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Measuring Selenoprotein Content in False Map Turtles (Graptemys pseudogeographica) Along the Missouri River

Ruby A. Hawks
University of South Dakota

Measuring selenoprotein content in False Map Turtles (*Graptemys pseudogeographica*) along the Missouri River

by

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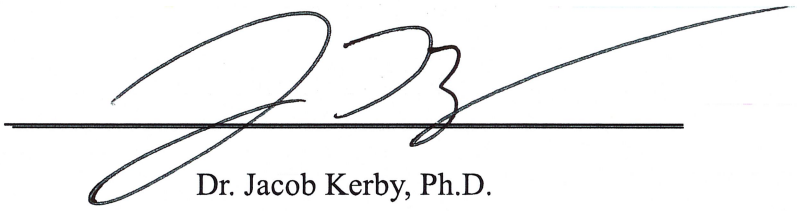
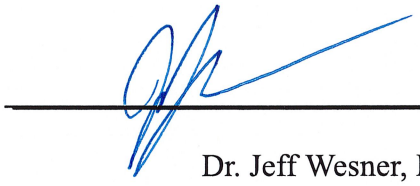


Dr. Jacob Kerby, Ph.D.
Chair of Biology
Committee Director



Dr. Jeff Wesner, Ph.D.
Associate Professor of Biology

Dr. David Swanson, Ph.D.
Professor of Biology

ABSTRACT

Metals and metalloids are becoming more prevalent in lakes and reservoirs of South Dakota which are toxic and hazardous in high concentrations or when biomagnified through trophic levels. Selenium is of particular concern as it can bind into the structures of proteins in place of other elements, changing protein structure and function within affected organisms. Zebra mussels (*Dreissena polymorpha*) are an invasive filter-feeding metal-bioaccumulating species that are rapidly spreading upstream through the Missouri River. They can take up selenium and directly transfer it to higher trophic level taxa. False map turtles (*Graptemys pseudogeographica*) are a common predator of zebra mussels and serve as a model organism to detect selenium levels in higher trophic level organisms. It was predicted that there would be a higher selenoprotein content in turtles residing in downstream vs upstream reservoirs of the Missouri River due the prevalence of zebra mussels downstream that may contribute to dietary selenium intake. To test this hypothesis, false map turtles were randomly sampled from Lake Francis Case (upstream) and the 59 Mile Stretch of the Missouri River (downstream). Blood samples were randomly selected from both sites and tested for selenoprotein concentration using an ELISA assay. Based on a Bayesian statistical model, there is a 96.3% probability that Lake Francis Case absorbance values are higher than the 59 Mile Stretch absorbance values. Higher absorbance values are indicative of higher concentrations of selenoprotein. These results suggest that the turtles of the 59 Mile Stretch are exposed to lower levels of selenium than those of Lake Francis Case. This indicates that there are other factors to consider. More work must be done to understand the reason for variance in selenoprotein absorbance values between sites and what impact the invasive zebra mussels truly have on other marine inhabitants.

Keywords: selenium, false map turtles, zebra mussels, bioaccumulation

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INTRODUCTION

Rationale

Metals and metalloids are becoming more prevalent in the lakes and reservoirs of South Dakota. Most often these substances are toxic and can cause greater hazard when they are high in concentration (DeForest et al. 2007). There is a history of heavy metal deposits in the sediments of the Missouri River (Pracheil, et al. 2010). Those of main concern are cadmium, copper, lead, mercury, selenium, and zinc (Deforest et al. 2007). Heavy metals are a big problem in part because of the lipophilicity of their common chemical forms found in the environment – this physical property ensures that they can bioaccumulate in organisms and through trophic levels. Their toxicity is due to their specific chemical and biochemical reactivities such as reacting with thiolates or substituting other crucial metal ions.

This project will focus on the effects of selenium as it has a particular affinity for binding in place of sulfur in amino acid structures such as methionine and cysteine (Daniels 1996). Amino acids are the building blocks of important enzymes and proteins. By changing even just one small molecule, the whole structure of a protein can be compromised. The linking between amino acids changes with the new properties of a metal like selenium being introduced; new bonds can be formed. An altered carbon chain of an amino acid can lead to alteration of shape and change its functionality. This may also result in change of behavior and health of the organism.

Selenium is naturally present in the soil and water (Sando & Neitzert 2003). However, additional build up of selenium in ecosystems can occur. Deeper soil that contains selenium left over from the glacial periods that carved the Great Plains is not disturbed. Because of the installation of tiling systems popular in South Dakota, deep selenium is accessed and mobilized.

