream to downstream sites. Restoration of the flood plain seems to have removed the risk of metal accumulation in songbird blood, as the contaminated site has much higher levels of heavy metals in songbird blood than remediated,

restored, or reference sites (Figure 10). However, selenium is elevated at the restored site (i.e. Warm Springs), even more so than the fully contaminated site (i.e. Forcella Ranch). Selenium is likely elevated at the restored site (i.e. Warm Springs) due to its proximity to a gold mine that may serve as a continuous point source of selenium input into the river. Considering these differences in blood metal concentration and our results on relative telomere length, it is possible that selenium exposure is driving differences in telomere length in part. If selenium was the main factor in impacting relative telomere length, we would expect that songbird relative telomere length at the restored (i.e. Warm Spring) and the impacted site (i.e. Forcella Ranch) would be the shortest. While the restored site does have shorter relative telomere lengths compared to the reference site, it has comparable relative telomere lengths to the remediated site, which has low levels of selenium.

It is also possible that while riparian songbirds are impacted by metals, they have a threshold of tolerance. This concept is referred to as the "ceiling effect" in the literature and refers to the idea that past a certain concentration of metals, songbirds may be equally negatively impacted (Boyd & Rajakaruna 2013). If this were true, we might expect relative telomere length to be similar across restored, remediated, and impacted sites and shorter than those at the reference site. Our results tentatively support this idea, as we observed both restored and remediated sites to have similar relative telomere length; however, without relative telomere length data from the impacted site, we are unable to support or reject the "ceiling effect."

Our results are best explained by an interaction of selenium concentration and habitat quality. Poor habitat quality at the remediated site could drive the observed shorter telomere length, while the shorter relative telomere length at the restored site could be explained by high blood concentrations of selenium.

Our results indicate that metal exposure and habitat degradation from mining contamination likely play a role in the pre-mature degradation of telomeres. While the explicit mechanism remains unclear, these data warrant further research to determine the physiological impacts of metal exposure on riparian songbirds. With these data, it seems that our current restoration efforts may not be restoring full ecosystem function as we detected negative fitness effects on organisms as measured by relative telomere length in restored habitats.

References:

Alberts, J. M., Sullivan, S. M., & Kautza, A. (2013). Riparian swallows as integrators of landscape change in a multiuse river system: Implications for aquatic-to-terrestrial transfers of contaminants. *Science of The Total Environment*, *463-464*, 42–50. https://doi.org/10.1016/j.scitotenv.2013.05.065

Aubert, G., & Lansdorp, P. M. (2008). Telomeres and aging. *Physiological Reviews*, *88*(2), 557–579. https://doi.org/10.1152/physrev.00026.2007

Baos, R., Blas, J., Bortolotti, G. R., Marchant, T. A., & Hiraldo, F. (2006). Adrenocortical response to stress and thyroid hormone status in free-living nestling white storks (ciconia ciconia) exposed to heavy metal and arsenic contamination. *Environmental Health Perspectives*, *114*(10), 1497–1501. https://doi.org/10.1289/ehp.9099

Baxter, C. V., Fausch, K. D., & Saunders, C. W. (2005). Tangled webs: Reciprocal flows of invertebrate prey link streams and riparian zones. *Freshwater Biology*, *50*(2), 201–220. https://doi.org/10.1111/j.1365-2427.2004.01328.x

Boyd, R. S., & Rajakaruna, N. (2013). Heavy Metal tolerance. *Oxford Bibliographies Online Datasets*. https://doi.org/10.1093/obo/9780199830060-0137

Breuner, C. W., Patterson, S. H., & Hahn, T. P. (2008). In search of relationships between the acute adrenocortical response and fitness. *General and Comparative Endocrinology*, *157*(3), 288–295. https://doi.org/10.1016/j.ygcen.2008.05.017

Geum Environmental. 2015. Upper Clark Fork River Basin Aquatic Resources Restoration Plan Monitoring and Maintenance Plan. Prepared for the Natural Resource Damage Program, Montana Department of Justice, Helena, Montana.

Grunst, M. L., Grunst, A. S., Pinxten, R., & Eens, M. (2020). Anthropogenic noise is associated with telomere length and carotenoid-based coloration in free-living nestling songbirds. *Environmental Pollution*, *260*, 114032.

Haussmann, M. F., Winkler, D. W., & Vleck, C. M. (2005). Longer telomeres associated with higher survival in birds. *Biology Letters*, *1*(2), 212–214. https://doi.org/10.1098/rsbl.2005.0301

Heidinger, B. J., Blount, J. D., Boner, W., Griffiths, K., Metcalfe, N. B., & Monaghan, P. (2012). Telomere length in early life predicts lifespan. *Proceedings of the National Academy of Sciences*, *109*(5), 1743–1748. https://doi.org/10.1073/pnas.1113306109

Herborn, K. A., Heidinger, B. J., Boner, W., Noguera, J. C., Adam, A., Daunt, F., & Monaghan, P. (2014). Stress exposure in early post-natal life reduces telomere length: An

experimental demonstration in a long-lived seabird. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1782), 20133151. https://doi.org/10.1098/rspb.2013.3151

Labocha, M. K., & Hayes, J. P. (2012). Morphometric indices of body condition in birds: a review. *Journal of Ornithology*, *153*, 1-22.

Lin, J., Smith, D. L., Esteves, K., & Drury, S. (2019). Telomere length measurement by qPCR - Summary of critical factors and recommendations for assay design. *Psychoneuroendocrinology*, *99*, 271–278. https://doi.org/10.1016/j.psyneuen.2018.10.005

Lottermoser, B. G. (2010). *Mine wastes characterization, treatment and environmental impacts*. Springer.

Pegan, T. M., Winkler, D. W., Haussmann, M. F., & Vitousek, M. N. (2019). Brief increases in corticosterone affect morphology, stress responses, and telomere length but not postfledging movements in a wild songbird. *Physiological and Biochemical Zoology*, *92*(3), 274-285.

Powolny, T., Bassin, N., Crini, N., Fourel, I., Morin, C., Pottinger, T. G., Massemin, S., Zahn, S., & Coeurdassier, M. (2020). Corticosterone mediates telomere length in raptor chicks exposed to chemical mixture. *Science of The Total Environment*, *706*, 135083. https://doi.org/10.1016/j.scitotenv.2019.135083

Walters, D. M., K. M. Fritz, and R. R. Otter. 2008. The Dark Side of Subsidies: Adult Stream Insects Export Organic Contaminants to Riparian Predators. Ecological Applications 18:1835–1841

Wilbourn, R. V., Moatt, J. P., Froy, H., Walling, C. A., Nussey, D. H., & Boonekamp, J. J. (2018). The relationship between telomere length and mortality risk in non-model vertebrate systems: A meta-analysis. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *373*(1741), 20160447. https://doi.org/10.1098/rstb.2016.0447