Title: Influence of Metal(loid) Bioaccumulation and Maternal Transfer on Embryo-Larval Development in Fish Exposed to a Major Coal Ash Spill

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Abstract

In December 2008, an earthen retaining wall at the Tennessee Valley Authority (TVA) Kingston Fossil Fuel Plant failed and released 4.1 million m³ of coal ash to rivers flowing into Watts Bar Reservoir in Tennessee, U.S.A.. As part of a comprehensive effort to evaluate the risks to aquatic resources from this spill – the largest in U.S. history – we compared bioaccumulation and maternal transfer of selenium (Se), arsenic (As), and mercury (Hg) in adult redear sunfish (*Lepomis macrolophus*) collected two years after the spill from both coal-ash exposed and non-exposed areas of the Emory and Clinch Rivers with the success of embryo-larval development in their offspring. Whole body and ovary concentrations of Se in female sunfish at three study sites downstream of the spill were significantly elevated (site means = 4.9–5.3 and 6.7–9.0 mg/kg d.w. whole body and ovary concentrations, respectively) compared with concentrations in fish from reference sites upstream of the spill site (2.2–3.2 mg/kg d.w. for whole bodies and 3.6–4.8 mg/kg d.w. for ovaries). However, Se concentrations in coal ash-exposed areas remain below proposed U.S. Environmental Protection Agency (USEPA) criteria for the protection of aquatic life. Site-to-site variation in fish concentrations of As and Hg were not well-correlated with ash-exposure, reflecting the multiple sources of these metal(loid)s in the affected watersheds. In 7-day laboratory tests of embryos and larvae derived from in vitro crosses of eggs and sperm from these field-collected sunfish, fertilization success, hatching success, embryo-larval survival, and incidences of developmental abnormalities did not differ significantly between ash-exposed and non-exposed fish. Furthermore, these developmental endpoints were not correlated with whole body or ovary concentrations of Se, As, or Hg in the maternal fish, or with fish size, ovary weight, or gonadal-somatic indices. Results from this and related studies associated with the Kingston ash spill appear consistent with proposed USEPA fish-based water quality criteria for Se, and to date continue to suggest that long-term exposures to sediment containing residual coal ash from the Kingston spill may not present a significant chronic risk to fish populations exposed to this major coal ash release.

1. Introduction

Coal combustion waste from coal-fired electrical power generating plants — often referred to as coal ash — constitutes one of the largest waste streams produced in the United States (USEPA, 2014a). Although approximately 40% of this ash is currently re-used for beneficial purposes, including incorporation into a variety of building materials, the remainder is typically stored or disposed in surface impoundments or landfills, many of which are unlined. A complex mixture of combustion byproducts, coal ash is enriched in potentially toxic substances such as selenium (Se), arsenic (As), and mercury (Hg) that can bioaccumulate or biomagnify in aquatic organisms and wildlife (Yudovich and Ketris, 2005a, b; Yudovich and Ketris, 2006; Reash, 2012; Otter et al, 2012). Releases of coal ash or ash leachates to the environment can pose significant risks to the health of aquatic organisms and terrestrial wildlife, as well as to human health (Baumann and Gillespie, 1986; Sorensen, 1988, Besser et. al., 1996; Rowe et al., 2002; Dorman et. al., 2010; Lemly and Skorupa, 2012; Ruhl et al., 2012; Mayfield et al., 2013; Rice et al., 2014).

Environmental releases of coal ash or ash constituents can occur through a variety of routes, including: (1) air-borne contamination from open surface storage impoundments; (2) wastewater discharges from coal-fired power plants; (3) runoff from ash stilling ponds or surface impoundments; (4) leaching from landfills, impoundments, or ash ponds to surface waters or groundwater; and (5) the failure of retaining structures (USEPA, 2014). An extreme example of the latter occurred in December 2008 with the collapse of an earthen retention wall at the Tennessee Valley Authority's (TVA) Kingston Fossil Fuel Plant in Tennessee, U.S. The resulting spill — the largest in U.S. history — released approximately 4.1 million m³ of wet coal fly ash and bottom ash onto surrounding land and into adjacent waterways including the Emory, Clinch, and Tennessee Rivers that flow into the TVA Watts Bar Reservoir (Arcadis, 2012; Walls et. al., 2015).

The environmental risks associated with chronic releases of coal ash or coal ash leachates from coal-fired plants or coal ash storage impoundments are well-known (Baumann and Gillespie, 1986; Besser et al., 1996; Lemly, 1993, 1996; Hamilton, 2004; Young et al., 2010). For instance, fish populations in the vicinity of coal-fired power plants often have elevated body burdens of Se compared with fish at locations further removed from power plants (Young et al., 2010). Furthermore, in some well-characterized cases such as Hyco and Belews Lakes in North Carolina, such Se contamination has been associated with reproductive impairments in fish populations, significant increases in the incidences of developmental abnormalities in fish larvae, and even the local extinction of sensitive fish species (Cumbie and Van Horn,

1978; Lemly, 2002). In light of these well-documented adverse outcomes to fish populations from chronic Se exposures attributable to coal ash-related contamination, understandable concerns were raised as to the potential for significant impacts to fish communities in the affected river systems and Watts Bar Reservoir from Se and other toxic constituents in the ash released by the catastrophic Kingston spill (Babiyak et al., 2009; Ruhl et al., 2009; Lisenby et al., 2009). To systematically evaluate the ecological and human health risks of the ash spill, and provide data for the formulation of a long-term strategy for managing the affected resources, TVA and the U.S. Environmental Protection Agency (USEPA) initiated a diverse environmental assessment program in the immediate aftermath of the spill (Jacobs Engineering, 2010, 2012).

In a series of related studies associated with this comprehensive research and monitoring program, Se was demonstrated to be significantly elevated in fish in areas of the Emory and Clinch Rivers affected by coal ash from the Kingston spill, with highest concentrations of this metal(loid) observed in redear sunfish (*Lepomis macrolophus*) among the various fish species examined (Adams et al., 2012; Otter et al., 2012; Mathews et al., 2014). Arsenic and Hg were also elevated in fish affected by the ash spill (Rigg et al., 2015), but site-to-site variation in these metal(loid)s also appeared to reflect additional sources of these metal(loid)s, including various Department of Energy (DOE) facilities in Oak Ridge, TN (Turner et al., 1985; Campbell et al., 1997). However, to date few if any adverse effects on fish health (Adams et al. 2012; Bevelhimer et al. 2014), fish reproduction and embryo-larval development (Greeley et al., 2012; Kay and Baker, 2012; Stanley et al., 2013; Greeley et al., 2014a,c; Rigg et al., 2015), or fish population and community structures (Rigg et al., 2015) have been conclusively linked in either field- or laboratory-based studies to the Kingston ash spill or to increased metal(loid) concentrations in fish.

