ADAPTIVE TOLERANCE TO ZINC IN FRESHWATER SNAILS (*PHYSA ACUTA*) ACROSS A CONTAMINATION GRADIENT

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Abstract: The Tri-State-Mining district is an area of northeast Oklahoma, southwest Missouri, and southeast Kansas in which zinc and lead mines operated for over 100 years. Metal contamination from wastes left behind by these historic mining operations has polluted the Neosho and Spring Rivers, the Grand Lake o' the Cherokees, and resulted in the EPA designating Tar Creek a superfund site in 1983. The receiving watershed has a gradient of contamination from likely toxic concentrations of zinc to background concentrations. The purpose of this study was to determine if native populations of freshwater snails have developed tolerance to environmental metal concentrations and, if present, the extent of the metals tolerance across a downstream gradient from the metals-contaminated area. Snails (*Physa acuta*) were collected from sites representing the gradient of metals contamination and field sediment and water samples were analyzed for zinc. These populations were cultured in the lab and zinc toxicity tests were conducted with F1+ juveniles collected from those cultures. Snails cultured from populations collected from contaminated, upstream sites were more tolerant to zinc exposure than snails cultured from populations collected from clean, downstream sites. Additionally, zinc tolerance was found in snails collected from a site that represented a midpoint geographically, although environmental zinc levels were below levels likely to cause toxicity. My results suggest that, despite past studies showing sediments from Grand Lake to be relatively nontoxic to sediment-dwelling organisms due to low bioavailability; aquatic organisms may still be experiencing physiological stress and selective pressures because of metals contamination.

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TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

History of the Tri-State Mining District

The Tri-State Mining District (TSMD) is an area of northeast Oklahoma, southwest Missouri, and southeast Kansas in which lead and zinc mining began in the 1850s. Mining reached its peak in 1925 and slowly declined after the 1950s until the abandonment of the mines in the mid-1970s (Andrews et al. 2009). The Oklahoma portion of the TSMD is called the Picher Mining District and includes portions of Ottawa County. The TSMD drains into the Neosho and Spring Rivers, which meet at the northern end of the Grand Lake O' the Cherokees; a reservoir created and managed the Grand River Dam Authority (GRDA) (Figure 1). TSMD mining operations resulted in extensive heavy metals contamination, specifically zinc, lead, and cadmium, with zinc being the metal found in greatest concentration. The three metals are typically found in mixture with a Zn:Pb:Cd ratio of 100:15:1.5 in sediments, making Zn and Pb the metals of greatest environmental concern to aquatic invertebrates (Morrison et al. 2019). Highest levels of contamination have been found in the northeast portion of the district, specifically in Tar Creek, which was declared an EPA Superfund Site in 1983. Two cities in the superfund site, Cardin and Picher, OK, were abandoned as a result of lead contamination and extensive undermining (US EPA 2018).

Metal contamination in the TSMD is primarily the product of massive piles of mining waste on the surface called "chat" as well as water rising from flooded mining tunnels (Johnson et al. 2016). Metals in these chat piles and abandoned mining sites may be dissolved and transported by flooding and runoff into the streams and river systems of the TSMD where they bind with sediments (Andrews et al. 2009). Areas with high levels of contamination correlate geographically with disposal sites for mine tailings, and terrestrial contamination is largely confined to those lands used for this purpose (Johnson *et al*., 2016). Funds intended for remediation of the area have historically been diverted to relocate residents, resulting in slower remediation and recovery than at other Superfund sites (Johnson *et al.*, 2016). However, metal contamination levels have been decreasing over the decades with measurements of metal concentrations taken from Tar Creek in Miami, OK in the mid-2000s being significantly lower compared to those taken from the same site in the mid-1980s (Andrews and Masoner 2011). Zinc, the primary contaminant and product from historic mining operations, had aqueous concentrations in the mid-2000s about 10-fold lower compared to the mid-1980s (~2,000 µg/L compared to ~20,000 µg/L) (Andrews and Masoner 2011).

Previous Studies in the Tri-State Mining District

Previous studies on the environmental effects of the TSMD metals contamination have demonstrated the potential for widespread adverse environmental impacts. Elevated levels of Pb, Cd, and Zn have been found in crayfish (*Orconectes spp*.) and fish (white crappie, *Pomoxis annularis*; common carp, *Cyprinus carpio*; channel catfish, *Ictalurus punctatus*; flathead catfish, *Pylodictis olivaris*; spotted bass, *Micropterus punctulatus*; and largemouth bass, *M. salmoides*) in the tributaries of the Spring River north of Grand

Lake (Schmitt et al. 2006). It was shown that the levels found in the most contaminated organisms could pose some risk of Pb and Zn toxicity to small wetland birds such as red-winged blackbirds (*Agelaius phoeniceus*), killdeer (*Charadrius vociferous*), or spotted sandpiper (*Actitis macularia*). Diets consisting entirely of the most contaminated fish or invertebrates would exceed NOAEL (no observed adverse effect level) values for daily consumption for lead by 10% and zinc by 740%, indicating harmful effects were possible (Schmitt *et al.*, 2006). Pb has also been identified as having pronounced effects on the activity of the enzyme d-aminolevulinic acid dehydratase (ALA-D) which has long been used as a biomarker for lead exposure. Inhibition of ALA-D can lead to anemia and oxidative stress. ALA-D was found to be inhibited by as much as 50% in channel catfish (*Ictalurus punctatus*) taken from contaminated sites in the Spring and Neosho Rivers (Schmitt *et al.*, 2005). Similar inhibition of ALA-D, as well as external and internal indicators of Pb and Zn poisoning, has also been documented in native passerines and waterfowl (Canada goose, *Branta canadensis*; and ducks, *Anas spp*.) in the TSMD (Beyer et al. 2004).

Despite elevated levels of heavy metals being found in some sediments, toxicological studies with sediment samples taken from Grand Lake have not been shown to cause significant harm to sediment-dwelling organisms. In toxicity tests with amphipods (*Hyalella azteca*), no significant correlations were detected between toxicity end points and metal concentrations found in Grand Lake sediments, despite 78% of sediment samples having metal concentrations that exceeded sediment quality guidelines (SQGs) for probable adverse effects (Ingersoll *et al.*, 2009). General SQGs consist of two thresholds to evaluate the potential risk posed by sediment-bound metals. The threshold

effect concentration (TEC, 121 mg/kg Zn) is the concentration below which adverse effects are not expected. The probable effect concentration (PEC, 459 mg/kg Zn) is the concentration above which adverse effects are considered likely to occur. Additionally, disturbance of shallow sediments typical of anthropogenic land and water use has not been shown to significantly increase the toxicity of metals-laden sediments (Morrison *et al*., 2013). This work has suggested that the risk of acute toxicity to sediment-dwelling organisms may be minimal, largely due to the lack of bioavailability of the metals and particular water chemistry of the watershed, but the effects of chronic or historic exposure to toxic metals are still unclear.

Adaptive Tolerance to Metals

Aquatic organisms living in polluted areas may develop adaptations that allow them to tolerate low levels of toxic metals or other contaminants in the environment (Blankespoor et al., 1985; Klerks & Weis, 1987; Lefcort et al., 2004; Luoma et al., 1983; Minghetti et al., 2008; Nevo et al., 1986). Such adaptations could manifest as inherited resistance, physiological response to the contaminant, or behavior. Adaptive tolerance to metals has been documented in bacteria, algae, annelids, molluscs, arthropods, and fish (Klerks and Weis 1987). For example, in grove snails (*Cepaea nemoralis*), biomineralization of zinc into shells has been correlated with environmental metals concentrations (Jordaens et al. 2006) and similar incidences of biomineralization have been proposed to be a mechanism of tolerance to metals contamination in garden snails (*Helix aspersa*) (Newman et al. 1994). Of particular interest in the case of the TSMD is whether populations of organisms that have experienced chronic or historic metal exposures are developing tolerances to the contaminant metals.

Purpose of Study

The objective of this study was to determine if native populations of *Physa acuta* have developed tolerance to environmental metal concentrations and if so, the extent of the metals tolerance along a downstream gradient from the metals-contaminated area. The presence of tolerance in moderately contaminated areas can provide insight into the historical and current extent of bioavailable metals contamination. The approach was to utilize a common freshwater snail species by collecting populations from sites throughout the study area and testing each population for chronic effects of zinc exposure. Zinc was chosen as the likely driver of toxicity in the system. Snail collection sites were chosen to reflect the expected gradient of contamination with the highest levels of metals contamination found upstream in Tar Creek and the lowest levels of metals contamination found downstream in Grand Lake, Spavinaw Lake, and Lake Hudson (Figure 1). Snails were cultured to establish lab-grown populations representing each collection site. Zinc toxicity tests were performed to determine tolerance to metals exhibited by each population, tracking mortality, egg mass production, and mass of the snails. Water and sediment samples from each collection site were analyzed so the tolerance to zinc of each population could be related to the zinc concentrations at collection sites.

CHAPTER II

MATERIALS AND METHODS

Materials

All acids used were trace metal grade and purchased from Avantor (Center Valley, PA). Zinc sulfate heptahydrate (≥99% purity) was purchased from Sigma-Aldrich (St. Louis, MO). An ICP-OES (iCAP 7400; Thermo Scientific, Waltham, MA) was used for all metals analysis. Calibration standards included a multi-element standard (CPI International, Santa Rosa, CA) and an internal yttrium standard (Peak Performance Inorganic, Yt Standard, CPI International, Santa Rosa, CA). All analytical reagents were trace metal grade. Water used for snail cultures, toxicity tests, and zinc treatment solutions was Oklahoma State University tap water, which originated from Lake Carl Blackwell, and was carbon filtered prior to use. Zinc concentration in tap water was detectable but < 33 µg/L (Limit of Quantitation). Ultra-pure water used for chemical analysis was created via a Millipore Integral water system (MilliporeSigma, Burlington, MA).

Study Species

Experiments were conducted with lab-cultured populations of *P. acuta* collected from experimental sites. *P. acuta* (Draparnaud, 1805) is a common freshwater snail

also commonly known as the tadpole snail or acute bladder snail. *P.acuta* is ubiquitous and reported to occur in North America, Europe, Africa, Australia, and Asia (Dillon et al. 2002; Paraense and Pointier 2003).

Animal and Sample Collection

Snails and sediment and water samples were collected from sites selected from portions of Grand Lake, Lake Hudson, Spavinaw Lake, and Tar Creek. The northernmost collection site was on Tar Creek at the Oklahoma-Kansas border while the southernmost collection site was at the southern end of Hudson Lake. Collection sites (n=7) were chosen for their expected levels of contamination, accessibility, and presence and successful development of snail laboratory cultures from the site. All collection sites were within walking distance (<50m) of roads, parking areas, or locations otherwise accessible via vehicle. However, these areas had low levels of urbanization and traffic and thus metals contamination was presumed to come from the TSMD. All snails were collected from sites in shallow water (<0.5m depth), typically on rocks, dead leaves, or other detritus.

