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Select metal and metalloid surveillance of free-ranging Eastern box turtles from Illinois and Tennessee (*Terrapene carolina carolina*)

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Abstract The Eastern box turtle (*Terrapene carolina carolina*) is a primarily terrestrial chelonian distributed across the eastern US. It has been proposed as a biomonitor due to its longevity, small home range, and reliance on the environment to meet its metabolic needs. Plasma samples from 273 free-ranging box turtles from populations in Tennessee and Illinois in 2011 and 2012 were evaluated for presence of heavy metals and to characterize hematologic variables. Lead (Pb), arsenic (As), zinc (Zn), chromium (Cr), selenium (Se), and copper (Cu) were detected, while cadmium (Cd) and silver (Ag) were not. There were no differences in any metal detected among age class or sex. However, Cr and Pb were higher in turtles from Tennessee, while As, Zn, Se, and Cu were higher in turtles from Illinois. Seasonal differences in metal concentrations were observed for Cr, Zn, and As. Health of turtles was assessed using hematologic variables. Packed cell volume was positively correlated with Cu, Se, and Pb in Tennessee. Total solids, a measure of plasma proteins, in Tennessee turtles were positively correlated with Cu and Zn. White blood cell count, a measure of inflammation, in Tennessee turtles was negatively correlated with Cu and As, and positively correlated with Pb. Metals are a threat to human health and the health of an ecosystem, and the Eastern Box Turtle can serve as a monitor of these contaminants. Differences established in this study can serve as baseline for future studies of these or related populations.

Keywords Box turtle · Terrapene · Heavy metal · Ecotoxicology · Reptile · Environment

Introduction

Elemental metals and metalloids have been shown to have adverse effects on the ecosystem, wildlife, and humans (Golden and Rattner 2003; Grillitsch and Schiesari 2010). These compounds persist in the environment and are potentially toxic to all organisms at various concentrations (Grillitsch and Schiesari 2010). Many of these elements are introduced into the environment by human action such as through pesticide and fertilizer use, waste streams, mining and smelting, fossil fuel combustion, and recreational shooting (Grillitsch and Schiesari 2010; Harmata and Restani 2013; Martinez-Lopez et al. 2010). Contamination may be important threats to reptiles, but little information exists in the literature (Gibbons et al. 2000; Grillitsch and Schiesari 2010).

Chelonians are proposed as biomonitors of environmental contamination due to their wide distribution, varying habitats and diets, and relatively long life spans (Golden and Rattner 2003; Yu et al. 2011). Many reports have demonstrated elevated levels of heavy metals in aquatic turtles (Ley-Quinonez et al. 2011; Yu et al. 2011). Furthermore, in response to lead toxicity in children in Mexico, Pellalo-Martinez et al. (2011) observed Sliders (*Trachemys scripta*) in the area to contain twice the lead

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concentration than in the children. They went on to hypothesize that turtles may provide an integrated approach to public health in that geographic area (Pellalo-Martinez et al. 2011). Moreover, at an ecosystem level, decreased turtle abundance was associated with increased concentrations of certain heavy metals within a superfund site in the US (Yu et al. 2013). Terrestrial chelonians, however, are less defined as biomonitors of contamination, in part because terrestrial systems are less homogenous than aquatic systems (Smith et al. 2007).

Eastern box turtles (*Terrapene carolina carolina*) are the most common and wide-spread terrestrial turtle in the eastern United States and are listed as vulnerable by the IUCN (International Union on the Conservation of Animals) (van Dijk 2011). They are long-lived, with lifespans ranging from 25 to more than 50 years (Dodd 2001), allowing the measurement of heavy metal exposure over greater periods of time. Furthermore, Eastern box turtles inhabit a wide variety of habitats from creeks to dry grassy fields; however they do not usually travel far and tend to occupy home ranges of a few hectares (Dodd 2001). These characteristics have led Eastern box turtles to be proposed as an important biomonitor (Sleeman 2008).

The objective of this study was (1) to determine the concentration of metals and metalloids in free-ranging Eastern box turtles in Illinois and Tennessee and (2) correlate those findings with basic health parameters commonly used for population monitoring.

Methods & materials

Animals

All activities were approved through the University of Illinois Institutional Animal Use and Care Committee (No. 10057). Free-ranging box turtles were sampled during ongoing collaborations with the Clinch River Environmental Studies Organization (CRESO) and the Illinois Natural History Survey (INHS) in 2011 and 2012. Individual box turtles were captured using human and canine search teams in Oak Ridge, TN (36.008°N, −84.22392°W), and east central Illinois including Middle Fork State Fish and Wildlife Area (MFSFWA; 40.2595°N, −87.7939°W), Kickapoo State Park (40.01167°N, −87.7359°W), and Forest Glen Preserve (40.0118°N, −87.5653°W). The Tennessee population is located near an urban/suburban center with a rock quarry at the edge of the site. Two of the sites in the Illinois population (Kickapoo and Collison) are in wooded reserves adjacent to agricultural fields that lie within a watershed containing a retired coal power plant and former strip mines, but not adjacent to any metropolitan center. The third site in Illinois (Forest Glen) is in a different watershed within a reserve furthest from any metropolitan center, but completely surrounded by corn and soybean agriculture. Both states offer differing risks of contamination and serve as baselines for ongoing conservation efforts. Upon capture, all turtles were weighed and received a unique ID and the GPS location was recorded.

Clinical examination

All physical examinations occurred in the field and included the evaluation of the carapace, plastron, oral cavity, nares, and when possible, all four limbs, tympanic membrane, and mass. Sex was determined based on secondary sex characteristics such as concavity of the plastron and tail length and assigned to the following categories: female, male, or unknown. Age class was determined based on mass and quantification of annular rings and coded as adult (mass >180 g or >8 annular rings) or juvenile.

Blood collection

Turtles were restrained in ventral recumbency and a 3.0 mL (or up to 0.8 % body weight) blood sample was collected from the subcarapacial sinus and divided between two lithium heparin microtainers (Becton–Dickinson, Franklin Lakes, NJ, USA). Samples were transported to the laboratory on wet ice. One tube was centrifuged immediately, and aliquots of plasma were frozen at −20 °C until sample analysis, while the other tube was utilized to test hematologic variables of health.