Oftentimes tiling systems lead to wetlands and other waterways that can create unnatural levels in the water (Henry et al. 2020). Many bodies of water are compromised due to this, because in high concentrations selenium can become toxic (Fan et al. 2002). The two most common inorganic forms of selenium in natural water are selenite and selenate (Benis et al. 2022). Selenite is the most toxic as it is the most bioavailable; it is released from coal-fired power plants and oil refining wastewaters. Selenate is the more common aquatic anthropogenic compound.

The concentration of selenium in the Lewis and Clark Lake, the most downstream reservoir on the Missouri River, has been confirmed to be at an elevated level by recent researchers (Pracheil et al. 2010). The selenium is released from surrounding soil into the waters during the annual high water/flooding cycle of the spring season (Pracheil et al. 2010). Climate change can make this seasonal flooding unpredictable. The increased concentration of selenium in the river increases its likelihood of being picked up by organisms living in the water. One such organism is the invasive zebra mussel species (*Dreissena polymorpha*), which is a known filter feeder that has a propensity for accumulating metals and other contaminants (Roditi & Fisher 1999).

Reproducing populations of zebra mussels were first confirmed in Lewis and Clark Lake by the South Dakota Game Fish and Parks in 2015, and they have since continued their spread to several other upstream reservoirs (SDGFP 2019). Juvenile zebra mussels are free-swimming larvae that can spread up the currents and rapidly proliferate (Whitney 2023). Major concerns of the state are the potential impact of invasive species on tourism, outdoor recreation, water quality, irrigation, and lake and river ecosystems (Whitney 2023).

Aside from their invasive habits, they influence the bioaccumulation of several trace elements in the water. Zebra mussels pump a large fraction of trace elements from the water

through their guts and gills, such as selenium, which can then be incorporated into their tissues and organs. In addition, zebra mussels are excellent at accumulating those elements and redistributing them to higher trophic levels (Roditi & Fisher 1999). Predators who are at higher trophic levels will experience this bioaccumulation the most.

According to DeForest et al. (2007), bioaccumulating factors (BAF) and trophic transfer factors (TTF) of metals including selenium are observed at high values even when aquatic concentrations are low. Freshwater selenium was observed on a concentration scale from 0.3 to nearly 5000 µg/L with BAF scores reaching their highest at nearly 100,000 at 0.3 µg/L. The BAF score is the ratio of a chemical concentration in an organism to the concentration in the water based on all possible routes of exposure (e.g., dietary absorption, transport across the respiratory surface). Similar relationships were found with TTF scores, the ratio of a substance concentration in an organism's tissue to its concentration in the organism's food item. This means that even when selenium concentration is low in the water, it can create big impacts as it transfers and accumulates from prey to predator. The important implication is that even though aquatic concentration evaluations can look low, they can be present in high dosages in organisms' tissues. Indeed several sites show a tolerable concentration of selenium in the water but a toxic threshold being reached in the tissues of native wildlife (DeForest 2007). These findings show that an analysis of the ecological health risk assessments is required for selenium and other metals.

Prior data on a geographically widespread turtle species, yellow-bellied sliders (*Trachemys scripta scripta*) collected from the southeastern United States supports the idea that experimental selenium exposure results in selenosis with varying degrees of severity depending on concentration levels (Haskins et al. 2018). These turtles were treated with

seleno-L-methionine. They found deleterious histological effects in the kidney, claws, and muscles of these organisms. This provides more evidence that selenium has a negative effect on organisms exposed to selenium in waterways. The drawback of the yellow-bellied slider turtle study is that it was experimentally driven with concentrations of selenium that were much higher than the range of what these slider turtles were expected to consume in their natural contaminated habitats. Using field data, rather than experimentally exposing captured turtles, our project aims to determine if natural habitats have selenium concentrations necessary for selenium toxicity and protein turnover.

The particular organism of interest along the Missouri River is the state-threatened false map turtle (*Graptemys pseudogeographica*), as they are a common predator of zebra mussels in Lewis and Clark Lake and have been confirmed by the Kerby Laboratory to readily eat zebra mussels in both experimental and wild settings (unpublished data). Due to the elevated dissolved selenium levels in the water and zebra mussel propensity for bioaccumulation, we hypothesize that high levels of selenium would also be present in blood samples from false map turtles. If there are high enough concentrations of selenium, there may also be proteins that have selenium-containing amino acids. If there is a detectable selenium content in the proteins of false map turtles, it will confirm that high concentrations of selenium contribute to uptake into the proteins themselves.

The Kerby Laboratory is also interested in determining the concentration of unbound selenium in the blood of false map turtles due to zebra mussel consumption. That data will be compared to the work of this specific project through comparisons of unbound selenium content to that which is folded within the proteins. All together we hope to discover whether the effect of

biomagnification of selenium through trophic levels is present in false map turtles in the Missouri River.