Concerns remain, however, that elevated Se in particular, accumulated by adult fish through their diet and maternally transferred to their offspring, might still impact fish populations in the affected bodies of water as the Se and other ash-related metal(loid)s continue to work through the aquatic food web (Mathews et al, 2014). Although remaining below proposed draft water quality criteria, Se concentrations in certain fish species from the vicinity of the Kingston ash spill site, again in particular the redear sunfish (Adams and Fortner, 2012; Otter et al., 2012; Mathews et al., 2014), exceed concentrations associated in some studies with adverse effects on fish reproductive or developmental processes (USEPA, 2014b). To address these concerns, we examined through a combined field and laboratory study the potential relationships between: (1) the bioaccumulation of Se, As, and Hg by wild adult redear sunfish exposed in situ to ash from the Kingston spill and sampled while spawning; (2) the maternal transfer of these

metal(loid)s to their offspring during oogenesis; and (3) potential adverse outcomes to the survival and early development of offspring from continuing exposures of fish to residual coal ash from the spill.

The primary objective of this investigation was to determine if redear sunfish affected by the Kingston coal ash spill transferred ash-related contaminants to their eggs in sufficient quantities and chemical forms to affect fertilization success or induce teratogenic abnormalities in F1-generation embryos and larvae. A secondary objective was to evaluate the potential direct or synergistic toxic contributions of water from ash-exposed study sites to the developing embryos and larvae. As reproduction and development provide key linkages between the effects of environmental stressors on individual organisms and adverse outcomes manifested at higher levels of biological organization (Rose et al., 1999), the results of this study are expected to assist in assessing the risks to fish populations from residual coal ash remaining in the Watts Bar Reservoir system following remediation. The results are also expected to further the general understanding of the toxicity and risks of coal ash and coal ash-associated contaminants such as Se, As, and Hg to aquatic organisms under real-life exposure conditions, and examine the protectiveness of proposed fish-based water quality criteria for Se. A preliminary report concerning these results was previously submitted to TVA for purposes of the Kingston spill investigation (Greeley et al., 2014b).

2. Materials and Methods

2.1 Setting and study sites

The Kingston Fossil Fuel Plant is located on a peninsula formed by the confluence of the Emory and Clinch Rivers approximately 6 km upstream of the confluence of the Clinch and Tennessee Rivers in the TVA Watts Bar Reservoir (Fig. 1). Upon the failure of a retaining wall, coal ash stored on-site since the opening of the facility in 1955 was released into the Emory River in the vicinity of Emory River mile (ERM) 2.2. The ash front surged both upstream and downstream in the Emory River and downstream into the Clinch River, with additional downstream movement into the Tennessee River and Watts Bar reservoir proper during subsequent high-water events. More detailed descriptions of the ash spill and its extent can be found in Jacobs Engineering (2010, 2011), Arcadis (2012), and Walls et al. (2015).

For the purposes of this study, several ash-exposed sites and unexposed reference sites in the Emory and Clinch Rivers were selected to represent various levels of fish exposure to coal ash (Fig. 1). Fish and water sampling locations in this study included upstream reference sites at Emory River mile (ERM) 8.0 and Clinch River mile (CRM) 8.0, and ash-exposed sites at ERM 3.0 (in the immediate vicinity of the ash

release), ERM 0.9 (in the lower reaches of the Emory River downstream of the spill site), and CRM 1.5 (downstream of the spill site and the confluence of the Emory and Clinch Rivers). Neither of the upstream reference site can be considered pristine: the ERM 8.0 reference site is affected by urban and rural runoff and legacy contamination from a former paper mill, while the CRM 8.0 reference site experiences current and legacy contamination from nearby DOE facilities in Oak Ridge in addition to urban and rural runoff. However, each reference site is located upstream of the furthest extent of the ash spill on the respective rivers, and both share common non-coal ash related exposure and water quality conditions with the downstream coal ash-exposed study sites.

2.2 Target fish species

Redear sunfish were selected as the target species for this study for a variety of reasons, including: (1) the relative abundance of this species of fish at each of the proposed study locations, as verified by preliminary sampling; (2) sufficient body and ovary sizes to provide tissues for metal analyses as well the number of eggs needed for crossing trials; (3) relatively high body burdens of Se at the coal-ash exposed study sites (Arcadis, 2012; Otter et al., 2012; Mathews et al., 2014); (4) a known sensitivity of this species to the adverse reproductive effects of Se exposure from coal ash settling basin discharges (Sorensen, 1988); and (5) a relatively compact home-range which contributes to a much greater site-fidelity than larger top-predators in the area such as largemouth bass. [Although the home-range of the redear sunfish is not well-documented, sunfish in general demonstrate a relatively high degree of site-affinity (Etnier and Starnes, 1994), with the home range of the closely-related pumpkinseed (*Lepomis gibbosus*) being estimated to be only about 1 hectare (Warren, 2009).

2.3 Fish sampling

Where applicable and achievable, specific spawning locations for redear sunfish within each study site were identified by preliminary sampling during the previous breeding season. For study purposes, fish were collected by boat electroshocking in cooperation with TVA from these locations during the spring 2011 breeding season according to protocols approved by the Oak Ridge National Laboratory (ORNL) Animal Care and Use Committee. Following capture, fish were tagged with a unique ID number, placed in an aerated live well on the collecting boat, and then transported in large ice-chests with aeration to a wet-laboratory facility at nearby ORNL where the in vitro spawning crosses and subsequent embryonic-larval tests were performed.

2.4 Water sampling

Site and reference water samples for the laboratory tests were collected from the water surface at the various study sites in I-CHEM certified 10-L CUBITAINERS® (Thermo Fisher Scientific, Rockwood, Tennessee, U.S.). After collection, water samples were pre-filtered through a 0.45 µm pore-size filter to remove large particulates, sterile-filtered at 0.2 µm, and stored at ≤ 4 °C in the dark until needed. Prior to use, temperature was raised to room temperature and analyzed for pH, conductivity, alkalinity, hardness, and dissolved oxygen (DO).

2.5 Study design and crossing procedures

The study design was adapted from procedures for identifying Se-induced deformities in early life stages of freshwater fish (Janz and Muscatello, 2008) and USEPA guidelines for evaluating the effects on fish embryo-larval development of contaminants in effluents and receiving waters (USEPA, 2002). The methodology included two-way ANOVA-based crossings involving fish and water collected from study sites affected by the spill as well as from reference sites located upstream of the influences of the spill. Evaluation endpoints included fertilization success, hatching success, embryo-larval survival, and incidences of developmental abnormalities during seven-day embryo-larval cultures in both site and reference waters, as well as the concentrations of Se, As, and Hg in the whole bodies and ovaries of the female parental fish supplying the eggs for the crosses.