Element quantification

Plasma samples were thawed at room temperature for 30 min before digestion. Each sample was then vortexed for 5 s and 400–500 mg of sample was transferred to a Teflon digestion vial. A blank was composed of 500 mg of Nanopure water (Labconco Corp., Model Water Pro Plus, Kansas City, MO, USA) and 400 mg of Dorm-2 (Dogfish Muscle from National Research Council Canada; Institute for Environmental Chemistry, Ottawa Canada) was utilized as a certified reference material. The masses of the plasma samples, the blank, and the digestion reference material (Dorm-2) were digested based on US EPA method 3015A: 2.5 mL of nitric acid (HNO_3) and 0.25 mL of hydrogen peroxide (H_2O_2) were added to the vessel and digested for 30 min at room temperature. Samples were then digested using a microwave digester (Milestone Inc., Model Ethos Touch Control, Monroe, CT, USA) with the following digestion program: step 1 at 1000 W and 170 °C, steps 2 and 3 at 800 W and 180 °C, and step 4 at 600 W and 180 °C; each step was 10 min. After digestion, the samples were quantitatively transferred to tubes and diluted to 12.5 mL with nanopure water. Dilutions were made using 2 % nitric acid. An analytical duplicate and analytical spike was made from one plasma sample for every 11 samples. All digested samples were stored at 4 °C until analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (VG Elemental, Model PQ Excell). ICP analysis was based on US EPA method 6020A.

Standard solution of combined metals was made at following concentrations: 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, and 100 µg/L from silver (Ag), arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), selenium (Se), and zinc (Zn) stock standard solutions (Spex Certprep; Assurance, Metuchen, NJ 08840, USA and VHG Labs, Manchester, NH 03103, USA) in 2 % nitric acid. Check standards were made at following concentrations: 0.3, 3.0, and 30.0 µg/L using independent stock standard solutions of the same metals stated above in 2 % nitric acid. The internal standard was made at 100 µg/L concentration using bismuth (Bi), rhodium (Rh), Scandium (Sc), and yttrium (Y) standard stock solutions in 2 % nitric acid. The instrument was calibrated daily with the standard solutions mentioned above and was verified by analysis of the check standards. Instrumental drift and matrix effects were monitored and corrected for by analysis of the internal standard. ICP-MS operating standards include a power of 1300 W, neb flow of 2.8–2.9 bar, pump flow rate of 1.0 mL/min with internal standard line, and expansion pressure of 1.7 mbar. Final results were calculated from the instrument results taking into account any dilution factors, the final digestion dilution volume, and the sample mass utilized in sample preparation. Level of detection (LOD) for each of the metals that were in this category were: 0.05 mg/kg for Cr, Zn, As, Se, and Ag (2012) and 0.005 mg/kg for Cu, Cd, and Pb. The LOD for Ag in 2011 samples was slightly greater, 0.1 mg/kg, due to performance of the ICP-MS instrumentation.

Hematology

Plasma collected from the hematocrit tubes was used to determine total solids using a refractometer (Amscope RHC-200ATC refractometer, National Industry Suppy, Torrance, CA, USA). Total white blood cell (WBC) count was determined using the Avian Leukopet (Vet lab Supply, Palmetto Bay, FL, USA) following manufacturer's protocol. Briefly, heterophils and eosinophils were selectively stained in a 20 µl sample of whole blood and then counted on a hemacytometer (Bright line). Blood smear slides were stained with a modified Wright's Geimsa stain and one hundred white blood cell differential counts were performed by a single observer. Total white blood cell count was then calculated using the following equation: {(Total heterophils and eosinophils counted on hemacytometers)*1.1*16*100]/(% Heterophils + % Eosinophils from the differential count).

Statistical methods

Normality of data was assessed using the Shapiro–Wilk test. All metal concentrations, mass, WBC, and TS were standardized first using a z-transformation so they were all on equivalent scales. Next, we conducted a series of multivariate general linear models with season (spring, summer, fall), sex (female, male, unknown), state (Tennessee, Illinois), stage (adult, juvenile), and year (2011, 2012) as the main effects and turtle mass, WBC, and TS as covariates. Our global model included all main effects, covariates and all two-way interactions. Subsequent models included all possible model combinations, again only up to two-way interactions. We chose not to examine three-way or higher level interactions because of sample size limitations and loss of degrees of freedom. We then used an information theoretic approach (AIC: Burnham and Anderson 1998) to determine which model from our candidate set performed best. Once the best model was selected we examined significant effects following a MANOVA/MANCOVA process using univariate F-tests and Bonferroni means comparison tests. For all significant effects, we then back-transformed the data. All data analysis was conducted in Statistical analysis was performed using SPSS (Version 22, IBM Statistics, Chicago, IL) and nominal alpha levels were set at $p = 0.05$.

Results

Clinical examinations and blood samples were collected on 273 Eastern box turtles in 2011 (n = 127) and 2012 (n = 146). Turtles were sampled in both Tennessee (n = 219) and Illinois (n = 54) during the spring (n = 102), summer (n = 101), and fall (n = 70). There were 87 females, 155 males, and 30 turtles of unknown sex, which consisted of 255 adults and 16 juveniles. In Illinois, turtles were sampled at three separate locations: MFSFWA (n = 22), Kickapoo (n = 15), and Forest Glen (n = 17). All Tennessee turtles were from the same location. There were no abnormalities identified in any of the turtles sampled. All values for Cd, and all but two values for Ag were below LOD and were not included in the analysis.