Snails were placed in mason jars filled with water taken from the collection site. Sediment and water samples were collected in separate 50 mL centrifuge tubes. All snails and environmental samples from a single site were collected within a 10m diameter area. All samples and snails were refrigerated in a cooler with ice packs transferred to Oklahoma State University within 48 hours. Snails from each collection site were then transferred to individual culture tanks specific to their collection site. Water and sediment

samples were frozen (-40 °C) for storage before analysis. The number of snails collected from each collection site averaged 27 per collection site, ranging from 18 to 38.

Animal Husbandry

Snails were cultured in approximately 25-30 L of water in tanks lined with crushed coral (Caribsea Geo-marine Florida Crushed Coral, Fort Pierce, FL). Tanks were kept at room temperature (27 ºC) and on a 15:9h light:dark photoperiod. Water in the tanks was continuously aerated and water changes performed every 2 weeks. Snails in culture tanks were provided algae wafers (Hikari USA, Hayward CA) three times per week. Prior to the initiation of toxicity testing, each snail culture was allowed to reproduce to establish a population sizable enough to sustain harvests for testing; typically a minimum of 3-4 months. All snails used in testing were offspring or later generations descended from the collected snails.

Zinc Treatments

A zinc treatment stock solution was created by dissolving zinc sulfate heptahydrate and 1 mL of 20% nitric acid solution into 200 mL of carbon-filtered water yielding a concentration of 2.7 mg Zn/mL. This stock solution was then diluted in carbon-filtered water to create spiking solutions. To obtain treatment concentrations, 1 mL of the corresponding spiking solution was added to 800 mL of carbon-filtered water contained in the test chamber at the initiation of each test and after water changes. Nominal concentrations of Zn were 200, 400, 800, 1600, and 3200 µg/L respectively.

Toxicity Tests

Toxicity tests consisted of 5 treatments and a negative control. Each treatment was replicated 4 to 5 times. Test tanks (experimental units) were plastic containers with approximately 50% of their bottom thinly lined with crushed coral and filled with 800 mL of carbon-filtered water. Each test tank was stocked with 5 snails. The mean initial mass of all snails used was 28.74±7.62 mg. Each tank received 50 mg of crushed algae wafers daily. Water in test tanks was changed and zinc renewed every 72 hours. Tests were concluded on day 21.

Replicates were staggered due to the large number of snails and replicates required for testing, the availability of lab space, and time requirements for setting up and maintaining toxicity tests. Due to these considerations and the unpredictable maturation of snail cultures, a randomized block design was not possible. Each replicate consisted of a single tank for each treatment and the control for a given snail population (six tanks in total). For each replicate, snails were sorted by mass and assigned to tanks so that the mass of snails between tanks was similar. Replicates were staggered as necessary to account for available laboratory space and the availability of a sufficient number of individuals from the snail cultures (30 snails of appropriate size per replicate).

Data Collection

All snails were weighed (±0.01 mg) using a Mettler Toledo Xs205 balance at the initiation of each toxicity test and surviving snails were weighed at the termination of each test. The number of living and dead snails was documented every 72 hours. Egg masses laid on the sides of the tank were counted, marked, and photographed on days 6,

12, 18, and 21. Egg masses laid on the coral substrate or on the shells of snails were not counted or photographed. Marking egg masses on the side of the tank ensured that no egg mass was counted twice. At the conclusion of each test, surviving snails were weighed and, along with the shells of deceased snails, collected and frozen (-40 C). Approximately 10 mL of sample water were collected from each test tank at the conclusion of the test, acidified, and stored at room temperature in a 15 mL centrifuge tube for analysis. A subset of these samples representing approximately 1/3 of all tests run were analyzed via USEPA method 6010B (USEPA SW-846, 2018) to quantify zinc recovery and compare to nominal concentrations.

Quality Control

Temperature, pH, and dissolved oxygen (DO) data were collected throughout the duration of the experiment. The temperature of the room in which culture tanks and experimental tanks were kept was documented on one day of each week. One measurement of pH and DO was collected from at least 4 tanks each week using Vernier LabQuest pH and DO probes.

Field Water and Sediment Collection and Analysis

Water samples were collected at each snail collection site in 50 mL centrifuge tubes. Samples were refrigerated at the time of collection and transported back to Oklahoma State University and frozen at -40 ºC. Samples were thawed and acidified with 20% nitric acid solution at a 1mL acid:10 mL sample ratio prior to analysis via ICP-OES using USEPA method 6010D (USEPA 2018).

Sediment samples were collected at each snail collection site in 50 mL centrifuge tubes. Samples were frozen at -40 ºC. Prior to digestion, samples were homogenized with an acid-rinsed spatula. EPA method 3050B was followed to accomplish sediment digestion (USEPA 1996). Analysis was performed via ICP-OES with USEPA method 6010D (USEPA 2018).

Statistical Analysis

Snail survival days represented the mean number of days survived by each snail in a tank and was calculated by tracking the number of living snails in each tank on 7 days corresponding with water changes and egg mass counts and at the conclusion of the experiment (days 3, 6, 9, 12, 15, 18, and 21). The number of all observations of living snails was summed, multiplied by 3 to account for the interval between observation events, and 1.5 days was added for each deceased snail to mitigate underrepresentation of survival caused by the 72-hour observation interval. This value was then divided by 5 to arrive at a mean survival days per snail. The formula for calculating survival days is below. In the formula, n = snails observed alive at each observation (i) and x = the number of deceased snails on day 21.

$$Survival\ days = \frac{3\left(\sum_{i=1}^{7} n_i\right)+1.5x}{5} \qquad \text{Equation 1}$$

In addition to counting the total number of egg masses produced, reproduction was quantified by combining survival days and egg mass production data to create a value for daily egg masses produced per capita. This serves as a measure of zinc inhibition on reproductive output, uncoupled from increases in mortality, which would

affect overall egg mass production. I calculated this value by dividing the total egg masses produced by the survival days to create a new unit; daily egg masses per capita.

$$Daily\ egg\ masses\ per\ capita = \frac{egg\ masses\ sum}{lifespan} \qquad \text{Equation 2}$$

Growth for each tank was quantified by dividing the mean mass of surviving snails at the conclusion of each toxicity test by the mean mass of the snails at the initiation of that toxicity test and expressing this as a percentage change using the following formula:

$$Growth = \frac{mean\ final\ mass}{mean\ initial\ mass} * 100 \qquad \text{Equation 3}$$

Baseline similarities among collection sites were verified by using one-way ANOVAs to compare the effect of each collection site on survival days, reproduction, and growth in experimental control tanks (0 µg/L Zn). The effect of zinc treatment and collection site on survival days, reproductive output, and growth were evaluated with two-way analysis of variance (ANOVA) with interaction and a Tukey's Honestly Significant Differences test (α=0.05) as a post hoc test using JMP Pro®, Version 13 (SAS Institute Inc., Cary, NC 1989-2019). Each two-way ANOVA included collection site and zinc treatment as independent variables as well as their interaction.

Data were examined for normality via the Shapiro Wilk W test and homogeneity of variance via Levene's test. Violations of the assumptions of normality and homogeneity of variance were detected in the growth data. To address these issues, a square root transformation was performed prior to analysis for the two-way ANOVA. A constant value of 10 was added to all data prior to transformation to account for the

presence of negative values (the lowest being -8.5) (McDonald 2014). Transformation was not deemed necessary for any other analyses.

Data were also examined for a post-hoc temporal blocking effect by grouping toxicity tests into blocks based on start-date such that toxicity tests beginning in January or February were pooled into a block and subsequent tests were pooled into their own corresponding two-month blocks, resulting in a total of 6 blocks. The final block consisted only of tests beginning in November because no tests began in December. A one-way ANOVA detected significant differences in the means between blocks for all endpoints; survival days ($p < 0.0028$), egg sum ($p < 0.001$), eggs per capita ($p < 0.001$), and growth ($p < 0.001$) (See Appendix C).

Concentration-response relationships were further assessed for each site by calculating the lowest observed effective concentration (LOEC) and regression analysis. LOECs were determined using one-way ANOVAs for zinc concentrations within each collection site followed by a Dunnett's test to determine which treatments significantly differed from within-site controls ($\alpha=0.05$). The lowest concentration that was significantly different from the controls was the LOEC. Regressions using log concentration were performed to further explore the extent to which zinc exposure could explain changes in endpoints within each site and assess sensitivity of sites. Nonlinear log (inhibitor) vs. response (three parameters) regressions were used when possible. If a non-linear fit failed to converge, a linear regression was applied instead. Regressions were performed with GraphPad Prism, Version 7 (GraphPad Software, Inc., La Jolla, CA).

CHAPTER III

RESULTS

Field Water and Sediment

Elevated levels of zinc were found in either the water or sediment in the 3 northern-most collection sites: TC Border, TC Miami, and Twin Bridges (Table 1). The highest concentrations of zinc were found at TC Border, the second highest was TC Miami, and the third was Twin Bridges. This pattern followed the gradient of contamination that I had anticipated. Sediment zinc concentrations at the two Tar Creek sites were 17 times higher than the sediment concentration at Twin Bridges. Twin Bridges sediment zinc concentration was 5 to 14 times higher than sediment zinc concentrations found in sites further south (Table 1). TC Border had 3.5 times more aqueous zinc than TC Miami which in turn had 5.4 times aqueous zinc than Twin Bridges. The other four collection sites did not have concentrations of zinc in either water or sediment samples that exceeded environmental criteria to suggest concern. No sites south of Twin Bridges had detectable levels of aqueous zinc (Table 1; Figure 1).

Toxicity Tests

Quality Control

Zinc recovery at the conclusion of toxicity tests showed a reduction in aqueous

zinc concentration when compared to nominal treatment concentrations. Mean measured concentrations of zinc at the end of tests for controls, 200, 400, 800, 1600, and 3200 µg/L zinc treatments were < 11, < 33, 69, 194, 433, and 1222 µg/L zinc, respectively. I found initial zinc treatment concentrations to be within 4% of nominal values and zinc was undetected in controls (<11 µg/L). I found evidence of rapid reductions in aqueous zinc over the course of the toxicity test with a loss ranging from >83% to 62% compared to nominal concentrations after 72h with about half of that loss occurring after 6h. (Table 2). Average temperature of the room in which the toxicity tests were performed was 26.5 ± 1.4 °C, pH averaged 7.8 ± 0.8, and DO averaged 7.6 ± 0.8 mg/L.