Significance

Finding that selenium is not just present in blood samples but is actually bound within the proteins of false map turtles gives direction for future projects. If we find that false map turtles are at risk of selenium toxicity, we can then better advocate for the removal of zebra mussels and extensive monitoring along the Missouri River for selenium.

There are several other fish and bird species that also consume zebra mussels that may be at risk of selenium bioaccumulation, biomagnification, and toxicity. If false map turtles have a large detectable amount of selenium bound proteins, this may hold true for other mussel predators along the Missouri, such as blue gills, pumpkin seeds, and catfish which are fish species known to consume zebra mussels. These species could be bioaccumulating selenium just as the turtles are. The reservoirs around the state are common recreational and fishing areas; having zebra mussels facilitate selenium bioaccumulation and trophic transfer could also significantly impact human health. Tested turtles will serve as a valid proxy for whole ecosystem health.

Additionally, there are very few studies comparing unbound and bound selenium within the body, and even fewer examining direct transfer of selenium from a specific prey item to a specific predator with ecosystem-wide implications.

The objective of this project is to determine whether there is a detectable concentration of selenium in the protein of false map turtles, most presumably assimilated from the consumption of zebra mussels. Additionally, we sought to determine if there was a difference in concentration

of those proteins in false map turtles collected from downstream vs. upstream Missouri River reservoirs based on the presence of zebra mussels who have spread upstream.

MATERIALS AND METHODS

Field Sampling

Field-based analysis of false map turtles along the Missouri River was used to identify prime locations for trapping. Optimal trapping sites have non-stagnant water and nearby vegetation with downed branches or logs. Field observations of false map turtles sun-bathing on logs or rocks gave indication of their presence at several locations.

Three locations were identified where false map turtles were previously prevalent. Additionally, these locations had been identified by prior Kerby Lab field trips as areas where the zebra mussel population had spread upstream, because we wanted to sample turtles at upstream and downstream reservoirs to analyze whether the presence of mussels has an impact on selenium bioaccumulation. The three sampled locations from upstream to downstream were Lake Sharpe downstream of the Oahe Dam in Pierre, SD, Lake Francis Case near Platte, SD, and the 59 Mile Stretch near Yankton, SD (Figure 1).

False map turtles were sampled between June and August of 2021. The first excursion was in mid-June, collecting data from both Lake Francis Case and Lake Sharp on a 10-day trip. The next excursion to the 59 Mile Stretch was in early August. Five days were spent at each site, using the first 2-3 days for setting traps and the last 2-3 days for retrieving and sampling. Field data were successfully collected from two of the three sites: Lake Francis Case and the 59 Mile Stretch. While false map turtles were sighted along the bank of Lake Sharpe and have been trapped in the area before, they did not respond to the bait nor enter the traps.

The turtles were caught using partially submerged hoop nets baited with salted pork and fish scraps. The traps were set along vegetation for 24-48 hours and GPS coordinates were recorded. Traps were set to ensure that there remained an air pocket for any captured organisms.

Unwanted organisms that were captured were released immediately, such as fish or other turtle species. The traps were removed after the above interval and all false maps turtles were processed on dry land to ensure accurate data collection. The turtles were released on-site after sample and data collection. All data collection was done under an approved Institutional Animal and Use Committee protocol.

Several data were collected: carapace length and width, plastron length and width, mass, sex, tissue sample, and blood sample. Tissue samples were cut from the webbing between indices of the front right foot – back legs are used to dig nests so front leg samples are preferred. Blood samples were collected via the dorsal coccygeal vein using a comfort point 1 mL insulin syringe. 1-3 mL of blood were collected from each individual. The blood samples were stored in 2 mL microcentrifuge tubes using heparin as an anticoagulant. They were then put on ice that was interchanged frequently throughout the field excursions. Upon returning to the main lab, these samples were transferred to a -10° Celsius freezer. All captured turtles were given an ID number using the numerical coding system for hard shell turtles (Figure 2; Ernest et al. 1974). Recaptured organisms were noted.

Assay Protocol

Using a random number generator for each of the two sites, 24 individual false map turtles were chosen, 12 from each site. The collected blood samples were thawed and centrifuged. The separated plasma was pipetted into a new microcentrifuge tube, taking care that at least 300 µL of sample plasma was available for use in the ELISA protein assay.

