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Reproduction and hatchling performance in freshwater turtles associated with a remediated coal fly-ash spill ☆

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ABSTRACT

In 2008 an impoundment retaining wall failed at the Tennessee Valley Authority's coal burning plant in Kingston, Tennessee, releasing large quantities of coal-fly ash into the Emory River. Following extensive remediation of the spill, we captured (in 2011 and 2012) gravid turtles of multiple species in three rivers (two impacted and one reference) within the vicinity of the spill to determine whether there was evidence of the spill influencing reproduction. There was little evidence that river of origin affected reproductive output, hatching success, hatchling size, or hatchling locomotor performance. Although hatching success and hatchling righting ability of pond sliders, *Trachemys scripta*, was higher in our reference river than in the Emory or Clinch River, respectively, these differences could not be attributed to differences in individual element concentrations in turtle tissues and effect sizes were relatively small. For example, hatching success was reduced by 11% in the spill zone compared to the reference river, an effect that is unlikely substantial enough to influence local population dynamics in light of turtle life history. Our results suggest that residual contamination that remains in the Emory–Clinch system after its remediation poses low risk of excessive element exposure and limited adverse reproductive effects to freshwater turtles. Future monitoring could reveal whether the observed reduction in hatching success gradually attenuates with time, or whether any long-term effects of chronic exposure to low-level contamination emerge over time.

1. Introduction

Coal combustion is a widespread source of global energy, accounting for approximately 30% of the world's power in 2011 (British Petroleum, 2012). A notable byproduct of coal combustion is fly ash; in the United States alone, over 140 million tons of fly ash were produced in 2005 (US EPA, 2010). Depending on the composition of the coal and the combustion technologies used, fly ash may contain a wide variety of elements, including arsenic (As), mercury (Hg), and selenium (Se; Izquierdo and Querol, 2012). Approximately 40% of the fly ash annually produced in the United States is stored in aquatic surface impoundments (ACAA, 2010). Ash-derived elements within these impoundments may contaminate surrounding bodies of water through leaching, discharge and spills or after the failure of impoundment retaining walls or dikes (Benito et al., 2001; Rowe et al., 2002; Ugurlu 2004; National Research Council, 2002, 2006; Lemly, 2015).

The release of elements from coal fly ash and other industrial processes into aquatic systems can negatively affect aquatic organisms and ecological processes (Rowe et al., 2002; National Research Council, 2006; Rowe, 2014). Certain elements are of particular concern because they can influence reproduction and/or survival at high concentrations (e.g., Sorensen, 1986; Lemly, 2002; Hopkins et al., 2013a). In addition, females may maternally transfer elements to their young during reproduction and post-hatching provisioning, potentially resulting in compromised physiology of offspring (Nagle et al., 2001), increased malformation prevalence (Ohlendorf et al., 1986; Rowe et al., 1996) and altered offspring behavior (Bergeron et al., 2011; Chin et al., 2013).

Turtles are good organisms to study element accumulation from coal fly ash and associated reproductive effects (e.g., Nagle et al., 2001; Yu et al., 2011; Hopkins et al., 2013a) because they live long lives (Congdon et al., 1993, 1994) and have relatively small

☆Capsule Abstract: We captured gravid turtles to determine if environmental contamination at a remediated coal-fly ash spill influenced reproduction. There was little evidence of adverse effects.

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home ranges (e.g., Obbard and Brooks, 1981); as a result, turtles may be exposed to local contaminants over a long period of time. In addition, turtles possess attributes that make them logistically tractable for study such as high population densities (Iverson, 1982) and inducible oviposition (Ewert and Legler, 1978). Turtles also allocate similar quantities of elements to all eggs within a clutch, reducing the variability of embryonic exposure to contaminants among siblings (Van Dyke et al., 2013b); therefore, entire clutches do not need to be sacrificed to obtain representative samples for toxicological studies. Negative effects of environmental contamination on freshwater turtles are particularly important to study because their populations are sensitive to anthropogenic sources of mortality and many species are highly imperiled (Rhodin et al., 2010). Negative reproductive effects observed among turtles associated with environmental contaminants could include reduced clutch sizes or decreased hatching success (e.g., Bishop et al., 1991; Hopkins et al., 2013a). In addition, hatchlings exposed to contaminants may develop abnormalities (Bishop et al., 1998) or altered locomotor behavior (i.e., speed, Burger et al., 1998), which may affect their survival because the speed of a hatchling is thought to be positively associated with its ability to avoid predation (Janzen et al., 2000a, 2000b, but see Congdon et al. (1999)).

In 2008, a retaining wall of a fly-ash impoundment failed at the Tennessee Valley Authority (TVA) coal-burning plant in Kingston, Tennessee, USA, resulting in over 4.1 million cubic meters of fly ash being released into the Emory River. Following the 2008 spill, TVA instituted an intensive remediation effort that included mechanical/hydraulic dredging and off-site disposal of ~2.5 million cubic meters of fly ash by August 2010 (TVA, 2009, 2011). Despite remediation efforts, residual ash remains in some areas of the system (Carriker et al., 2011) and element concentrations in the area may be sufficiently elevated to be of environmental concern (e.g., Jackson, 2011; Ruhl et al., 2010). Thus, it is important to determine whether remaining ash poses any risks to wildlife. In 2011 and 2012, we sampled turtles in two rivers (i.e., the Clinch and Emory Rivers) downstream of the spill and also the Tennessee River upstream of its confluence with the Clinch River, where turtles were unlikely to be affected by the spill (Fig. 1). Initial work suggested that females in the area near the remediated spill were maternally transferring some elements (i.e., As, Hg, Se, Sr, and Zn) to their offspring (Van Dyke et al., 2014). Therefore, the goal of our study was to determine whether gravid female turtles inhabiting the impacted area experienced adverse reproductive effects.

