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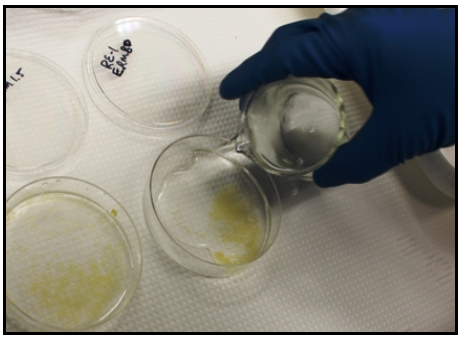
EVALUATING THE EFFECTS OF THE KINGSTON FLY ASH RELEASE ON FISH REPRODUCTION AND EARLY LIFE STAGES: *IN VITRO* SPAWNING STUDY



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1. INTRODUCTION

On December 22, 2008, a dike containing coal fly ash and bottom ash at the Tennessee Valley Authority's (TVA) Kingston Fossil Plant in East Tennessee failed, releasing over four million cubic meters of ash into the adjacent Emory River. The area influenced by the spill was widespread, with ash deposits extending upstream in the Emory River approximately 4 miles, downstream past the confluence of the Emory River with the Clinch River, and at least 4 additional miles downstream past the confluence of the Clinch River with the Tennessee River. A byproduct of coal combustion, coal ash contains a variety of substances which, at sufficient concentrations and in specific forms, can be toxic to biological systems. More specifically, coal ash contains various metals and metalloids such as selenium, arsenic, and mercury which, due to their relative toxicity and potential to bioaccumulate in the aquatic foodchains, have made them particular contaminants of concern in the case of the Kingston ash spill.

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Of the contaminants mentioned above, selenium in particular poses a known risk to the reproduction and/or early development of exposed fish populations (Lemly 1993; Besser and others 1996; USEPA 2004). Elevated body burdens of selenium in female fish have been associated with increased frequencies of atretic (dead or damaged) oocytes and reductions in the overall abundance of immature eggs in female fish ovaries. Similarly, increased incidences of developmental abnormalities have been reported in larval fish exposed to selenium by maternal transfer of this contaminant to developing eggs in both laboratory studies and at selenium-contaminated locations (Woock and others 1987, Lemly 1999, Muscatello and others 2006, Jezierska and others 2009), including several sites where coal ash from power plants was the primary source of the selenium contamination.

Reproduction is generally considered to be the most critical life function affected by environmental contamination. Successful reproduction is essential for the establishment and maintenance of healthy populations of fish and other aquatic organisms. From a regulatory perspective, the issue of potential contaminant-related effects on fish reproduction from the Kingston fly ash spill has particular significance because the growth and propagation of fish and other aquatic life is a specific classified use of the affected river systems.

To address the potential effects of fly ash from the Kingston spill on the reproductive health of downstream fish populations, researchers at the Oak Ridge National Laboratory (ORNL) have undertaken a series of studies in collaboration with and funded by TVA, including: (1) a field study of metal bioaccumulation in ovaries and other fish tissues of sentinel fish species in reaches of the Emory and

Clinch Rivers affected by the fly ash spill (Adams and others 2012); (2) an associated study of the reproductive condition of the female fish collected for bioaccumulation analyses during the spring breeding seasons (Greeley and others 2012a); (3) laboratory tests of the potential toxicity of fly ash from the spill area on fish embryonic and larval development (Greeley and others 2012b); (4) additional laboratory experimentation focused on the potential effects of long-term exposures to fly ash on fish survival and reproductive competence (Greeley and others 2012c); and (5) a combined field and laboratory project which examined the developmental success of embryos and larvae obtained from fish exposed *in situ* for over two years to ash in the Emory and Clinch Rivers and spawned *in vitro* in the laboratory (the current study).