Of the 218 models examined, the main effects model performed best, explaining the variance observed in relation to sample size and number of parameters estimated (Table 1; Wilk's $\lambda = 0.939$, $F_{6,228} = 2.486$, $p = 0.024$). Although simpler models including the effects of season, sex, state, stage, and TS were within the top five, they had

Table 1 The top five and global models sorted by ΔAIC for the GLM analysis on the plasma concentrations of metals and metalloids for 273 Eastern box turtles (*Terrapene carolina carolina*) sampled in Tennessee and Illinois in 2011 and 2012

Rank	Models	Det.	*K*	AIC	ΔAIC	w_i	Σw_i
1	Main effects	0.080	66	−558.71	0.00	1.00	1.00
2	S + X + Y + TS	0.136	36	−473.59	85.12	≪0.001	1.00
3	T + S + Y + TS	0.145	30	−467.27	91.44	≪0.001	1.00
4	N + X + T + TS	0.133	42	−466.80	91.90	≪0.001	1.00
5	T + Y + TS	0.152	24	−465.60	93.11	≪0.001	1.00
27	Global	0.023	306	−419.15	139.55	≪0.001	1.00

Det. is the determinant of the pooled error sums of squares cross-product matrix divided by sample size, *K* is the number of parameters, w_i is the Akaike weight, and Σw_i is the cumulative sum of the Akaike weights. For the models, N is season, X is sex, S is stage, T is state, Y is year, and TS is total solids. The global model includes all main effects and two-way interactions and the main effects model includes all main effects and no interactions. The metal and metalloid concentrations and co-variates (mass, TS, and WBC) were standardized using a z-transformation

little support in terms of Akaike Weights (Table 1). The global model also ranked low in comparison with other models (Table 1). This suggests a more complex multivariate effect with the main effects but no evidence of interactions (Table 1). For the main effects model, we found significantly different between subject effects for Cu ($F_{10,233} = 6.936$, $p \ll 0.001$), Cr ($F_{10,233} = 5.038$, $p \ll 0.001$), Pb ($F_{10,233} = 3.198$, $p = 0.001$), Se ($F_{10,233} = 5.229$, $p \ll 0.001$), Zn ($F_{10,233} = 32.927$, $p \ll 0.001$), but not As ($F_{10,233} = 1.109$, $p = 0.356$).

Effects of season

Season showed a significant multivariate effect (Fig. 1; Wilk's $\lambda = 0.706$, $F_{12,456} = 7.235$, $p \ll 0.001$). Significant differences were observed between seasons for Cu (Table 2; $F_{2,233} = 3.282$, $p = 0.039$), Cr (Table 2; $F_{2,233} = 5.080$, $p = 0.007$), and Zn (Table 2; $F_{2,233} = 31.960$, $p \ll 0.001$) but not for Pb ($F_{2,233} = 1.590$, $p = 0.206$), Se ($F_{2,233} = 2.016$, $p = 0.136$), and As ($F_{2,233} = 0.131$, $p = 0.878$). Copper concentrations were higher in spring than summer (Mean diff. = 0.347, $p = 0.033$). Chromium concentrations were higher in fall than spring (Mean diff. = 0.490, $p = 0.013$) and summer (Mean diff. = 0.486, $p = 0.013$). For Zn, concentrations in spring were lower than summer (Mean diff. = −0.362, $p = 0.001$) but higher than fall (Mean diff. = 0.579, $p \ll 0.001$). In addition, Zn concentrations for summer were higher than fall (Mean diff. = 0.940, $p \ll 0.001$). Thus it appears that the Cu, Cr, and Zn levels are driving a seasonal trend in the overall model, with Cr and Zn predominantly driving the effects of season as their confidence intervals do not bound zero (Table 2).

Effects of sex

Sex did not show a significant multivariate effect (Fig. 1; Wilk's $\lambda = 0.916$, $F_{12,456} = 1.694$, $p = 0.065$). There were significant sex effects for Cu ($F_{1,233} = 6.031$, $p = 0.003$) and Zn ($F_{1,233} = 3.059$, $p = 0.049$) but not for Cr ($F_{1,233} = 0.190$, $p = 0.827$), Pb ($F_{1,233} = 1.954$, $p = 0.144$), Se ($F_{1,233} = 0.178$, $p = 0.837$), or As ($F_{1,233} = 1.222$, $p = 0.296$). The only pair-wise effects we found were that females had lower levels of Cu than males (Mean diff. = −0.446, $p = 0.004$). However, although the effect is explaining enough variance to be included in the model, because the confidence intervals for the sex parameters are bounding zero (Table 2), it is providing no predictive power for any metal or metalloid.

Effects of state

State showed a significant multivariate effect (Fig. 1; Wilk's $\lambda = 0.771$, $F_{6,228} = 11.309$, $p \ll 0.001$). Significant differences were observed between states for Se (Table 2; $F_{1,233} = 16.801$, $p \ll 0.001$) and Zn (Table 2; $F_{1,233} = 55.498$, $p \ll 0.001$) but not for Cu ($F_{1,233} = 0.389$, $p = 0.533$), Cr ($F_{1,233} = 1.234$, $p = 0.268$), Pb ($F_{1,233} = 1.238$, $p = 0.267$), and As ($F_{1,233} = 2.726$, $p = 0.100$). Turtles from Tennessee had lower concentrations of both Se (Mean diff. = −0.849, $p \ll 0.001$) and Zn (Mean diff. = −1.118, $p \ll 0.001$) compared to turtles from Illinois. However, the predictive power of the state effect should only be attributed to Zn as the confidence intervals for the parameter bound zero for Se (Table 2).

Effects of age class

Age class did not show a significant multivariate effect (Fig. 1; Wilk's $\lambda = 0.983$, $F_{6,228} = 0.638$, $p = 0.669$). Although it explains some variance, we may lack the resolution to determine why it remained as a main effect in the most parsimonious model. Stage also lacks any predictive power in the model as all parameter estimates bound zero (Table 2).