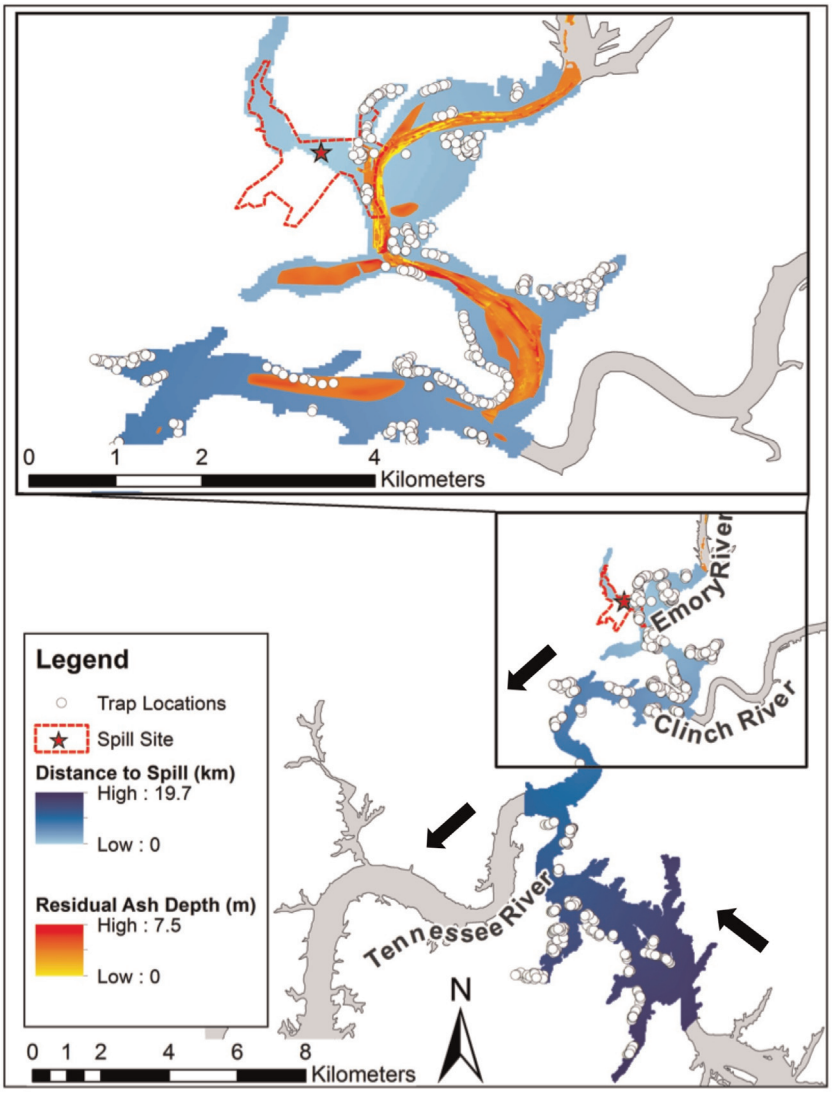

Fig. 1. Segments of the Clinch, Emory, and Tennessee Rivers trapped for turtles in 2011 and 2012. The Emory and Clinch rivers were impacted by the spill, but trap locations on the Tennessee River were upstream of the confluence with the Clinch River and thus served as an unimpacted reference river. Arrows indicate flow direction.

2. Methods

2.1. Study species

We focused on four common turtle species: eastern musk turtle (i.e., stinkpot; *Sternotherus odoratus*), pond slider (*Trachemys scripta*), spiny softshell turtle (*Apalone spinifera*), and snapping turtle (*Chelydra serpentina*). However, we did not capture enough gravid *C. serpentina* to include them in any statistical analyses and only captured enough gravid *A. spinifera* to include them in analyses pertaining to clutch and egg size. Turtles likely accumulate elements primarily through the ingestion of food (Storelli and Marcotrigiano, 2003) thus a species' diet is an important determinant of trace element accumulation (Storelli et al., 1998). The species we captured have variable natural history traits, and in particular possess disparate foraging ecologies that may influence their relative vulnerability to element accumulation (Van Dyke et al., 2013a). *Sternotherus* is a small, primarily carnivorous generalist while *Trachemys scripta* is an intermediate-sized habitat and dietary generalist. Although previous studies have described accumulation of coal ash-derived elements in *T. scripta* (Nagle et al., 2001), there has been little comparable information generated for *Sternotherus odoratus* (but see Bergeron et al. (2007) and Hopkins et al. (2013a) in reference to Hg accumulation). *Apalone spinifera* is primarily carnivorous and therefore occupies a relatively high trophic level (Van Dyke et al., 2013a). *Chelydra serpentina* is a relatively large omnivorous species that may be particularly vulnerable to accumulation of elements, based on results from other ecotoxicology studies (e.g., Bergeron et al., 2007).

2.2. Turtle trapping

From April–July 2011 and April–August 2012 we trapped turtles using hoop traps baited with fish, cat food and/or chicken. All hoop traps were fitted with Styrofoam floats to elevate traps during periods of high water, which allowed turtles continuous access to air. Trapping was concentrated along discrete sections of the Emory (river km 0.0–5.5), Clinch (river km 0.0–7.0) and Tennessee Rivers (river km 914–922). Throughout the study, we generally maintained at least 15 traps at various sites along the Emory and Clinch Rivers and 15–20 traps along the Tennessee River (45–50 total traps per day). Traps were re-baited every three days and were rotated among trapping locations depending upon trapping success.

Turtles were collected from traps daily unless weather or flow conditions prevented river access. We determined species and sex (based on secondary sexual characteristics) of all captured turtles. Targeted species were placed in water-filled plastic tubs for transport back to a field laboratory. For all turtles brought to the lab, we measured mass (g) with Pesola® scales (Baar, Switzerland), and carapace length (cm), carapace width (cm), and plastron length (cm) using forestry calipers. Turtles were individually marked by scute notching (Cagle, 1939) except for *A. spinifera*, which received passive integrated transponders (PIT tags; Biomark, Boise, Idaho). We removed 2–3 mm from each claw on a single foot for element analysis in this study because blood–tissue turnover rates are much faster than those of claws (Longnecker et al., 1993), and thus claws provided a more integrative measure of maternal element concentrations (Hopkins et al., 2013a, b; Van Dyke et al., 2014). We used maternal element concentrations specifically to determine whether maternal exposure to ash-derived trace elements was related to maternal reproductive allocation decisions such as egg size and clutch size. Gravid turtles were held in the lab to induce oviposition and did not have their tissues sampled until after egg collection to minimize stress. After clutch completion (see below), we collected tissue samples from females, measured them, and released them at their point of capture.

2.3. Egg oviposition and Incubation

Gravid turtles were injected intraperitoneally with 20 IU kg-1 of oxytocin solution once a day for three consecutive days to induce oviposition (Ewert and Legler, 1978). After the first oxytocin injection, *A. spinifera*, *C. serpentina*, and *T. scripta* were placed in large Rubbermaid® containers with enough dechlorinated water to allow animals to completely submerge. Container bottoms were lined with 2×3 cm^2 wire mesh $\sim$3 cm from the bottom that protected eggs from being accidentally broken by the mother. *Sternotherus odoratus* were handled similarly, but in smaller plastic tubs without the wire mesh. Females were monitored every 2–3 h for egg deposition and we collected eggs as they were laid. If a female did not lay a complete clutch over three days (based on whether eggs were felt during physical palpation or visible via X-ray), we waited several days and repeated the injection cycle.

Eggs were weighed to the nearest 0.01 g, measured in length and width to the nearest 0.01 cm, and marked with the maternal ID and the egg number. In 2011, the first egg of every clutch was frozen for element analysis, as were any eggs that were cracked during oviposition or measurements. Remaining intact eggs were placed in plastic containers filled with a 1.2:1 mixture of vermiculite and water in 2011. Egg containers were then placed in incubators (Hovabator, Model #1602 N; G.Q.F. Manufacturing Company, Savannah, GA, USA) set at 25 °C. Due to low hatching success for *A. spinifera* in 2011, eggs of this species were placed in incubators set at 27.5 °C in 2012. Incubator temperature was monitored every 15 min with Microlite® USB temperature dataloggers (Fourier Systems, Fairfield, CT). We determined how much water had evaporated in egg containers by weighing the containers every four days and we sprayed eggs and vermiculite with water until they returned to their previous masses. Eggs were frozen if embryos died during development, as determined by candling or the presence of extensive mold or discoloration.