Female redear sunfish possessing mature eggs or nearly mature vitellogenic oocytes were collected along with adult male fish from each study site at the beginning of the 2011 breeding season (Fig. 2), approximately 2 ½ years after the ash spill, and transferred to ORNL for mating crosses. Female sunfish with ovulated eggs were used immediately for in vitro spawning purposes upon return to the laboratory. Females that had well-developed vitellogenic oocytes, but lacked mature eggs at the time of collection, were injected intramuscularly with 0.5 µl/gm of a commercial ovulating/spermiating agent (Ovaprim® from Western Chemical Inc.) and held in the laboratory for 24-hours in attempts to induce final egg maturation for crossing trials. Unlike male sunfish, which responded well to such injections by producing copious amounts of sperm within 24 hours, Ovaprim® did not reliably induce final maturation and ovulation in female sunfish collected for this study. As a consequence, only the relatively few female sunfish collected and checked during this effort that already had ovulated eggs in their ovaries were employed in these spawning trials. Sperm for crosses was obtained on the day of the procedures, either from males newly-collected from field sites or those held in the laboratory for up to 3-d following

1 injection of Ovaprim®. Milt was pooled from at least four different male sunfish collected from the same
2 field sites as the female fish, and kept on ice for up to an hour before use in fertilizing ovulated eggs from
3 one or more females.
4

5 Prior to these crossing trials, female sunfish were lightly anesthetized with tricane methanesulfonate
6 (MS-222) and patted dry with clean towels. Mature ovulated eggs were obtained by applying gentle
7 pressure in a stroking motion to the abdomen using gloved fingers, with eggs from each fish collected
8 directly into a pair of newly-opened sterile 100x15 mm polystyrene petri dishes. Extraneous material
9 such as blood or excrement was carefully removed from the eggs with clean forceps or plastic pipettes.
10 Full-strength pooled milt (40 µl) was then added to each dish and mixed with the eggs along with gentle
11 stirring, followed by the addition of just enough sterile-filtered water from either the site of fish collection
12 or the primary reference site (ERM 8.0) to cover the eggs and bottom of the dishes. The dishes were
13 loosely covered, and immediately gently swirled by hand to facilitate the fertilization process and initiate
14 egg activation. After the addition of sufficient water (from the same site or from the ERM 8.0 reference
15 site, as applicable) to fill the dishes approximately half-full, the now fertilized eggs were allowed to
16 water-harden undisturbed for one hour before further handling.
17

18 Randomization of embryos within each dish was accomplished by gentle swirling. Twenty-five
19 embryos from each dish were subsequently transferred using a clean plastic pipette into each of 4
20 replicate dishes containing 30 ml of clean sterile-filtered water from either the site of fish collection or the
21 primary reference site at ERM 8. These procedures resulted in a two-way (crossover) ANOVA
22 experimental design, with embryos from each cross being separately fertilized and cultured in both site
23 and reference water. In the case of fish collected from the reference site at ERM 8.0, embryos were
24 fertilized and cultured both in site water (which in this case was the ERM 8.0 reference water), and in
25 water from the spill site at ERM 3.0 for comparison purposes. Embryos and eventually larvae were
26 subsequently incubated for 7-days, a period extending to the absorption of the larval yolk sac, without
27 feeding in environmental chambers which were maintained at $25 \pm 1^\circ \text{C}$ with a 16L:8D photoperiod.
28

29 Approximately 90% of the culture water was changed — and embryos/larvae were checked — daily in
30 each culture dish during the 7-day duration of the tests. Fertilization success was evaluated at the first
31 daily following egg activation. Embryos or hatched larvae were inspected daily for survival, hatching
32 status, and the presence of obvious developmental abnormalities as specified in Greeley et al. (2014c),
33 with dead embryos or larvae being removed and discarded when found. At test termination,
34 approximately 60% of the test solution was removed from each test chamber without disturbing the

1 remaining embryos and larvae, then an overdose of MS-222 was added to each chamber to immobilize
2 and euthanize the test organisms. Embryos and/or larvae were immediately scored for final
3 determinations of survival and incidences of developmental abnormalities, and then stored in formalin for
4 archiving. For the purposes of data analysis and presentation, developmental abnormalities scored at the
5 time of test termination were considered lethal and added to the final totals for larvae mortality as
6 specified in USEPA method 1001.0 (USEPA, 2002).

7
8 Initial target sample sizes for the in vitro crosses were at least four clutches of eggs per sit, as
9 recommended in Janz and Muscatello (2008). Eventually, at least six crosses were evaluated for each of
10 the five study sites during the active portion of the 2011 redear sunfish breeding season.

11 12 *2.6 Fish processing*

13
14 Once eggs were obtained for crosses, the female fish supplying eggs for this study were euthanized with
15 an overdose of MS-222. Total length was recorded to the nearest 0.1 cm and body weight measured to the
16 nearest 0.01 g. Gonadosomatic indices (GSI) were calculated as:

$$17 \qquad \qquad \qquad \text{GSI} = (\text{ovary weights/body weights}) \times 100$$

18
19
20 Ovaries, minus the relatively few mature eggs used for spawning purposes, and gut tissues were removed
21 from the body cavity and weighed to the nearest 10 mg, following which the gut tissues were discarded.
22 Ovaries and fish carcasses were rinsed with distilled water before being placed individually into plastic bags
23 and stored frozen ($< -10^{\circ}\text{C}$) prior to shipment to the analytical laboratory for measurement of metal(loid)
24 concentrations.

25 26 *2.7 Metal(loid)s analyses*

27
28 Fish samples were shipped frozen ($< -10^{\circ}\text{C}$) to Pace Analytical Services, Inc. (Green Bay, WI) for
29 homogenization, moisture determination, and analysis of Se and As by USEPA method SW846-6020 and Hg
30 by USEPA method SW846-7473. All quality assurance procedures (e.g., blanks, matrix spikes) were
31 performed as specified in the respective analytical methods. Method detection limits and the use of standard
32 reference materials were as described by Mathews et al. (2014). Sample data were reported by the laboratory
33 on a wet weight (w.w) basis and converted to dry weight (d.w.) values, where appropriate, using the moisture
34 content reported for each sample. Concentrations for whole body determinations were adjusted to reflect the

relative contributions of each metal(loid) in (1) ovaries/eggs (hereafter referred to only as ovaries) and (2) carcasses minus the ovaries, gut tissues, and their contents. Four ovary samples — including three from fish collected at the ERM 3.0 study site and one from ERM 0.9 — were unable to be analyzed after a shipping container was damaged during transit to the analytical laboratory, so for these fish the whole body metal(loid) concentrations were estimated to be equivalent to the carcass concentrations for graphical purposes.

