Environmental Pollution 194 (2014) 38–49

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Maternal transfer and embryonic assimilation of trace elements in freshwater turtles after remediation of a coal fly-ash spill

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ARTICLE INFO

Article history:
Received 6 April 2014
Received in revised form
16 June 2014
Accepted 1 July 2014
Available online

Keywords:
Bioaccumulation
Nondestructive sampling
Maternal effects
Egg production
Embryogenesis

ABSTRACT

Oviparous vertebrates maternally transfer elements to their offspring during egg production. Maternal transfer occurs because elements mimic, or are incorporated into, nutrients allocated to eggs, but likely differs among species depending on the quantities of specific nutrients allocated to eggs. Developing embryos are often assumed to assimilate all of the elements allocated to eggs, but this assumption has rarely been tested. We tested the hypothesis that maternal transfer and embryonic assimilation of trace elements differed between two species of freshwater turtles exposed to a recently-remediated coal fly-ash spill. *Sternotherus odoratus* transferred As, Se, and Zn, while *Trachemys scripta* transferred As, Hg, Se, Sr, and Zn. Logarithmic non-linear relationships between hatchling and egg concentrations indicated that turtles partially assimilated elements present in eggs. In systems contaminated with multiple trace elements, our data show that maternal transfer and embryonic assimilation are element- and species-specific, and may be inconsistent even among closely-related species.

1. Introduction

Maternal transfer is the primary route of exposure to bioaccumulative metals, metalloids, and trace elements (collectively referred to as trace elements for simplicity hereafter) in developing embryos of oviparous vertebrates (di Giulio and Tillitt, 1999). In oviparous amniote vertebrates (birds and most reptiles), trace elements are primarily transferred to offspring during egg formation, via vitellogenesis (yolk production), albumin deposition, or shell deposition (reviewed by Van Dyke et al., 2013a). Maternal transfer often occurs because trace elements have similar biochemical properties to essential elements with the same core charge. For example, Sr can replace Ca directly in biological tissues, while Se can replace S in the structure of some amino acids, like cysteine (reviewed by Van Dyke et al., 2013a). Thus, maternal transfer occurs because trace elements replace, or are incorporated into, essential nutrients that must be transferred to eggs to nourish developing offspring. However, maternal transfer also differs among trace elements (Chin et al., 2013; Guirlet et al., 2008; Hopkins et al., 2013a; Nagle et al., 2001; Páez-Osuna et al., 2010; Xu et al., 2006) because different trace elements replace or are incorporated into different nutrients. As a result, whereas the mechanisms of egg production, such as vitellogenesis, are conserved across oviparous amniotes, among-species differences in maternal transfer might be expected due to among-species differences in the proportions of specific nutrients allocated to eggs (Speake and Thompson, 1999, 2000; Thompson et al., 1999). Understanding these sources of variation in maternal transfer is therefore fundamental to determining which species inhabiting a contaminated area are likely to suffer reproductive consequences (Van Dyke et al., 2013a).

During development, embryos are usually assumed to assimilate maternally-transferred trace elements as they assimilate nutrients from yolk, albumin, and components of eggshell during embryogenesis. To our knowledge, only one study has examined whether embryos assimilate all of the trace elements present in eggs. Green sea turtle (*Chelonia mydas*) embryos from Malaysia bioaccumulated Cr, Cu, Mn, Se, and Zn from yolk, but the assimilation efficiency differed among elements (Ikonomopoulou et al., 2013). Hatchling and egg concentrations of Cr, Mn, and Zn were approximately equal, while hatchling concentrations of Cu were twice those of eggs, and hatchling concentrations of Se were 1.5 times greater than those of eggs (Ikonomopoulou et al., 2013).

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http://dx.doi.org/10.1016/j.envpol.2014.07.005
0269-7491/

These data suggest that embryos may differentially assimilate maternally-transferred trace elements from yolk and albumin, which is a potentially overlooked source of variation in studies of ecotoxicological effects on reproduction. Additional studies are needed to determine whether this pattern is widespread in oviparous vertebrates, and if so, to identify whether selective assimilation (i.e., selective uptake or excretion by embryos) is the cause.

In the current study we quantified concentrations of 13 trace elements in the blood and claws of adults as well as the eggs and hatchlings of two species of freshwater turtles exposed to the 2008 Kingston, TN coal fly-ash spill. In December, 2008, the Kingston Fossil Plant accidentally discharged over 4 million m^3 of coal fly-ash into the Emory-Clinch-Tennessee River system in eastern Tennessee (TVA, 2009). Remediation efforts subsequently removed most of the ash by May, 2010 (~1 year prior to our study), but 400,000 m^3 of ash still remained in the system (Yankee et al., 2011) and trace elements in the ash may enter local food webs. We used this event as an opportunity to test three hypotheses regarding maternal transfer and embryonic assimilation of fly ash-derived trace elements. First, we examined maternal claw-hatchling and maternal blood-hatchling trace element concentration relationships to determine whether either maternal blood or claw concentrations were correlated with hatchling concentration, and thus indicated whether trace elements were maternally transferred to offspring in a concentration-dependent manner. Second, we compared maternal claw-hatchling and maternal blood-hatchling relationships between species to determine whether maternal transfer, based on these indices, differed. Third, we compared egg-hatchling trace element relationships between species to determine whether embryos of either species differentially assimilated trace elements from yolk. This analysis also allowed us to test whether hatchlings can be used for future maternal transfer analysis, rather than eggs, so that entire clutches can be incubated to examine hatching success. For maternal transfer analyses, we focused on blood and claws because they have been commonly used to develop nondestructive indices of trace element bioaccumulation in vertebrates (e.g., Bearhop et al., 2003; Hopkins et al., 2013b, 2007). Blood typically represents a snapshot of trace element exposure, while claws represent integrated exposure over long periods of time (~12 months; Aresco, 2005; Hopkins et al., 2013b). We focused on turtles because they are long lived, relatively sedentary, and often feed in association with the benthos (Ernst and Lovich, 2009), and are therefore particularly likely to bioaccumulate trace elements in areas affected by large-scale industrial spills. In addition, turtles produce all eggs within a clutch simultaneously and as a result have low within-clutch variance in maternally transferred trace elements, making them useful for comparing eggs and hatchlings from a single clutch (Van Dyke et al., 2013a).

2. Methods

2.1. Sample collection

From April–July 2011 and 2012, we trapped turtles in the vicinity of the Kingston, TN Fossil Plant using hoop traps baited with sardines and/or chicken. We set traps in shallow-water areas (<1 m deep) in microhabitats suitable for turtles. We concentrated trapping among sections of the Emory (river km 0–5.5) and Clinch (river km 3–7) Rivers impacted by the coal fly ash spill (impacted), and within a section of the Tennessee River (river km 914–922) that was not impacted by the spill (reference; Fig. 1). Throughout the study, we maintained at least 15 traps at various sites along both the Emory and Clinch Rivers, and 15–20 traps at sites along the Tennessee River (45–50 total traps per day). We documented trap locations using GPS. We rebaited traps every 3 days, and rotated them among trapping locations depending upon trapping success. We collected turtles from traps once per day. Throughout the study area, we targeted gravid *Sternotherus odoratus* and *Trachemys scripta*, because *S. odoratus* is a benthic forager and is likely to ingest sessile fly ash directly, and *T. scripta* is by far the most commonly trapped turtle in the system. Prior data also suggest that their diets may overlap (Van Dyke et al., 2013b). Both species exhibit high site fidelity, and rarely travel more than 400 m among points of capture (Ernst, 1986; Schubauer et al., 1990), so turtles inhabiting areas impacted by a spill are likely to forage exclusively in the impacted area.

We processed turtles at a laboratory facility in Kingston, TN. We determined whether female turtles were gravid via palpation and/or x-ray radiography (Ecoray Ultralight 9020 HF). We weighed gravid female turtles with Pesola® scales (Baar, Switzerland) and then induced oviposition via subcutaneous injection of 20 mg/kg of oxytocin dissolved in deionized water (Ewert and Legler, 1978; Tucker et al., 2007). We placed injected females in plastic tubs with ~2 cm of dechlorinated water (Ewert and Legler, 1978), and placed the tubs in a dark room at ~25 °C. We checked females for deposited eggs every 2 h. When eggs were present, we gently dried and weighed them to the nearest 0.01 g. In 2011, we froze one egg from each clutch at −20 °C. We labeled all other eggs laid in 2011 and 2012 with their maternal ID and egg number, and incubated all eggs in hovobators at 25 °C with a substrate of 1:1 vermiculite:water (by mass). Upon hatching, we weighed and measured all hatchling turtles, and euthanized the first turtle to hatch via an overdose of isoflurane or MS-222. Other hatchlings were eventually released at the point of maternal capture. Because we previously demonstrated that within-clutch variation in concentrations of trace elements is low (Van Dyke et al., 2013a), we paired the single egg and hatchling from each clutch to examine the bioaccumulation of egg trace elements by embryos.