Samples were run using an Aviva Systems Biology Selenop ELISA Kit (OKEH02413 2021). The kit is based on sandwich enzyme-linked immuno-sorbent assay technology. A

96-well plate was used, pre-coated with an antibody specific for Selenoprotein P. This particular protein serves as a marker for the bound selenium content. Standards were first added to the wells with three replicates each, leaving room for a blank. 100 µL test samples were added to the wells, 12 from each site with three replicates each for a total of 72 sample wells. The plate was sealed with the provided plate sealer and incubated at 37°C for two hours. After incubation, the liquid was flicked off the plate into an acceptable waste bin, taking care to gently blot any remaining liquid from the wells by inverting the plate on the covered benchtop.

A prepared biotinylated Selenop detector antibody was added and the plate was recovered to be incubated again at 37°C for one hour. Again, the liquid was flicked from the wells after incubation. Without allowing the wells to ever dry completely, three washes were performed using a prepared wash buffer. 300 µL of wash buffer was applied each time allowing incubation for one minute between each wash.

Next, 100 µL avidin horseradish peroxidase (HRP) conjugate was added to each well. The plate was covered and allowed to incubate at 37°C for one hour. Again the liquid was flicked from the wells after incubation. Without allowing the wells to ever dry completely, five washes were performed using the same prepared wash buffer to wash away any unbound conjugate. 300 µL of wash buffer was applied each time allowing incubation for one minute between each wash.

Next, 90 µL of TMB substrate was added to the wells. The plate was covered with the plate sealer and placed under aluminum foil to be incubated in the dark for 30 minutes. The substrate was catalyzed by HRP to produce a blue color in an enzymatic reaction. Incubation time can vary, but seeing the variable blues across the standards is a good indicator. Finally, 50 µL of Stop Solution was added to each well taking care to follow the same well order as the TMB substrate was added. The color immediately turned yellow due to the acidity of the Stop

Solution. The density of the yellow coloration indicates the amount of Selenop captured in the wells.

Within 5 minutes of applying the stop solution, the plate was read by a microplate reader at an absorbance of 450 nm to quantify the samples. Relative optical density of the standards and samples was calculated. (Relative OD_{450}) = (Well OD_{450}) – (Mean Blank Well OD_{450}). The standard curve was generated by plotting the relative optical density vs standard concentration. If done correctly, each sample's concentration could be interpolated using linear regression along the standard curve.

RESULTS

Two sites yielded usable data along the Missouri River: Lake Francis Case and the 59 Mile Stretch. While we attempted to obtain additional samples from a third location at Lake Sharpe, the false map turtles did not respond to the bait nor enter the traps. From the two successful sites, 100s of turtles were sampled. 24 false map turtle samples were randomly chosen to be assayed, 12 from each of the two sites. Tested samples came from turtles that were a mix of male and female, from three years of age on. Their masses ranged from 1100 to 1800 g, with carapace lengths from 10-25 cm. These ranges in sex and size aimed to help report a broad population of false map turtles within the two sample areas that can be used to generalize the populations along the Missouri River.

The mean selenoprotein absorbance derived from the turtle samples at the two sites were Lake Francis Case at 0.21 and the 59 Mile Stretch at 0.158. The difference between the means of the two group's absorbance is 0.052 (Figure 3). A Bayesian statistical analysis was used to interpret the data (Figure 4). Based on this model, there is a 96.3% probability that Lake Francis Case absorbance values are higher than the 59 Mile Stretch of the Missouri River absorbance values. Higher absorbance values are indicative of higher concentrations of selenoprotein. Mean absorbance values are compared in Figures 3 and 4.

DISCUSSION

The results show a higher selenoprotein absorbance in the samples from Lake Francis Case. This suggests that the turtles of the 59 Mile Stretch carry lower levels of selenium than those of Lake Francis Case. These results appear despite the predicted higher concentration due to the higher availability of invasive zebra mussel prey downstream. We hypothesized that false map turtles are exposed to high enough concentrations of selenium through their diet that a detectable amount of Selenoprotein P would be present in their blood samples. Additionally, it was hypothesized that turtles of Lake Francis Case, being upstream, would have lower concentrations of Selenoprotein P than those of the 59 Mile Stretch due to the upstream spread of the zebra mussel population. The difference between our hypothesis and results indicates that there are other factors to consider.

It must be noted that absorbance values were used in data analysis rather than quantified concentrations. Rough estimates of the quantity differences signify a very low difference in concentration. The interpolated data gives a difference of less than 1ug/mL. However, the experimental assay's standard curve gave mixed results that did not allow direct usage of the trendline. Absorbance values are viewed as more reliable in this project. While unable to quantify the selenoprotein on a standard scale, the two sites could still be compared based on absorbance values.