This report focuses on the results of an *in vitro* spawning study conducted in the spring of 2011 that evaluated the potential for fish from the ash spill site and other locations within the Emory and Clinch River systems to examine the viability of offspring produced by fish exposed to the ash release. Study procedures were adapted from *in vitro* spawning procedures in Janz and Muscatello (2008). Fish for these studies were collected during the spring 2010 (preliminary studies only) and spring 2011 (definitive study) breeding seasons from five sites in the Emory River (Emory River Mile [ERM] 8.0, ERM 3.0, and ERM 0.9) and Clinch River (Clinch River Mile [CRM] 8.0 and CRM 1.5). Redear sunfish (*Lepomis microlophus*) was the preferred species for this study, both because of the abundance of these fish at each of the study locations and the tendency of this species to accumulate high concentrations of selenium (Adams and others 2012). This particular species of sunfish also has a body size sufficient to provide the requisite number of eggs needed for developmental trials. Similar spawning trials were also attempted with bluegill sunfish (*Lepomis macrochirus*), a species known to be particularly susceptible to the selenium constituent of fly ash, but success with this species was limited, primarily by the small numbers of females of this species in appropriate reproductive condition that were able to be collected from the study sites.

Data generated from this and related fish reproduction and early life stage studies provide direct input to ecological risk assessment efforts associated with the Kingston ash spill and complement and support other phases of a more comprehensive and integrative biological monitoring program.

2. APPROACH AND METHODS

Objectives

The primary objective of this study was to evaluate whether female fish exposed in situ in the Emory and Clinch Rivers to ash from the Kingston ash spill transfer contaminants to their eggs in quantities and forms sufficient to adversely affect the early development of the offspring. A secondary objective was to examine the potential for river water from ash-exposed study sites to directly impact the early development of offspring as compared with water from reference sites unaffected by the ash spill.

Sampling sites

Fish and site water were sampled by TVA and ORNL personnel from five locations in the Emory and Clinch rivers (Fig. 1) during the spring 2010 and 2011 breeding seasons. Sites included upstream reference sites unaffected by the ash spill (ERM 8.0 and CRM 8.0) and sites influenced by the fly ash release (ERM 3.0, ERM 0.9, and CRM 1.5).

Study species

Redear sunfish were the preferred species for this task for several reasons: (1) their relative abundance at each of the proposed study locations; (2) a presumed tendency, based on observations made during previous field studies, for this species to have mature gametes for a relatively extended duration of time during the proposed study period; (3) a sufficient body size to provide the requisite number of eggs for developmental trials and tissues for metal analyses; and (4) a tenedency to accumulate selenium selenium (Adams and others 2012). Bluegill sunfish, a species which is widely considered to be particularly sensitive to the adverse effects of selenium (Woock and others 1987, USEPA 2004), was also included as a secondary target species. However, it was recognized from preliminary sampling efforts that: (1) reproductively mature female bluegill sunfish were not as readily available at the proposed study sites as redear sunfish females, and (2) the relatively small body sizes of female bluegill sunfish from these locations might not be sufficient to allow the full performance of studies that could be conducted with redear sunfish.