Effects of year

Year showed a significant multivariate effect (Fig. 1; Wilk's $\lambda = 0.753$, $F_{6,228} = 12.454$, $p \ll 0.001$). Significant

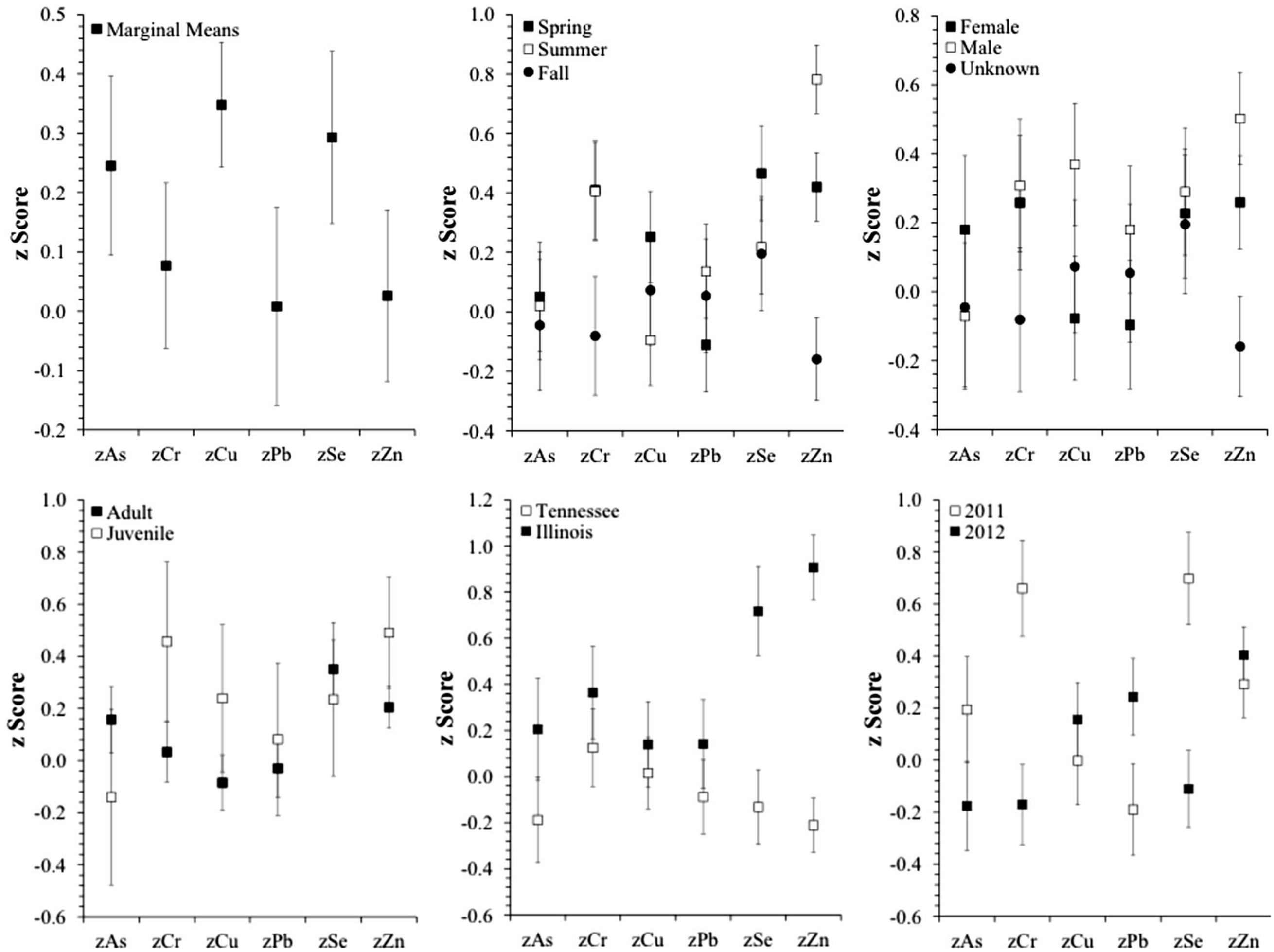

Fig. 1 Estimated marginal means for z-transformed metal and metalloid (mg/kg) variables in eastern box turtles (*Terrapene carolina carolina*) from the best fit multivariate model. Means were evaluated at z-transformed co-variate values of 0.0113 for mass, −0.0018 for white blood cell counts, and −0.0033 for total solids

differences between years were found for As (Table 2; $F_{1,233} = 4.610$, $p = 0.033$), Cr (Table 2; $F_{1,233} = 28.092$, $p \ll 0.001$), Pb (Table 2; $F_{1,233} = 8.304$, $p = 0.004$), Se (Table 2; $F_{1,233} = 28.738$, $p \ll 0.001$) but not for Cu ($F_{1,233} = 1.178$, $p = 0.279$) and Zn ($F_{1,233} = 1.056$, $p = 0.305$),. Turtles from 2011 had higher concentrations of As (Mean diff. = 0.372, $p = 0.033$), Cr (Mean diff. = 0.831, $p \ll 0.001$), and Se (Mean diff. = 0.809, $p \ll 0.001$) but lower concentrations of Pb (Mean diff. = −0.433, $p = 0.004$) than turtles in 2012. Yearly parameters for As, Cr, Pb, and Se all had good predictive power as their confidence intervals did not bound zero (Table 2).

Effects of mass

Mass did not show a significant multivariate effect (Fig. 2; Wilk's $\lambda = 0.972$, $F_{6,228} = 1.091$, $p = 0.368$). We also found no association between mass and any of the metal or metalloid variables (Fig. 2) as all mass parameters had large confidence intervals and bounded zero (Table 3).

Effects of white blood cell counts

WBC did not show a significant multivariate effect (Fig. 2; Wilk's $\lambda = 0.987$, $F_{6,228} = 0.503$, $p = 0.806$). Although it does appear that WBC might be associated with a few of the elements (Fig. 2), confidence intervals for WBC parameter were wide and bounded zero (Table 3).