Once turtles had laid all of the eggs we had counted via initial x-rays, we palpated and x-rayed females again to ensure they had deposited all eggs in their clutches. We then measured carapace length (cm), carapace width (cm), and plastron length (cm) of females using forestry calipers. We removed the tips (top 2–3 mm) of all claws on the right rear foot (if present) from turtles for trace element analysis. We sampled blood (0.5–1.0 ml) from the cervical sinus for trace element analysis, using heparinized 1-ml tuberculin syringes fitted with 26.5-gauge hypodermic needles. After sampling, we released female turtles at the site of capture.

We froze and stored all blood, claw, egg, and hatchling samples at −20 °C. Prior to further manipulation, we removed eggshells from eggs and homogenized egg contents (yolk and albumin) by vortexing samples with Teflon beads. We freeze-dried all egg, claw, and hatchling samples to asymptotic mass. We homogenized hatchling turtles by grinding them with mortars and pestles while the hatchlings were submerged in liquid nitrogen. We then freeze-dried hatchling samples again to remove any water that condensed on supercooled samples during homogenization. All samples were shipped to Dartmouth College for trace element analysis.

2.2. Trace elements analysis

We quantified concentrations of As, Ba, Cd, Cr, Cu, Fe, Mn, Hg, Se, Sr, Tl, V, and Zn in blood, claw, egg, and hatchlings using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) at the Trace Element Analysis Core at Dartmouth College. Prior to analysis, claws were washed in Triton X-100, rinsed in distilled water, and dried to remove external contamination. Claw, egg, and hatchling samples were weighed into a pre-weighed VWR trace metal clean polypropylene centrifuge tube and 0.5 ml of 9:1 HNO_3:HCl (Optima Grade, Fisher Scientific) was added. Individual egg and hatchling subsample weights were variable but were generally <0.05 g.

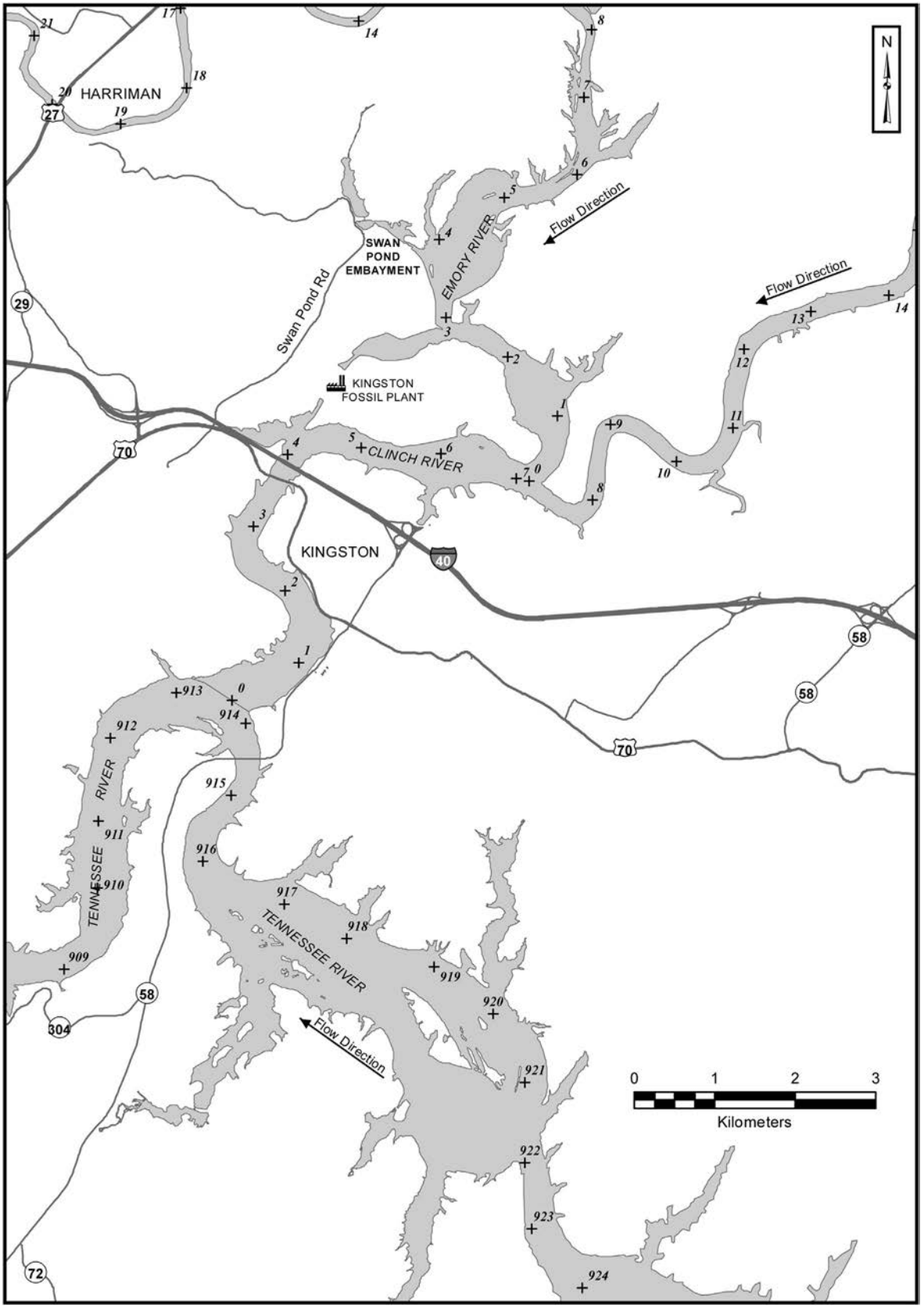

Fig. 1. Map of the turtle sampling area near Kingston, TN. Note that the Emory River is a tributary of the Clinch River, which is itself a tributary of the Tennessee River. Markers (+) and associated numbers indicate river kilometers, which are reported as distances from the downstream terminus of each river. The coal fly-ash spill of 22 December 2008 occurred at approximately Emory River kilometer 4, in and around the Swan Pond Embayment, which flows into the Emory River proper. All of the sampling reported in this study occurred from Emory River kilometers 5.5-0 (Impacted), Clinch River kilometers 7-3 (Impacted), and Tennessee River kilometers 914-922 (Reference).

Tissue samples were prepared for acid digestion in batches of 100 samples along with five each of blank, certified reference material, and fortified blank quality control samples. We also digested and analyzed matrix duplicates and matrix duplicate spikes at a frequency of 1 duplicate and spike for each 20 samples. All tubes were lightly capped and placed into a CEM MARS Express (Mathews, NC) microwave digestion unit for an open vessel digestion. A fiber optic temperature probe was placed into one of the sample tubes to provide temperature feedback to the MARS unit and the samples were heated to 95 °C with a 15-minute ramp to temperature, held at 95 °C for 45 min, then were allowed to cool. Following initial digestion, 0.1 ml of H_2O_2 (Optima Grade, Fisher Scientific) was added and the samples were taken through another microwave heating program. The samples were then brought up to 10 ml with deionized water (Element QPod, Millipore, Billarica, MA). All measurements were recorded gravimetrically.

Digested samples were analyzed by collision cell ICP-MS (7700x, Agilent, Santa Clara, CA). Selenium (78) was measured in hydrogen mode (2.8 ml min-1), Hg, Pb and Tl in no gas mode and all other analytes in He mode (4.8 ml min-1). Analytical procedures followed the general protocols outlined in EPA 6020A; the instrument was calibrated with NIST-traceable standards and calibration was verified with a second source traceable standard.