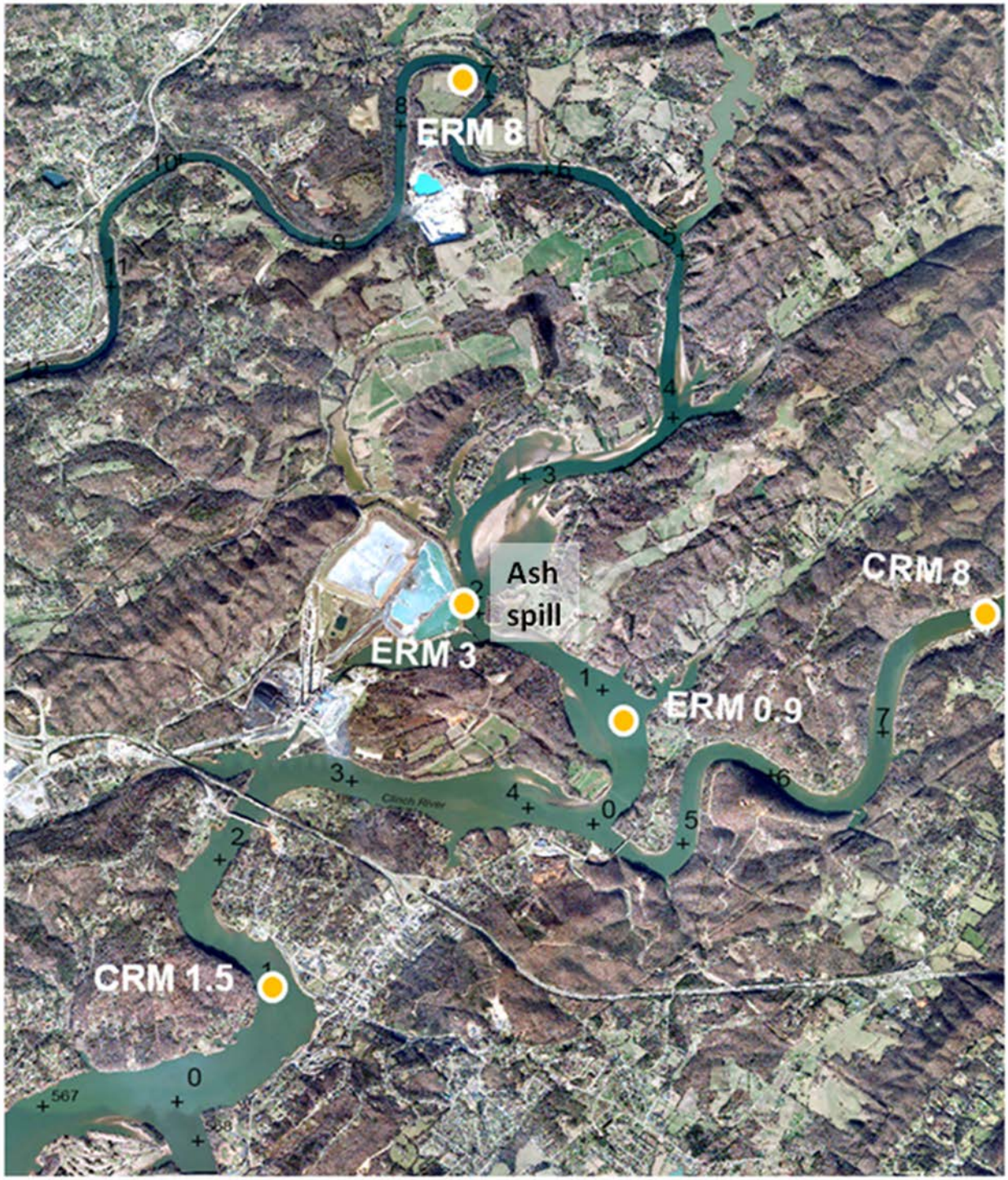

Figure 1. Map showing fish and site water sampling locations in the Emory and Clinch Rivers for fish *in vitro* spawning studies associated with the Kingston fly ash release. ERM = Emory River Mile; CRM = Clinch River Mile.

Experimental procedures

Female fish in spawning or near-spawning condition were collected by electroshocking from each study site (Fig. 2) and brought to ORNL for spawning trials. Female sunfish used for the *in vitro* spawning trials either already had mature ovulated eggs when collected or were in near-spawning condition based on the gross extended appearance of the abdomen and the condition of the oocytes in biopsies of ovary tissues obtained through the ovipositor. Fish with nearly mature eggs were injected into the musculature with 0.5 µl/gm of a commercial spawning hormone solution (Ovaprim® from Western Chemical Inc.) and held in the laboratory for 24-h to induce final egg maturation for *in vitro* spawning purposes. Original target sample sizes were four clutches of eggs per species per site as recommended in Janz and Muscatello (2008), although in the case of redear sunfish we eventually successfully spawned at least six clutches from each of the five study sites.

Sperm for *in vitro* spawning trials was obtained by pooling milt from at least four different male sunfish which were collected from the same sites as the females being spawned either on the day of spawning or up to 7-d prior to spawning with temporary housing in the laboratory (Fig. 3). Milt was always obtained fresh on the day of an *in vitro* spawn and kept on ice for up to an hour before use to fertilize ovulated eggs from one or multiple females (Fig. 3).