We incubated eggs until they began to hatch. One hatchling per clutch was euthanized by immersion in a buffered MS-222 solution and frozen in an -80 °C freezer after the turtle was non-responsive. There is limited intra-clutch variability in turtle egg element concentrations (Van Dyke et al., 2013b), and egg and hatchling element concentrations are tightly correlated (Van Dyke et al., 2014), so element concentrations within the single hatchling we sacrificed should be representative of element concentrations within other eggs and hatchlings of its clutch. The element concentrations from sacrificed hatchlings were used to determine relationships between concentrations and measures of hatchling performance. We temporarily housed remaining hatchlings collectively by clutch in plastic tubs filled with $\sim$3 cm of water and released hatchlings within two months of hatching at the river mile nearest the point of maternal capture. For all hatchlings, we measured carapace and plastron length (to 0.1 cm) and obtained mass (to 0.01 g). We also quantified (1) clutch size (i.e., the number of eggs within a clutch), and (2) hatch success (i.e., the proportion of eggs within a clutch that hatched). When calculating hatch success, we did not include the single egg we sacrificed from clutches or any eggs that were damaged.

2.4. Turtle locomotor performance

Because it is difficult to use turtle hatchling performance results obtained in the laboratory to extrapolate to what may occur under natural conditions, it is important to test several different measures of performance (Delmas et al., 2007). In 2012, we used two primary measures, locomotor performance and righting

response, to assess hatchling performance. The second hatchling to emerge from each clutch was housed in a plastic cup containing water at 25 °C for 21 days after hatching. We delayed testing locomotor performance for this period of time to allow hatchlings to completely internalize external yolk and reabsorb a portion of residual yolk because its presence may affect locomotor performance (Miller et al., 1987). We used 21 days because this amount of time has been reported in the literature for reabsorbtion of most yolk in at least one species, *Apalone mutica* (Van Dyke et al., 2011), though species with larger residual yolks may require more time (Finkler et al., 2002). Maximum sprint speed was measured on a photo cell lined racetrack interfaced to a laptop computer that produced velocity measurements every 10 cm. Turtles were conditioned to the track on their 21st day by racing them at 25 °C and locomotor performance was measured the following day (Finkler, 1999). During both conditioning and measurement runs, turtles were placed in a gated section of the track and allowed to acclimate for two minutes. Afterwards, the gate was lifted and turtles were gently prodded near the tail with forceps to coerce them to move forward. After initiating movement, turtles were prodded whenever they stopped moving for more than two seconds. If a turtle turned 90° (i.e., faced the wall of the racetrack), it was picked up and turned forward without changing its relative position on the track. In addition to maximum velocity, we recorded the number of prods and turns for each turtle. This procedure was used until turtles traversed a total distance of 0.8 m.

2.5. Turtle righting response

To determine whether there was a relationship between river of origin and a hatchling's ability to right itself when placed on its carapace (Delmas et al., 2007), we evaluated hatchling righting response in 2012. We tested the righting response of the second turtle to hatch from each clutch on the day following its locomotor performance trial. We placed hatchlings in a plastic container filled with sand to a depth of approximately 2.5 cm. After two minutes of acclimation, the turtle was placed on its carapace. Trials were recorded on video and later viewed to calculate the (A) total number of righting attempts, and (B) total time that elapsed until righting occurred. Trials ended after one hour if turtles did not right themselves. Three righting response trials occurred for each hatchling, but we only statistically analyzed righting performance data from one trial for each turtle. For turtles that did successfully right, we used the trial that included the fastest righting time with the fewest attempts in the analyses. If a hatchling did not successfully right itself in any trial, we included the trial that included the most attempts in the analyses.

2.6. Sample processing

Claw samples were shipped overnight on dry ice to the Trace Element Analysis Core at Dartmouth College where they were first washed to remove external contamination. Individual claws were transferred to a 7 ml polyethylene vial, 2 ml 1% solution of Triton X-100 was added, and the vial was then placed in an ultrasonic bath for 20 min. The claw sample was then washed five times with deionized water and then dried in the vial in a dry box.

Hatchlings frozen for element analysis were freeze-dried at Virginia Tech to a constant mass and dry-stored at −80 °C prior to homogenization. Hatchlings were then homogenized using ceramic mortars and pestles that were washed in an acid bath and stored in an −80 °C freezer between samples. Each hatchling was placed in a mortar, immersed in liquid nitrogen, and homogenized by grinding with the pestle. Homogenized turtle tissues were again freeze-dried to eliminate any condensation that accumulated during homogenization and were stored in dessicators with drierite. Both maternal claw samples and homogenized hatchlings were shipped overnight on dry ice to the Trace Element Analysis Core at Dartmouth College.

Arsenic, Ba, Cd, Cr, Cu, Fe, Mn, Hg, Se, Sr, Tl, V, and Zn concentrations were quantified using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Dried tissues were stored at 4 °C prior to sample preparation and analysis. Each sample was weighed in a VWR trace metal clean polypropylene centrifuge tube and 0.5 ml of 9:1 HNO_3:HCl (Optima Grade, Fisher Scientific) was added (0.25 ml for samples <5 mg). Individual subsample weights were variable but were generally <0.05 g. 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. 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 over a 15 min period and held at this temperature for 45 min. The samples were then allowed to cool and 0.1 ml of H_2O_2 (Optima Grade, Fisher Scientific) was added and the samples were taken through a further microwave-heating program. The samples were then brought up to 10 ml (5 ml for <5 mg samples) with deionized water (Element QPod, Millipore, Billarica, MA). All measurements were recorded gravimetrically.

We analyzed samples by collision cell ICP-MS (7700x, Agilent, Santa Clara, CA). Selenium (78) was measured in hydrogen mode (2.8 ml min^{-1}), Hg and Tl in normal mode, and all other analytes in He mode (4.8 ml min^{-1}). Analytical procedures followed the general protocols outlined in EPA 6020 A; the instrument was calibrated with NIST-traceable standards and calibration was verified with a second source traceable standard. Reporting limits were checked after each calibration by running standards at 1, 2 and 3X of the reporting limit concentration. A continuing calibration check and blank was run every 10 samples. Analysis duplicates and spikes were run at a frequency of 1 duplicate and spike for each 20 samples. All element concentrations are reported as dry mass (mg/kg). Detection limits for each tissue sample varied because the mass of each sample varied.

2.7. Statistical analysis

We sought to determine how the fly ash spill and associated elements influenced turtle reproduction and performance of hatchlings. Because fly ash contains a complex mixture of elements that are not independent of one another, the ideal statistical analysis would consider the collective influence of the suite of elements on health response variables. Thus, we initially attempted to use principal component analysis to reduce the number of explanatory variables. Unfortunately, none of the resulting principal components explained a sufficient proportion of the variance to allow us to reduce the number of explanatory variables (5 eigenvectors were required to explain more than 95% of the cumulative variance). This suggested that trace element concentrations were not consistently elevated only in areas contaminated by the coal fly-ash spill. Moreover, the spill and dredging caused considerable physical disturbance which itself could influence local food webs and wildlife health independently of contaminant effects. Thus, to determine whether the fly-ash spill and associated elements influenced turtle reproduction, we used a two tier analytical approach. In the first tier, we used maternal river (i.e., where gravid females were captured) as the main factor in our initial statistical models (details of models below). In doing so, we assumed that the river that we captured the turtle in was the river in which the turtle had acquired her trace elements. This assumption is supported by what is generally known regarding the

spatial ecology of our study species (summarized in Ernst and Lovich (2009)). If the fly-ash spill negatively influenced turtle reproduction, we would expect negative effects, if they occurred, to be most pronounced in the Emory River, followed by the Clinch River, relative to the Tennessee River (Fig. 1).