2.8 Statistical analyses

Statistical analysis procedures followed the recommendations of USEPA (2002) and Janz and Muscatello (2008). Homogeneity of variance was verified by a Bartlett's test and normality of distribution by the Shapiro-Wilk procedure. If homogeneous and normally distributed, two-way Analysis of Variance (ANOVA) was used to test for site and water source effects on fertilization success, hatching success, embryo-larval survival, and incidences of embryo-larval deformities at the termination of the 7-day tests after arcsine transformation. If the ANOVA rejected a null hypothesis of equal means, then the Holm-Sidak method was used to test for significant site differences in response parameters. For data with non-homogeneous variances and abnormal distributions, differences between means were examined by the Kruskal-Wallis One Way Analysis of Variance in Ranks followed by Dunn's Pairwise Multiple Comparison Procedures. Correlations between concentrations of Se, As, and Hg in whole bodies and ovaries of adult females and the developmental success of offspring were examined by Pearson Product Moment Correlation following arcsine transformation of the embryo-larval test endpoints. Analyses were conducted in SigmaPlot, with the significance level for rejecting the hypothesis of equal means between sites set at $\alpha = 0.05$.

3. Results

3.1 Whole body and ovary selenium

Whole body and ovary concentrations of Se were significantly elevated (Fig. 2) in adult female redear sunfish collected for this study from two sites on the Emory River (Fig. 1: ERM 3.0 and ERM 0.9) and one site on the Clinch River (CRM 1.5) that were directly affected by the Kingston coal ash spill. Selenium concentrations were nearly twice as great in fish from ash-exposed sites [4.9–5.3 mg/kg (d.w.) site means for whole body Se analyses and 6.7–9.0 mg/kg Se (d.w.) for ovary/egg analyses] as compared with fish from two reference locations at ERM 8.0 and CRM 8.0 that were well upstream on the respective rivers from any influences of the ash spill [2.2–3.2 mg/kg (d.w.) for whole bodies and 3.6–4.8

mg/kg (d.w.) for ovaries]. Concentrations of Se in individual fish at the ash-exposed sites ranged up to 6.5 mg/kg (d.w.) for whole body analyses and 11.2 mg/kg (d.w.) for the ovaries. However, even the relatively higher concentrations of Se in some individual fish at the ash-contaminated sites remained below proposed Se freshwater ambient water quality chronic criteria for the protection of aquatic life of 8.1 mg/kg (d.w.) for fish whole bodies and 15.2 mg/kg (d.w.) for fish ovary and eggs (USEPA, 2014).

The proportions of Se concentrations in redear sunfish ovaries compared with whole bodies (not including the ovaries) varied in these female sunfish from 1.44 – 1.72 (Fig. 2) at the various study sites, with an overall mean proportion across all study sites of 1.54. Ratios greater than 1 suggest active sequestration of Se in the ovaries, demonstrating a maternal transfer of this metal(loid) to the offspring of fish exposed in situ to coal ash the Kingston spill.

3.2 Whole body and ovary arsenic

As with body burdens of Se, As concentrations were higher in adult female sunfish collected for this study from sites affected by ash from the Kingston spill than from the reference site located upstream of the ash spill on the Emory River at ERM 8.0 [Fig.3: 0.2 mg/kg (d.w.) for whole bodies and 0.3 mg/kg (d.w.) for ovaries from fish collected from ERM 8.0]. However, fish with the highest concentrations of As were actually collected from the Clinch River reference site at CRM 8.0 [1.0 mg/kg (d.w) for whole bodies and 1.6 mg/kg (d.w) for ovaries], with fish from the ash-affected study sites falling in between these extremes. Statistically significant differences between site-means at $p < 0.05$ occurred only in the cases of (1) lower whole body concentrations of As in fish collected from ERM 8.0 compared with fish from each of the other study sites; and (2) lower concentrations in the ovaries of fish collected from ERM 8.0 compared with fish collected from ERM 0.9 and CRM 8.0.

Formal fish tissue criteria for adverse health effects are not available for this metal(oid). However, published effects levels for As in fish tissues of 18 mg/kg (d.w.) [converted from 5.4 mg/kg (w.w.): McGeachy and Dixon, 1992; Hinck et al., 2006] are much higher than the whole body and ovary As concentrations measured in the current study (Fig. 3).

Proportions of As in redear sunfish ovaries compared with As in whole bodies (minus ovaries) ranged from 0.74 to 1.58 (site means), with an overall mean proportion of 1.02 across all five study sites (Fig.3). Ovary to whole body ratios of about 1 indicate concentrations of As are in approximate equilibrium between the ovary and other fish tissues, and imply that maternal transfer of this metal(loid) to the

offspring through the developing oocytes and eggs may be occurring, but just not at a level that would appreciably depurate the adult fish or particularly magnify larval exposures to this metal(loid) (with the apparent exception of fish from CRM 8.0)

3.3 Whole body and ovary mercury

The majority of Hg in fish tissues occurs in the form of methylmercury (MeHg) (Cabanero et al., 2007), so the current total Hg analyses can be assumed to approximate MeHg concentrations in both whole bodies and ovary tissue. Mean concentrations of Hg in the whole bodies of the redear sunfish collected for this investigation [expressed in this case in terms of w.w. to facilitate comparison to published MeHg wildlife effects thresholds (USEPA, 2001)] were highest in fish from the two Clinch River sites at CRM 8.0 and CRM 1.5 [0.11 and 0.09 mg/kg (w.w.), respectively] and the upstream Emory River reference site at ERM 8.0 [0.09 mg/kg (w.w.)] (Fig. 4). In comparison, whole body Hg concentrations were lowest at the two ash-exposed study sites on the Emory River at ERM 3.0 and ERM 0.9 [0.06 mg/kg (w.w.) and 0.07 mg/kg (w.w.), respectively]. Ovary concentrations of Hg were conservatively estimated to be < 0.017 mg/kg (w.w.) at all sites, with over 75% of analyses for individual fish actually being reported as being less than the sensitivity limits of the assay (non-detects were set to the sensitivity limit for graphical comparison in Fig. 4).

Proportions of Hg in redear sunfish ovaries to Hg in whole bodies (minus the ovaries) were conservatively estimated to be ≤ 0.18 (Fig.4), with the caveat that the actual concentrations of Hg in ovaries were probably lower than the method sensitivity values used for these calculations. Ovary to whole body ratios less than 1 indicate Hg is not being actively concentrated in redear sunfish ovaries or maternally transferred to the offspring to any significant degree (EPA, 1980; WHO, 2001).