Female sunfish were lightly-anesthetized with tricane methanesulfonate (MS-222) and patted dry with clean towels before use. Mature eggs for fertilization purposes were obtained by applying gentle pressure in a stroking motion to the abdomen with gloved fingers, with eggs collected directly into a pair of 100x15 mm polystyrene petri dishes (Fig. 3). Any extraneous material such as blood or excrement was carefully removed from the eggs with clean forceps or plastic pipettes. Milt (40 µl) was then added to each dish and mixed with the eggs by gentle stirring, followed by the addition of just enough sterile-filtered water from either the site of collection or the primary reference site (ERM 8.0) to cover the eggs and bottom of the dishes. Dishes were loosely covered and immediately gently swirled by hand to complete fertilization and initiate egg activation. After the addition of sufficient water to fill the dishes approximately half-full, eggs were allowed to develop undisturbed for one hour before fertilization success was visually checked.

Twenty-five embryos from each pairing were then transferred using a clean plastic pipette into each of four replicate dishes containing 30 ml of clean sterile-filtered water from

Figure 2. Fish sampling from the Emory and Clinch Rivers and short-term fish housing at Oak Ridge National Laboratory (ORNL). Fish collections by electrofishing by TVA and ORNL personnel (upper left); male [top] and female [bottom] redear sunfish (upper right); wet-lab facilities at ORNL (lower right); male redear sunfish, separated by site, temporarily housed for 0-7 days indoors (bottom left) as sources of milt for *in vitro* spawning purposes.