Baseline similarities among populations

There were no significant differences in survival days, total egg masses, daily egg masses per capita, or growth among collection sites in experimental control tanks (0 µg/L Zn). Control mortality averaged 23% (±16%) at the conclusion of the 21d test across all collection sites ranging from a mean of 10% (HL South) to 30% (TC Border) (ANOVA, $p = 0.5534$). This mortality translated to an average survival days of 18.6d (±2d) ranging from a mean of 16.9d (Spavinaw State Park) to 19.6d (HL South) (ANOVA, $p = 0.5204$). Total egg mass production averaged 46 egg masses (±13) laid in each test tank over 21d in controls (ANOVA, $p = 0.916$). Daily egg masses laid per capita averaged 2.5 (±0.7) (ANOVA, $p = 0.9025$). Growth averaged 288% (±126%) increase in mass (ANOVA, $p = 0.8031$).

Survival Days

Collection site ($p < 0.001$) and zinc treatment ($p < 0.001$) both had a significant effect on survival days and the interaction between collection site and treatment was not significant ($p = 0.1563$) across all zinc concentrations. Spavinaw State Park snails were shortest-lived (mean survival days 13d) but did not differ significantly from HL North (15d) or HL South (15.1d) snails (Table 3, Table 4). The longest-lived snails were from the TC Border population (17.5 survival days), living an average of 34% longer than Spavinaw State Park snails (Table 4). Adverse effects on survival days were observed at 1600 and 3200 µg/L zinc treatments (Table 3). Compared to controls, survival days was reduced by 21% in the 1600 µg/L zinc treatment and by 28% in the 3200 µg/L zinc treatment (Table 5).

When evaluating each population independently, zinc exposure resulted in a significantly reduced survival days at only the two southern-most sites at any concentration: Spavinaw State Park ($p = 0.008$) and HL South ($p = 0.0082$) both with an LOEC value of 3200 µg /L zinc (Dunnett's, $p = 0.0016$ and $p = 0.0012$, respectively) (Figure 2). Despite not having a significant interaction in the overall statistical model, differences among sites were primarily only observed at the high zinc concentrations.

Populations from the TC Border and Twin Bridges had survival days greater than 18 days at the highest zinc treatment concentration, which was very similar to controls, and TC Miami and GL Bernice had survival days greater than 15 days while populations from the 2 southern-most sites had mean survival days of less than 7.5 days (Figure 3). Based on regressions, zinc treatments could account for 25% to 52% of the variation in survival days in HL North, Spavinaw State Park, and HL South snails ($R^2 = 2535$; $R^2 = 0.518$; and $R^2 = 0.4759$, respectively). Zinc treatments could explain only 1% to 16% of variation in TC Border, TC Miami, Twin Bridges, and GL Bernice snails ($R^2 = 0.0104$, $p = 0.6353$; $R^2 = 0.1575$; $R^2 = 0.9847$; and $R^2 = 0.1486$, respectively) (Figure 3).

Reproductive Output

Both collection site ($p < 0.001$) and treatment ($p < 0.001$) had a significant effect on total egg mass production and the interaction between collection site and treatment was not significant ($p = 0.3915$) across all zinc concentrations. Among collection sites, mean total egg mass production was greatest in TC Border snails (mean egg masses sum 46.2). Snails from HL South (23.8) produced the fewest total egg masses on average, followed by those from Spavinaw State Park (26.3), producing 48% and 43% fewer egg masses than TC Border, respectively (Table 4). The 5 northern-most sites did not significantly differ from one another in total egg mass production (Table 3). Adverse effects on total egg mass production were observed at zinc concentrations of 1600 and 3200 µg/L (Table 3). Compared to controls, the total number of egg masses laid was

reduced by 36% in the 1600 µg/L zinc treatment and by 53% in the 3200 µg/L zinc treatment (Table 5).

Evaluating within-site differences among zinc treatments showed there were significant differences in total egg mass production at both Hudson Lake sites across zinc treatments: HL North (ANOVA, $p = 0.0463$) and HL South (ANOVA, $p = 0.0108$) and the LOEC was 3200 µg/L zinc at both of these sites (Dunnett's, $p = 0.0125$ and $p = 0.004$, respectively) (Figure 2). Although there was no significant interaction between collection site and zinc treatment in the overall model, differences among sites were primarily only observed at the high zinc concentrations. At the highest zinc treatment, snails from TC Border produced 43.8 egg masses, TC Miami 32.4, Twin Bridges 33.8, GL Bernice 21.5, HL North 9.8, Spavinaw State Park 5.8, and snails from HL South produced zero egg masses.

Based on regressions, zinc treatments could explain 21% to 52% of the variation in total egg mass production in the four southern-most sites (GL Bernice, $R^2 = 0.2138$; HL North, $R^2 = 0.2633$; Spavinaw State Park, $R^2 = 0.3723$; HL South, $R^2 = 0.5154$). In all other, contaminated sites, zinc treatments could explain < 1% to 3% of variation (TC Border, $R^2 = 0.0003$, $p = 0.9311$; TC Miami, $R^2 = 0.03495$; Twin Bridges, $R^2 = 0.0213$, $p = 0.4963$) (Figure 4).

Both collection site ($p < 0.001$) and zinc treatment ($p < 0.001$) had a significant effect on daily egg masses per capita and the interaction of collection site and treatment

was not significant ($p = 0.5255$). Snails collected from the southern-most site, HL South, produced the fewest egg masses per capita (1.4); significantly (42%-48%) fewer than those collected from TC Border (2.7), Twin Bridges (2.4), and GL Bernice (2.5) (Table 3, Table 4). Adverse effects on daily egg masses laid per capita were observed 3200 µg/L zinc treatment (Table 3). Compared to controls, daily egg masses laid per capita was reduced by 48% in the 3200 µg/L zinc treatment (Table 5).

Evaluating treatment differences within each site showed there were significant differences in daily egg masses per capita only in snails from HL South (ANOVA, $p = 0.0129$) with an LOEC of 3200 µg /L (Dunnett's, $p = 0.015$) (Figure 2) . Based on regression, zinc treatments could explain 11% to 49% of the variation in daily egg masses per capita in the four southern-most and clean sites (GL Bernice, $R^2 = 0.1124$; HL North, $R^2 = 0.1599$; Spavinaw State Park, $R^2 = 0.2839$; HL South, $R^2 = 0.4907$). In the three contaminated sites, zinc treatments could explain less than 1% of variation (TC Border, $R^2 = 0.0028$, $p = 0.8072$; TC Miami, $R^2 = 0.0092$, $p = 0.6143$; Twin Bridges, $R^2 = 0.0048$, $p = 0.7478$) (Figure 5).

Growth

Collection site ($p = 0.0122$) and treatment ($p < 0.001$) both had a significant effect on growth, but the interaction of collection site and treatment was not significant ($p = 0.9946$). Snails from the two southern-most collection sites, Spavinaw State Park and HL South, grew the least with an average increase in mass of 190.1% and 197.3%,

respectively (Table 4). Snails from two zinc-contaminated sites, TC Border and Twin Bridges, had the greatest growth with an increase in mass of 267.6% and 293.2%, respectively (Table 4). Snails from the two southernmost sites experienced 26%-35% less growth than those from TC Border or Twin Bridges (Table 4). Adverse effects on growth were observed at 1600 and 3200 µg/L (Table 3). Compared to controls, growth was reduced by 38% in the 1600 µg/L zinc treatment and by 44% in the 3200 µg/L zinc treatment (Table 5).

Comparing the effect of zinc treatments within each population found no evidence of differences in mean growth between treatments at any of the collection sites. The coefficients of determination for regressions with growth did not closely correspond to relative site geography or environmental zinc concentrations as it had with other endpoints. Zinc treatments could explain 8% to 32% of the variation in growth in snails from TC Border, Twin Bridges, HL North, Spavinaw State Park, and HL South (R^2 = 0.1415; R^2 = 0.08165; R^2 = 0.245; R^2 = 0.09644; R^2 =0.3238, respectively)). In TC Miami and GL Bernice snails, zinc treatments could explain only 2% of variation (R^2 = 0.0219, p = 0.4441; and R^2 = 0.01701, respectively) (Figure 6)

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CHAPTER IV

DISCUSSION

Environmental Zinc Concentrations

The highest levels of zinc were found in the northernmost collection sites (TC Border, TC Miami, and Twin Bridges), with a clear gradient of lower environmental zinc concentrations as one moves south through the Tar Creek and Grand Lake watershed. This is consistent with environmental concentrations reported in previous studies (Andrews et al. 2009; Ingersoll et al. 2009; Andrews and Masoner 2011; Johnson et al. 2016; Morrison et al. 2019). Of the sites from which snails and environmental samples were collected, Tar Creek is the closest in proximity to historic mining sites and the presence of mining waste piles, locally referred to as "chat". Previous studies have established that metals contamination is closely correlated with the presence of these piles of mining waste (Andrews et al. 2009; Johnson et al. 2016). Zinc was not detected in water samples taken from any sites south of Twin Bridges (Table 1). Sediment and water concentrations found at Twin Bridges were similar to those reported by Ingersoll et al. in 2009; a mean of 807 mg/kg zinc sediment concentration and 72.1 μg/L zinc water concentration (Ingersoll et al. 2009). South of this location, the mine drainage from Tar Creek and the Neosho and Spring Rivers enters Grand Lake.

In addition to being a greater distance from the contamination source, the lake provides the conditions for dilution and reduced stream flow. This is consistent with previous findings that aqueous metals contamination in the TSMD is highly correlated with stream flow, likely due to resuspension of metal particulates bound to sediment (Andrews et al. 2009). Sediment concentrations of Zn were highest at the TC Border and TC Miami collection sites and surpassed the general PEC of 459 mg/kg (MacDonald et al. 2000) by more than 21 fold. They also surpassed the TSMD-specific PEC of 2083 mg/kg (MacDonald et al., 2009) by more than 4.5 fold, suggesting likely impacts even when the ameliorating effects of local water chemistry are considered. The Twin Bridges sediment sample surpassed general PECs but not TSMD-specific PECs suggesting that, although zinc levels are elevated, they are not high enough to cause adverse effects. All sites south of Twin Bridges had concentrations below general TEC, defined as 121 mg/kg zinc (MacDonald et al. 2000) and may be regarded as uncontaminated or clean sites.

Toxicity Tests and Evident Zinc Tolerance

My experiments produced LOECs of 3200 µg/L Zn in snails from the 3 southern-southern sites (Figure 2). Past studies have largely focused on the acute toxicity of zinc, making direct comparisons to my data difficult. Hoang and Tong (2015) compared acute toxicity data for numerous snail species, two of which belonged to the genus Physa (*P. gyrina* and *P. heterstropha*) and they both had 96h LC50 values of about 3400 µg/L Zn. This is similar to my highest concentration zinc treatment. Given the 21d duration of my toxicity tests and the reductions in dissolved zinc concentrations between each spiking interval, the high LOEC seems reasonable if *P. acut*a has a similar baseline resilience to zinc as the other two Physa species.