3.4 Fertilization and hatching success, embryo-larval survival, and percent deformities

The results of in vitro spawning crosses involving gametes from female and male redear sunfish collected from reference and ash-exposed locations on the Emory and Clinch Rivers are shown in Table 1, along with various measures of developmental success scored at the termination of the 7-day embryo-larval tests. Mean fertilization success in these trials ranged from 93.0 % for eggs and sperm from fish collected at CRM 8.0 and incubated in water from the same location (referred to as “site” water) to 99.3 % for eggs from fish collected from the ash-exposed site at CRM 1.5 and incubated in same-site water

(Table 1). There were no statistically significant differences ($p < 0.5$) in fertilization success between any combination of fish and water sources.

Mean hatching success of embryos derived from these in vitro crossings ranged from 95.3%, for embryos from fish collected from the ERM 8.0 reference site and incubated in water from ERM 3.0 (for the ERM 8.0 primary reference site only, water from ERM 3.0 served as reference water and water from ERM 8.0 as site water) to 99.0% for embryos from fish collected at the CRM 8.0 secondary reference site and incubated in water collected from the ERM 8.0 primary reference site (Table 1). As with fertilization success, there were no significant differences in hatching success between any combination of fish or water source used for these crosses.

Mean percent survival, the primary test endpoint that describes larvae that hatch and are alive without obvious developmental abnormalities (that are presumed to be eventual lethal) at 7-days post-fertilization, ranged from a low of 79.4 % — for the offspring of fish collected from ERM 0.9 and incubated in water from the same location — to a high of 95.8 % for embryos from fish collected at CRM 1.5 and cultured in same-site water (Table 1). There were again no significant differences in survival attributable to the sources of either the fish or water used for these crosses.

The subset of embryos/larvae that were scored as mortalities in the survival analysis but were actually still alive but deformed at the termination of the Larvae alive at the end of the 7-d embryo-larval cultures that exhibited various developmental deformities, ranging from mild edema to severe circulatory skeletal abnormalities, were noted in every embryo-larval grouping involving the various combinations of parental fish and incubation water sources (Table 1). It is likely that some larvae scored as being abnormalities that were observed during daily embryo-larval checks died prior to the end of the 7-day trial periods, and therefore were not included in the deformity statistics compiled at the end of the tests but rather were included as mortalities in the survival statistics. In other cases, notably where mild edema or spinal curvatures were observed soon after hatching, apparent abnormalities noted during daily checks did not persist to be counted as deformities in final tallies. The percentages of larval deformities scored at the end of the 7-day embryo-larval cultures ranged from 1.0 % — for larvae from crossings involving fish from CRM 1.5 and using water from the ERM 8.0 reference site — to 3.8 % for larvae from crossings involving fish from CRM 8.0 and water from the same source (Table 1). There were no statistically significant differences in percent deformities due to the sources of either parental fish or culture water. For perspective, deformity rates $< 5\%$ are sometimes considered ecotoxicologically irrelevant (Lemly, 1997a; Janz et al., 2010).

3.5 *Correlations between metal(loid) concentrations and embryo-larval success*

Results of the embryo-larval development trials and the metal analyses conducted on ovaries and whole bodies of the parental fish demonstrated no significant correlations between (1) concentrations of Se, As, or Hg in the maternal parents, and (2) fertilization success, hatching success, embryo-larval survival, or incidences of developmental abnormalities in the offspring of either ash-exposed fish or reference fish (Table 2).

To verify that other adult fish-related factors were not sources of bias in interpreting these results, the embryo-larval results were also subjected to correlation analysis against body lengths of the parental female fish, body weights, ovary weights, and the GSI. As shown in Table 3, no significant correlations were observed between any of these adult fish-related factors and fertilization success, hatching success, embryo-larval survival, or incidences of developmental abnormalities.

4. Discussion

The metal(loid)s Se, As, and Hg were contaminants of particular toxicological concern for the health of aquatic biota in downstream reaches of the Emory, Clinch, and Tennessee Rivers and Watts Bar Reservoir following the massive December 2008 coal ash spill at the TVA Kingston Fossil Fuel Plant. Concerns with these metal(loid)s were due primarily to the relative enrichment of these metal(loid)s in coal ash, their tendency to bioaccumulate in aquatic organisms, and their potential for significant toxicity to aquatic organisms. Of particular concern were the potential effects of Se on fish populations in the bodies of water impacted by the coal ash spill, as Se has been shown in both laboratory and field studies to be capable of causing developmental abnormalities, reproductive impairments, and even the local extirpation of sensitive fish species (Woock et al., 1987; Lemly, 1996; Muscatello et al., 2006; Jezierska et al., 2009; Thomas and Janz, 2014), including at locations where coal ash from power plants was the primary source of the Se contamination (Sorensen, 1988; Lemly, 2002).

That the highest concentrations of Se occurred in fish from the three ash-exposed study sites and the lowest concentrations at the two reference sites located well upstream of the influence of the ash spill supports an assumption that the Kingston coal ash spill was the primary source of fish exposure to this metal(loid) in the broad study area. The relatively small home ranges and high site-affinities of sunfish species in general (Etnier and Starnes, 1994), and of the closely related pumpkinseed in particular

(Warren, 2009), do not necessarily preclude there being some limited movement of redear sunfish between relatively closely spaced study sites (for example, between ERM 3.0 and ERM 0.9). But these life-history characteristics do suggest that significant overlap between sunfish from the ash-exposed sites and reference sites located well upstream on both the Emory and Clinch Rivers is highly unlikely. Thus the adult redear sunfish collected for the purposes of the current study are likely well-representative of the range of fish exposures to coal ash and ash-associated metal(loid)s to be encountered at each of the various study sites, despite there being no way to conclusively verify the exact history and movements of each individual fish supplying gametes for these in vitro crosses. Thus, for older resident sunfish at the three study sites impacted by the Kingston coal ash spill, whole body metal(loid) burdens could be result of greater than two years' exposure to ash and ash-derived metal(loid)s in the interval between the spill and the conduct of this study. In contrast, ovary concentrations of Se and the other metal(oid)s almost certainly reflect much more recent exposures occurring primarily during ovarian recrudescence.