Effects of total solids

TS showed a significant multivariate effect (Fig. 2; Wilk's $\lambda = 0.816$, $F_{6,228} = 8.549$, $p \ll 0.001$). We found TS values were associated with concentrations of Cu ($F_{1,233} = 48.692$, $p \ll 0.001$) and Zn ($F_{1,233} = 11.715$, $p = 0.001$) but not for Cr ($F_{1,233} = 0.198$, $p = 0.657$), Pb ($F_{1,233} = 0.594$, $p = 0.442$), Se ($F_{1,233} = 1.093$,

Table 2 Back-transformed estimated marginal means for significant metal and metalloid (mg/kg) variables in Eastern box turtles (*Terrapene carolina carolina*) by season, state, and year from the best fit multivariate model

Season	Mean	95 % CI	
		Lower	Upper
Cr			
Spring	0.152	0.121	0.182
Summer	0.151	0.121	0.182
Fall	0.105	0.068	0.142
Cu			
Spring	0.697	0.636	0.758
Summer	0.627	0.566	0.688
Fall	0.661	0.588	0.735
Zn			
Spring	2.136	1.941	2.332
Summer	2.447	2.252	2.642
Fall	1.639	1.404	1.875
State			
Se			
Tennessee	0.187	0.158	0.217
Illinois	0.266	0.231	0.301
Zn			
Tennessee	1.595	1.396	1.792
Illinois	2.554	2.317	2.791
Year			
As			
2011	0.033	0.005	0.061
2012	0.007	0.000	0.031
Cr			
2011	0.175	0.141	0.209
2012	0.097	0.068	0.126
Pb			
2011	0.003	0.000	0.009
2012	0.010	0.005	0.015
Se			
2011	0.264	0.232	0.296
2012	0.189	0.162	0.216

Means were evaluated at co-variate values at a mass of 377.79 g, white blood cell count of 9234 cells, and total solids of 3.52 g/dl. Sample sizes were spring = 102, summer = 101, fall = 70, Tennessee = 219, Illinois = 54, 2011 = 127, and 2012 = 146

$p = 0.297$), and As ($F_{1,233} = 0.002$, $p = 0.961$). The confidence intervals of the TS parameters for Cu and Zn were relatively narrow and positive suggesting an increase in TS with respect to these elements (Table 2). The parameter estimates for the remaining elements had poor predictive capacity with larger confidence intervals bounding zero (Table 3).

Discussion

Heavy metals and metalloids are ubiquitous in natural environments and contamination has led to population declines involving numerous taxa of free-ranging vertebrates (Davidson 2004; Friend and Franson 1999; Sparling et al. 2001). In recent years, reports of metals in reptiles have increased, but the impact on populations is unknown (Burger et al. 1998; Hays and McBee 2010; Yu et al. 2013), particularly in regard to terrestrial North America chelonians (Beresford et al. 1981).

Lead has been linked to wildlife population declines, including in reptiles (Grillitsch and Schiesari 2010). In the current study, there was only a significant effect observed between years, not for sex, age class, state, or season. Several surveys have been performed in chelonians in various habitats that provide reference to the current study. Spur-thighed tortoises (*Testudo graeca*) in Europe and Africa (0.06–0.12 µg/g) had similar concentrations of Pb in whole blood compared to the plasma of box turtles in the current study (Martinez-Lopez et al. 2010). But, box turtles in Missouri near a smelting operation had whole blood concentrations (0.11 µg/g) double the concentration of turtles in the Tennessee population of the current study (Beresford et al. 1981). Plasma concentrations from 97 Alligator Snapping Turtles (*Macrochelys temminckii*) in Georgia were all below the LOD (Chaffin et al. 2008). The variability seen between species highlights the need for more monitoring of lead in free-ranging populations and experimental studies that equate these results to effects on individuals.

Acute toxicity of Pb may include substantial hematological effects (Borkowski 1997; Friend and Franson 1999) and, therefore, subclinical toxicity may lead to subtle hematological changes that could have long-term effects. There were no significant associations between Pb and any health factor, although a trend towards a decrease in WBC was observed with increasing Pb. Similarly, other reports in free-ranging turtles observed a negative relationship between Pb and WBC (Yu et al. 2011). Reductions in WBC suggest a possible immunosuppressive effect, which has been supported by work in mallards (Rocke and Samuel 1991). Further experimental work is needed in turtles to determine the effect of increasing Pb concentrations on immune function.

Elevated levels of As, Cd, Cr, and Se have been proposed to be associated with coal ash (Hopkins et al. 1998). Despite the low values in this study, Se concentrations were significantly higher in plasma from turtles in Illinois compared to Tennessee. There were no differences in concentrations between turtles in Illinois and Tennessee for As or Cr, but the data were trending towards higher