We analyzed the effects of maternal river, year, and maternal carapace length on clutch size and mean egg mass in *A. spinifera*, *S. odoratus*, and *T. scripta* using separate multivariate analyses of covariance (MANCOVA) for each species in PROC GLM. We included maternal carapace length as a covariate to account for effects of maternal body size, and included year to test for between year-differences in maternal production of eggs.

We tested for effects of maternal river on hatching success in *S. odoratus* and *T. scripta* using separate within-species binomial comparisons in PROC GLIMMIX. We included year in the models to test for between year-differences in egg mortality caused by changes in environmental conditions (e.g., bioavailability of contaminants) or other factors (e.g., maternal effects or infertility rates) between years.

We tested for effects of maternal river on mean hatchling mass, carapace length, carapace width, and plastron length in *S. odoratus* and *T. scripta* using separate MANCOVAs in PROC GLM. We included mean egg mass as a covariate to account for egg size, and also tested for between-year differences. We tested for differences on clutch mean hatchling size metrics specifically because models would not converge if all hatchlings were included in the analysis, thus clutch was the experimental unit in these analyses.

We tested for effects of the ash spill on variables of righting response in separate analyses. First, we tested for effects of maternal river and hatchling carapace length on righting success (% of turtles that successfully righted) by modeling a logistic regression in PROC GLIMMIX. We included hatchling carapace length as a covariate to account for hatchling size. Second, we tested for effects of maternal river and hatchling carapace length on the number of righting attempts by modeling an ANCOVA in PROC GLIMMIX. We specified a Poisson distribution in the analysis because number of righting attempts was an ordinal variable. Finally, we tested the effects of maternal river on the total time turtles required to right themselves. Again, we included hatchling carapace length as a covariate.

Similar to righting response, we tested for effects of the ash spill on variables of terrestrial locomotor performance in separate analyses. We examined the effects of maternal river on number of prods necessary to encourage turtles to walk the length of the track, and reversals made by turtles by modeling mixed-effects ANCOVAs in PROC GLIMMIX. In both analyses, we included hatchling carapace length as a covariate. We specified a Poisson distribution in these analyses because numbers of prods and turns were both ordinal variables. We then examined the effects of hatchling element concentration on sprint speed (fastest 0.1 m) using ANCOVA in PROC GLM, with hatchling carapace length as a covariate.

In cases where there were significant among-river differences in a reproductive variable that indicated that it might have been affected by the fly-ash spill, we then conducted a second tier analysis with element concentrations in maternal females or hatchlings, as appropriate, included as independent variables (rather than river) in an attempt to determine whether any element could be responsible for the effect. We found significant among-river differences in both hatching success and righting success within *T. scripta*. The second tier analyses for both of these variables was the same. We tested for effects of hatchling trace element concentrations (As, Hg, Se, Sr, and Zn) on both hatching and righting success by modeling separate logistic regressions in PROC GLIMMIX. In these analyses, we focused on concentrations of As, Hg, Se, Sr, and Zn, because these were the only elements maternally transferred to offspring in a complementary study (Van Dyke et al., 2014). We also dropped all other factors (i.e., year, carapace length) from these follow-up analyses if they were not significant in the original models.

We did not compare any reproductive variables among species because egg and clutch size differ among the species we examined independent of any coal fly-ash effect (Ernst and Lovich, 2009). Hatching success, offspring size, and performance measurements are all fundamentally linked to either clutch or egg size (e.g., Miller et al., 1987; Gutzke and Packard, 1985), so the among-species differences in egg and clutch size are likely to bias among-species comparisons of our dependent variables related to reproductive success. When conducting analyses in PROC GLM and PROC MIXED. We used Shapiro–Wilk Tests and normal probability plots to assess normality and homoscedasticity of variance, respectively. Sample sizes were not large enough to test for violations of multivariate normality in MANCOVA models, so we used Pillai's Trace as a test statistic in all multivariate analyses because it is the most

Table 1
Mean maternal claw element concentrations (mg/kg dry mass) from three species of turtles inhabiting contaminated (Clinch and Emory Rivers) and reference (Tennessee River) sites in the vicinity of a remediated coal fly-ash spill in Kingston, Tennessee, USA. All values are ± 1 SE. *A. spinifera* sample sizes from the Clinch River were not large enough to calculate SE.

Species	Year	River	*N*	As (mg/kg)	Hg (mg/kg)	Se (mg/kg)	Sr (mg/kg)	Zn (mg/kg)
Apalone spinifera	**2011**	**Tennessee**	9	0.14 ± 0.02	2.48 ± 0.38	0.97 ± 0.05	0.10 ± 0.03	445.18 ± 23.15
		Emory	11	0.76 ± 0.18	3.04 ± 0.47	1.79 ± 0.05	0.18 ± 0.06	437.25 ± 26.44
		Clinch	2	0.94	2.41	2.01	0.15	401.74
	2012	**Tennessee**	7	0.31 ± 0.04	1.92 ± 0.26	0.93 ± 0.05	0.25 ± 0.04	450.72 ± 15.96
		Emory	3	1.32 ± 0.69	4.99 ± 2.61	2.21 ± 0.42	0.20 ± 0.02	399.10 ± 38.72
		Clinch	1	0.26	4.61	1.44	0.18	405.77
Sternotherus odoratus	**2011**	**Tennessee**	10	0.24 ± 0.06	0.73 ± 0.11	0.53 ± 0.06	37.17 ± 9.92	516.35 ± 26.38
		Emory	17	0.81 ± 0.12	0.98 ± 0.11	1.27 ± 0.09	47.65 ± 6.69	491.38 ± 16.69
		Clinch	13	0.75 ± 0.07	0.89 ± 0.08	1.53 ± 0.13	50.24 ± 10.13	499.81 ± 26.73
	2012	**Tennessee**	23	0.33 ± 0.05	0.61 ± 0.10	0.94 ± 0.16	20.81 ± 6.17	525.71 ± 24.80
		Emory	26	0.81 ± 0.10	0.54 ± 0.07	1.09 ± 0.05	16.37 ± 3.39	544.73 ± 19.47
		Clinch	29	0.72 ± 0.06	0.42 ± 0.05	1.33 ± 0.08	15.28 ± 3.05	501.90 ± 11.09
Trachemys scripta	**2011**	**Tennessee**	12	0.39 ± 0.27	1.70 ± 0.27	0.48 ± 0.06	2.32 ± 0.54	347.46 ± 13.59
		Emory	17	5.09 ± 1.57	1.79 ± 0.31	1.10 ± 0.16	1.13 ± 0.50	309.43 ± 10.45
		Clinch	23	1.63 ± 0.26	1.78 ± 0.21	0.96 ± 0.05	0.24 ± 0.07	328.01 ± 12.68
	2012	**Tennessee**	30	0.46 ± 0.04	0.90 ± 0.15	0.41 ± 0.02	0.53 ± 0.16	299.25 ± 8.55
		Emory	15	1.65 ± 0.41	1.56 ± 0.25	0.71 ± 0.04	0.14 ± 0.01	325.91 ± 11.04
		Clinch	8	0.87 ± 0.17	1.04 ± 0.25	0.75 ± 0.09	0.14 ± 0.02	290.89 ± 13.38

Table 2
Mean hatchling element concentrations (mg/kg dry mass) from two species of turtles inhabiting contaminated (Clinch and Emory Rivers) and reference (Tennessee River) sites in the vicinity of a remediated coal fly-ash spill in Kingston, Tennessee, USA.