Initial batches of blood samples were prepared by dilution with 1% TMAOH (Tetramethylammonium hydroxide), 4% butanol, 0.1% EDTA, 0.1% Triton X-100, 0.2% HCl (50X dilution, 200 μl sample, 9.8 ml of diluent). Later batches were prepared by acid digestion (0.5 ml of 9:1 optima HNO_3:HCL to 0.25 ml of sample, heated in an open vessel microwave digestion at 100 °C for 30 min). We then added 200 ul of H_2O_2 and heated the sample again at 100 °C for 20 min. Following digestion, the sample was diluted to 10 ml and analyzed by ICP-MS as described above for claw, hatchling and egg samples, with identical sample and analysis quality control. In general, sample masses for digestion were quite low (ca. 50 mg or less), and all samples appeared to be completely digested, regardless of tissue type.

Ideal reference materials do not exist for every biological sample type. For this study we chose the Japanese National Institute of Environmental Studies (NIES) Human Hair reference material #13 to validate the claw analysis, DORM 3 (National Research Council of Canada, Ottawa, Canada) and Oyster 1566b (National Institute of Standards and Technology, Gaithersburg, USA) to validate the hatchling and egg data and Seronorm level 1 and level 2 whole blood reference materials (Seronorm, Billingstad, Norway) to validate the whole blood analysis. Aliquots of these reference materials were digested and analyzed with the sample batches over a two year period. The number, type, certified value, % recovery and CV for these analyses are shown in Table S1 (supplementary material). In general recoveries were good, being within 90–110% of the certified value and precision was also good with coefficients of variation being generally ca. 10% or less. This level of accuracy and precision was typical of the sample digestion duplicates and spikes.

Detection limits for each tissue sample varied because the mass of each tissue sample used in the analysis varied. If the trace element concentration was below the detection limit, for statistical comparisons we assigned that sample a concentration of half of the detection limit. Trace elements whose concentrations were below detectable limits (BDL) in ≥50% of samples were excluded from analysis. If less than 50% of samples were BDL, then we designated 50% of the detection limit as the concentration for samples that registered BDL.

2.3. Statistical analysis

To test for maternal transfer of trace elements, we examined relationships between both maternal claw and maternal blood trace element concentrations with those of hatchlings using individual ANCOVA models for each element, where maternal claw (or blood) concentration was the covariate, species was a fixed effect, and hatchling concentration was the dependent variable. We used a similar approach to examine relationships between hatchling and egg trace element concentrations, where egg trace element concentration was the covariate. In all statistical tests, we used Shapiro–Wilk Tests and normal probability plots to assess normality and homoscedasticity of variance, respectively. We log-transformed all data to improve normality and homoscedasticity of variance, and to linearize relationships. All statistical tests were performed using SAS (ver. 9.3, SAS Institute, Cary, NC). Significance was judged at $\alpha = 0.05$.

3. Results

In total, we sampled claws, blood, and hatchlings from 75 *Sternotherus odoratus* and 85 *Trachemys scripta*. Of these 160 individuals, we also sampled eggs from 29 *S. odoratus* and 51 *T. scripta*. Cadmium, Cr, Tl, and V concentrations were below detection limits (BDL) in more than 50% of both blood and claw samples in both species. These elements were therefore dropped from further statistical analysis. In addition, Cd, Cr, Hg, and V concentrations were BDL in more than 50% of eggs in each species; therefore we did not compare the relationships of egg and hatchling concentrations of these elements between species. Mean tissue concentrations for each trace element are reported in Table 1. Across the study, clutch sizes averaged 3.25 ± 0.16 for *S. odoratus* and 9.41 ± 0.37 for *T. scripta*.

3.1. Maternal and hatchling concentration relationships

Relationships between hatchling and maternal trace element concentrations varied in slope depending on trace element, species, and maternal tissue. In *S. odoratus*, hatchling concentrations of As, Se, and Zn all increased with maternal claw concentrations (Table 2; Fig. 2). In *T. scripta*, hatchling concentrations of As, Hg, Se, Sr, and Zn increased with maternal claw concentration (Table 2; Fig. 2). Although hatchling As, Se, and Zn concentrations increased with maternal concentrations in both species, the slopes of these relationships were significantly greater in *T. scripta* than in *S. odoratus* (Table 2). Hatchling concentrations of Ba, Cu, and Mn did not covary with maternal claw concentrations, but did differ between *S. odoratus* and *T. scripta* (Table 2). Hatchling Ba concentrations were significantly higher in *S. odoratus* than in *T. scripta* ($P < 0.001$; Table 2; Fig. 2), while hatchling Cu and Mn concentrations were significantly higher in *T. scripta* than in *S. odoratus* (Cu: $P < 0.001$; Mn: $P < 0.001$; Table 2; Fig. 2). Hatchling Fe concentrations did not covary significantly with maternal claw concentration ($P = 0.340$), and did not differ between species ($P = 0.657$; Table 2).

Hatchling concentrations of As, Hg, Se, and Sr increased with maternal blood concentrations in both *S. odoratus* and *T. scripta* (Table 3; Fig. 3). The slopes of relationships did not differ between species for As, Hg, or Se. However, the slope of the relationship between Sr concentrations in maternal blood and hatchlings was significantly higher in *T. scripta* than in *S. odoratus* ($P = 0.030$; Table 3). Hatchling concentrations of Ba, Cu, Mn, and Zn did not covary with maternal blood concentrations, but did differ between *S. odoratus* and *T. scripta* (Table 3; Fig. 3). Barium and Zn concentrations were significantly higher in *S. odoratus* hatchlings than in *T. scripta* hatchlings (Ba: $P < 0.001$; Zn: $P < 0.001$; Table 3; Fig. 3), while Cu and Mn concentrations were significantly higher in *T. scripta* than in *S. odoratus* hatchlings (Cu: $P < 0.001$; Zn: $P = 0.006$; Table 3; Fig. 3). Hatchling Fe concentrations did not

Table 1
Mean trace element concentrations of maternal claws, maternal blood, eggs, and hatchlings of *S. odoratus* and *T. scripta* from the vicinity of a coal fly-ash spill. All trace elements that were below detectable limits are listed as BDL. "Impacted" refers to samples collected from turtles from the Emory and Clinch Rivers, while "Reference" refers to samples collected from turtles from the Tennessee River (Fig. 1). Across all tissues, detection limits (mg/kg) were as follows: As: 0.022 ± 0.001; Ba: 0.032 ± 0.003; Cu: 0.159 ± 0.010; Fe: 5.803 ± 0.331; Hg: 0.105 ± 0.007; Mn: 0.060 ± 0.005; Se: 0.092 ± 0.008; Sr: 0.024 ± 0.002; Tl: 0.006 ± 0.001; Zn: 1.053 ± 0.092.