Measured environmental concentrations were relatively indicative of the ability of snails to tolerate zinc. The impact of zinc on suvival days and reproduction differed among the different populations. Populations collected fromsouthern sites, compared to those collected from more northern contaminated sites, were more sensitive to zinc suggesting that adaptation has occurred in northern populations (Figures 3 and 4). Both the total number of egg masses produced over the course of the test and the number of egg masses produced daily per capita were quantified. The total egg mass production within each test tank could be considered representive of the ecological impact that zinc exposure may have on a particular population; when assessing the population-level effects of a contaminant on an organism, the impact on survival and reproduction are of primary concern. The limitation of this approach is that it is tied to the survival of snails within each test tank; a reduction in total egg masses produced may be the result of premature mortality rather than an inhibition in the egg mass production of individual snails. Daily egg masses per capita, the other measure that was considered, is a means of accounting for the effect of mortality and allows for a better measure of inhibition of reproduction. The reproductive output data largely mirrored the survival days data and further supports the general conclusion that snails found further south, particularly those south of Grand Lake (represented by collection sites HL North, Spavinaw State Park, and HL South), are less tolerant of zinc exposure. Environmental sediment and water sample analysis also suggests that they may be considered relatively naïve to zinc.

At the initiation of the experiment, it was thought that inhibition in growth may be a dose-dependent and sensitive indicator of chronic zinc exposure and toxicity as had been found in the land snail, *Helix aspersa* (Laskowski and Hopkin 1996; Gomot-De

Vaufleury 2000; Gomot-de Vaufleury and Bispo 2002) and has been observed in *Pomacea paludosa* after chronic copper exposure (Rogevich et al. 2009). This makes sense, as organisms exposed to toxicants must dedicate resources to the metabolism and elimination of those substances and, as a result, have fewer resources for other essential processes such as growth and reproduction. Growth was significantly lower in HL South snails compared to TC Border and Twin Bridges snails in the overall model (Table 3). However, within-site analysis of treatment effects did not prove to be statistically significant. This may be due to the unpredictable effects of mortality on the mean mass of surviving snails at the conclusion of a toxicity test. Because individual snails were not tracked during the test, the mean mass of all living snails was used for data at the initiation and the conclusion of each toxicity test. Variability between individuals could be quite high over the course of the test, perhaps due to monopoliztion of food resources by individual snails or other individual behavioral or physiological variations. Should a particularly large or particularly small snail die before the end of the test, the mean mass of the snails in the tank could swing dramatically. This may have been especially true for high zinc treatments on relatlively sensitive snails, resulting in very high mortality and unrepresentative final mass data for that tank. The total increase in mass of snails prior to mortality may have had significant effects on the mean mass of snails that was not accounted for in my experimental design, limiting the conclusions that may be drawn from what growth data was collected. Additionally, total mortality in some tanks (particularly those receiving 3200 µ/L zinc treatments) reduced the available data for analysis. For example, at the 3200 µ/L zinc treatment, GL Bernice had only 2 tanks with surviving snails that could be weighed at the conclusion of the toxicity test. Spavinaw

State Park and HL South both had only one tank with any snails surviving 3200 µ/L zinc treatments.

This study demonstrtates that there are signficant difference in tolerance to zinc exposure, both in regards to mortality and reproductive capability, observed in snails from sites ranging from Tar Creek at the Oklahoma-Kansas border to the southern end of Lake Hudson, within the same water shed and separated by approximately 55 miles. The observed trend is for snails to be increasingly zinc-sensetive at sites further downstream to the south, and increasingly zinc-tolerant as you move upstream to the north where higher concentrations of zinc are found in both the sediments and the water. The location of these metals-contaminated collection sites is also correlated with proximity to historic mining operations and particularly with proximity to mining waste disposal sites.

Snails from Tar Creek (TC Border and TC Miami), Twin Bridges, and GL Bernice were found to be tolerant to zinc exposure. Finding zinc tolerance in Tar Creek snails was unsurprising given the high levels of zinc contamination that was anticipated at those sites, but tolerance found in snails from Twin Bridges and GL Bernice is more curious. Previous work in the TSMD demonstrated wide-ranging zinc contamination in sediments that exceeded general PECs, but not TSMD-specific PECs, including Twin Bridges (Morrison et al. 2019). This study's finding of tolerant snails at sites with environemtnal metals concentrations between general and TSMD-specific values suggests that organisms may experience physiological effects and selective pressures at levels thought to be below environmental concern or that snails may be dispersing from more contaminated sites further upstream.

The GL Bernice site showed little sensitivity to zinc for any of the measured endpoints, despite having low environmental zinc concentrations. GL Bernice has an environmental zinc contamination profile more similar to the southern, clean sites but in terms of sensetivity to zinc, GL Bernice snails are much more similar to those found at metals-contaminated sites. If envrionmental concentrtions were entirey predictive of sensetivity or tolerance, snails from GL Bernice should be more similar to those from the naïve sites. This inconsistency suggests that something has prompted the development or persistence of zinc tolerance in a population that currently does not live in a zinc-contaminated site. There are several possibilities for how this may occure; gene flow, acute pulses combined with genetic drift, and historic environmental contamination (Blankespoor et al. 1985; Marten et al. 2006; Bohonak 2010). Gene flow may occur if metals-tolerant snails are washed downstream from northern populations occupying metals-contaminated sites and immigrate into southern downstream populations where there is less metals contamination; a regular influx of metals-tolerance genes may allow the trait to persist in populations where it is not needed. Secondly, there may be acute pulses of envrionmental zinc contamination associated with weather events as mining waste sites flood, allowing zinc particulates trapped in waste ponds to seep and mix with the creeks and rivers downstream (Andrews et al. 2009). Pulses of zinc from flooding events might be acutely or subchronicly toxic to snails in naïve populations who lack some degree of zinc tolerance, creating periodic bottleneck events. Thirdly, this tolerance found in what should be a naïve population could be evidence of historically higher levels of zinc in sediments and water at those sites in past decades. Previous studies have shown a trend for metals contamination levels in the TSMD to lessen with time (Andrews and

Masoner 2011). A past history of a site having a higher concentration of environmental metals would suggest that the population would have been zinc tolerant in the past, just as populations from contaminated sites are tolerant presently. If this is the case, then the tolerance seen today could be a vestigial characteristic still expressed by these populations that now find themselves in relatively uncontaminated environments.

Metals tolerance has been observed in molluscs generally and snails specifically at other sites with environmental contamination (Luoma et al. 1983; Blankespoor et al. 1985; Lefcort et al. 2004). Snails collected from mining sites have demonstrated increased sensitivity and avoidance of meatls-polluted water that included zinc, lead, cadmium, and other metals; this sensitivity and increased avoidance behavior was also shown to be possessed, to a lesser degree, by their offspring (Lefcort et al. 2004). Freshwater snails collected from Lake Houghton in Michigan, which was treated for 40 years with copper sulfate molluscicide, were shown to be significantly more tolerant to copper exposure than snails collected from a lake that had not been treated with copper (Blankespoor et al. 1985). Perhaps most similarly to the present study, populations of the bivalve *Macoma balthica* collected from different stations within San Francisco Bay were shown to have differing levels of tolerance to copper (Luoma et al. 1983). This was attributed to different environmental concentrations, but the tolerances found did not entirely correlate with the relative concentrations between sites. It was shown, however, that populations need not be geographically isolated to have significantly different responses to toxic metals which is supported by my findings. To my knowledge, there are no other studies demonstrating a geographic and contaminant gradient that corresponds with metals tolerance in freshwater snails.

Uncertainties

Due to the experimental design, a number of factors inherent in the system may present confounding factors to the data. The age of snails at the beginning of the toxicity test was unknown. The criteria for snails to be used in testing was their mass and if snails from a culture tank were growing more slowly or quickly than in other culture tanks, it's possible there may have been significant differences in the ages of snails tested among sites which could have unknown effects on survival, reproduction, or growth. The 3-day interval between survival observations makes the exact date of a snail's death unknown and it may have occurred at any point within that 72 hour period. I attempted to mitigate this with the equation for survival days by adding 1.5 days per deceased snail at the conclusion of the test to the numerator; this estimates the mortality to have occurred midway between the most recent day the snail was confirmed alive and the observation that it was dead. Another variable that could not be accounted for was the effect of population density within culture tanks and toxicity test tanks on reproduction and growth as has been observed in apple snails (*Pomacea canaliculata)* (Tanaka et al. 2002). Lastly, the cause of the lack of zinc recovery observed after 72h in samples taken from toxicity test tanks is not known for certain but is likely due to rapid sorption to the coral substrate used to line the bottom of the test tanks (Kitano et al. 1976).

The evident blocking effect seems to be potentially temporal, as subsequent pairs of months were assigned to blocks, and factors such as daylight through two windows in the lab area or unrecorded fluctuations in culture tank conditions may have influenced tests run during different blocks. However, the blocks with the highest mean endpoints tend to correspond with the start dates of toxicity tests run with what were found to be the

most tolerant cultures (TC Border, TC Miami, and Twin Bridges) while blocks with the lowest mean endpoints correspond with start dates pertaining mostly to toxicity tests with more sensitive cultures (HL North, Spavinaw, and HL South) (See Appendix D). This suggests that the evident blocking effect may be an artifact of the non-randomized distribution of start dates for toxicity test replicates and the cultures from which the snails came.

Potential Mechanisms for Zinc Tolerance

The snails used in this study were all F1 or later, meaning that the tolerance shown in those from zinc-tolerant populations was a heritable trait and not the result of some prior zinc exoposure in that individual snail's lifetime at the original collection site. This suggests that these populations have adapated to the presence of zinc in the TSMD watershed. There are two ways in which organisms may adapt to such contamination; physiological and behavioral. Likely physiological adaptations include the sequestration of metals in the shells of snails via biomineralization or an upregulation in the production or activity of metallothionein (MT). Behavioral adaptations would manifest as avoidance of zinc-contaminated waters and sediments.

Sequestration and bioaccumulation of metals into the shells in molluscs is well documented (Newman et al. 1994; Jordaens et al. 2006). This trait of molluscs may be protectively adaptive by preventing metals from being metabolically active and causing damage to the living tissues of the organism (Newman et al. 1994). Varying rates of tissue:shell zinc deposition have been reported in different species of freshwater and terrestrial snails. When comparing populations of snails from sites with different levels of

zinc contamination in their sediments, freshwater snail species *Brotia costula* and *Melanoides tuberculata* collected from sites with greater metals-contamination demonstrated a heightened preference for deposition of zinc into their shells rather than into tissues (Lau et al. 1998), mirroring previous findings with lead (Newman et al. 1994). A negative correlation between shell zinc content and shell strength has been reported in *Cepaea nemoralis*, which may increase predation risk (Jordaens et al. 2006). If the sequestration of metals is truly adaptive, it may come at the cost of reduced fitness in the face of other, competing selective pressures.