The concentrations of Se in the fish collected for this investigation were similar to concentrations previously reported for redear sunfish whole bodies and muscle tissue at these and other nearby coal ash-exposed sites in related bio-assessment efforts associated with the Kingston ash spill (Adams et al., 2012a; Mathews et al., 2014). Both whole body and especially ovary concentrations of Se at these locations have remained below proposed freshwater ambient water quality chronic criteria for aquatic life of 15.2 mg/kg (d.w.) in fish ovary/eggs and 8.1 mg/kg for fish whole bodies (USEPA, 2014). However, adverse effects have also previously been reported in fish with lower Se concentrations much lower than the proposed criteria, with Hinck et al. (2006) — citing studies and reviews by Hamilton (2004) and Lemly (1996, 2002) — suggesting Se effects thresholds for avoiding toxicity to larval fish as low as 3 mg/kg (d.w.) and as low as 4 mg/kg (d.w.) for ensuring the protection of piscivorous wildlife. Both thresholds were exceeded by many of the fish collected for the current study.

Concentrations of Se were even higher in the ovaries of the female fish used in this study, with an average ratio of Se in ovaries to Se in whole bodies of 1.54:1 (Fig. 2). These comparative ratios suggest significant maternal transfer of this metal(loid) to the ovaries and developing oocytes during seasonal oogenesis. Although the primary route of Se exposure to adult fish is through the food chain (Coyle et al., 1993; USEPA, 2014), Se supplied to the offspring through maternal transfer, and therefore the potential teratogenic effects of Se on the F1-generation, may not necessarily require the intermediate step of Se bioaccumulation by the tissues (Conley et al., 2014), further suggesting the possibility of potential developmental effects from Se exposure at even lower concentrations than currently expected.

1 Arsenic concentrations were also elevated in both whole bodies and ovaries of fish at sites directly
2 affected by the Kingston spill (Fig.3). However, site-to-site variation in both whole body and ovary
3 concentrations also suggested there to be at least one additional significant source of this metal(loid)
4 upstream of the coal ash-affected areas on the Clinch River, most likely the DOE facilities in Oak Ridge,
5 TN (Turner et al., 1985; Campbell et al., 1997). An average ratio of As in redear sunfish ovaries and
6 whole bodies of nearly 1:1 indicated relative equilibrium in concentrations between these tissue types.

8 Arsenic exposure is capable of adversely affecting a variety of organ systems (USEPA, 1980; WHO,
9 2001), including In fish various reproductive and developmental impairments such as the perturbation of
10 vascular development in zebrafish (McCollum et al., 2014). However, such adverse effects are typically
11 observed only at concentrations rarely encountered in aquatic environs (EPA, 1980; WHO, 2001). In this
12 particular case-study, both whole body and ovary concentrations of As measured in the adult fish used for
13 in vitro crosses were far lower than published effects thresholds for fish (Fig. 3: McGreachy and Dixon,
14 1992; Hinck et al, 2006), and were apparently insufficient to cause any discernible adverse effects on
15 embryo-larval development in redear sunfish exposed in situ to the Kingston coal ash spill.

17 Coal ash is also enriched in Hg (Yudovich and Ketris, 2005b), although the patterns of Hg
18 concentrations in the whole bodies of the redear sunfish collected for this investigation are consistent with
19 the existence of other significant sources of this particular metal(loid) on both the Emory and Clinch
20 Rivers that are apparently unrelated to the coal ash spill, especially the DOE facilities in Oak Ridge, TN
21 (Turner et al., 1985; Campbell et al., 1997). Mercury concentrations in whole fish from the two upstream
22 reference sites approached or exceeded MeHg effects-levels noted in other fish studies. For instance,
23 Birge et al. (1979) associated tissue concentrations of Hg in the range of 0.07-0.1 mg/kg with increased
24 embryo mortality, and proposed that egg concentrations of approximately 0.5 mg/kg could inhibit both
25 larval survival and fish reproduction. There is even some evidence that Hg concentrations as low as 0.01
26 mg/kg might be capable of reducing fertilization success in the offspring of exposed fish (Matta et al.,
27 2001), suggesting that even the relatively low levels of Hg observed in the ovaries of at least some of the
28 females fish supplying eggs for these current in vitro crosses might be sufficient to disrupt fish
29 reproduction.

31 However, despite concentrations of Se and Hg in whole bodies or ovaries that approach or exceed
32 reported effects levels in fish from either ash-affected or reference sites in this investigation, no
33 significant adverse effects were observed during the current study in fertilization success, hatching
34 success, embryo-larval survival, or incidences of developmental abnormalities in the offspring of ash-

1 exposed versus reference fish cultured either in water from the site of origin of the parental fish or
2 reference water. Nor were these developmental endpoints correlated with the size of the maternal fish,
3 ovary weights, gonadal-somatic indices, or whole body and ovary concentrations of Se, As, and Hg.
4 These results suggest that prolonged in situ exposure of redear sunfish in the Emory and Clinch Rivers to
5 coal ash from the Kingston ash spill, although sufficient to elevate Se significantly in fish tissues and eggs
6 compared with non-exposed reference fish, had no obvious adverse effects on developmental processes in
7 the offspring of exposed fish. Neither was the water from any site obviously toxic to redear sunfish
8 embryos or larvae.

10 We should mention the potential, however slight the effect may be, for survivor-bias in this
11 study. Some dead fish were observed in the days immediately following the spill, both in the
12 water and on the river banks, and some fish were collected at that time that appeared to be in
13 distress [see Rigg et al. (2015) for discussion]. Other fish potentially could have suffered
14 sufficiently severe effects from subsequent direct or indirect exposure to ash-related toxicants to
15 later die unobserved, and so would not have been available to be included in this study.
16 However, it should be acknowledged that there have been no indications from the many
17 intensive studies conducted throughout the area since the ash spill of either subsequent fish kills
18 or significant adverse fish health effects following the initial impacts, so the degree to which
19 survivor-bias might have affected the current results cannot be determined at this time.

21 In general, the current results are consistent with those from other studies associated with the
22 Kingston coal ash spill which report few if any adverse effects from the Kingston coal ash spill on the
23 health (Adams et al. 2012; Bevelhimer et al. 2014), reproduction and development (Greeley et al., 2012;
24 Kay and Baker, 2012; Stanley et al., 2013; Greeley et al., 2014a,c; Rigg et al., 2015), or population and
25 community structures (Rigg et al., 2015) of fish exposed to the ash spill. So an obvious question arising
26 from these studies is “Why has this major coal ash spill had so few obvious adverse effects on the fish in
27 the Watts Bar reservoir system, considering the magnitude of the spill and the well-characterized effects
28 of coal ash contamination and Se contamination on fish populations in other aquatic systems?”