Species	Year	River	N	As (mg/kg)	Hg (mg/kg)	Se (mg/kg)	Sr (mg/kg)	Zn (mg/kg)
Sternotherus odoratus	**2011**	**Tennessee**	11	0.025 ± 0.004	0.035 ± 0.007	1.99 ± 0.05	27.98 ± 2.80	107.16 ± 2.16
		Emory	11	0.034 ± 0.005	0.044 ± 0.005	3.17 ± 0.20	35.20 ± 2.20	116.42 ± 2.78
		Clinch	7	0.042 ± 0.004	0.043 ± 0.008	4.05 ± 0.43	31.49 ± 3.11	118.08 ± 3.55
	2012	**Tennessee**	12	0.028 ± 0.004	0.06 ± 0.006	1.77 ± 0.08	27.51 ± 1.36	109.79 ± 2.73
		Emory	19	0.047 ± 0.004	0.058 ± 0.008	3.02 ± 0.15	32.29 ± 1.69	110.82 ± 1.65
		Clinch	21	0.063 ± 0.007	0.057 ± 0.004	3.65 ± 0.11	32.03 ± 0.95	105.65 ± 1.82
Trachemys scripta	**2011**	**Tennessee**	11	0.013 ± 0.002	0.139 ± 0.026	1.66 ± 0.07	18.68 ± 2.59	61.17 ± 1.56
		Emory	19	0.059 ± 0.018	0.102 ± 0.014	2.79 ± 0.28	16.48 ± 1.79	62.59 ± 1.85
		Clinch	20	0.025 ± 0.003	0.134 ± 0.021	2.41 ± 0.09	10.33 ± 0.85	59.71 ± 1.76
	2012	**Tennessee**	25	0.015 ± 0.002	0.069 ± 0.008	1.26 ± 0.06	32.37 ± 6.31	61.00 ± 1.89
		Emory	12	0.032 ± 0.006	0.122 ± 0.023	1.70 ± 0.10	16.32 ± 1.85	62.97 ± 2.01
		Clinch	5	0.025 ± 0.005	0.138 ± 0.035	2.14 ± 0.22	11.27 ± 0.84	61.89 ± 1.69

Table 3
Mean clutch size and egg mass from three species of turtles inhabiting contaminated (Clinch and Emory Rivers) and reference (Tennessee River) sites in the vicinity of a remediated coal fly-ash spill in Kingston, Tennessee, USA. All values are ± 1SE. Clinch River *A. spinifera* sample sizes were not large enough to calculate SE.

Species	Year	River	*N*	Clutch size (#)	Egg mass (g)
Apalone spinifera	**2011**	**Tennessee**	9	14.44 ± 0.93	10.73 ± 0.69
		Emory	11	15.82 ± 0.99	10.33 ± 0.38
		Clinch	2	19.00	11.09
	2012	**Tennessee**	7	12.57 ± 0.92	11.17 ± 0.83
		Emory	3	15.67 ± 2.96	10.26 ± 1.37
		Clinch	1	21.00	11.53
Sternotherus odoratus	**2011**	**Tennessee**	10	3.6 ± 0.27	4.24 ± 0.16
		Emory	17	2.88 ± 0.21	4.19 ± 0.15
		Clinch	13	3.46 ± 0.29	4.29 ± 0.16
	2012	**Tennessee**	23	3.3 ± 0.23	4.08 ± 0.12
		Emory	26	3.04 ± 0.18	4.13 ± 0.11
		Clinch	29	3.72 ± 0.27	4.12 ± 0.11
Trachemys scripta	**2011**	**Tennessee**	12	9.58 ± 0.76	10.57 ± 0.35
		Emory	17	9.89 ± 0.6	10.65 ± 0.3
		Clinch	23	9.83 ± 0.41	11.51 ± 0.28
	2012	**Tennessee**	30	9.43 ± 0.37	11.66 ± 0.24
		Emory	15	8.67 ± 0.37	11.25 ± 0.35
		Clinch	8	9.13 ± 0.69	11.25 ± 0.49

robust to violations of multivariate normality (Scheiner, 2001). In all statistical tests, we attempted to examine the effects of higher-order interactions first. All statistical tests were performed using SAS (ver. 9.3, SAS Institute, Cary, NC). Statistical significance was judged at $\alpha = 0.05$. In all ANCOVA analyses, we LOG-transformed continuous variables to ensure that relationships were linear.

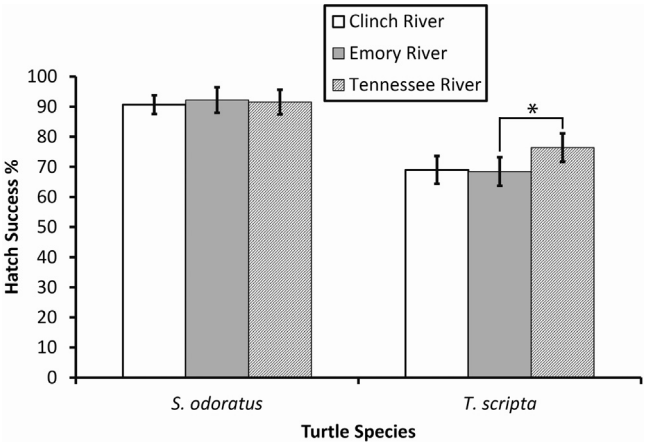

Fig. 2. Mean hatching success of two species of turtles inhabiting an area impacted by a recently-remediated coal fly-ash spill. The Clinch and Emory Rivers were impacted by the spill and the Tennessee River was a reference site. Sample sizes (total clutches) are as follows: *S. odoratus*: Clinch River: 27; Emory River: 28; Tennessee River: 23; *T. scripta*: Clinch River: 25; Emory River: 30; Tennessee River: 30. Asterisks (*) indicate significant differences. Error bars represent ± 1 SE.

Table 5
Results of binomial tests comparing the effects of river, year, and the river x year interaction on hatching success within *S. odoratus* and *T. scripta*. Significant factors are bolded and identified with asterisks.