Metallothioneins are small, non enzymatic proteins which have metal-binding properties. They are categorized into 3 classes; classes I, II, and III. Each metallothionein has 20 cysteines which fold to bind to up to 7 metal ions in a cooperative fashion; typically zinc or cadmium ions (Klaassen et al. 1999). Some species of snails have developed two metal-specific MT proteins that specialize in binding copper or cadmium. The copper-specific MT can bind up to 12 copper ions (Gehrig et al. 2000) and may have evolved as a means of protecting copper-reliant metabolic pathways from cadmium (Palacios et al. 2011).

The exact function and importance of MTs is not entirely understood, but it is thought that they play an important role in protecting organisms from excessive metals exposure. Single-metal-specific MTs are particularly important for snails; copper-specific MTs serve as reservoirs that allow snails to maintain high levels of copper which is needed for the synthesis of the oxygen transporter, hemocyanin, while cadmium-specific MTs protect against cadmium toxicity (Dallinger et al. 1997). Non-specific MTs are capable of absorbing zinc from the intracellular environment and releasing it when it is needed by the

cell (Klaassen et al. 1999; Kręzel and Maret 2007). MTs also function to scavenge free radicals from the environment and bind to reactive oxygen species, which reduces the risk of oxidative stress. Zinc has been found to inhibit glutathione reductase (GR), which is necessary to produce glutathione (GSH) which in turn is critical in combating oxidative stress (Trevisan et al. 2014). An increase in the production of MTs may be doubly beneficial for organisms living in zinc-contaminated sites because it not only increases the organism's ability to store zinc without ill effect, but also buffers the organism against the effects of oxidative stress that may be caused by toxic levels of zinc.

Perceptual sensitivity to heavy metals has been demonstrated in snail populations collected from sites polluted from metal mining operations. This allows those snails to sense and preferentially choose less-contaminated environments. This trait has also been shown to be heritable and is present in F1 snails (Lefcort et al. 2004). This experiment was not designed to explore such behavioral adaptations and would not explain the differences found in our tests as no refuge was available.

Ecological Implications

That I found evidence of metals-tolerant populations in the study area has both positive and negative implications. The positive is that it demonstrates that ecosystems in this metals-contaminated region are resilient and may persist and adapt to environmental hazards introduced by the historic mining operations that took place here. Despite environmental concentrations well above those expected to cause effects, snail popluations persist in the streams of the TSMD. However, tolerance may not be an entirely cost-free trait and those snails that exhibit tolerance are likely to experience physiological stress as

a result of whatever mechanism allows them to live and reproduce in metals-contaminated sites. For tolerant populations, additional stressors such as an unseasonably warm or dry season may present enough additional stress to put the population at risk.

Of particular intersest is the presence of a relatively zinc-tolerant population at the GL Bernice site. Though zinc was not detected in the water and sediment concentrations were below levels of environmetnal concern, the tolerance exhibited by these snails may be evidence of a long history of chronic exposure to low concentrations of zinc. This would suggest more ecological concern for zinc contamination may be warranted than has been previosuly suggested by zinc toxicity testing (Ingersoll et al. 2009; Morrison et al. 2019). The alternative explanation for the adaptive tolerance of GL Bernice snails, that it is the reuslt of gene flow from more northern, metals-tolerant populations, has its own implications for the health of the snail populations. Given that tolerance may be indicative of additional physiological costs expended by an organism and is and of itself a stressor, a constant influx of tolerance-genes into populations where that tolerance is not necessary may put those populations at greater risk to additional environmental stressors.

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Table 1. Field sediment and water zinc concentrations[a]

Collection Site	Sediment mg/kg Zn	Water µg/L Zn
TC Border	9813[bcd]	1735[e]
TC Miami	9659[bcd]	489[e]
Twin Bridges	575[bc]	89
GL Bernice	55	<11
HL North	65	<11
Spav. St. Park	40	<11
HL South	117	<11

[a] Concentrations of zinc found in sediment and water at 7 collection sites in Lake o' the Cherokees watershed in Oklahoma (Figure 1).

[b] Sediment concentration exceeds general TEC.

[c] Sediment concentration exceeds general PEC.

[d] Sediment concentration exceeds TSMD-specific PEC.

[e] Water concentration exceeds USEPA water quality criteria for chronic toxicity in aquatic organisms.

TEC = threshold effect concentration; PEC = probable effect concentration; TSMD = Tri-State Mining District, USEPA = United States Environmental Protection Agency.

Table 2. Zinc recovery from selected test tanks after zinc renewal[a]

	Zinc recovery (µg/L)[a]					Percent of nominal concentration				
Time	0h	6h	24h	48h	72h	0h	6h	24h	48h	72h
Nominal (µg/L)										
200	192 (±6)	115 (±19)	84 (±7)	60 (±30)	< 33 (±6)	96	57	42	30	< 17
400	385 (±6)	264 (±70)	171 (±9)	119 (±23)	68 (±21)	96	66	43	30	17
800	774 (±6)	547 (±73)	383 (±55)	278 (±42)	188 (±53)	97	68	48	35	24
1600	1556 (±56)	1171 (±15)	806 (±16)	631 (±167)	452 (±129)	97	73	50	39	28
3200	3075 (±92)	2637 (±205)	1868 (±277)	1471 (±171)	1213 (±348)	96	82	58	46	38

[a] Mean zinc (±SD) recovery from selected test tanks during toxicity testing with freshwater snails (*Physa acuta*). n = 3 for all groups except 72h (n = 10).

Table 3. Analysis of the effects of collection site, zinc treatment, and their interaction on survival, reproduction, and growth in freshwater snails (*Physa acuta*)[a]

	p values			Collection site[b]							Treatment (µg/L Zn)[b]					
	Main Effects		Interaction													
Endpoint	site	treatment	treatment * site	TC Border	TC Miami	Twin Bridges	GL Bernice	HL North	Spav. St. Park	HL South	0	200	400	800	1600	3200
Survival days	<0.001	<0.001	0.156	A	A	A	A	AB	B	AB	A	AB	AB	AB	BC	C
Egg masses sum	<0.001	<0.001	0.391	A	ABC	AB	AB	ABC	BC	C	A	AB	AB	AB	BC	C
Egg masses/capita	<0.001	<0.001	0.525	A	AB	A	A	AB	AB	B	A	A	A	A	AB	B
Growth	0.012	<0.001	0.994	A	AB	A	AB	AB	AB	B	A	A	AB	AB	B	B

[a] Results of two-way ANOVA main effects tests and Tukey HSD analysis of zinc toxicity tests performed to determine differences in survival, reproduction, and growth among freshwater snails (*P. acuta*). Snails were cultured from individuals collected from 7 sites representing a gradient of environmental zinc contamination in northeast Oklahoma within the same watershed. Sites are arranged according to geography and corresponding environmental zinc concentrations with TC Border being the northernmost, most contaminated site. HL South is the southernmost site and, along with GL Bernice, HL North, and Spav. St. Park has no appreciable zinc contamination.

[b] Multiple comparisons of endpoints across sites via Tukey HSD. Groups not connected by the same letter significantly differ from one another.

Table 4. Overall mean, standard error, and sample size for all endpoints among all treatments by collection site[a]

	Collection site																		
	TC Border			TC Miami			Twin Bridges			GL Bernice			HL North			Spavinaw State Park			HL South		
Endpoint	x̄	SE	n	x̄	SE	n	x̄	SE	n	x̄	SE	n	x̄	SE	n	x̄	SE	n	x̄	SE	n
Survival days[b]	17.5	0.5	24	16.8	0.7	30	17.4	0.4	24	17.2	0.7	24	15.0	0.9	24	13.0	0.8	24	15.1	1.1	24
Egg mass sum[c]	46.1	2.4	24	37.0	4.0	30	41.0	3.0	24	42.0	4.5	24	30.0	4.4	24	26.3	4.7	24	23.8	4.3	24
Egg masses per capita[d]	2.7	0.1	24	2.1	0.2	30	2.4	0.2	24	2.5	0.3	24	1.8	0.2	24	1.9	0.3	24	1.4	0.3	24
Growth (%)[e]	267.6	19.6	24	216.8	26.8	28	293.2	25.0	24	237.3	34.0	22	226.6	34.6	20	190.1	32.0	20	197.3	39.7	21

[a] Mean survival days, egg mass sum, egg masses per capita, and growth for lab-cultured freshwater snails (*Physa acuta*) after 21d zinc toxicity tests by collection site. Toxicity tests included controls and 5 levels of zinc treatment (200, 400, 800, 1600, and 3200 μg/L) and were conducted in test tanks that each had 5 snails at the initiation of the test.

[b] The average number days survived by snails during the toxicity test.

[c] Total number of egg masses laid by snails (5 snails at test initiation) in a test tank over 21d.

[d] Average number of eggs laid per day per snail.

[e] Mean increase in snail mass within a test tank after 21d, expressed as a percentage.

Table 5. Overall mean, standard error, and sample size for all endpoints among all snail collection sites by treatment[a]

	Treatment (μg/L Zn)																	
	0			200			400			800			1600			3200		
Endpoint	x̄	SE	n	x̄	SE	n	x̄	SE	n	x̄	SE	n	x̄	SE	n	x̄	SE	n
Survival days[b]	18.6	0.4	29	16.5	0.6	29	16.7	0.6	29	16.2	0.6	29	14.7	0.7	29	13.4	1.0	29
Egg mass sum[c]	45.8	2.4	29	39.1	3.9	29	38.3	3.8	29	37.3	3.8	29	29.3	3.8	29	21.4	4.1	29
Egg masses per capita[d]	2.5	0.1	29	2.5	0.3	29	2.2	0.2	29	2.2	0.2	29	1.9	0.2	29	1.3	0.2	29
Growth[e]	288.3	23.4	29	293.3	30.8	27	229.0	25.6	29	229.9	25.6	28	177.5	26.9	26	162.6	30.5	20

[a] Mean survival days, egg mass sum, egg masses per capita, and growth for lab-cultured freshwater snails (*Physa acuta*) after 21d zinc toxicity tests by treatment. Snails were cultured from populations collected from 7 sites in a mining-contaminated watershed in northeast Oklahoma. Toxicity tests were conducted in test tanks that each had 5 snails at the initiation of the test.