There does appear to be a slightly higher concentration of selenium in false map turtles of Lake Francis Case. While this is a real difference, it may not be biologically significant. In prior selenium exposure experiments, turtles were exposed to selenium differing in far greater concentration levels to produce histological results. Haskins et al. (2018) used concentrations of 0 mg/kg, 15 mg/kg, and 30 mg/kg to determine negative biological effects. In other organisms,

between 1 and 5 mg/kg body weight are considered toxic (USDA 2018). Further studies must be completed to determine whether smaller differences in concentration would yield similar results.

Limitations:

One practical limitation of this study was the number of sites sampled. The Missouri River is a vast body of water and sampling all along it is not a capability possible in our lab due to funding and time. While three sites were originally chosen to be sampled, only two sites were viable due to turtles below the Oahe Dam being trap shy. However, we view that even with two sites, one upstream and one downstream, we were still able to sample and analyze data from a broad range of false map turtles within those two areas that can be used to generalize the populations along the Missouri River.

An additional limitation was blood processing in the field. Our team pulled blood samples with a syringe using heparin as an appropriate anticoagulant according to the ELISA assay protocol. However, the assay protocol outlines additional processing that includes centrifugation within the first 30 minutes of collection, and prompt assay or freezer storage of the samples. We did not have access to a centrifuge or freezer while out in the field. Our blood samples were not immediately centrifuged, but rather put on ice until they could be stored at the appropriate temperature back in the main lab several days later.

Next Steps:

With the additional data from future blood sample analysis, these results will be further examined through a linear regression comparing blood levels of bound selenoproteins to unbound selenium. To grasp the full picture of selenium transfer through trophic levels it would

also be beneficial to test zebra mussel samples for selenoproteins at the same sites. This would help researchers determine what amount of selenium exposure is due to their diet and what is simply due to being in contaminated water. While it is possible that zebra mussels are the main contributor, there may be additional sources.

The results of this project show that there are higher levels of selenoprotein in Lake Francis Case turtles. Our hypothesis that downstream reservoirs would expose false map turtles to more selenium was based on the assumption that the spread of zebra mussels was moving upstream through their own means as swimming juvenile larvae. However, recently the spread of zebra mussels has been documented across unconnected waterways and isolated lakes in areas as far out west in South Dakota as Lake Pactola (Whitney 2023). When a boat owner refrains from draining their boat, power washing it with hot water, and allowing it to completely dry for several days, zebra mussels can survive the trip to the next body of water (SD Least Wanted n.d.). One female zebra mussel can release over 1 million eggs per year, meaning mass contamination is possible with just a small population transferred (Whitney 2023). This could mean that the basis for which the hypothesis was set on is outdated and that the spread upstream is not as key an observation as once thought. Perhaps the invasive zebra mussels are all along the Missouri River with no greater or less contamination in any one area.

Additionally, the differences seen may be more so due to the flow of the site rather than whether it is a downstream or upstream site along the Missouri River. Turtles tested from Lake Francis Case were collected from areas with comparably stagnant water as it is a reservoir site. Turtles collected from the 59 Mile Stretch were collected from free-flowing riverine water. Zebra mussel populations may be better suited to rapid reproduction in the reservoir water when they aren’t swept away by the current. There is also much more vegetation available as a dietary

means at Lake Francis Case – selenium-accumulating plants may have also contributed to the high selenoprotein values in the false map turtles.

Another possible explanation for the variance in selenoprotein absorbance readings between Lake Francis Case and the 59 Mile Stretch is that the two reservoirs have different selenium concentrations present baseline in the water. The difference in flow of the two sites could impact the way selenium is deposited. Selenium may be less available as it flows through the 59 Mile Stretch. Actual water test results are still pending but research-driven assumptions can be made. Contributing factors for different absorbance readings might include natural and anthropogenic selenium sources.

Natural glacially deposited selenium present in soils is carried to waterways through seasonal flooding cycles and agricultural tiling (Pracheil et al. 2010). This can be further exacerbated by the introduction of selenium as a mineral in livestock feeds. Selenium is an essential nutrient in trace quantities for many organisms. Many selenoproteins play important roles in reproduction, thyroid hormone metabolism, DNA synthesis, and protection from oxidative damage and infection. The suggested intake of selenium in humans is ~200 µg/day, but just a marginally higher intake of >400 µg/day on a regular basis is considered toxic (Benis et al. 2022). Many livestock owners recognize the benefits of dietary selenium and introduce small amounts of this metal into feed for their animals for efficient digestion and enzyme production. The addition of selenium in mineralized feed contributes to more selenium being washed away through the tiling systems.

Again, elevated levels of selenium result in deleterious effects so cattle raisers use a sort of Goldilocks approach – not too much, not too little. Selenium-accumulating plants grow in areas with soil rich in selenium; they are found throughout South Dakota (USDA 2018).