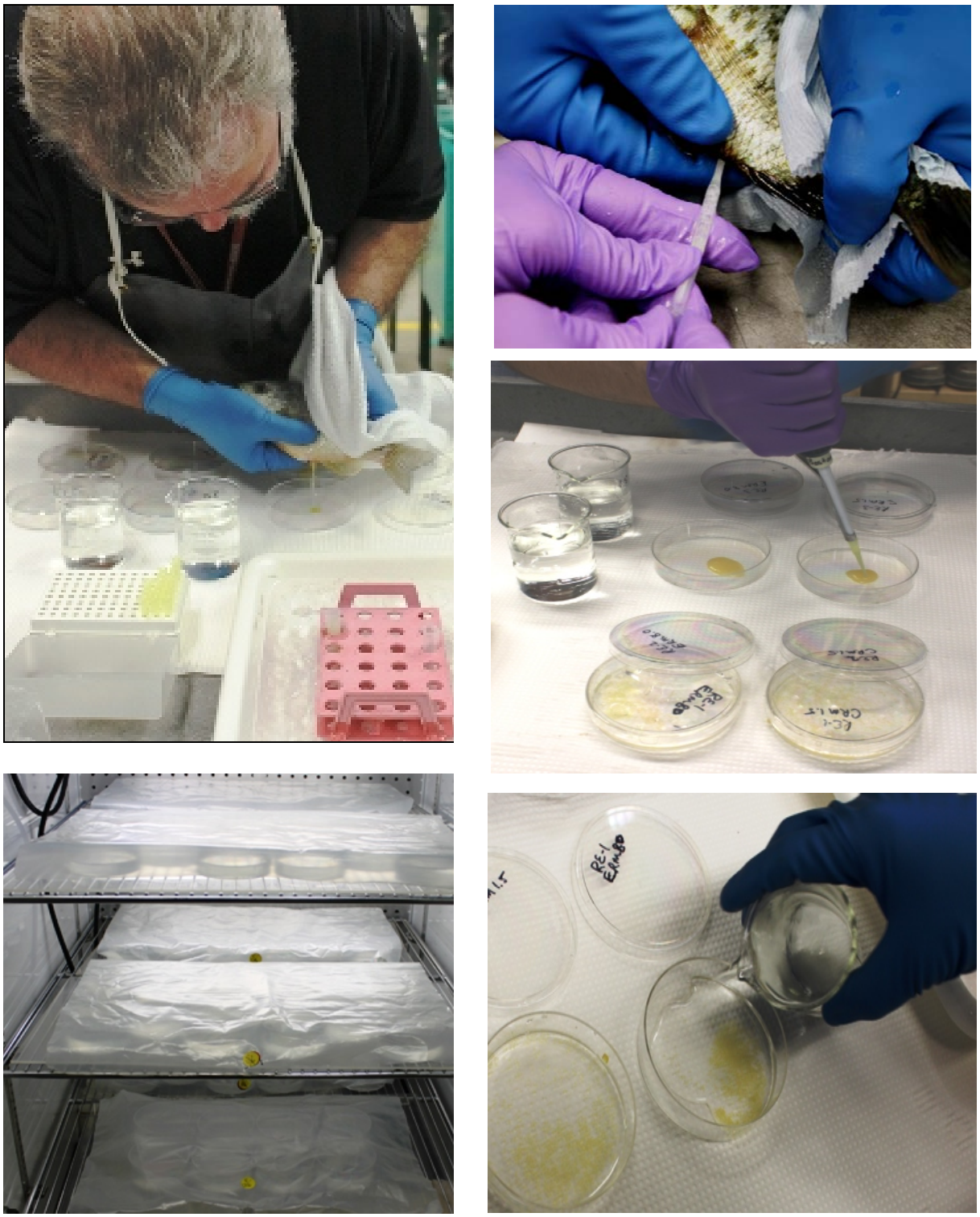

Figure 3. *In vitro* spawning of sunfish from the Emory and Clinch Rivers. Eggs stripped from female fish (upper left); milt being obtained from male fish (upper right); eggs and sperm mixed (middle right); water added (bottom right); embryos/larvae incubated for 7-d (bottom left).

either the site of fish collection or the reference site in a two-way (crossover) ANOVA experimental design. Embryos and larvae were incubated for 7-days, through the absorption of the larval yolk sac, without feeding in environmental chambers maintained at 25 ± 1 ° C with a 16L:8D photoperiod. Approximately 90% of the culture water was changed daily in each dish. Embryos or hatched larvae were inspected daily for survival, hatching status, and the presence of obvious developmental abnormalities including stunted growth, skeletal curvatures, fin anomalies, abnormal eye development, jaw and craniofacial deformities, circulatory irregularities, edema, and assorted miscellaneous abnormalities, and dead embryos or larvae were removed and discarded. At test termination, approximately 60% of the test solution was removed from each test chamber without disturbing the remaining embryos and larvae. An overdose of MS-222 was added to each chamber to immobilize and euthanize the test organisms. Embryos and/or larvae were scored for final determinations of survival and developmental abnormalities, and then stored in formalin for archiving purposes. For the purposes of data analysis and presentation, developmental abnormalities scored at the time of test termination were considered lethal and added to the final totals for larvae mortality (USEPA 2002).

Fish processing and metal analyses

Female fish supplying eggs for this study were euthanized with an overdose of MS-222 before further processing. Total length was recorded to the nearest 0.1 cm and body weight to the nearest 0.01 g. Ovary (minus the relatively few mature eggs used for spawning purposes) and gut tissues and contents were removed from the body cavity and weighed to the nearest 10 mg. Gut tissues were discarded. Ovaries and fish carcasses were rinsed with distilled water before being placed individually into plastic bags and stored frozen until shipment to Pace Analytical for metal/metalloid analyses. Contaminant concentrations as reported in the current report for whole bodies were adjusted to reflect the relative contributions of ovaries and the carcasses minus the ovaries. Mercury concentrations were adjusted to account for tissue type and analytical method (William Rogers, TVA, personal communication). Non-detects were estimated at the respective method sensitivity limits.

It should be noted that a shipping container was damaged during shipment and the custody seals broken upon arrival at the analytical laboratory. Although the container remained closed and all tissue samples with the exception of a few ovaries were readily identifiable and rinsed with distilled water before analysis, these results should not be used for compliance or legal purposes.