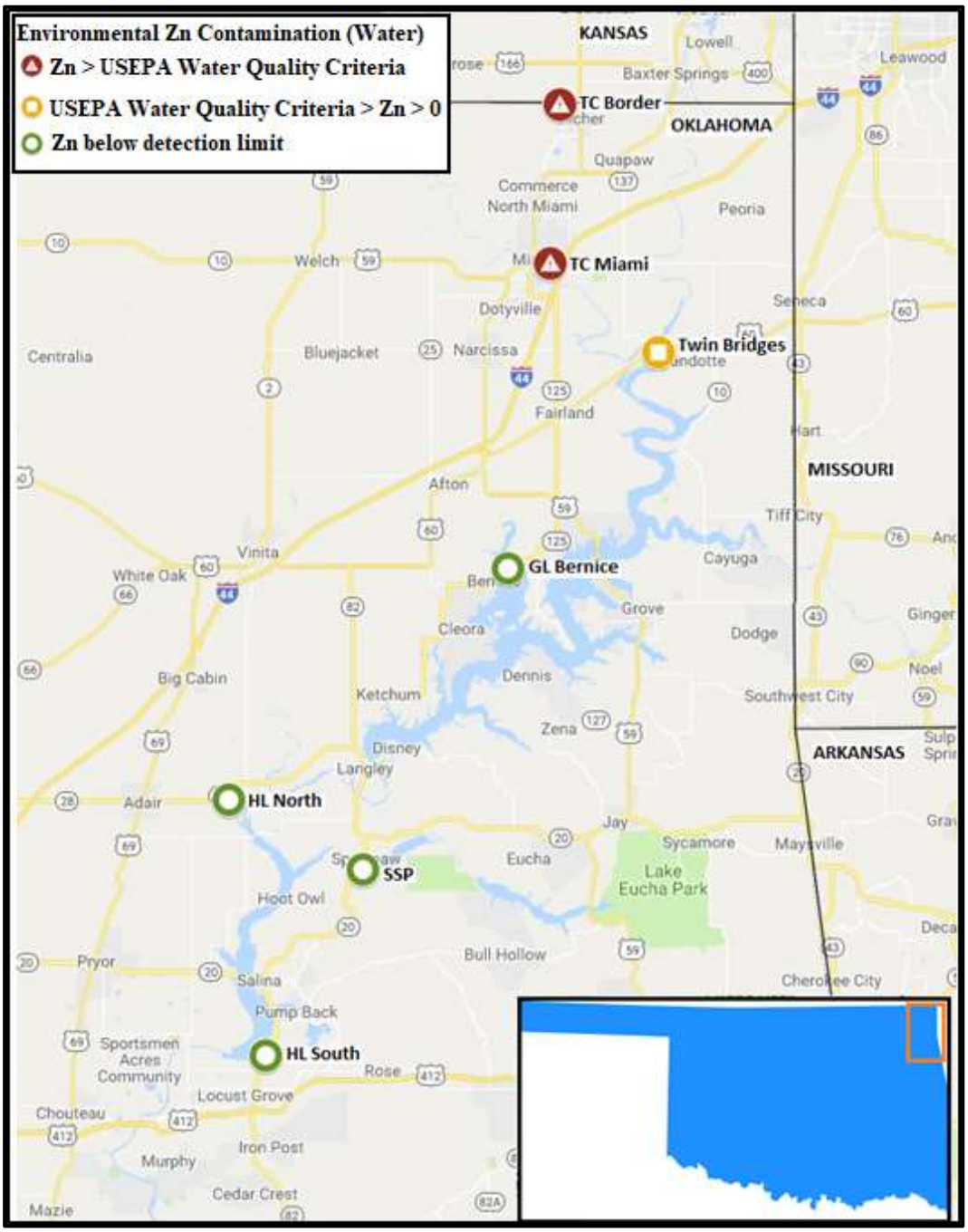
[b] The average number days survived by snails during the toxicity test.

[c] Total number of egg masses laid by snails (5 snails at test initiation) in a test tank over 21d.

[d] Average number of eggs laid per day per snail.

[e] Mean increase in snail mass within a test tank after 21d, expressed as a percentage.

Figure 1. Seven sites from which snails and water and sediment samples were collected in the Lake o' the Cherokees watershed, which includes drainage from the Neosho and Spring Rivers in northeastern Oklahoma. Sites represent a gradient of zinc concentrations and were chosen to assess the presence of adaptive metal tolerance in collected populations. Environmental concentrations relative to USEPA Water Quality Criteria are denoted in red, yellow, and green (see legend).



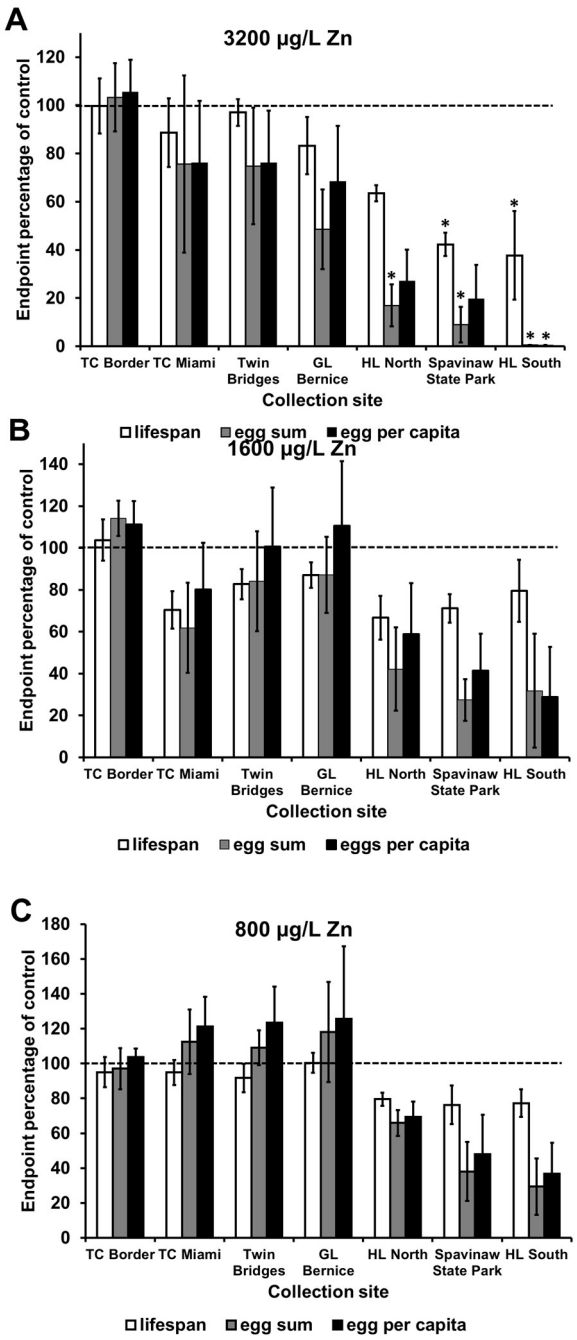


Figure 2. Mean survival days, mean total egg mass production, and mean daily per capita egg mass production at 3200 (**A**), 1600 (**B**), and 800 (**C**) µg/L zinc treatments across all sites arranged from north to south, with endpoints expressed as a percentage of the means of controls within that site. A trend of reduction in endpoints in the three most southern collection sites (HL North, Spavinaw State Park, and HL South) can be seen at 1600 µg/L zinc (**B**) as well as 800 µg/L treatments (**C**). Error bars show standard error. An asterisk denotes values that significantly differ from control (Dunnett's test, $p \leq 0.05$) (*).

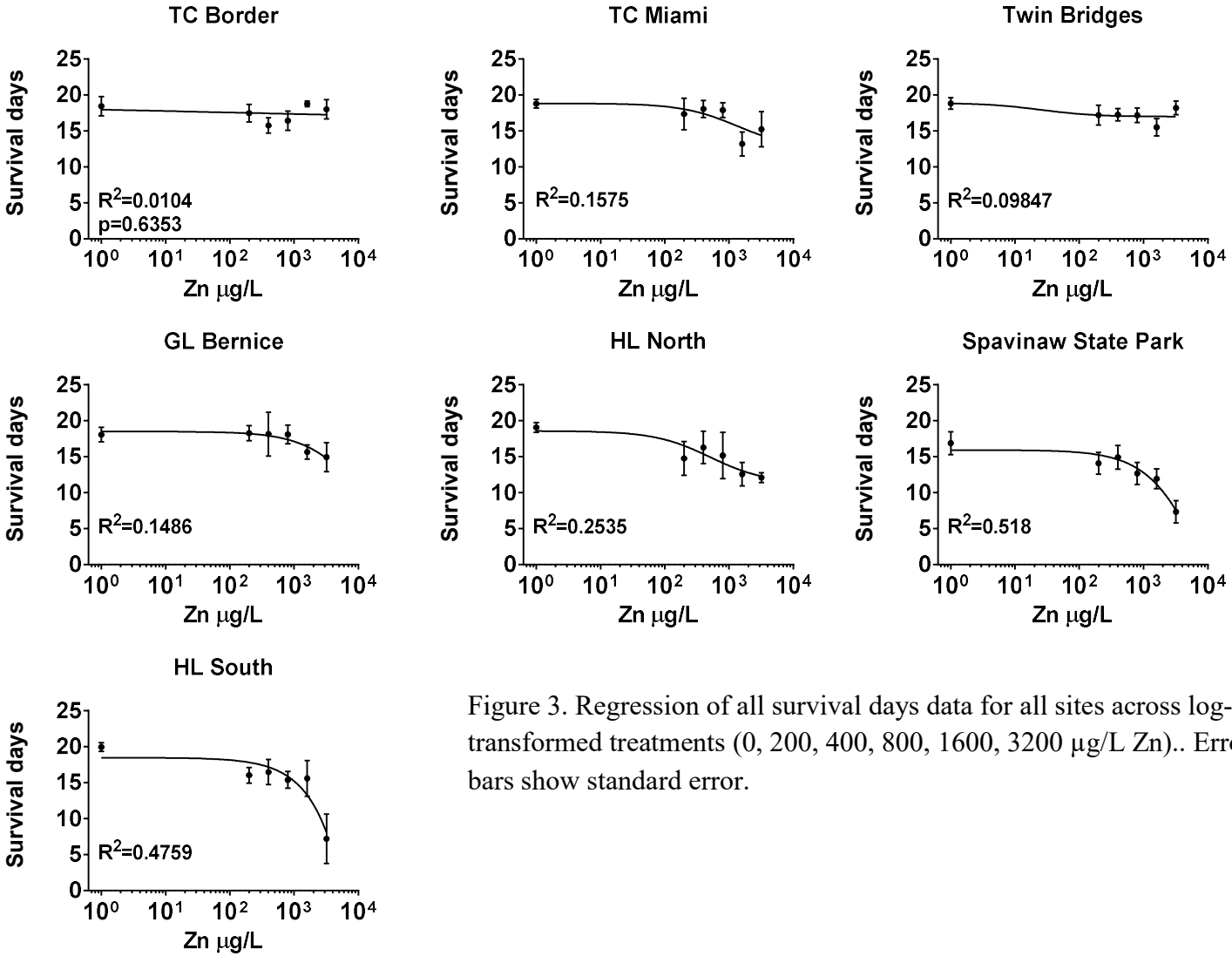


Figure 3. Regression of all survival days data for all sites across log-transformed treatments (0, 200, 400, 800, 1600, 3200 µg/L Zn).. Error bars show standard error.