Species	Site	As	Ba	Cu	Fe	Hg	Mn	Se	Sr	Tl	Zn
Claw trace element concentrations (mg/kg dry mass)											
S. odoratus	Impacted	0.815	22.216	2.203	202.981	0.652	10.653	1.258	22.164	BDL	514.703
	±SE	0.059	3.540	0.336	57.980	0.059	2.291	0.054	3.682		10.773
	Reference	0.271	24.490	1.983	158.939	0.560	26.600	0.488	25.810	BDL	513.282
	±SE	0.029	5.403	0.273	30.715	0.081	16.232	0.035	5.691		26.317
T. scripta	Impacted	2.790	0.833	1.460	52.334	1.694	2.685	0.939	0.489	BDL	317.219
	±SE	0.573	0.144	0.154	8.661	0.152	0.915	0.061	0.173		7.388
	Reference	0.431	0.868	1.148	86.603	1.179	1.792	0.436	0.820	BDL	322.398
	±SE	0.043	0.181	0.169	20.872	0.157	0.308	0.020	0.372		8.740
Blood trace element concentrations (mg/kg dry mass)											
S. odoratus	Impacted	1.864E-02	0.414	0.485	168.555	0.321	2.370E-02	0.547	0.376	BDL	3.724
	±SE	1.807E-03	0.044	0.022	9.882	0.042	1.594E-03	0.035	0.029		0.169
	Reference	7.183E-03	0.325	0.526	167.200	0.275	1.448E-02	0.309	0.324	BDL	3.875
	±SE	1.105E-03	0.036	0.026	14.578	0.033	2.111E-03	0.021	0.023		0.187
T. scripta	Impacted	1.556E-02	0.082	0.346	98.597	0.041	3.458E-02	0.201	0.200	BDL	1.903
	±SE	2.638E-03	0.009	0.023	9.063	0.005	5.087E-03	0.017	0.022		0.149
	Reference	4.493E-03	0.087	0.302	96.636	0.041	1.814E-02	0.121	0.348	BDL	1.866
	±SE	8.005E-04	0.011	0.026	11.199	0.005	3.506E-03	0.014	0.045		0.200
Egg trace element concentrations (mg/kg dry mass)											
S. odoratus	Impacted	0.114	41.885	2.998	68.189	BDL	1.059	3.220	11.489	7.868E-02	80.148
	±SE	0.014	5.292	0.184	2.478		0.097	0.227	0.748	9.783E-03	4.580
	Reference	0.060	29.488	3.160	66.132	BDL	0.888	1.749	11.574	1.990E-02	85.259
	±SE	0.008	3.305	0.266	2.543		0.060	0.115	1.119	3.923E-03	4.321
T. scripta	Impacted	0.138	10.685	3.582	36.637	BDL	1.448	2.780	9.466	5.679E-02	45.048
	±SE	0.038	1.091	0.163	1.288		0.125	0.141	1.047	1.088E-02	2.383
	Reference	0.033	11.926	3.111	37.532	BDL	1.303	1.890	13.161	1.185E-02	43.153
	±SE	0.003	1.263	0.211	2.838		0.130	0.092	2.148	9.718E-04	3.972
Hatchling trace element concentrations (mg/kg dry mass)											
S. odoratus	Impacted	4.912E-02	57.262	3.174	98.948	2.302	5.484E-02	3.372	32.609	0.109	110.441
	±SE	3.598E-03	3.639	0.103	3.882	0.273	3.511E-03	0.104	0.921	0.014	1.327
	Reference	2.672E-02	43.256	2.881	92.193	1.682	4.775E-02	1.872	27.387	0.031	108.861
	±SE	3.015E-03	3.539	0.116	2.158	0.132	5.584E-03	0.057	1.548	0.007	1.899
T. scripta	Impacted	3.861E-02	16.075	3.909	171.569	4.183	1.239E-01	2.391	13.892	0.081	61.053
	±SE	7.140E-03	0.864	0.102	14.588	0.249	1.133E-02	0.125	0.951	0.011	1.044
	Reference	1.365E-02	19.579	3.971	190.198	4.092	9.309E-02	1.424	25.732	0.022	60.684
	±SE	1.072E-03	1.616	0.283	20.651	0.335	1.386E-02	0.054	3.464	0.011	1.558

covary with maternal blood concentrations ($P = 0.562$), and did not differ between species ($P = 0.152$; Table 3).

Overall r-square values were qualitatively higher and indicated better goodness-of-fit for claw-hatchling concentration relationships than for blood-hatchling concentration relationships in As (*T. scripta*), Hg (*T. scripta*), Se (both species), and Zn (both species; Fig. 2; Fig. 3). In contrast, the r-square value of the blood-hatchling relationships for Hg (*S. odoratus*) and Sr (both species) were higher and indicated better goodness-of-fit than did the r-square values of the claw-hatchling relationships (Fig. 2; Fig. 3). R-square values were similar for both relationships in As (*S. odoratus*; Fig. 2; Fig. 3). In addition, slopes of relationships between maternal claw and hatchling concentrations were greater than those of relationships between maternal blood and hatchling concentrations in As (both species), Hg (*T. scripta*), Se (both species), and Zn (both species; Tables 2; Table 3). Slopes of relationships between maternal blood and hatchling concentrations were greater than those of relationships between maternal claw and hatchling concentrations in Hg (*S. odoratus*) and Sr (both species; Table 2; Table 3). In all cases (all elements and both species), these slopes were less than 1.

3.2. Egg-hatchling relationships

Hatchling concentrations of As, Ba, Mn, Se, Sr, and Tl increased with egg concentrations in both *S. odoratus* and *T. scripta* (Table 4; Fig. 4). Slopes of these relationships did not differ between species for As, Mn, Sr, or Zn, but the slope of the relationship for Ba was significantly greater in *S. odoratus* ($P = 0.001$; Table 4), while the slope of the relationship for Se was significantly greater in *T. scripta* ($P = 0.012$; Table 4). Relative to egg concentrations, hatchling Mn concentrations were significantly higher in *T. scripta* than in *S. odoratus* ($P < 0.001$; Table 4; Fig. 4). In As (both species), Ba (*T. scripta*), Mn (both species), Se (*S. odoratus*), Sr (both species), and Tl (both species), slopes of egg-hatchling LOG-transformed concentration relationships were less than 1 (Table 4). In contrast, Ba (*S. odoratus*) and Se (*T. scripta*) concentration relationships had slopes approximately equal to 1 (Table 4). Hatchling concentrations of Cu, Fe, and Zn did not covary with egg concentrations, and did not differ between species (Table 4).

4. Discussion

Taken together, concentration relationships between hatchlings and maternal tissues indicated that As, Se, and Zn were maternally transferred in a concentration-dependent manner in *Sternotherus odoratus*, and As, Hg, Se, Sr, and Zn in *Trachemys scripta*. The rates of maternal transfer, estimated from the slopes of relationships between hatchling and maternal concentrations, differed among the five maternally transferred elements, and also differed between *S. odoratus* and *T. scripta*. Finally, concentration relationships

Table 2
Results of ANCOVA comparisons of the relationships between log-transformed maternal claw and hatchling trace element concentrations between *S. odoratus* and *T. scripta* from the vicinity of a coal fly-ash spill. Factors that were significant in ANCOVA models are bolded. Parameter estimates were calculated from least-squares means regression estimates from ANCOVA comparisons. Within-species relationships with slopes significantly greater than zero are identified with asterisks. No parameter estimates are presented for Fe because neither species nor maternal claw concentration effects were significant. Parameter estimates are presented ±1 SE.

As				Ba				Cu			
Source	*F*	*df*	*P*	Source	*F*	*df*	*P*	Source	*F*	*df*	*P*
Maternal Claw As	90.34	1	<0.001	Maternal Claw Ba	0.03	1	0.874	Maternal Claw Cu	2.10	1	0.15
Species	62.24	1	<0.001	Species	110.21	1	<0.001	Species	40.38	1	<0.001
Maternal Claw As*Species	4.02	1	0.047	Maternal Claw Ba*Species	0.79	1	0.376	Maternal Claw Cu*Species	0.25	1	0.619
Relationship	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**	
S. odoratus	−1.34 ± 0.03	0.32 ± 0.08*		*S. odoratus*	1.71 ± 0.04	−0.03 ± 0.03		*S. odoratus*	0.47 ± 0.01	0.02 ± 0.04	
T. Scripta	−1.66 ± 0.02	0.50 ± 0.04*		*T. Scripta*	1.24 ± 0.02	0.02 ± 0.04		*T. Scripta*	0.58 ± 0.01	0.05 ± 0.03	
Fe				Hg				Mn			
Source	*F*	*df*	*P*	Source	*F*	*df*	*P*	Source	*F*	*df*	*P*
Maternal Claw Fe	0.92	1	0.340	Maternal Claw Hg	28.20	1	<0.001	Maternal Claw Mn	0.04	1	0.843
Species	0.20	1	0.657	Species	38.78	1	<0.001	Species	24.56	1	<0.001
Maternal Claw Fe*Species	0.93	1	0.335	Maternal Claw Hg*Species	36.21	1	<0.001	Maternal Claw Mn*Species	0.01	1	0.917
				Relationship	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**	
				S. odoratus	−1.33 ± 0.03	−0.04 ± 0.09		*S. odoratus*	0.29 ± 0.05	−0.01 ± 0.05	
				T. Scripta	−1.08 ± 0.02	0.59 ± 0.06*		*T. Scripta*	0.57 ± 0.02	−0.01 ± 0.06	
Se				Sr				Zn			
Source	*F*	*df*	*P*	Source	*F*	*df*	*P*	Source	*F*	*df*	*P*
Maternal Claw Se	373.10	1	<0.001	Maternal Claw Sr	4.49	1	0.036	Maternal Claw Zn	8.73	1	0.004
Species	22.85	1	<0.001	Species	11.56	1	<0.001	Species	6.17	1	0.014
Maternal Claw Se*Species	6.85	1	0.010	Maternal Claw Sr*Species	6.87	1	0.010	Maternal Claw Zn*Species	2.73	1	0.101
Relationship	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**	
S. odoratus	0.46 ± 0.01	0.50 ± 0.04*		*S. odoratus*	1.49 ± 0.02	−0.01 ± 0.02		*S. odoratus*	1.86 ± 0.15	0.07 ± 0.06	
T. Scripta	0.39 ± 0.01	0.66 ± 0.04*		*T. Scripta*	1.31 ± 0.05	0.13 ± 0.06*		*T. Scripta*	1.19 ± 0.21	0.24 ± 0.08*	

Individual species relationships with slopes significantly different from zero are identified with asterisks (*).

between hatchlings and freshly-laid eggs were logarithmic with slopes less than one for some elements, meaning that the rate of increase in hatchling concentration decreased as egg concentration increased (Fig. 5). Thus, embryonic turtles of both species assimilated only part of the total concentrations of elements present in eggs. Several important observations on maternal transfer and embryonic assimilation of fly ash-derived trace elements can be made from these results.