GSI as a reproductive indicator

The gonadsomatic index (GSI), or relative size of the gonad to the whole body, was calculated for each fish as one measure of reproductive readiness (see Fig. 4) along with the presence or absence of mature eggs. Changes in the GSI (generally downward) have been correlated with contaminant exposure in a number of field studies, including at sites contaminated with pulp and paper mill effluent (Munkittrick and others 1994; Janz and others 1997, van den Heuvel and others 2002), aromatic hydrocarbons, PCBs, and other chlorinated compounds (Johnson and others 1999). Because of the rapidity and ease with which this parameter can be measured, the GSI remains one of the most widely used reproductive indicators of environmental stress (Greeley 2002).

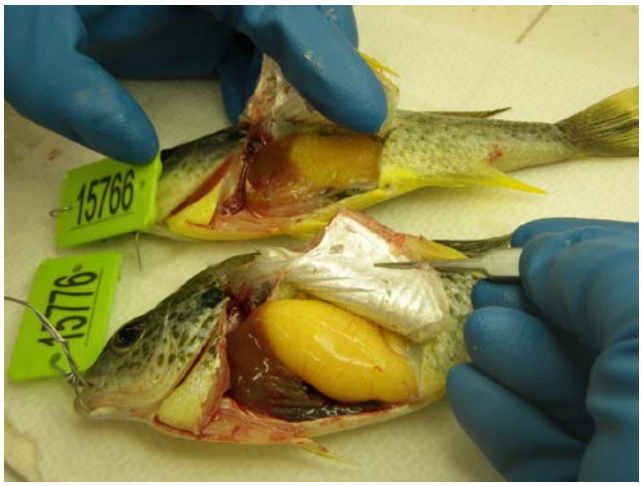

Figure 4. Comparison of a female redear sunfish with a well-developed ovary and relatively high gonadosomatic index (GSI) (front) and an immediately post-spawn fish with a comparatively low GSI (back). From Greeley and others 2012a).

Statistical analyses

Homogeneity of variance was verified by a Levene's Test (Sokal and Rohlf 1981), an F-distribution test that compares the ratios of the variances from two independent sample populations. If variances proved to be homogeneous, one-way Analysis of Variance (ANOVA) or two-way ANOVAs were used to test for site effects for the various reproductive parameters. If the ANOVA rejected a null hypothesis of equal means, then the Holm-Sidak method or other pairwise multiple comparison tests as appropriate were used to test for significant site differences

from the reference in response parameters. For data with non-homogeneous variances, 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 selected contaminant concentrations in whole bodies and ovaries of adult females and developmental success of offspring were examined by Pearson Product Moment Correlation, with analyses conducted only with samples possessing metal analyses for both carcasses and ovaries. In all cases, the significance level for rejecting the hypothesis of equal means between sites was set at $\alpha = 0.05$.

3. RESULTS AND DISCUSSION

Sunfish are spring-spawners that can produce multiple clutches of eggs for up to several weeks or even months under certain conditions (Breder and Rosen 1960). However, in a riverine or reservoir setting, it is our experience that both redear sunfish and bluegill sunfish generally spawn actively as a population for only a few weeks. It was apparent from preliminary studies conducted during the spring of 2010 that the spawning status of neither species was particularly well-synchronized within or between study sites, making *in vitro* spawning attempts with these fish much more difficult than would be the case with species with more-synchronous spawning habits. Preliminary sampling further established that: (1) adult females of both species were much less frequently collected by electrofishing at the study sites than were males, which could often be found in relatively large numbers in the shallows where spawning beds would eventually be maintained ; (2) sexually mature adult bluegill sunfish females were very small and not particularly abundant at any of the study locations; and (3) females of either species were rarely collected with mature ovulated eggs, except in the case of redear sunfish at specific locations within each broad study site where bedding activity by males had been previously detected and the location regularly monitored for spawning activity and/or the presence of females.