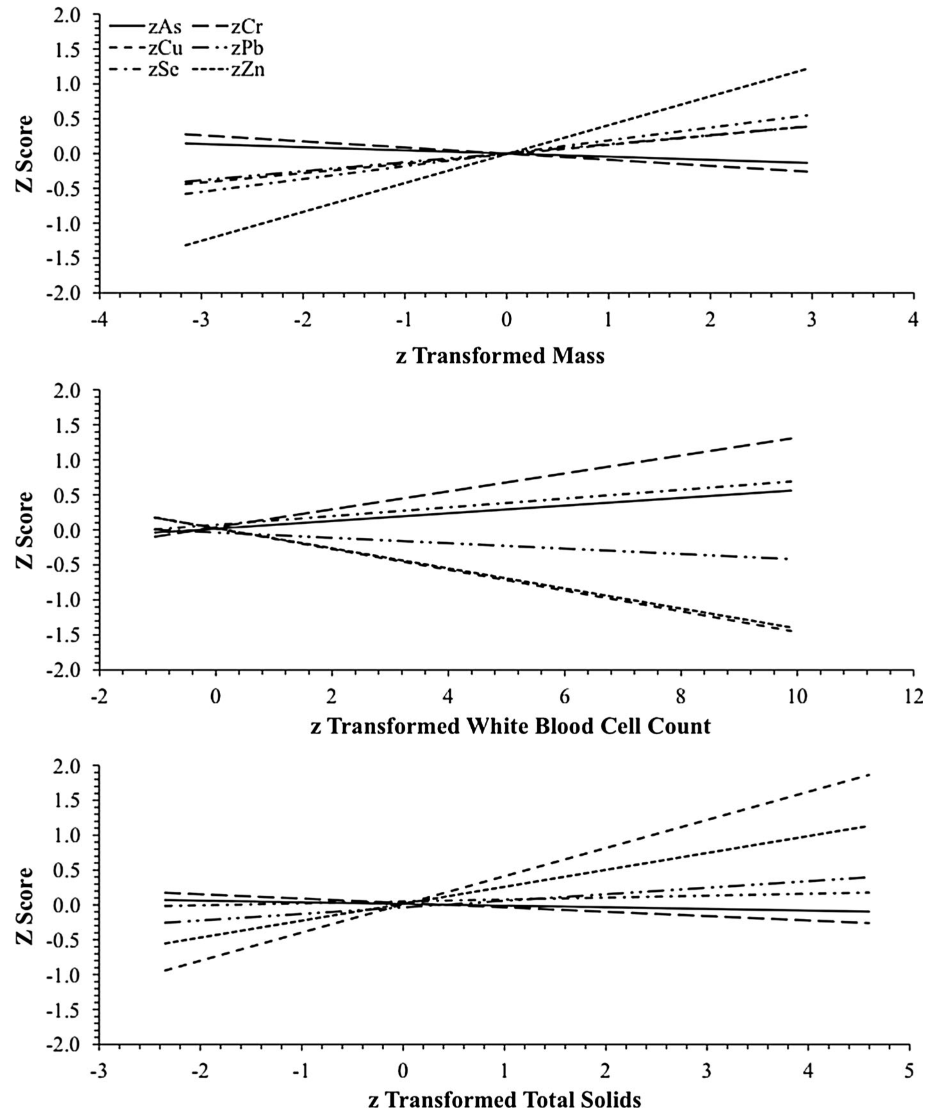

Fig. 2 Regression lines for the z-transformed covariates of mass, white blood cell counts, and total solids for z-transformed metal and metalloid (mg/kg) variables in eastern box turtles (*Terrapene carolina carolina*) from the best fit multivariate model

concentrations in Illinois. And Cd was below the LOD for both sites. While there is a paucity of information on contaminants of coal ash, specifically, As, Cd, and Se, in reptile literature, Alligator Snapping Turtles in Georgia had twice the concentration of both As (measured in whole blood) and Se (measured in serum) compared to the current study (Chaffin et al. 2008) and were not associated with coal ash contamination. Four species of Amazonian terrapins also had similar or higher Se concentrations (Burger et al. 2010). In addition to the potential direct toxicity, exposure to coal ash has been observed to cause sublethal effects in amphibians (Guthrie and Cherry 1979; Rowe et al. 1996; Hopkins et al. 1998). While no statistically significant differences were observed with health factors for any of these elements, both As and Cr demonstrated a trend for decreasing mass and TS with increasing concentration. Furthermore, all three elements (As, Cr, and Se) showed a positive relationship with WBC. Controlled experiments testing a range of concentrations on these potential sublethal effects on health are needed to elucidate if these trends are real.

Copper concentrations were higher in turtles in Illinois compared to Tennessee, with the highest concentration at two sites, both of which are in the Middle Fork watershed near the site of a retired coal–fired plant. Concentrations in the current study were greater than those found in Alligator Snapping Turtles in Georgia (0.342 μg/g plasma) (Chaffin et al. 2008). TS were significantly higher in association with greater Cu concentrations. Protein electrophoretic profiles were not pursued in the current study, but future studies should evaluate which protein fractions are driving this positive relationship.

Zinc concentrations in our study were lower than Zn concentrations in plasma of Alligator Snapping Turtles in

Table 3 Parameter estimates for the best GLM as determined by AIC for the plasma concentrations of metals and metalloids in the Eastern box turtle (*Terrapene carolina carolina*) populations in Tennessee and Illinois

Arsenic				Chromium			
Par.	Est.	SE	95 % CI	Par.	Est.	SE	95 % CI
Intercept	−0.274	0.370	−1.003, 0.455	Intercept	−0.242	0.335	−0.902, 0.418
β_{2011}	**0.372**	**0.173**	**0.031, 0.713**	**β_{2011}**	**0.831**	**0.157**	**0.522, 1.141**
β_{Spring}	0.096	0.188	−0.280, 0.467	**β_{Spring}**	**0.490**	**0.171**	**0.150, 0.826**
β_{Summer}	0.065	0.186	−0.300, 0.433	**β_{Summer}**	**0.486**	**0.169**	**0.150, 0.818**
β_{Ten}	−0.393	0.238	−0.860, 0.076	β_{Ten}	−0.239	0.215	−0.660, 0.185
β_{Female}	0.264	0.289	−0.300, 0.833	β_{Female}	0.091	0.261	−0.420, 0.606
β_{Male}	0.296	0.387	−0.520, 0.548	β_{Male}	−0.424	0.350	−0.340, 0.626
β_{Adult}	−0.151	0.100	−0.470, 1.058	β_{Adult}	−0.011	0.091	−1.110, 0.267
β_{zMass}	0.004	0.076	−0.350, 0.046	β_{zMass}	0.031	0.069	−0.190, 0.168
β_{zTS}	0.004	0.076	−0.150, 0.154	β_{zTS}	0.031	0.069	−0.110, 0.167
β_{zWBC}	−0.017	0.078	−0.170, 0.137	β_{zWBC}	0.037	0.070	−0.100, 0.176
Copper				Lead			
Intercept	0.238	0.310	−0.372, 0.848	Intercept	0.410	0.321	−0.222, 1.042
β_{2011}	−0.157	0.145	−0.443, 0.128	**β_{2011}**	**−0.433**	**0.150**	**−0.728, −0.137**
β_{Spring}	0.179	0.158	−0.130, 0.489	β_{Spring}	−0.165	0.163	−0.490, 0.156
β_{Summer}	−0.168	0.156	−0.480, 0.139	β_{Summer}	0.082	0.162	−0.240, 0.400
β_{Ten}	−0.124	0.199	−0.520, 0.268	β_{Ten}	−0.229	0.206	−0.640, 0.177
β_{Female}	−0.015	0.241	−0.490, 0.461	β_{Female}	−0.092	0.250	−0.580, 0.401
β_{Male}	−0.324	0.324	−0.020, 0.879	β_{Male}	−0.112	0.335	−0.280, 0.648
β_{Adult}	0.007	0.084	−0.960, 0.314	β_{Adult}	0.053	0.087	−0.770, 0.549
β_{zMass}	0.446	0.064	−0.106, 0.172	β_{zMass}	0.051	0.066	−0.120, 0.224
β_{zTS}	**0.446**	**0.064**	**0.320, 0.572**	β_{zTS}	0.051	0.066	−0.080, 0.182
β_{zWBC}	−0.057	0.065	−0.190, 0.071	β_{zWBC}	0.071	0.067	−0.060, 0.204
Selenium				**Zinc**			
Intercept	0.225	0.322	−0.410, 0.861	**Intercept**	**0.534**	**0.234**	**0.074, 0.994**
β_{2011}	**0.809**	**0.151**	**0.512, 1.106**	β_{2011}	−0.112	0.109	−0.328, 0.103
β_{Spring}	0.271	0.164	−0.050, 0.594	**β_{Spring}**	**0.579**	**0.119**	**0.340, 0.813**
β_{Summer}	0.023	0.162	−0.300, 0.343	**β_{Summer}**	**0.940**	**0.118**	**0.701, 1.172**
β_{Ten}	**−0.849**	**0.207**	**−1.260, −0.441**	**β_{Ten}**	**−1.118**	**0.150**	**−1.410, −0.822**
β_{Female}	−0.136	0.251	−0.630, 0.359	β_{Female}	−0.024	0.182	−0.380, 0.335
β_{Male}	0.117	0.337	−0.540, 0.394	β_{Male}	−0.286	0.244	−0.120, 0.556
β_{Adult}	0.092	0.087	−0.550, 0.781	β_{Adult}	0.106	0.063	−0.770, 0.195
β_{zMass}	0.070	0.067	−0.080, 0.264	β_{zMass}	0.165	0.048	−0.020, 0.231
β_{zTS}	0.070	0.067	−0.060, 0.201	**β_{zTS}**	**0.165**	**0.048**	**0.070, 0.260**
β_{zWBC}	−0.005	0.068	−0.140, 0.128	β_{zWBC}	0.044	0.049	−0.050, 0.141