30 Matthews et al. (2014) recently posed a similar and possibly related question, focused more on Se
31 bioaccumulation than fish health, which broadly paraphrased was “Why is selenium bioaccumulation not
32 higher in fish in areas affected by this coal ash spill, considering the magnitude of the spill and the degree
33 of Se contamination in the area?. One possible explanation put forward by these authors is that the

1 relatively lotic nature of the water bodies affected by this spill compared with the often lentic nature of
2 aquatic systems where Se bioaccumulation is often relatively higher, may be influencing Se
3 bioaccumulation by local fish populations. Another potential factor is the complex relationship between
4 Se, As, and Hg bioaccumulation and effects, which can be neutral, antagonistic, or even synergistic
5 depending on a variety of factors that include the species of fish, the specific species of the metal(loid)s,
6 and the concentrations involved (Mason et al., 2000; Peterson et al., 2009; Yang et al., 2010; Penglase et
7 al., 2014). Either explanation may also be applicable to the question of why adverse effects on fish
8 populations affected by the spill may be more limited than originally anticipated.

9
10 Whatever the explanation, Se bioaccumulation at this site remains below proposed fish tissue-based
11 water quality criteria for this metal(loid). With multiple sources of metal(loid)s within the affected
12 watersheds, it may be that existing contamination from sources other than the coal ash spill could be
13 influencing our ability to isolate and discern the more subtle effects of contaminants from the coal ash
14 spill on fish bioaccumulation, health, reproduction, and developmental success.

15
16 Although no significant effects on early development were noted in this study, investigations such as
17 this which include reproductive or developmental endpoints can be invaluable to the environmental risk
18 assessment paradigm. Such studies provide key linkages between the effects of environmental stressors —
19 including in this case to coal ash-derived metal(loid)s — on individual aquatic organisms and potential
20 adverse outcomes at higher levels of biological organization (Rose et al., 1999). Results from this and
21 related studies associated with the Kingston coal ash spill response are consistent with USEPA proposed
22 fish-based Se water quality criteria, and continue to suggest that lingering exposures to sediment
23 containing residual coal ash from the Kingston spill may not present a significant long-term risk to fish
24 populations in the affected waterways.

25
26 In summary, although metalloids including Se are elevated in fish affected by the TVA Kingston coal
27 ash spill both whole body and ovary concentrations of Se in redear sunfish inhabiting areas of upper
28 Watts Bar reservoir affected by the spill have remained below proposed freshwater ambient water quality
29 chronic criteria for aquatic life of 15.2 mg/kg (d.w.) in fish ovary/eggs and 8.1 mg/kg for fish whole
30 bodies (USEPA, 2014). Fertilization and hatching success, embryo-larval survival, and incidences of
31 developmental abnormalities in the offspring of sunfish exposed in situ to ash from the spill in
32 comparison with fish from unaffected reference sites. These largely negative results are in agreement with
33 other related bio-assessment studies associated with the Kingston spill response (Greeley et al., 2012a;
34 Kay and Baker, 2012; Stanley et al., 2013; Greeley et al., 2014a,c; Sherrard et al., 2015; Rigg et al., 2015)

1 in suggesting that continuing exposures to sediment containing residual coal ash from the Kingston spill
2 may not present a significant long-term risk to fish populations in the effected waterways. However,
3 concerns continue for the fish populations in areas affected by the ash as subtle chronic effects on fish
4 populations might manifest in the future as toxicants related to the spill continue to work through the food
5 web. Therefore, long-term biological monitoring will continue in the Emory and Clinch Rivers and
6 elsewhere in the upper Watts Bar Reservoir to ensure that potential chronic effects of the spill will not in
7 the future compromise the integrity of fish populations in these aquatic systems. More generally, these
8 results attest to the protectiveness of the proposed freshwater ambient water quality chronic criteria for
9 aquatic life (USEPA, 2014).

11 **5. Conclusions**

13 In December 2008, a dike at the Tennessee Valley Authority (TVA) Kingston Fossil Fuel Plant
14 abruptly failed, triggering the largest environmental release of coal ash in U.S. history. As part of a
15 comprehensive effort to evaluate the potential risks of this catastrophic ash release to fish in the Emory
16 and Clinch Rivers, we evaluated (1) the bioaccumulation and maternal transfer of Se and other metals and
17 metal(loid)s in female redear sunfish exposed in situ for over two years to coal ash from the TVA
18 Kingston coal ash spill; (2) the effects of this parental exposure on the ability of ash-exposed fish to
19 develop viable eggs; and (3) the effects of parental exposures on the early developmental success of F1-
20 generation offspring produced in experimental laboratory crosses with parental fish, including the
21 occurrence of developmental abnormalities in the offspring potentially attributable to elevated Se in the
22 coal ash. Selenium was significantly elevated in both whole bodies [maximum of 11.2 kg/mg (d.w.)] and
23 ovaries [(6.5 mg/kg (d.w.)) of fish from the ash-affected study sites, with relative whole body-ovary
24 concentrations indicative of significant maternal transfer of this metal(loid) from adult fish to their
25 offspring. However, both mean and maximum Se concentrations remained below proposed water quality
26 criteria for aquatic life effects. No significant adverse effects were observed in fertilization success,
27 hatching success, embryo-larval survival, or incidences of developmental abnormalities in the offspring of
28 ash-exposed versus reference fish. Results from this and related studies associated with the Kingston coal
29 ash spill response are consistent with USEPA proposed fish-based Se water quality criteria, and continue
30 to suggest that lingering exposures to sediment containing residual coal ash from the Kingston spill may
31 not present a significant long-term risk to fish populations in the affected waterways. More generally,
32 these results attest to the protectiveness of the proposed freshwater ambient water quality chronic criteria
33 for aquatic life (USEPA, 2014).

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Table 1

Fertilization success, hatching success, survival, and incidences of developmental deformities following 7-day cultures of redear sunfish embryos and larvae following in vitro crosses of fish collected from sites in the Emory and Clinch Rivers, TN., affected by coal ash from the 2008 TVA Kingston ash spill (ERM 3.0, ERM 0.9, and CRM 1.5) or from reference sites upstream of the influences of the ash spill (ERM 8.0 and CRM 8.0). Data are mean percentages $\pm$ SE.

Fish Sites ^a	Number of Crosses	<u>Fertilization Success</u>		<u>Hatching Success</u>		<u>Survival</u>		<u>Deformities</u>	
		<i>Water Source^b =</i>	<i>Site</i>	<i>Reference</i>	<i>Site</i>	<i>Reference</i>	<i>Site</i>	<i>Reference</i>	<i>Site</i>
ERM 8.0 (Ref 1)	7	98.7 ± 0.4	97.7 ± 0.4	96.1 ± 1.9	95.3 ± 2.9	90.6 ± 3.5	90.4 ± 4.9	1.7 ± 0.9	1.1 ± 0.6
ERM 3.0	14	95.0 ± 1.4	95.9 ± 0.9	97.3 ± 1.6	98.4 ± 0.6	83.9 ± 6.5	81.1 ± 7.5	2.9 ± 0.9	2.4 ± 0.8
ERM 0.9	8	93.6 ± 3.0	95.9 ± 1.3	98.3 ± 0.9	98.3 ± 0.9	79.4 ± 7.6	91.0 ± 2.1	2.0 ± 0.7	2.4 ± 0.7
CRM 8.0 (Ref 2)	6	93.0 ± 4.2	96.0 ± 0.9	97.2 ± 1.4	99.0 ± 0.5	83.3 ± 4.4	86.8 ± 4.3	3.8 ± 1.5	2.0 ± 0.5
CRM 1.5	8	99.3 ± 0.3	97.1 ± 1.5	98.9 ± 0.4	98.5 ± 0.5	95.8 ± 0.8	92.5 ± 2.2	1.9 ± 0.6	1.0 ± 0.4

2

3 ^a Fish collection site: ERM = Emory River mile and CRM = Clinch River mile.