During the spring of 2011, a total of 43 clutches of redear sunfish eggs from five study sites were successfully fertilized and cultured in the laboratory (Table 1). For these redear sunfish *in vitro* spawning trials, embryo-larval survival – a parameter which also includes embryos that were still alive but possessing one or more developmental abnormalities at test termination – proved to be the most useful of the measured endpoints. Hatching success (not shown) was captured within the embryo-larval survival statistic (non-hatched embryos at test termination were also considered dead for test purposes), and mean hatching was uniformly high for embryos that were not either dead or deformed at test termination. Mean embryo-larval success ranged from a low of approximately 74% for embryos from fish collected at ERM 0.9 and cultured in water from the same site, to a high of 94% for embryos from fish collected at CRM 1.5 and cultured in same-site water (Table 1). Embryo-larval survival in reference water obtained from ERM 8.0 varied from a high of 89% in the case of embryos obtained from fish collected at ERM 8.0 to a low of 77% for embryos obtained from fish collected at ERM 3.0. There were no statistically significant differences in embryo-larval survival in either site-water or reference water for any study site as compared with ERM 8.0 results. Nor were any statistically significant differences observed in embryo-larval survival in site-water versus survival in the ERM 8.0 reference water for any of the study sites.

Table 1. Survival of redear sunfish embryos and larvae during 7-d laboratory tests following *in vitro* spawning of parental fish collected from study sites in the Emory and Clinch river systems (TN) during the spring 2011 breeding season.

Location[a]	Number of Spawning Trials	Embryo-Larval Survival[b] (mean % ± SEM[c])	
		Site Water	*Reference water*[d]
ERM 8.0	7	−	89.4 ± 3.7
ERM 3.0	14	80.1 ± 6.3	77.4 ± 7.1
ERM 0.9	8	73.9 ± 7.2	87.3 ± 2.9
CRM 8.0	6	78.2 ± 5.3	83.2 ± 4.3
CRM 1.5	8	94.3 ± 0.8	86.6 ± 3.5

[a] ERM = Emory River Mile; CRM = Clinch River Mile
[b] Survival in site water or reference water at end of 7-d test; larvae with abnormalities on day 7are considered dead for test purposes
[d] SEM = Standard error of the mean
[c] Sterile-filtered water from ERM 8.0

A slight tendency towards reduced − but statistically insignificant − embryo-larval survival and higher variability was noted in the results of ERM 3.0 and ERM 0.9 spawning trials (Table 1), particularly in comparison with ERM 8.0 trials. This was largely due to the relatively poor embryo-larval survival in one or two isolated clutches of eggs tested for each of these sites (data not shown). Similar test results were occasionally observed for isolated clutches of embryos from fish collected at other sites as well. Whether these tendencies were due solely to chance, to actual site differences in embryo fitness and\or contaminant burdens, or to some other factor such as the spawning status of a particular female sunfish (for instance, whether the fish had already spawned, or not, prior to use in a trial) when collected and used for *in vitro* spawning trials is not clear at this time. There were some minor site-to-site variations in the reproductive status of the female fish used in these spawning trials as demonstrated by significant differences in the mean GSIs between ERM 8.0 and ERM 0.9 (Fig. 5). However, when the results were considered across all study sites, statistically significant correlations were not observed between embryo-larval survival

during in vitro spawning trials and the GSIs, body weights, body lengths, or ovary weights (unadjusted for body size) of the female parental fish (Table 2).