We observed concentration-dependent maternal transfer of As, Hg, Se, Sr, and Zn in the freshwater turtles examined here. Prior studies of maternal transfer of coal fly ash-derived trace elements have often reported that only Se and Sr are maternally transferred to offspring (Bryan et al., 2003; Hopkins et al., 2006; Metts et al., 2013; Nagle et al., 2001; Roe et al., 2004). Our observation that As was maternally transferred is of particular interest because most studies of fly ash-derived trace elements do not report maternal transfer of this element (Bryan et al., 2003; Guirlet et al., 2008; Metts et al., 2013; Nagle et al., 2001). In coal fly-ash, As is typically present in inorganic forms, especially arsenate, which are less available for bioaccumulation and maternal transfer than are organic forms (reviewed by Wang et al., 2009). In contrast, maternal transfer of As has been documented in systems that are contaminated by sources other than coal fly-ash, including in the eggs of pelagic seabirds (Kubota et al., 2002b) and across the placenta of pelagic marine mammals (Kubota et al., 2005) exposed to As from non-point sources in the western Pacific Ocean. In both of these studies, arsenobetaine was the primary form of As present in both adults and in eggs or fetus, and was suggested to be the species of As bioaccumulated by adults (Kubota et al., 2002a), while other As species were less abundant. Some As at the Kingston spill site may also be present as organic species that are more easily maternally transferred (N.E. Carriker, *personal communication*), potentially as a result of transformation by primary producers (e.g., Francesconi and Edmonds, 1997), but we lack As speciation data for this system to address this possibility.

Our results indicate that use of maternal blood and claw to predict concentrations of elements maternally transferred to eggs can produce variable results. In this study, maternal claw concentrations were usually (but not always) more strongly correlated with hatchling concentration than were maternal blood concentrations. During vitellogenesis, blood concentrations of maternally transferred trace elements, along with those of nutrients, should fluctuate as they are assimilated from the gut, mobilized from storage and then transported to the liver and ovaries for production of yolk and allocation to eggs, respectively (e.g., Van Dyke et al., 2012). However, we sampled blood after oviposition, which occurs weeks or even months after the bulk of vitellogenesis had occurred (e.g., McPherson et al., 1982; Mendonça, 1987). Thus the only reason to expect blood and hatchling trace element concentrations to be correlated is if maternal bioaccumulation remained similar between vitellogenesis and oviposition. This seems unlikely given the potentially fast turnover in blood element concentrations. In contrast, claw tissue turnover requires up to a year, and a single claw sample should represent a long-term integration of trace element bioaccumulation, independent of short-term fluctuations (Aresco, 2005; Hopkins et al., 2013b). Therefore, even though claw tissue cannot be mobilized and reallocated to offspring, a significant portion of claw tissue was likely generated during vitellogenesis, and should partially reflect the mean bioaccumulation during that period. As a result, we predicted that hatchling and maternal claw concentrations of trace elements should be better correlated than hatchling and maternal blood concentrations. Overall, this prediction was supported, with the notable exception of Sr, which was better correlated with blood concentrations than

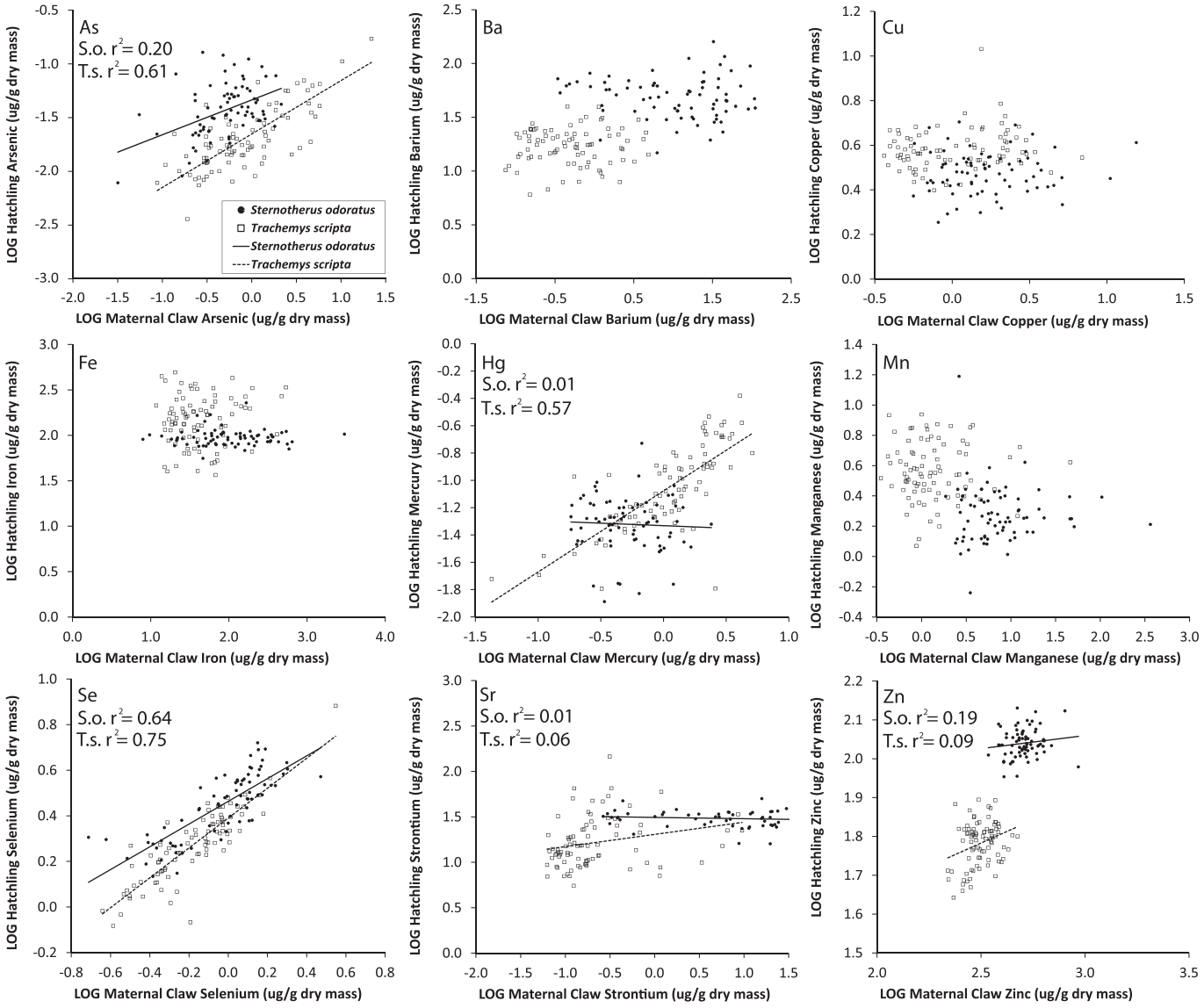

Fig. 2. Relationships between log-transformed maternal claw and hatchling concentrations in *S. odoratus* and *T. scripta* are plotted for As, Ba, Cu, Fe, Mn, Hg, Se, Sr, and Zn. Within-species best-fit regression lines are plotted for all elements that exhibited significant claw-hatchling relationships. R-square values are specific to species (S.o. for *S. odoratus* and T.s. for *T. scripta*).

with claws in both species. Strontium replaces calcium in metabolic pathways (Simkiss, 1961, 1962). Calcium is typically rare in the β-keratin matrices which form turtle claws (Espinoza and Baker, 2007). In lizards, the fibrous core of claws is rich in calcium (Gillespie et al., 1982), but this has not been investigated in turtles. In our study, Sr concentrations were low in the claws of *T. scripta*, but were two orders of magnitude higher in the claws of *S. odoratus* (Table 1). This large difference was not reflected in blood, hatchling, or egg concentrations (Table 1), so it is probably not the result of differences in bioaccumulation or exposure to ash-derived Sr between species. Instead, it may indicate that the composition of claws differs between *S. odoratus* and *T. scripta*. If turtle claws are similar in morphology to those of lizards, then the Sr concentration difference we observe may be a result of fibrous cores being larger in the claws of *S. odoratus* than in *T. scripta*. Claws of *S. odoratus* are smaller than those of *T. scripta*, so it is also possible that we sampled a larger proportion of the fibrous core in *S. odoratus* than *T. scripta*.