Effect	*Sternotherus odoratus*			*Trachemys scripta*		
	DF	***F***	***P***	***DF***	***F***	***P***
River	2	0.05	0.949	**2**	**3.57**	**0.033***
Year	1	0.01	0.974	1	0.25	0.617
River × year	2	0.13	0.874	2	0.23	0.794

Table 4
Results of MANCOVA comparisons of the effects of year, river, carapace length, and their interactions on egg size and clutch size in *A. spinifera*, *S. odoratus*, and *T. scripta*. Factors that were significant in each within-species model are bolded and identified with asterisks.

Effect	*Apalone spinifera*				*Sternotherus odoratus*				*Trachemys scripta*			
	Pillai's Trace	***DF***	***F***	***P***	***Pillai's Trace***	***DF***	***F***	***P***	***Pillai's Trace***	***DF***	***F***	***P***
Carapace length	**0.273**	**2**	**3.76**	**0.041***	**0.263**	**2**	**17.88**	**< 0.001***	**0.261**	**2**	**16.29**	**< 0.001***
River	0.148	4	0.84	0.508	0.007	4	0.18	0.951	0.021	4	0.49	0.743
Year	0.173	2	2.09	0.150	0.005	2	0.26	0.768	0.025	2	1.19	0.309
Carapace length × river	0.144	4	0.81	0.524	0.008	4	0.19	0.942	0.021	4	0.50	0.737
Carapace length × year	0.173	2	2.08	0.151	0.005	2	0.26	0.770	0.085	2	2.06	0.088
River × year	0.060	2	0.64	0.538	0.054	4	1.39	0.239	0.027	4	1.26	0.289
Carapace length × river × year	0.061	2	0.65	0.532	0.053	4	1.38	0.241	0.087	4	2.12	0.079

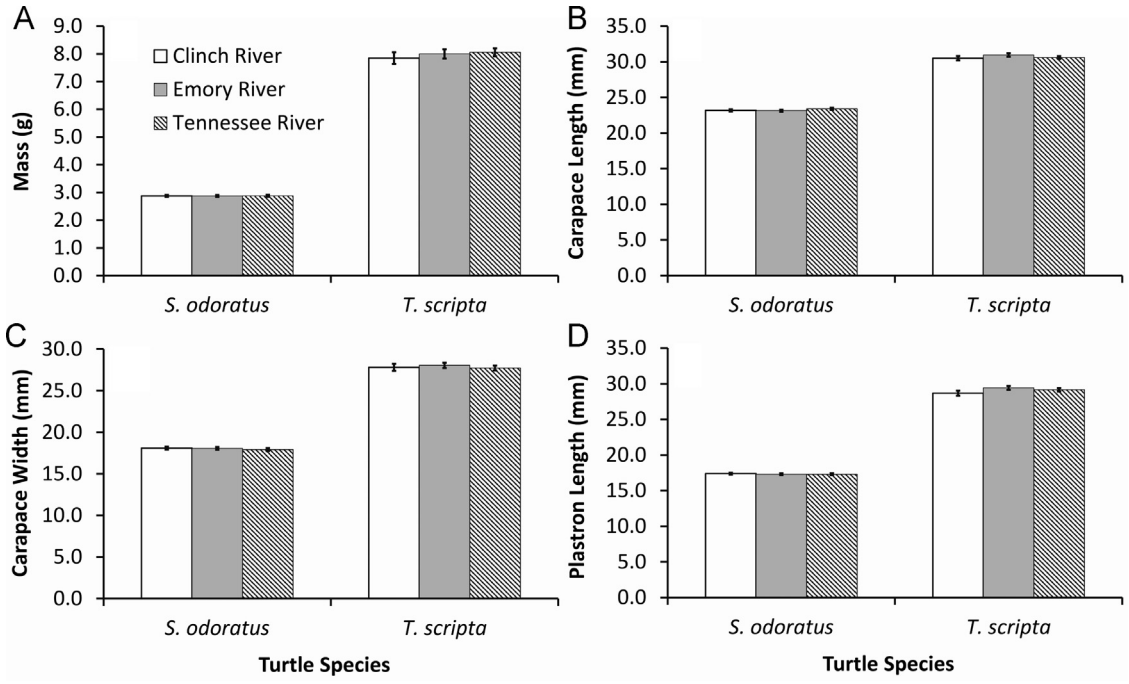

Fig. 3. Hatchling mass (A), carapace length (B), carapace width (C), and plastron length (D) of two species of turtles inhabiting an area impacted by a recently-remediated coal fly-ash spill. The Clinch and Emory Rivers were impacted by the spill and the Tennessee River was a reference site. No measurement of hatchling size differed among rivers in either species. Sample sizes (total clutches) are as follows: *S. odoratus*: Clinch River: 29; Emory River: 27; Tennessee River: 23; *T. scripta*: Clinch River: 24; Emory River: 30; Tennessee River: 30. Error bars represent ± 1 SE.

Table 6
Results of MANCOVA comparisons of the effects of egg mass, river, year, and interactions on hatchling mass, carapace length, carapace width, and plastron length in *S. odoratus* and *T. scripta*. Factors that were significant in each within-species model are bolded and identified with asterisks.

Effect	*Sternotherus odoratus*				*Trachemys scripta*			
	Pillai's Trace	*DF*	*F*	*P*	*Pillai's Trace*	*DF*	*F*	*P*
Egg mass	**0.751**	**4**	**45.15**	**< 0.001***	**0.357**	**4**	**9.59**	**< 0.001***
River	0.039	8	0.30	0.965	0.116	8	1.07	0.384
Year	0.011	4	0.17	0.953	0.067	4	1.23	0.306
Egg mass × river	0.040	8	0.31	0.962	0.112	8	1.04	0.409
Egg mass × year	0.010	4	0.15	0.962	0.063	4	1.15	0.340
River × year	0.036	8	0.28	0.971	0.072	8	0.66	0.728
Egg mass × river × year	0.040	8	0.31	0.962	0.070	8	0.64	0.747

3. Results

Mean element concentrations in maternal females and hatchlings are presented in Tables 1 and 2.

3.1. Reproductive output: clutch size and egg mass

Mean clutch sizes and egg masses are reported in Table 3. Neither clutch size nor egg mass differed among maternal rivers of origin or between years in *A. spinifera*, *S. odoratus*, or *T. scripta* (Table 4). Both clutch size and egg mass increased with maternal carapace length in all three species (Table 4). Neither clutch size nor egg mass were significantly affected by any interaction of maternal river, year, or carapace length in any species (Table 4).

3.2. Hatching success

In *S. odoratus*, hatching success did not differ between years or among maternal rivers of origin (Fig. 2; Table 5). In contrast, *T. scripta* hatching success significantly differed among maternal rivers (Fig. 2; Table 5). Post-hoc differences of means tests showed that hatching success was significantly lower in clutches from the Emory River than in those from the Tennessee River ($T=2.63$, $P=0.010$). Hatching success in the Tennessee River was ~11% higher than in the Emory River (Fig. 2). No other pairwise comparisons were significant ($T \leq 1.57$, $P \geq 0.120$). *Trachemys scripta* hatching success did not differ between 2011 and 2012, or with the interaction of river and year (Table 5). A second tier analysis testing the effects of hatchling As, Hg, Se, Sr, and Zn concentrations on hatching success in *T. scripta* found no significant effects of any of these individual elements or interactions between them ($F_1 \leq 3.48$, $P \geq 0.068$; Table S1).