Data is reported as mean, standard error, and 95 % confidence interval for each transformed element. Parameters are state, year, season, sex, age class, total white blood cell count (WBC), total solids (TS), and mass. The following parameters are classified as redundant, $\beta_{Ill.}$, β_{2012}, β_{Fall}, $\beta_{Unknown}$, and $\beta_{Juvenile}$. Parameters with confidence intervals not bounding zero are bolded

Georgia (6.56 μg/g) (Chaffin et al. 2008). Both Zn deficiency and toxicity have been reported to cause clinical signs in mammals, but little is known concerning Zn levels in reptiles (Fitzgerald and Vera 2006). The current study also observed increasing TS with increasing Zn concentrations, but also observed seasonal effects. Seasonal effects are not reported in previous studies of reptile or amphibians to the authors' knowledge. It is possible that seasonal changes in mobilizing Zn from bone are linked to reproductive cycling or changes in nutritional state. Future research should investigate these potential effects.

Establishing criteria of health in reptiles is complex, making correlations with concentrations of metals and metalloids difficult. Furthermore, investigating the health of reptiles in the context of environmental contamination is rarely pursued (Jacobson et al. 1991). In the current study, we utilized basic morphologic (mass) and hematologic (WBC, TS) variables as measures of health, which is commonly done but lacks specificity. However, the populations examined have been tested routinely (over 2100 captures in 7 years) and thus reference intervals are well established (Rose and Allender 2011) and consistent with the current study. While few statistically significant associations were observed, those that cause a potential decrease in WBC are most interesting (Cu, Pb, Zn), as this might be indicative of immune suppression. In Gopher Tortoises, these same hematologic (WBC, TS) variables were not affected by heavy metals (Cd, Cu, Fe, Hg, Pb, Se) (Jacobson et al. 1991). However, individual Gopher Tortoises in that study infected with *Mycoplasma* had higher concentrations of iron and mercury (Jacobson et al. 1991), thereby indicating that there may be sublethal effects of increasing metal concentrations not detected by hematology. If increases in these metals (Cu, Pb, and Zn) cause immunosuppression, then these animals may be more susceptible to infectious diseases, such as ranavirus. *Ranaviruses* have caused several mortality events in box turtles, and the mechanism of viral emergence is still unknown (Allender 2012). Future studies should continue to integrate ecotoxicology with health and disease for a more complete picture of the epidemiology. Furthermore, this study highlights that cross-sectional data may be important in guiding follow-up investigations into causality.

Age class and sex failed to explain any of the variation in element concentrations in this study. Accumulation of metals and metalloids likely occurs over long period of time resulting in the expectation that adults would have greater concentrations than juveniles. However, plasma concentrations may reflect mobilized elements rather than total body stores. Total body stores should be investigated, but prospective sampling of a declining wildlife species in this manner is not feasible. Therefore, opportunistic sampling should be encouraged to answer these questions. The lack of sex differences are not surprising, but total body stores of metals and metalloids that mobilize in fat or bone may be expected to be lower in females that deposit these compounds in eggs. This needs to be tested in experimental models.

In conclusion, box turtles may serve as important monitors of environmental contamination. Metals and metalloids are serious threats to human, domestic animal, and wildlife health and utilizing a widespread terrestrial species may aid in identifying the complexity with both spatial and temporal variations in contaminants. It is critical that future studies not only report the concentrations of contaminants, but relate them to animal health, and ideally in the context of ecosystem health. This reporting requires collaborations among biologists, ecologists, chemists, veterinarians, and industry leaders to develop long-term plans that sustain or restore ecosystem quality.

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Conflict of interest The authors declare that they have no conflict of interest.

Statement of Human and Animal Rights All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

Informed Consent There were no human subjects, thus informed consent is not applicable for this study.