Our results highlight the importance of selecting appropriate tissues when seeking to describe relationships between concentrations of contaminants in mothers and their offspring. Studies of maternal transfer should compare egg or neonate element concentrations to those of maternal tissues generated at the time reproductive allocation occurs, which can differ significantly from when an egg is laid or a neonate is born. For lecithotrophic species that ovulate macrolecithal eggs (e.g., Van Dyke et al., 2012), investigators should sample maternal tissues during vitellogenesis or, as in our study, sample tissues such as claw that were likely generated at some point during vitellogenesis. In contrast, for matrotrophic species that allocate nutrients to offspring during development (e.g., Van Dyke and Beaupre, 2012; Van Dyke et al., 2014a, In Press), investigators should sample maternal tissues during gestation, or sample tissues that were generated during gestation.

Sternotherus odoratus and *T. scripta* also exhibited differences in the concentrations of specific elements that were maternally transferred. Arsenic, Se, Sr, and Zn were more heavily concentrated, relative to maternal concentration, in hatchling *S. odoratus*, while Hg was more concentrated in hatchling *T. scripta.* Notably, these differences were independent of between-species differences in

Table 3
Results of ANCOVA comparisons of the relationships between log-transformed maternal blood and hatchling trace element concentrations between *S. odoratus* and *T. scripta* from the vicinity of a coal fly-ash spill. Factors that were significant in ANCOVA models are bolded. Parameter estimates were calculated from least-squares means regression estimates from ANCOVA comparisons. Within-species relationships with slopes significantly greater than zero are identified with asterisks. No parameter estimates are presented for Fe because neither species nor maternal claw concentration effects were significant. Parameter estimates are presented ±1 SE.

As				Ba				Cu			
Source	*F*	*df*	*P*	Source	*F*	*df*	*P*	Source	*F*	*df*	*P*
Maternal Blood As	41.81	1	<0.001	Maternal Blood Ba	3.31	1	0.071	Maternal Blood Cu	1.60	1	0.208
Species	0.32	1	0.571	Species	11.23	1	0.001	Species	15.81	1	<0.001
Maternal Blood As*Species	2.26	1	0.135	Maternal Blood Ba*Species	0.79	1	0.3759	Maternal Blood Cu*Species	1.03	1	0.312
Relationship	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**	
S. odoratus	−0.98 ± 0.13	0.23 ± 0.06*		*S. odoratus*	1.82 ± 0.04	0.27 ± 0.08*		*S. odoratus*	0.48 ± 0.03	0.01 ± 0.08	
T. Scripta	−0.87 ± 0.15	0.37 ± 0.07*		*T. Scripta*	1.26 ± 0.18	0.09 ± 0.14		*T. Scripta*	0.64 ± 0.03	0.11 ± 0.05	
Fe				Hg				Mn			
Source	*F*	*df*	*P*	Source	*F*	*df*	*P*	Source	*F*	*df*	*P*
Maternal Blood Fe	0.34	1	0.562	Maternal Blood Hg	48.16	1	<0.001	Maternal Blood Mn	0.01	1	0.988
Species	2.07	1	0.152	Species	1.22	1	0.272	Species	7.8	1	0.006
Maternal Blood Fe*Species	0.55	1	0.459	Maternal Blood Hg*Species	0.11	1	0.738	Maternal Blood Mn*Species	0.12	1	0.731
				Relationship	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**	
				S. odoratus	−0.63 ± 0.13	0.40 ± 0.07*		*S. odoratus*	0.26 ± 0.06	−0.02 ± 0.09	
				T. Scripta	−0.41 ± 0.12	0.36 ± 0.06*		*T. Scripta*	0.61 ± 0.11	0.02 ± 0.07	
Se				Sr				Zn			
Source	*F*	*df*	*P*	Source	*F*	*df*	*P*	Source	*F*	*df*	*P*
Maternal Blood Se	44.59	1	<0.001	Maternal Blood Sr	40.08	1	<0.001	Maternal Blood Zn	1.41	1	0.237
Species	0.04	1	0.85	Species	0.34	1	0.558	Species	126.05	1	<0.001
Maternal Blood Se*Species	0.40	1	0.529	Maternal Blood Sr*Species	4.78	1	0.030	Maternal Blood Zn*Species	0.06	1	0.808
Relationship	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**	
S. odoratus	0.55 ± 0.03	0.27 ± 0.07*		*S. odoratus*	1.60 ± 0.03	0.25 ± 0.06*		*S. odoratus*	2.03 ± 0.02	0.02 ± 0.03	
T. Scripta	0.56 ± 0.05	0.33 ± 0.06*		*T. Scripta*	1.56 ± 0.06	0.51 ± 0.08*		*T. Scripta*	1.78 ± 0.01	0.03 ± 0.02	

maternal bioaccumulation between *S. odoratus* and *T. scripta*, because hatchling concentrations differed even after maternal concentrations are taken into account via ANCOVA. Maternal transfer occurs as a result of trace elements mimicking, or being incorporated into, essential nutrients that are allocated to eggs. Thus, our results suggest that maternal transfer differences between *S. odoratus* and *T. scripta* are the result of between-species differences in the proportional allocation of essential nutrients to eggs (Speake and Thompson, 1999, 2000; Thompson et al., 1999).

Not all elements were assimilated by embryos in proportion to their abundance in eggs. Positive relationships between egg and hatchling As, Ba, Mn, Se, Sr, Tl, and Zn concentrations were largely consistent between *S. odoratus* and *T. scripta*. However, it is noteworthy that the slopes of some of these relationships, particularly Ba in *T. scripta*, Se in *S. odoratus*, and As, Mn, and Sr in both species, were less than 1, suggesting that not all of the maternally transferred element was assimilated into the hatchling (Table 4). In particular, slopes of relationships in As (*S. odoratus* only), Ba (*T. scripta* only), Mn (both species) were all less than or equal to 0.7. Because we examined relationships between LOG-transformed tissue concentrations, slopes less than 1 indicated that hatchling trace element concentrations increased with egg concentrations in a curvilinear fashion in which the *rate* of increase in hatchling concentration decreases as egg concentration increases (Fig. 5). Arsenic and Mn assimilation efficiencies were also low, with hatchlings concentrations averaging 39% (As) and 5% (Mn) of egg concentrations. In addition, the lack of significant relationships between egg and hatchling concentrations of Cu, Fe, and Zn indicated that these elements were not assimilated in proportion to their concentration in yolk. Notably, our method of comparing element concentrations between one egg and one hatchling within a clutch assumes that within-clutch variance in element concentration is low. We have previously provided strong support for this assumption in a study which found that egg element concentrations are both relatively invariable and highly repeatable in turtle clutches from this site (Van Dyke et al., 2013a). Furthermore, we did not measure element concentrations in eggshells, which may be a source of inorganic nutrients such as calcium (Stewart, 2013). However, if elements from the eggshell augmented those present in yolk and albumin, then using our method we would expect to see relative increases in element concentration from egg to hatchling. Instead, we observed the reverse. Thus, if eggshell does contribute elements to the hatchling, then our observation that embryonic turtles do not assimilate all of the elements present in their eggs is strengthened.