3.3. Hatchling size

No metric of hatchling body size differed among maternal rivers (Fig. 3) or between years in *S. odoratus* or *T. scripta* (Table 6). Hatchling mass, carapace length, carapace width, and plastron length increased with egg mass in both species (Table 6). No metric of hatchling body size was significantly affected by any interaction of maternal river, year, or egg mass in any species (Table 6).

Table 7
Results of statistical comparisons of the effects of maternal river and hatchling carapace length on righting performance of hatchling *S. odoratus* and *T. scripta*. Righting success was analyzed using a logistic regression, numbers of righting attempts were compared with mixed-effects ANCOVAs to account for discrete count data, and times to successful righting were compared with ANCOVAs. Factors that were significant in each within-species model are bolded and identified with asterisks.

Effect	*Sternotherus odoratus*			*Trachemys scripta*		
	DF	*F*	*P*	*DF*	*F*	*P*
Righting success						
Carapace length	Not tested because all turtles			1	0.05	0.830
River	successfully righted			**2**	**6.32**	**< 0.001***
Carapace length × river				2	0.01	0.988
Number of righting attempts						
Carapace length	1	0.68	0.413	1	0.10	0.751
River	2	2.10	0.137	2	0.53	0.597
Carapace length × river	2	2.16	0.129	2	0.51	0.606
Time to successful righting						
Carapace length	1	0.01	0.994	1	0.54	0.471
River	2	0.78	0.465	2	0.40	0.672
Carapace length × river	2	0.78	0.466	2	0.40	0.678

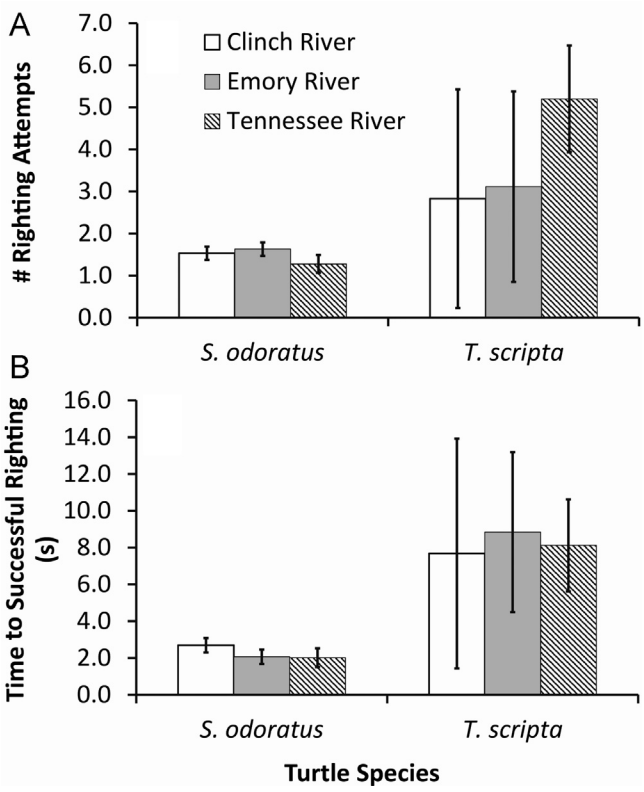

Fig. 4. Number of righting attempts (A) and time to successful righting (B) in two species of turtles inhabiting an area impacted by a recently remediated coal fly-ash spill. We considered the Clinch and Emory Rivers as impacted by the spill and the Tennessee River was a reference site. No aspect of righting performance differed among rivers in either species. Sample sizes (total clutches) are as follows: *S. odoratus*: Clinch River: 17; Emory River: 17; Tennessee River: 11; *T. scripta*: Clinch River: 4; Emory River: 8; Tennessee River: 17. Error bars represent ± 1 SE.

3.4. Righting response

All hatchling *S. odoratus* successfully righted, regardless of body size or element concentration, so they were not included in analyses investigating effects on righting success. The univariate full factorial logistic regression model testing the effects of carapace length and maternal river on ability to right in *T. scripta* revealed that the percentage of hatchling *T. scripta* that successfully righted significantly differed among rivers (Table 7). Specifically, 20% of *T. scripta* from the Clinch River successfully righted, while 100% of *T. scripta* from the Emory and Tennessee Rivers righted. Differences of least-squares means tests found that Clinch River *T. scripta* righting success was significantly lower than *T. scripta* from the Emory River ($T=3.71$, $P=0.002$) and the Tennessee River ($T=3.27$, $P=0.003$). Carapace length did not affect righting success (Table 7). A second tier analysis testing the effects of As, Hg, Se, Sr, and Zn hatchling concentrations on righting success in *T. scripta* found no significant effects of any of these elements ($F_1 \leq 3.01$, $P \geq 0.095$; Table S2).

Number of righting attempts did not differ among maternal rivers in either *S. odoratus* or *T. scripta* (Fig. 4A; Table 7). However, there was considerable variance around the mean number of righting attempts in hatchling *T. scripta* from all three rivers (Fig. 4A). Carapace length did not affect the number of righting

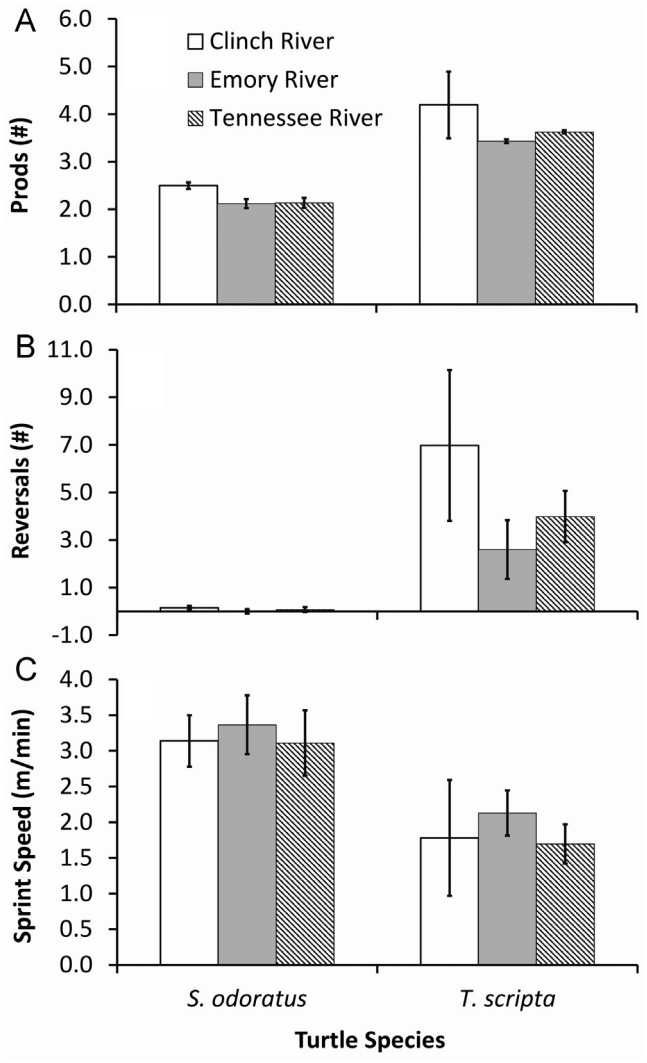

Fig. 5. Number of prods (A), number of reversals (B), and sprint speed (C) in of two species of turtles inhabiting an area impacted by a recently remediated coal fly-ash spill. We considered the Clinch and Emory Rivers as impacted by the spill and the Tennessee River was a reference site. No aspect of locomotor performance differed among rivers in either species. Sample sizes (total clutches) are as follows: *S. odoratus*: Clinch River: 19; Emory River: 17; Tennessee River: 12; *T. scripta*: Clinch River: 5; Emory River: 20; Tennessee River: 27. Error bars represent ± 1 SE.