References

Allender MC (2012) Characterizing the epidemiology of *Ranavirus* in North American chelonians: diagnosis, surveillance, pathogenesis, and Therapy. PhD Dissertation, University of Illinois

Beresford WA, Donovan MP, Henninger JM, Waalkes MP (1981) Lead in the bone and soft tissues of box turtles caught near smelters. Bull Environ Contam Toxicol 27:349–352

Borkowski R (1997) Lead poisoning and intestinal perforations in a snapping turtle (Chelydra serpentine) due to fishing gear ingestion. J Zoo Wildl Med 28:109–113

Burger J, Carruth-Hinchey C, Ondroff J, McMahon M, Gibbons JW, Gochfeld M (1998) Effects of lead on behavior, growth, and survival of hatchling slider turtles. J Toxicol Environm Health Part A 55:495–502

Burger J, Jeitner C, Schneider L, Vogt R, Gochfeld M (2010) Arsenic, cadmium, chromium, lead, mercury, and selenium levels in blood of four species on turtles from the Amazon in Brazil. J Toxicol Environ Health Part A 73:33–40

Burnham KP, Anderson DR (1998) Model selection and inference: a practical information-theoretic approach. Springer, New York

Chaffin K, Norton TM, Gilardi K, Poppenga R, Jensen JB, Moler P, Cray C, Dierenfeld ES, Chen T, Oliva M, Origgi FC, Gibbs S, Mazarro L, Mazet J (2008) Health assessment of free-ranging alligator snapping turtles (*Macrochelys temminickii*) in Georgia and Florida. J Wildl Dis 44:670–686

Davidson C (2004) Declining downwind: amphibian population declines in California and historical pesticide use. Ecol Appl 14:1892–1902

Dodd CK (2001) North American box turtles: a natural history. University of Oklahoma Press, Norman

Fitzgerald K, Vera R (2006) Reported toxicities in reptiles. In: Mader D (ed) Reptile Medicine and Surgery. Elsevier Publishing, St. Louis, pp 1068–1080

Friend M, Franson JC (1999) Lead. In: Ciganovich (ed) Field manual of wildlife diseases. USGS Biology Resources Division, Washington, pp 317–334

Gibbons JW, Scott DE, Ryan TJ, Buhlmann KA, Tuberville TD, Metts BS, Greene JL, Mills T, Leiden Y, Poppy S, Winne CT (2000) The global decline of reptiles, Deja Vu Amphibians. Bioscience 50:653–666

Golden NH, Rattner BA (2003) Ranking terrestrial vertebrate species for utility in biomonitoring and vulnerability to environmental contaminants. Rev Environ Contam Toxicol 176:67–136

Grillitsch B, Schiesari L (2010) The ecotoxicology of metals in reptiles. In: Sparling D, Linder G, Bishop CA, Krest SK (eds) Ecotoxicology of Amphibians and Reptiles, 2nd edn. CRC Press, Boca Raton, pp 337–448

Guthrie RK, Cherry DS (1979) Trophic level accumulation of heavy metals in a coal ash basin drainage system. Water Res Bull 25:244–248

Harmata AR, Restani M (2013) Lead, mercury, selenium, and other trace elements in tissues of golden eagles from southwestern Montanta, USA. J Wildl Dis 49:114–124

Hays KA, McBee K (2010) Population demographics of red-eared slider turtles (*Trachemys scripta*) from the Tar Creek superfund site. J Herpetol 44:441–446

Hopkins WA, Mendonca MT, Rowe CL, Congdon JD (1998) Elevated trace element concentrations in southern toads, *Bufo terrestris*, exposed to coal combustion waste. Arch Environ Contam Toxicol 35:325–329

Jacobson ER, Gaskin JM, Brown MB, Harris RK, Gardiner CH, LaPointe JL, Adams HP, Reggiardo C (1991) Chronic upper respiratory tract disease of free-ranging desert tortoises (*Xerobates agassizii*). J Wildl Dis 27:296–316

Ley-Quinonez C, Zavala-Norzagaray AA, Espinosa-Carreon TL, Peckham H, Marquez-Herrera C, Campos-Villegas L, Aguirre AA (2011) Baseline heavy metals and metalloid values in blood of loggerhead turtles (*Caretta caretta*) from Baja California Sur, Mexico. Mar Pollut Bull 62:1979–1983

Martinez-Lopez E, Sousa AR, Maria-Mojica P, Gomez-Ramirez P, Guilhermino L, Garcia-Fernandez AJ (2010) Blood δ-ALAD, lead, and cadmium concentrations in spur-thighed tortoises (*Testudo graeca*) from southeastern Spain and northern Africa. Ecotoxicology 19:670–677

Pellalo-Martinez NA, Ilizaliturri-Hernandez CA, Espinosa-Reyes G, Carrizales-Yanez L, Gonzalez-Mille DJ (2011) Assessment of exposure to lead in humans and turtles living in an industrial site in Coatzacoalcos Veracruz, Mexico. Bull Environ Contam Toxicol 86:642–645

Rocke TE, Samuel MD (1991) Effects of lead shot ingestion on selected cells of the mallard immune system. J Wildl Dis 27:1–9

Rose BMR, Allender MC (2011) Health assessment of wild Eastern box turtles (Terrapene carolina carolina) in East Tennessee. J Herpetol Med Surg 21:107–112

Rowe CL, Kinney OM, Fiori AP, Congdon JD (1996) Oral deformities in tadpoles (*Rana catesbeiana*) associated with coal ash deposition: effects on grazing ability and growth. Freshw Biol 36:723–730

Sleeman J (2008) Use of wildlife rehabilitation centers as monitors of ecosystem health. In: Fowler ME, Miller RE (eds) Zoo and wild animal medicine, current therapy 6. Saunders Elsevier, St. Louis, pp 97–104

Smith PN, Cobb GP, Godard-Codding C, Hoff D, McMurry ST, Rainwater TR, Reynolds KD (2007) Contaminant exposure in terrestrial vertebrates. Environ Poll 150:41–64

Sparling DW, Fellers GM, McConnell LL (2001) Pesticides and amphibian population declines in California, USA. Environ Toxicol Chem 20:1591–1595

Van Dijk PP (2011) Terrapene carolina. IUCN red list of threatened species. IUCN, Cambridge

Yu S, Halbrook RS, Sparling DW, Colombo R (2011) Metal accumulation and evaluation of effects in a freshwater turtle. Ecotoxicology 20:1801–1812

Yu S, Halbrook RS, Sparling DW (2013) Correlation between heavy metals and turtle abundance in ponds near the Paducah gaseous diffusion plant, Kentucky USA. Arch Environ Contam Toxicol 65:555–566