That embryonic turtles do not assimilate all elements from yolk should not be surprising. The mechanisms of yolk assimilation are similar to those of digestion in the gut (Holdsworth and Wilson, 1967), and assimilation efficiencies of trace elements from diet are not always 100% (Reinfelder et al., 1998). Furthermore, partial bioaccumulation might be expected for essential micronutrients, like Cu, Fe, Mn, Se, and Zn, which in elemental form, may be selectively assimilated from yolk via active transport mechanisms (e.g., Lichten and Cousins, 2009; Manis and Schachter, 1962; Wurmli et al., 1989). A prior study found evidence of similar disparities between egg and hatchling trace element concentrations in sea turtles (Ikonomopoulou et al., 2013), but the mechanisms underlying these disparities are unknown. The fate(s) of trace elements present in yolk but not assimilated by the embryo is also unclear because an egg and its developing embryo are largely a closed system except for exchange of water and respiratory gases. Turtles consume all of the yolk allocated to eggs via the yolk sac, either during or just after hatching (i.e., residual yolk; Van Dyke et al., 2011), so any elements remaining in yolk after hatching should be included in our measurements. Thus, it is likely that any non-assimilated elements are initially absorbed or assimilated from

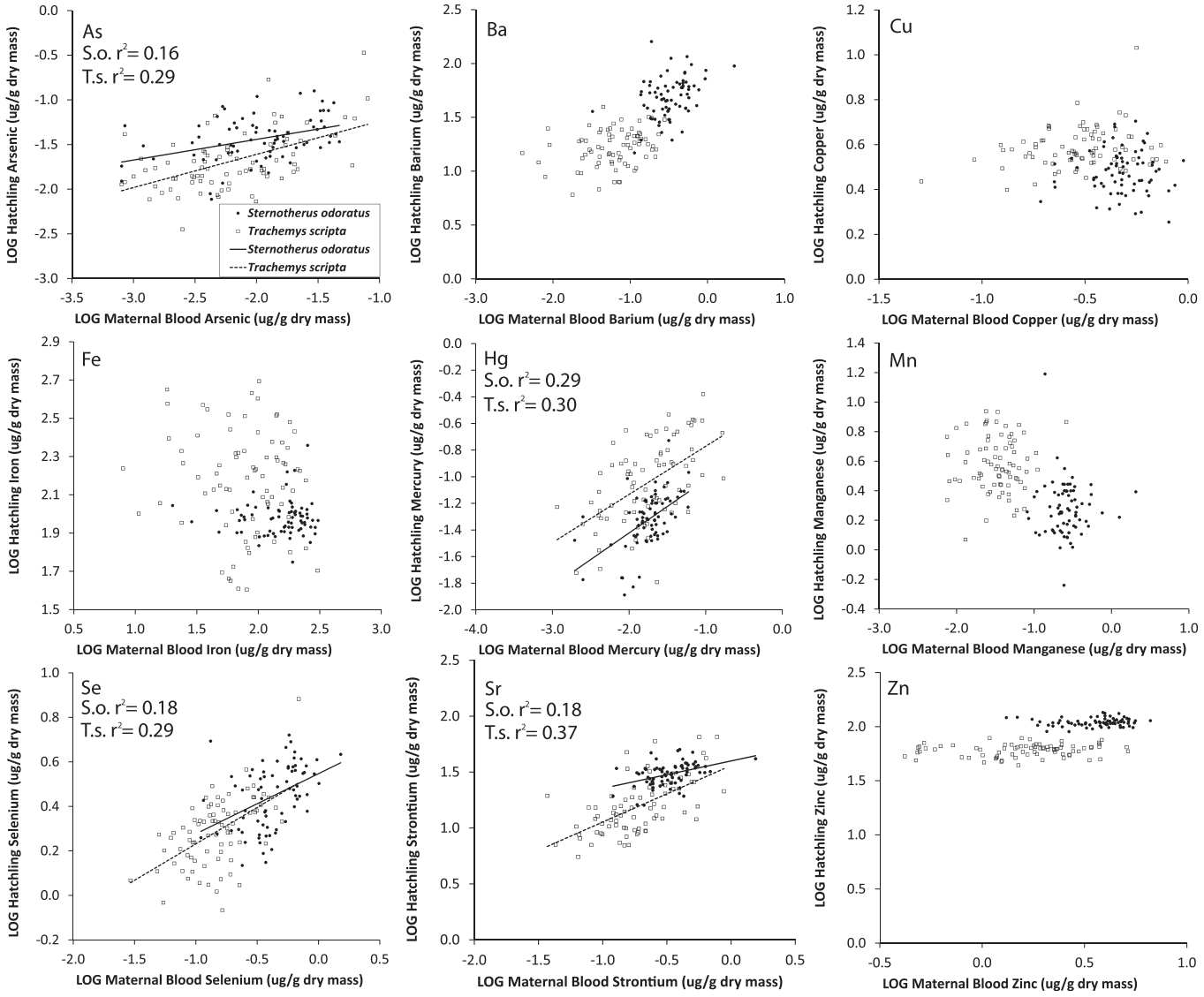

Fig. 3. Relationships between log-transformed maternal blood and hatchling concentrations in *S. odoratus* and *T. scripta* are plotted for As, Ba, Cu, Fe, Mn, Hg, Se, Sr, and Zn. Within-species best-fit regression lines are plotted for all elements that exhibited significant claw-hatchling relationships. R-square values are specific to species (S.o. for *S. odoratus* and T.s. for *T. scripta*).

the yolk, but then excreted into the allantois along with nitrogenous wastes, or could diffuse from the embryo to the surrounding amniotic fluid. Alternatively, they may be selectively allocated to the chorionic, allantoic, and amniotic membranes, which are left in the egg when the turtle hatches (Cobb et al., 2003; Cobb and Wood, 1997). Further study is needed to determine whether the apparent tendency to partially bioaccumulate trace elements from eggs we report here is widespread in other taxa and other systems. Our site is characterized by relatively low bioaccumulation of trace elements as a result of rapid remediation of the spill (e.g., Van Dyke et al., 2013a; Van Dyke et al., 2013b), and it is unclear whether the patterns we observed would remain if contamination were more severe. However, given the curvilinear nature of some of the egg-hatchling relationships (discussed above), it is possible that the disparities between egg and hatchling concentrations could become more pronounced as egg concentrations increase even further. If the trends we report do occur in other systems, or are exacerbated at higher concentrations, then further study is also needed to elucidate the biochemical mechanisms responsible for partial assimilation of egg trace elements by embryos.

The observation that embryos partially, and perhaps even selectively, assimilate trace elements from yolk raises a potential source of uncertainty in studies attempting to predict effects of maternal transfer on embryogenesis. If embryos only partially assimilate elements that are present in yolk, then the quantity of elements maternally transferred to eggs may be significantly higher than the quantity that is actually assimilated by the embryo. Thus, freshly-laid eggs and newly-hatched offspring may not always provide equivalent estimates of maternal transfer. Analyzing hatchlings instead of eggs may be preferred in studies seeking to link concentrations to developmental effects because it allows whole clutches/litters to be used for simultaneous examination of maternal transfer and developmental effects (e.g., Chin et al., 2013). Such approaches are particularly valuable in studies of species like *S. odoratus*, which have very small clutch sizes. However, our study indicates that this approach may need to account for differences in element concentrations between eggs and offspring to accurately report the concentrations of elements that are maternally transferred during egg production.

Table 4
Results of ANCOVA comparisons of the relationships between log-transformed egg and hatchling trace element concentrations between *S. odoratus* and *T. scripta* from the vicinity of a coal fly-ash spill. Factors that were significant in ANCOVA models are bolded. Parameter estimates were calculated from least-squares means regression estimates from ANCOVA comparisons. Within-species relationships with slopes significantly greater than zero are identified with asterisks. No parameter estimates are presented for Cu, Fe, or Zn because neither species nor maternal claw concentration effects were significant. Parameter estimates are presented ±1 SE.