Table 8
Results of statistical comparisons of the effects of maternal river and hatchling carapace length on terrestrial locomotor performance of hatchling *S. odoratus* and *T. scripta*. Numbers of prods and reversals were compared with mixed-effects ANCOVAs, and sprint speed was compared with ANCOVAs. Factors that were significant in each within-species model are bolded and identified with asterisks.

Effect	*Sternotherus odoratus*			*Trachemys scripta*		
	DF	*F*	*P*	*DF*	*F*	*P*
Number of prods						
Carapace length	1	0.38	0.543	1	1.76	0.191
River	2	0.56	0.576	2	0.55	0.579
Carapace length × river	2	0.50	0.608	2	0.58	0.567
Number of reversals						
Carapace length	1	0.01	0.931	**1**	**5.47**	**0.024***
River	2	0.24	0.789	2	1.46	0.244
Carapace length × river	2	0.47	0.499	2	1.56	0.220
Sprint speed						
Carapace length	1	0.90	0.348	1	0.06	0.812
River	2	0.01	0.989	2	0.79	0.460
Carapace length × river	2	0.01	0.988	2	0.78	0.466

attempts in either species (Table 7). Within *T. scripta*, turtles that successfully righted did not make significantly more righting attempts than those that did not successfully right ($F_{1,26}=0.01$, $P=0.97$). Time to successful righting was not significantly affected by maternal river in either *S. odoratus* or *T. scripta* (Fig. 4; Table 7). Carapace length did not affect righting success in either species (Table 7).

3.5. Hatchling terrestrial locomotor performance

The number of prods did not differ among maternal rivers, with carapace length, or with the interaction between carapace length and river in either *S. odoratus* or *T. scripta* (Fig. 5A; Table 8). However, there was considerable variance around the mean number of prods in *T. scripta* from the Clinch River (Fig. 5A). In *S. odoratus*, the number of reversals also did not differ among maternal rivers or with carapace length or the interaction between the two (Fig. 5B; Table 8). In contrast, the number of reversals significantly increased with carapace length in *T. scripta*, but also did not differ among maternal rivers of origin (Table 8). Hatchling sprint speed did not differ among maternal rivers or with carapace length in either hatchling *S. odoratus* or hatchling *T. scripta* (Fig. 5C, Table 8).

4. Discussion

After remediation of the 2008 coal fly-ash spill in Kingston, Tennessee, female turtles collected from impacted rivers accumulated modestly elevated concentrations of trace elements in their tissues and maternally transferred some of these elements to their eggs (Van Dyke et al., 2014). However, we found little evidence that the remediated fly-ash spill influenced reproductive output, hatching success, or hatchling performance. In cases where we found significant differences in reproductive and hatchling traits, effect sizes were relatively small and we could not attribute these differences to specific element concentrations in turtle tissues. Among elements associated with coal fly ash, Hg and Se are of particular concern because they are known to cause negative reproductive effects in oviparous wildlife (e.g., Coyle et al., 1993; Skorupa, 1998; Janz et al., 2010; B.C. Hopkins et al., 2013a; Metts et al., 2013). Our results suggest that the 2008 coal fly-ash spill affected concentrations of these elements in turtle tissues (Table 1), but accumulation resulted in concentrations below those needed to elicit adverse effects at the time we conducted our study multiple years after remediation.

Female turtles from rivers that were impacted by the fly-ash spill produced clutch, egg, and hatchling sizes similar to those observed in our nearby reference river. Clutch size, egg mass, and hatchling size increased with maternal female body size, consistent with what is generally known for freshwater turtles (Congdon and Gibbons, 1985). Although it remains unknown whether turtle reproductive output was compromised immediately following the spill, our results suggest that it was not influenced by the low element concentrations remaining after remediation of the coal fly-ash spill.

Hatching success was generally > 70% among turtles in our study, but in *T. scripta* it was approximately 11% lower in clutches from the Emory River than in clutches from our reference river. Life history tables for similar species suggest that this relatively modest difference is unlikely to have major population-level effects (Congdon et al., 1993, 1994; Heppell, 1998). Furthermore, we found no relationship between concentrations of the elements maternally transferred to hatchlings and hatching success. Therefore, if the spill is responsible for the observed reduction in hatching success in the Emory River, it is more likely mediated through a mechanism other than direct embryotoxicity. Differences in egg quality due to local resource conditions as well as among-river differences in male fertility, indirect effects of element exposure (e.g., food resource availability), physical disturbance from the spill and ash removal, or other unmeasured ecological differences could have also contributed to our observations. For example, although the effects of ash-derived contaminants on sperm production or quality in turtles are not known, decreases in either could presumably affect hatching success independently of direct effects of maternal transfer of elements to eggs (e.g., Harrison et al., 1997; Olsson and Madsen, 1998).

Although exposure to coal ash reduces swimming performance of both fish (Hopkins et al., 2003) and tadpoles (Hopkins et al., 2000) elsewhere, we found limited evidence the coal fly-ash spill altered turtle hatchling development or performance. For example, all *S. odoratus* successfully righted themselves, regardless of river of origin. Although the ability of *T. scripta* hatchlings to successfully right themselves was higher in hatchlings from the Tennessee River than hatchlings from the Clinch River, this difference was unrelated to element concentrations. Similarly, we did not find among-river differences in locomotor performance for either species. The lack of a pronounced effect in this study is consistent with the relatively low element concentrations in turtle tissues at the time of our study.

Overall element concentrations were low in the turtle tissues we examined and adverse reproductive effects in the area near the spill were limited to significantly lower hatching success or righting ability for *T. scripta* in the Emory or Clinch River, respectively, as compared to the Tennessee River. It remains unclear whether the few differences we observed among rivers are the result of the spill event, natural variation among rivers in this system, or perhaps affected by historical and/or atmospheric sources of contamination (Brooks and Southworth, 2011). Because turtles may live for decades and some elements may bioaccumulate or biomagnify (e.g., Hamilton, 2004), future monitoring may be warranted to determine whether the observed reduction in hatching success gradually attenuates with time, or whether any long-term effects of chronic exposure to low contaminant concentrations emerge over time.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2015.01.024.

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