Several studies have suggested that ingestion of high selenium concentrations in cattle are the cause of negative conditions like alkali disease, which is characterized by lack of energy, anemia, sloughing of hooves, damage to keratinized tissues, and lameness (Daniels 1996)(Haskins et al. 2018). These conditions are much like those seen in the experimentally exposed yellow-bellied slider turtles that had negative histological effects in their claws, kidneys, and muscles (Haskins et al. 2018).

Still, selenoprotein content could be amplified by zebra mussels's ability to filter and therefore transfer selenium to predators. It is hard to say what the dietary makeup of each sampled site is without having done a lavage test. While the Kerby lab has determined that false map turtles ingest zebra mussels in both the lab and the wild, future studies could focus on determining whether turtles prefer to eat zebra mussels. The variability between the two sample sites could be due to dietary preferences at each site. It would be interesting to see if the turtles have a sense for the toxicity that the zebra mussels may be transmitting to them and whether that has an impact on the downstream turtles who have had more experience with zebra mussel populations.

Supporting evidence of this trophic transfer in false map turtles could mean detrimental effects to other organisms in aquatic ecosystems along the Missouri River and beyond. Many aquatic reptiles have higher life expectancies, meaning their exposure to selenium could be longer lasting in comparison to other vertebrates (Haskins et al. 2018). Studies have found that oviparous organisms appear to be most sensitive to selenium toxicity, particularly through their prey items. Reptiles, amphibians, and fish exposed to elevated levels of selenium experience histopathological abnormalities, reduced egg viability and hatching rates, and maternal transfer of organic selenium (Janz et al. 2010). Oviparous organisms make up most of the species

diversity along the Missouri River, meaning the whole ecosystem is at high risk of selenium toxicity.

More work must be done to understand the reason for variance in selenoprotein absorbance values between tested sites and what impact the invasive zebra mussels truly have on other marine inhabitants. In providing a relationship between threatened false map turtles and the zebra mussels species, we can further understand the impacts of invasive species compounding with human activity that releases heavy metals to our Missouri River ecosystems.

APPENDIX

Figure 1: Map of chosen sampling sites along the Missouri. From upstream to downstream: Lake Sharpe, Lake Francis Case, 59 Mile Stretch. Note: only Lake Francis Case and the 59 Mile Stretch were sampled