As				Ba				Cu			
Source	*F*	*df*	*P*	Source	*F*	*df*	*P*	Source	*F*	*df*	*P*
Egg As	273.15	1	<0.001	Egg Ba	139.88	1	<0.001	Egg Cu	0.64	1	0.425
Species	1.59	1	0.212	Species	8.14	1	0.006	Species	3.78	1	0.055
Egg As*Species	3.44	1	0.068	Egg Ba*Species	12.07	1	0.001	Egg Cu*Species	0.05	1	0.827
Relationship	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**					
S. odoratus	−0.76 ± 0.08	0.70 ± 0.07*		*S. odoratus*	0.15 ± 0.11	0.99 ± 0.07*					
T. Scripta	−0.62 ± 0.61	0.88 ± 0.05*		*T. Scripta*	0.65 ± 0.09	0.54 ± 0.09*					
Fe				**Mn**				**Se**			
Source	*F*	*df*	*P*	Source	*F*	*Df*	*P*	Source	*F*	*df*	*P*
Egg Fe	0.86	1	0.356	Egg Mn	6.65	1	0.012	Egg Se	441.44	1	<0.001
Species	0.04	1	0.833	Species	43.33	1	<0.001	Species	24.68	1	<0.001
Egg Fe*Species	0.17	1	0.679	Egg Mn*Species	0.92	1	0.340	Egg Se*Species	6.63	1	0.012
				Relationship	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**	
				S. odoratus	0.30 ± 0.03	0.51 ± 0.20*		*S. odoratus*	0.13 ± 0.03	0.80 ± 0.07*	
				T. Scripta	0.60 ± 0.03	0.23 ± 0.14		*T. Scripta*	−0.06 ± 0.03	1.03 ± 0.06*	
Sr				**Tl**				**Zn**			
Source	*F*	*df*	*P*	Source	*F*	*df*	*P*	Source	*F*	*df*	*P*
Egg Sr	87.17	1	<0.001	Egg Tl	1348.99	1	<0.001	Egg Zn	2.44	1	0.122
Species	3.14	1	0.081	Species	1.90	1	0.172	Species	1.70	1	0.200
Egg Sr*Species	0.01	1	0.967	Egg Tl*Species	3.11	1	0.082	Egg Zn*Species	0.01	1	0.973
Relationship	**Intercept**	**Slope**		**Relationship**	**Intercept**	**Slope**					
S. odoratus	0.68 ± 0.13	0.77 ± 0.13*		*S. odoratus*	0.07 ± 0.06	0.93 ± 0.04*					
T. Scripta	0.37 ± 0.07	0.78 ± 0.07*		*T. Scripta*	−0.03 ± 0.05	0.84 ± 0.03*					

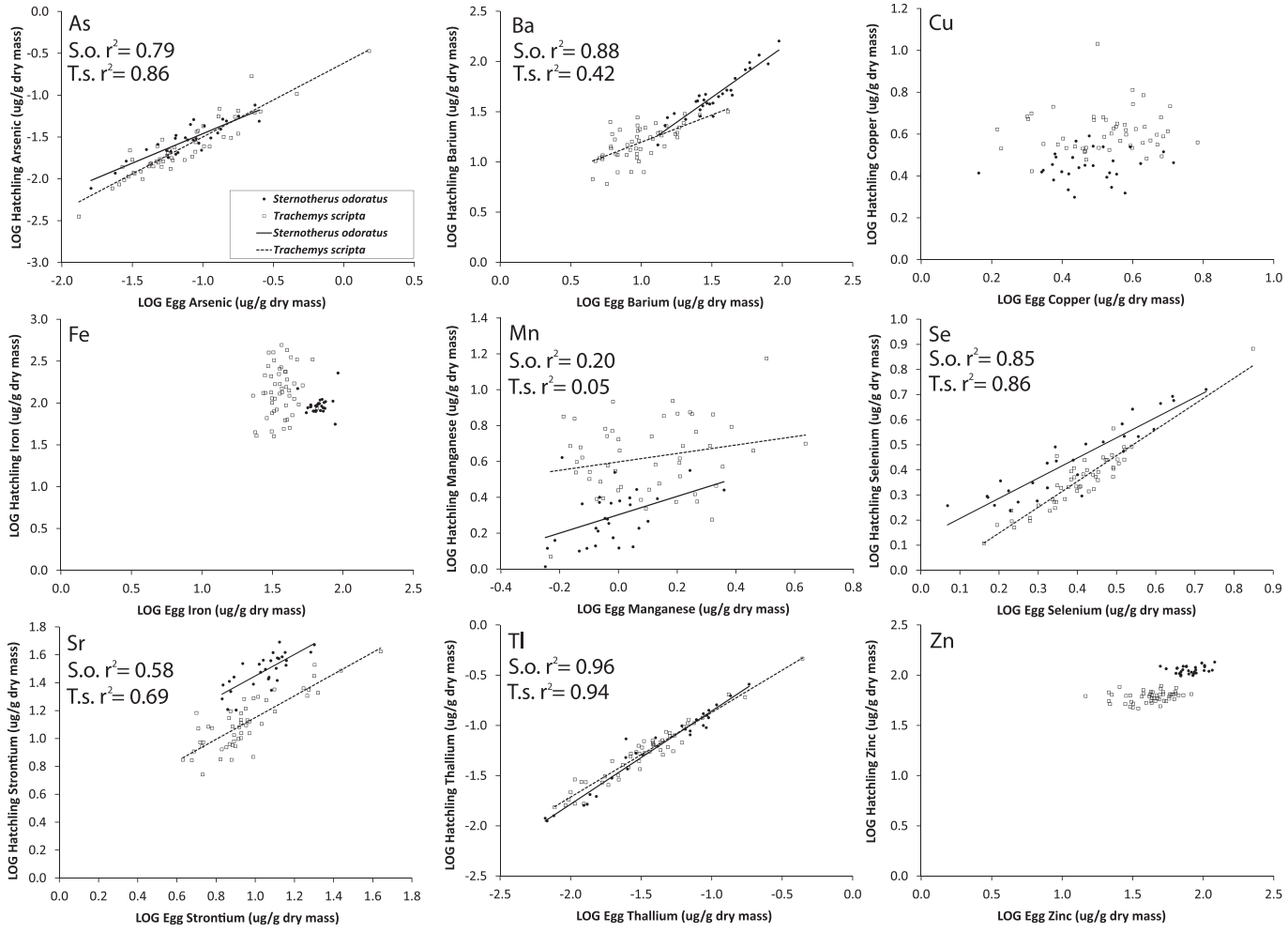

Fig. 4. Relationships between log-transformed egg and hatchling concentrations in *S. odoratus* and *T. scripta* are plotted for As, Ba, Cu, Fe, Mn, Se, Sr, Tl, and Zn. Within-species best-fit regression lines are plotted for all elements that exhibited significant claw-hatchling relationships. R-square values are specific to species (S.o. for *S. odoratus* and T.s. for *T. scripta*).

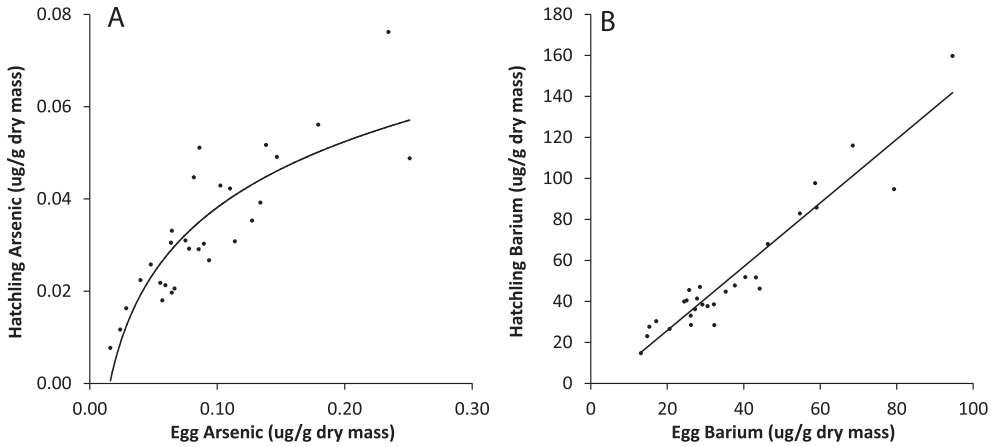

Fig. 5. Raw relationships between egg and hatchling As (A) and Ba (B) concentrations in *S. odoratus* are presented to demonstrate how hatchling trace element concentrations increase curvilinearly when the slope of the log-transformed relationship is less than 1 (A), and linearly when the slope is equal to 1 (B). The equation of the line in A is hatchling = $0.17\ egg^{0.70}$, while the equation of the line in B is hatchling = $1.41\ egg^{1.00} - 5.22$.

Acknowledgments

We thank H. Vogel, M. Hepner, M. Dziadzio, K. Webb, T. Parker, J. Harris, M. Knoerr, J. Hallagan, J. Botero, D. Drewett, S. Chin, A. Wilson, A. Love, M. Keeven, V. Alaasam, and A. Walker for assistance in the field and the lab. J. Favara, S. Young, W. James, N. Carriker, R. Crawford, and M. Beck provided logistical support. We would also like to thank D. Hawley for providing help in the field, advice, and logistical support. We thank N.E. Carriker for constructive comments on the manuscript. Turtles were collected and sampled in compliance with appropriate permits in Tennessee, and sampling methods complied with Virginia Tech's animal care and use protocols. This research was supported financially by the Tennessee Valley Authority.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2014.07.005.

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