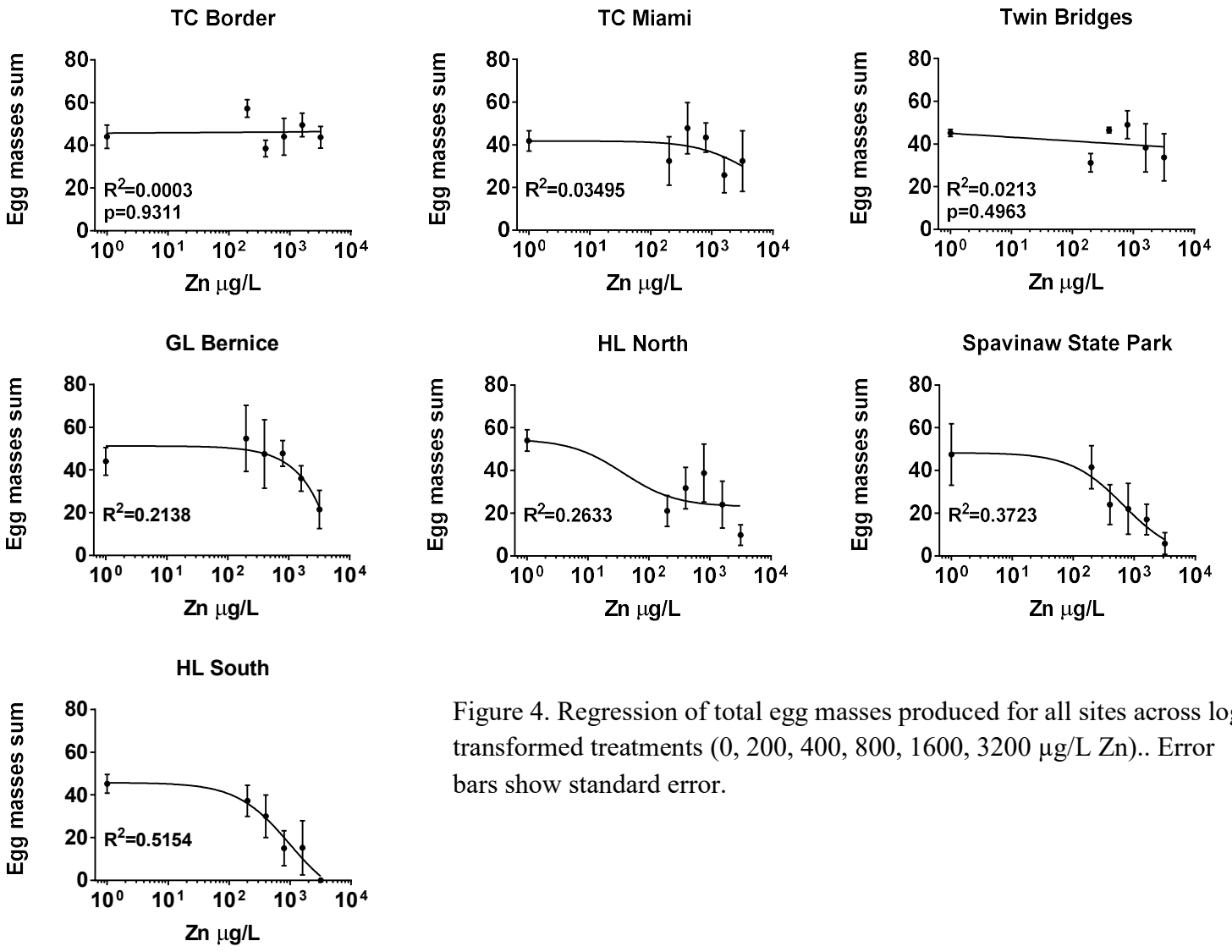


Figure 4. Regression of total egg masses produced for all sites across log-transformed treatments (0, 200, 400, 800, 1600, 3200 µg/L Zn).. Error bars show standard error.

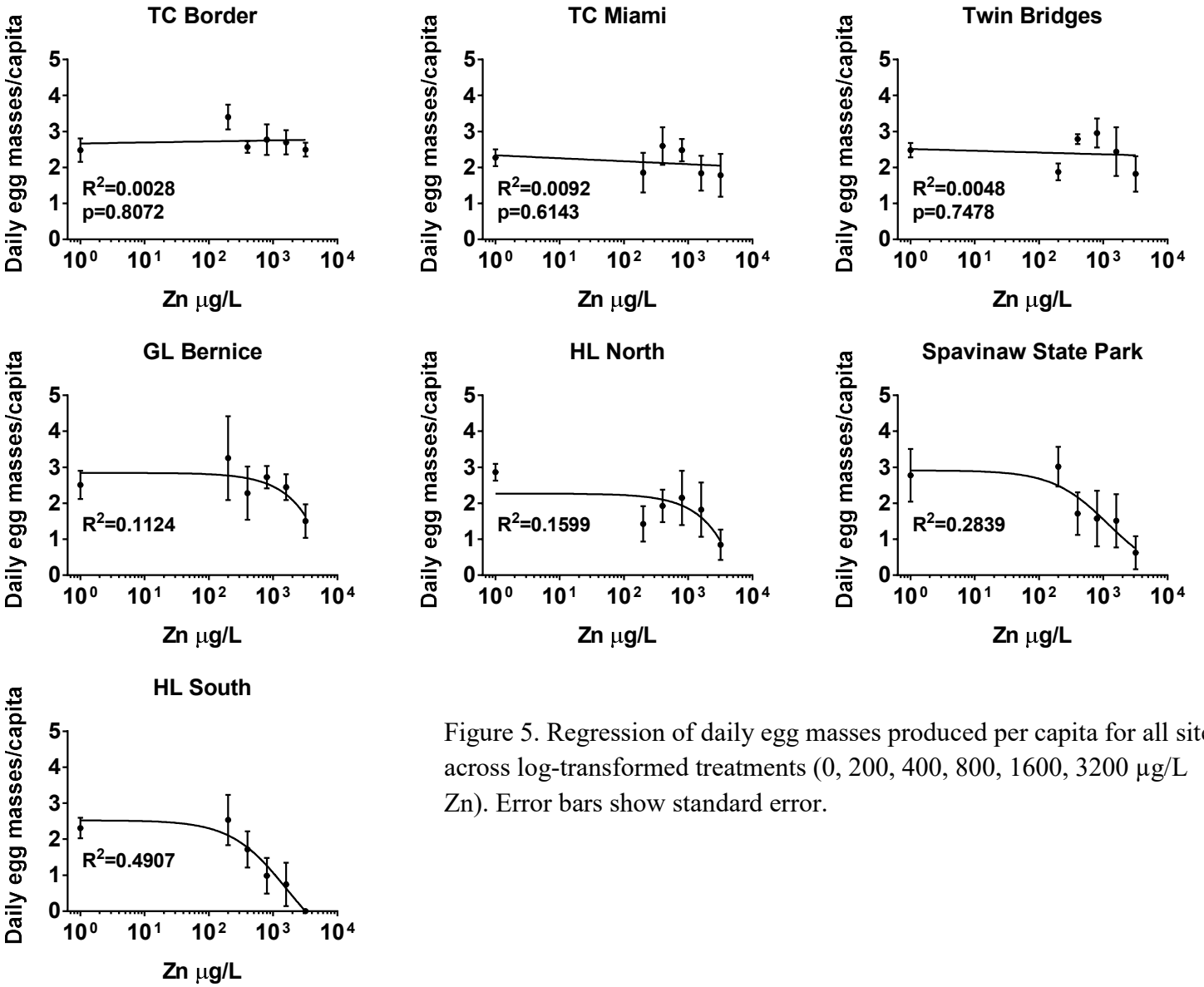


Figure 5. Regression of daily egg masses produced per capita for all sites across log-transformed treatments (0, 200, 400, 800, 1600, 3200 µg/L Zn). Error bars show standard error.

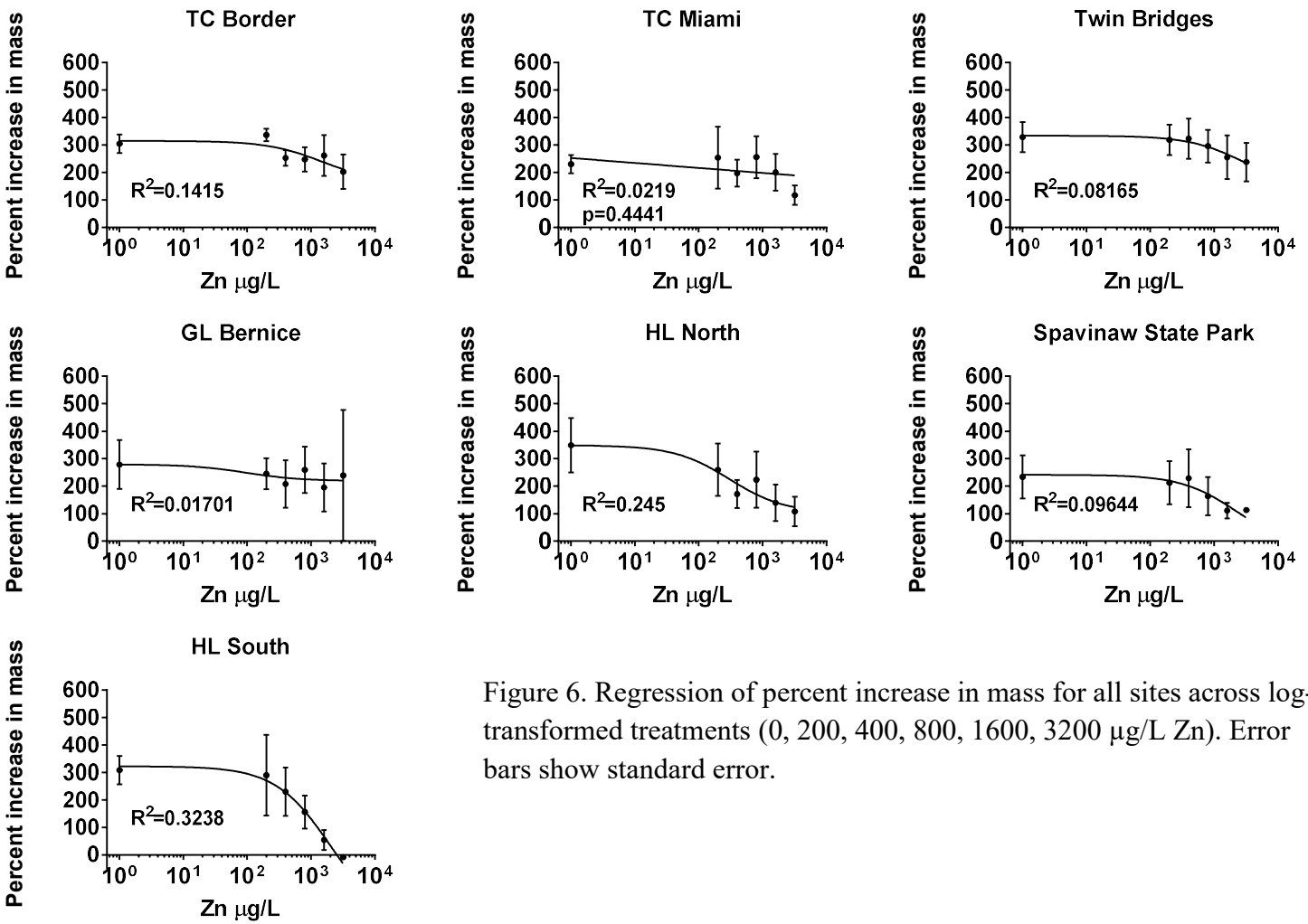


Figure 6. Regression of percent increase in mass for all sites across log-transformed treatments (0, 200, 400, 800, 1600, 3200 µg/L Zn). Error bars show standard error.