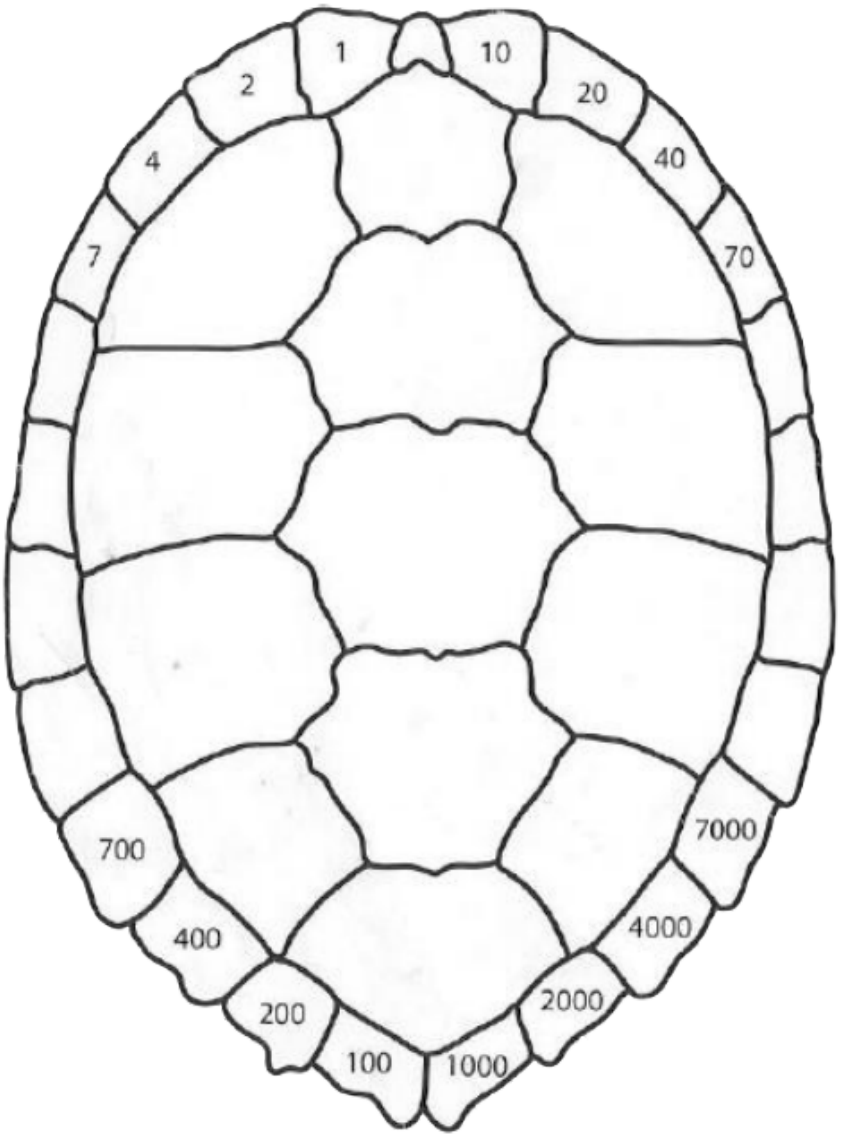

Figure 2: Diagram of the numerical coding system used to notch individuals to assign them an special identification number (Ernst et al. 1974).

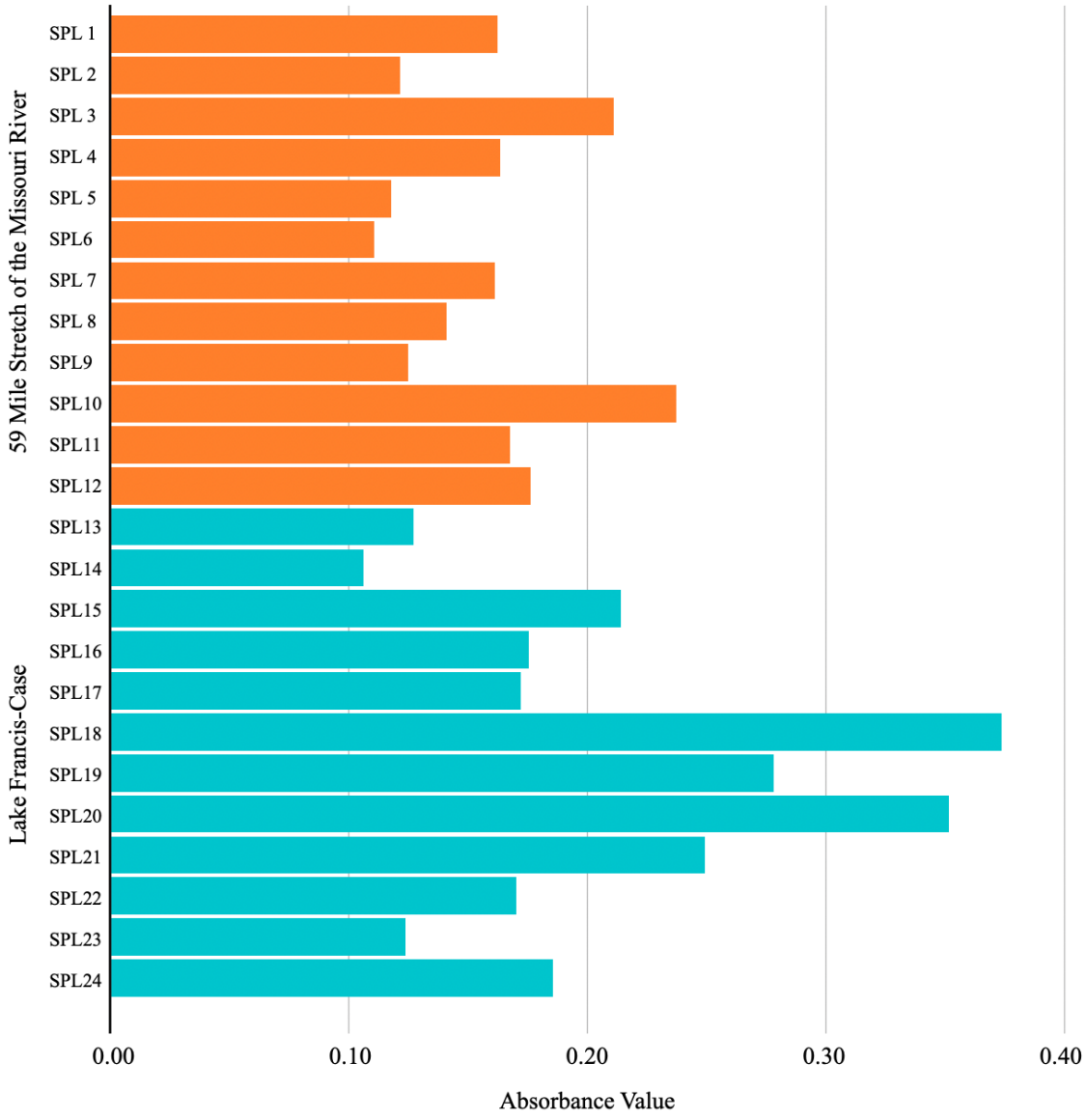

Figure 3: Bar graph comparing absorbance values of each turtle from the two sites. The difference between the means of the two group's absorbance is 0.052.