4 ^b Source of water used for fertilization and embryo cultures: Site = same source as parental fish; Reference = ERM 8.0 (with the
5 exception of ERM 8.0, for which “reference” water for culture purposes was obtained from the ash spill site at ERM 3.0)

6 There were no significant differences between site means in two-way ANOVA analysis at $p < 0.5$.

7

Table 2

Pearson Product Moment Correlation Analyses for the relationship of fertilization and hatching success, survival, and incidences of larval deformities in ash-exposed and reference redear sunfish embryos and larvae versus total Se, As, and Hg concentrations (mg/kg dry wt.) in whole bodies and ovaries of female redear sunfish used for in vitro crossing trials. Data are correlation coefficients/*p* values; *n* = 39 for ovaries and 43 for whole bodies, respectively.

Test Result	Whole Body			Ovary		
	Se	As	Hg	Se	As	Hg ^a
Fertilization Success (%)	-0.0470	-0.1630	0.2690	0.2590	-0.2770	---
in <i>Site Water</i>	0.765	0.298	0.081	0.112	0.088	
Fertilization Success (%)	0.0758	-0.1390	0.1980	0.1950	-0.2150	---
in <i>Reference Water</i>	0.633	0.374	0.203	0.235	0.188	
Hatching Success (%)	0.1820	0.1030	-0.0799	0.2770	-0.0002	---
in <i>Site Water</i>	0.242	0.509	0.611	0.088	0.999	
Hatching Success (%)	0.0578	0.1760	-0.0169	0.1520	0.0987	---
in <i>Reference Water</i>	0.713	0.260	0.914	0.356	0.550	
Embryo-larval Survival (%)	0.1350	-0.0274	0.1210	0.2210	-0.2910	---
in <i>Site Water</i>	0.387	0.862	0.440	0.177	0.072	
Embryo-larval Survival (%)	-0.0254	0.0219	0.0702	0.0417	-0.0127	---
in <i>Reference Water</i>	0.872	0.889	0.655	0.801	0.939	
Larvae Deformities (%)	-0.0304	0.0014	-0.1040	0.0855	0.0092	---
in <i>Site Water</i>	0.846	0.993	0.506	0.605	0.956	
Larvae Deformities (%)	0.0669	0.0471	-0.0909	0.0784	0.0472	---
in <i>Reference Water</i>	0.670	0.764	0.562	0.635	0.775	

^a Were not subjected to correlation analysis because the majority of results were below the method sensitivity limits.

There were no significant correlations at *p* < 0.5.

Table 3

Pearson Product Moment Correlation Analyses for the relationships between fertilization and hatching success, embryo-larval survival, and incidences of larval deformities versus body lengths and weights, ovary weights, and gonadosomatic indices (GSIs) of female redear sunfish used for in vitro crossing trials. Data are correlation coefficients/*p* values; *n* = 43.

Test Result	Total Body Length (cm)	Body Weight (g)	Ovary Weight (g)	GSI
Fertilization Success (%)	0.0013	-0.0110	-0.1860	-0.1950
in <i>Site Water</i>	0.994	0.944	0.231	0.210
Fertilization Success (%)	0.1040	0.0907	-0.0407	-0.0943
in <i>Reference Water</i>	0.505	0.563	0.796	0.548
Hatching Success (%)	-0.0333	-0.0182	-0.0271	-0.0403
in <i>Site Water</i>	0.832	0.908	0.863	0.797
Hatching Success (%)	0.0506	0.0735	0.0078	-0.0387
in <i>Reference Water</i>	0.747	0.639	0.960	0.805
Embryo-larval survival (%)	-0.0007	-0.0323	0.0826	0.1430
in <i>Site Water</i>	0.962	0.837	0.598	0.361
Embryo-larval survival (%)	0.1500	0.1370	0.1400	0.0773
in <i>Reference Water</i>	0.339	0.381	0.371	0.622
Larvae deformities (%)	-0.0941	-0.1130	-0.1020	0.0220
in <i>Site Water</i>	0.549	0.470	0.516	0.889
Larvae deformities (%)	-0.0764	-0.1080	-0.1360	-0.0339
in <i>Reference Water</i>	0.626	0.492	0.386	0.829

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Figure Legends

Figure 1. Sampling locations in the Emory and Clinch Rivers in East Tennessee (U.S.) for adult redear sunfish and site water used for *in vitro* spawning studies. ERM = Emory River Mile; CRM = Clinch River Mile. Adapted from source map supplied by the Tennessee Valley Authority.

Figure 2. Total selenium in the whole bodies and ovaries/eggs of female redear sunfish supplying eggs for *in vitro* spawning trials. Presented are concentrations of Se in individual fish (shaded circles) at each study site, expressed as mg/kg on a dry weight (d.w.) basis, and site-means (bars), with shaded bars identifying ash-exposed sites and non-shaded bars the upstream reference sites. Dissimilar letters above each bar indicate statistically significant differences between site-means at $p < 0.05$.

Figure 3. Total arsenic in whole bodies and ovaries of female redear sunfish supplying eggs for *in vitro* spawning. Presented are concentrations of As in individual fish (shaded circles) at each study site, expressed as mg/kg on a dry weight (d.w.) basis, and site-means (bars), with shaded bars identifying ash-exposed sites and non-shaded bars the upstream reference sites. Dissimilar letters above each bar indicate statistically significant differences between site-means at $p < 0.05$.

Figure 4. Total mercury in whole bodies and ovaries of female redear sunfish supplying eggs for *in vitro* spawning. Presented are concentrations of Hg in individual fish (shaded circles) at each study site, expressed as mg/kg on a dry weight (d.w.) basis, and site-means (bars), with shaded bars identifying ash-exposed sites and non-shaded bars the upstream reference sites. Dissimilar letters above each bar indicate statistically significant differences between site-means at $p < 0.05$.

Fig. 1.

Fig. 2

Fig. 3

Fig.4