APPENDIX A

Summary statistics for survival days and growth for all combinations of collection site and zinc treatment[a]

	Collection site																		
	TC Border			TC Miami			Twin Bridges			GL Bernice			HL North			Spav. St. Park			HL South		
Zn (µg/L)	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n
Survival days[b]																					
0	18.5	1.3	4	18.8	0.6	5	18.8	0.8	4	18.1	1.0	4	19.1	0.7	4	16.9	1.6	4	20.0	0.6	4
200	17.5	1.2	4	17.3	2.2	5	17.2	1.4	4	18.3	1.0	4	14.8	2.4	4	14.1	1.5	4	16.0	1.1	4
400	15.8	1.1	4	18.1	1.2	5	17.3	0.9	4	18.2	3.1	4	16.3	2.3	4	14.9	1.7	4	16.5	1.8	4
800	16.4	1.3	4	17.9	1.0	5	17.2	1.0	4	18.1	1.3	4	15.2	3.2	4	12.7	1.5	4	15.4	1.2	4
1600	18.8	0.4	4	13.2	1.7	5	15.5	1.2	4	15.7	1.0	4	12.6	1.6	4	11.9	1.4	4	15.6	2.5	4
3200	18.0	1.3	4	15.2	2.5	5	18.2	1.0	4	15.0	2.0	4	12.1	0.7	4	7.4	1.6	4	7.2	3.4	4
Growth[c]																					
0	304.7	34.1	4	230.1	33.6	5	329.1	54.7	4	278.4	89.0	4	348.8	99.0	4	233.7	78.8	4	308.1	52.0	4
200	336.3	23.1	4	318.0	120.0	4	318.8	55.6	4	245.1	56.6	4	345.9	57.1	3	212.6	78.9	4	289.7	146.9	4
400	252.9	29.1	4	197.1	49.1	5	322.9	73.1	4	207.6	86.2	4	171.5	51.9	4	229.0	105.7	4	229.9	88.6	4
800	247.4	44.6	4	255.5	76.4	5	295.6	59.8	4	259.4	83.8	4	223.4	102.7	3	163.9	69.4	4	156.2	60.1	4
1600	261.6	74.6	4	200.3	67.5	4	255.0	79.9	4	195.1	87.8	4	139.4	66.5	3	110.8	28.6	3	54.3	36.6	4
3200	202.8	62.3	4	116.9	35.5	5	237.8	70.2	4	238.8	238.2	2	107.9	53.6	3	112.7	-	1	-8.5	-	1

[a] Summary statistics for 21d zinc toxicity tests conducted with freshwater snails (*Physa acuta*) cultured from populations collected from 7 collection sites within the same mining-contaminated watershed. The most contaminated and northern-most site is TC Border (left) and the southernmost collection site is HL South (right). Sites are arranged by relatively geographical relationship. Elevated levels of zinc were found at TC Border, TC Miami, and Twin Bridges. Zinc levels were not elevated at other sites.

[b] The average number days survived by snails during the 21d zinc toxicity test.

[c] Mean increase in snail mass within a test tank after 21d, expressed as a percentage.

APPENDIX B

Summary statistics for egg masses sum and egg masses per capita for all combinations of collection site and zinc treatment[a]

	Collection site																			
	TC Border			TC Miami			Twin Bridges			GL Bernice			HL North			Spav. St. Park			HL South		
Zn (µg/L)	x̄	SE	n	x̄	SE	n	x̄	SE	n	x̄	SE	n	x̄	SE	n	x̄	SE	n	x̄	SE	n
Egg masses sum[b]																					
0	44	5.5	4	42	4.8	5	45	1.7	4	44	6.5	4	54	5.0	4	48	14.4	4	45	4.3	4
200	57	4.2	4	32	11.3	5	31	4.3	4	55	15.5	4	21	7.3	4	42	10.1	4	37	7.4	4
400	39	3.9	4	48	12.0	5	47	1.3	4	48	16.0	4	32	9.7	4	24	9.4	4	30	10.0	4
800	44	8.6	4	43	6.8	5	49	6.5	4	48	6.1	4	39	13.6	4	22	12.0	4	15	8.2	4
1600	50	5.6	4	26	8.4	5	38	11.3	4	36	6.0	4	24	11.0	4	17	7.2	4	15	12.7	4
3200	44	5.1	4	32	14.3	5	34	11.0	4	22	8.9	4	10	4.8	4	6	5.1	4	0	0.0	4
Egg masses per capita[c]																					
0	2.5	0.3	4	2.3	0.2	5	2.5	0.2	4	2.5	0.4	4	2.9	0.2	4	2.8	0.7	4	2.3	0.3	4
200	3.4	0.3	4	1.9	0.5	5	1.9	0.2	4	3.3	1.2	4	1.4	0.5	4	3.0	0.6	4	2.5	0.7	4
400	2.6	0.2	4	2.6	0.5	5	2.8	0.1	4	2.3	0.7	4	1.9	0.5	4	1.7	0.6	4	1.7	0.5	4
800	2.8	0.4	4	2.5	0.3	5	3.0	0.4	4	2.7	0.3	4	2.2	0.8	4	1.6	0.8	4	1.0	0.5	4
1600	2.7	0.3	4	1.8	0.5	5	2.4	0.7	4	2.4	0.4	4	1.8	0.8	4	1.5	0.7	4	0.7	0.6	4
3200	2.5	0.2	4	1.8	0.6	5	1.8	0.5	4	1.5	0.5	4	0.8	0.4	4	0.6	0.5	4	0.0	0.0	4

[a] Summary statistics for zinc toxicity tests conducted with freshwater snails (*Physa acuta*) cultured from populations collected from 7 collection sites within the same mining-contaminated watershed. The most contaminated and northern-most site is TC Border (left) and the southernmost collection site is HL South (right). Sites are arranged by relatively geographical relationship. Elevated levels of zinc were found at TC Border, TC Miami, and Twin Bridges. Zinc levels were not elevated at other sites.

[b] Total number of egg masses laid by snails (5 snails at test initiation) in a test tank over 21d.

[c] Average number of eggs laid per day per snail.

APPENDIX C

Blocking effect and mean, standard deviation, and sample size for each testing block[a]

		Survival days			Egg sum			Eggs per capita			Growth		
Blocking effect p value		0.0028			< 0.001			< 0.001			< 0.001		
Start date	Block	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n
January, February	A	14.7	0.6	42	22.9	3.0	42	1.4	0.2	42	197.5	23.0	33
March, April	B	15.0	0.6	36	32.2	3.3	36	2.1	0.2	36	210.9	23.3	32
May, Jun	C	17.2	0.5	60	44.0	2.5	60	2.6	0.1	60	307.3	17.2	59
July, August	D	17.8	1.1	12	45.3	5.7	12	2.6	0.3	12	219.9	38.1	12
September, October	E	17.5	1.1	12	34.3	5.7	12	2.0	0.3	12	194.9	38.1	12
November	F	14.4	1.1	12	34.6	5.7	12	2.3	0.3	12	77.3	39.8	11

[a] Blocking effect and means, standard, deviations, and sample sizes for zinc toxicity tests conducted with lab-cultured freshwater snails (Physa acuta) cultured from populations collected from 7 sites in a mining-contaminated watershed in northeastern Oklahoma.

APPENDIX D

Zinc toxicity test start date and block assignments[a]

Replicate by collection site	Start Date	Block
TC Border		
2	4/2/2018	B
3	5/18/2018	C
4	6/12/2018	C
6	10/19/2018	E
TC Miami		
1	3/5/2018	B
2	4/4/2018	B
3	5/29/2018	C
4	6/22/2018	C
6	8/20/2018	D
Twin Bridges		
1	1/10/2018	A
3	5/15/2018	C
4	6/1/2018	C
5	7/2/2028	D
GL Bernice		
1	1/5/2018	A
2	1/5/2018	A
4	4/5/2018	C
6	11/23/2018	F
HL North		
1	1/12/2028	A
4	4/9/2018	B
5	6/14/2018	C
7	11/24/2018	F
Spavinaw State Park		
1	2/27/2018	A
2	3/25/2018	B
3	5/28/2018	C
4	6/27/2018	C
HL South		
1	2/11/2018	A
2	2/11/2018	A
3	3/11/2018	B
7	9/27/2018	E

[a] Start dates and block assignments for replicates of zinc toxicity tests conducted with lab-cultured freshwater snails (Physa acuta) cultured from populations collected from 7 sites from a mining-contaminated watershed in northeastern Oklahoma.

APPENDIX E

Collection date and site location information for snails (*Physa acuta*) used to establish lab cultures[a]

Collection Site	Date Collected	Water Body	Snails Captured	GPS
TC Border	10/8/2017	Tar Creek	32	36°59'55.4"N 94°51'04.6"W
TC Miami	10/7/2017	Tar Creek	26	36°52'14.3"N 94°51'39.1"W
Twin Bridges	6/6/2017	Grand Lake	18	36°47'57.5"N 94°45'11.3"W
GL Bernice	6/6/2017	Grand Lake	22	36°37'30.8"N, 94°54'15.0"W
HL North	10/22/2017	Lake Hudson	22	36°26'19.0"N 95°11'03.1"W
Spavinaw State Park	10/22/2017	Spavinaw Creek	38	36°22'57.2"N 95°02'59.4"W
HL South	10/22/2017	Lake Hudson	28	36°13'55.0"N 95°08'51.9"W

[a] Collection site location and date information for snails used to establish lab-grown cultures used for zinc toxicity tests in 2018. All collection sites are from within a mining-contaminated watershed in northeastern Oklahoma.

VITA

Joel Hickey

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Master of Science

Thesis: ADAPTIVE TOLERANCE TO ZINC IN FRESHWATER SNAILS (*PHYSA ACUTA*) ACROSS A CONTAMINATION GRADIENT

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