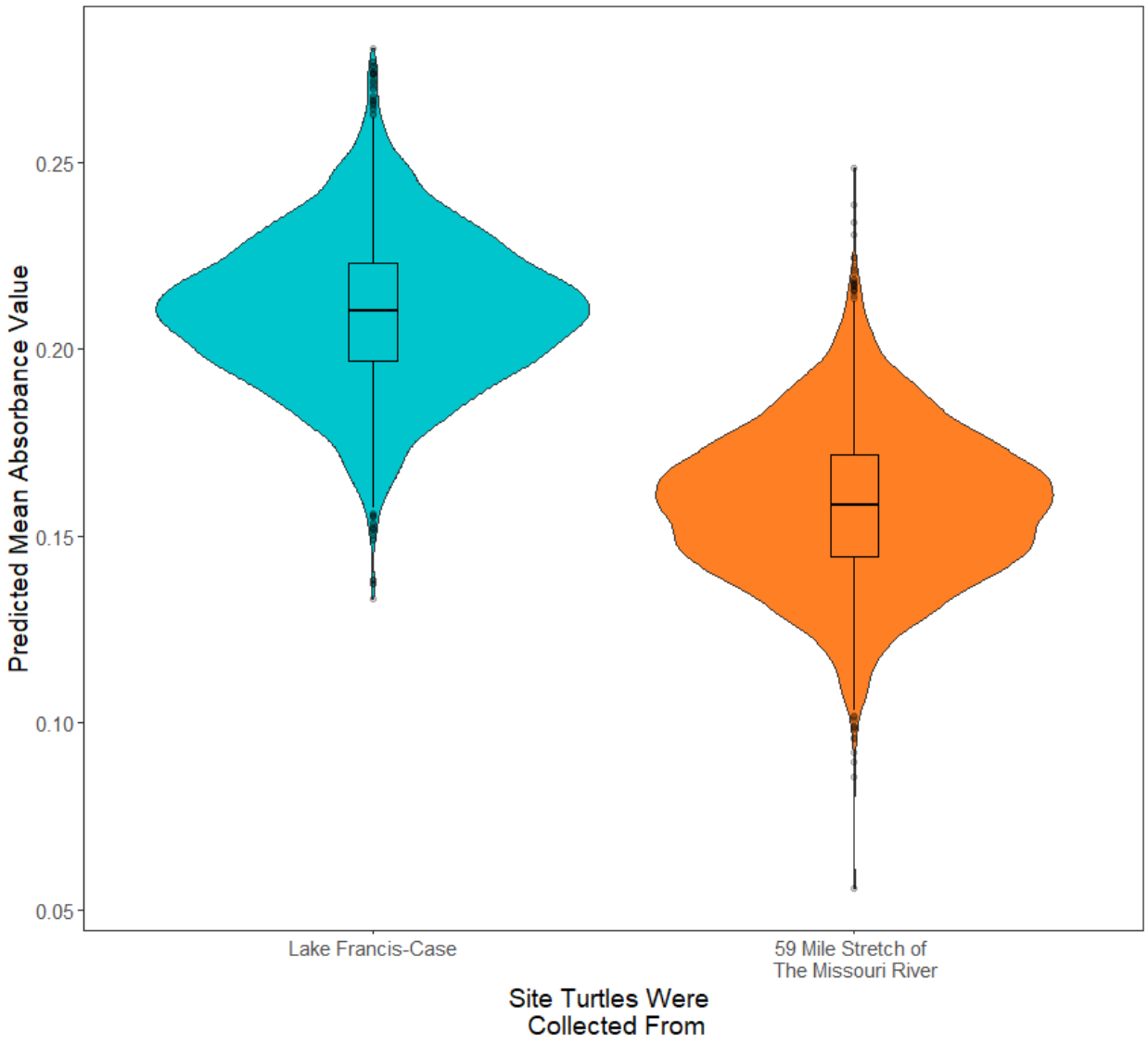

Figure 4: Bayesian plot comparing absorbance value means from the two sites. There is a 96.3% probability that Lake Francis Case absorbance values are higher than the 59 Mile Stretch of the Missouri River absorbance values. The difference between the means of the two group's absorbance is 0.052.

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