Statistically-significant site differences in both whole body and ovary concentrations of selected contaminants in the female redear sunfish used for these *in vitro* spawning trials was noted, an observation reflected by fish bioaccumulation results from other studies associated with the Kingston fly ash release (Adams and others 2012). Of the various contaminants analyzed in these fish tissues, arsenic, mercury, and selenium were contaminants of particular concern for the Kingston ash spill. Whole body and ovary concentrations of arsenic proved to be higher in female fish collected from sites influenced by the ash release (ERM 3.0, ERM 0.9, and CRM 1.5) than from the ERM 8.0 reference site located upstream of the spill (Fig. 6). However, concentrations were even higher at the CRM 8.0 reference site, indicative of an upstream source of this contaminant on the Clinch River which could also be influencing not only the Clinch River study sites but the ERM 0.9 in the lower Emory River as well. Concentrations of total mercury in both whole bodies and ovaries did not differ significantly between study sites upstream of the ash release on the Emory River at ERM 8.0 or downstream at ERM 3.0, ERM 0.9, and CRM 1.5 (Fig. 7). Whole body mercury concentrations were significantly higher at CRM 8.0 than at ERM 3.0, and there was a trend towards higher concentrations at Clinch River sites as compared with Emory River sites − again indicative of an upstream source on the Clinch River –although none of the parental fish had whole body mercury concentrations approaching the U.S. EPA National Recommended Water Quality Criterion of 0.3 mg/kg (wet weight). Selenium concentrations were significantly higher in both whole bodies and ovaries at sites affected by the ash release (ERM 3.0, ERM 0.9, and CRM 1.5) as compared with the upstream reference site at ERM 8.0 (Fig. 8), but did not differ significantly between the ERM 8.0 and CRM 8.0 reference sites. Although selenium concentrations expressed on a wet weight basis were elevated in fish from sites affected by the ash release, maximum values in the parental fish, when adjusted for moisture content, remained well below draft whole body criteria for selenium effects on aquatic life (USEPA 2004).

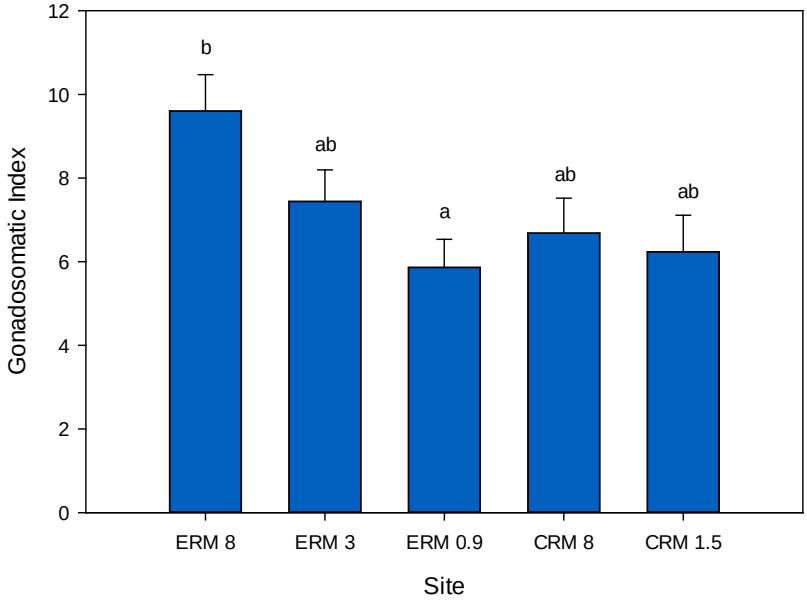

Figure 5. Gonadosomatic indices (GSIs) of female redear sunfish supplying eggs for the *in vitro* spawning study. Presented as means ± standard errors. Similar letters above error bars indicate the absence of significant differences between means at $\alpha = 0.05$.

Table 2. Pearson Product Moment Correlation Analyses for embryo-larval survival in site water and reference water versus body lengths and weights, ovary weights, and GSIs of parental female redear sunfish from sites in the Emory River and Clinch River used for in *vitro* spawning trials during the spring of 2011. Presented as the correlation coefficient[a]/*p* value[b]/number of samples. Larvae with obvious developmental abnormalities at the end of each test were considered dead for test purposes.

Test Result	Body Weight (g)	Total Body Length (cm)	Ovary Weight (g)	GSI
Embryo-larval survival (%)	-0.0396	-0.015	0.0339	0.104
in *Site Water*	0.801	0.925	0.829	0.507
	43	43	43	43
Embryo-larval survival (%)	0.160	0.187	0.160	0.082
in *Reference Water*	0.306	0.229	0.305	0.601
	43	43	43	43

[a] Positive correlation coefficients and *P* values below 0.05 tend to increase together; negative correlation coefficients and *P* values below 0.05 suggest an inverse relationship between variables

[b] *P* values greater than 0.050 indicate there is no significant relationship between the two variables

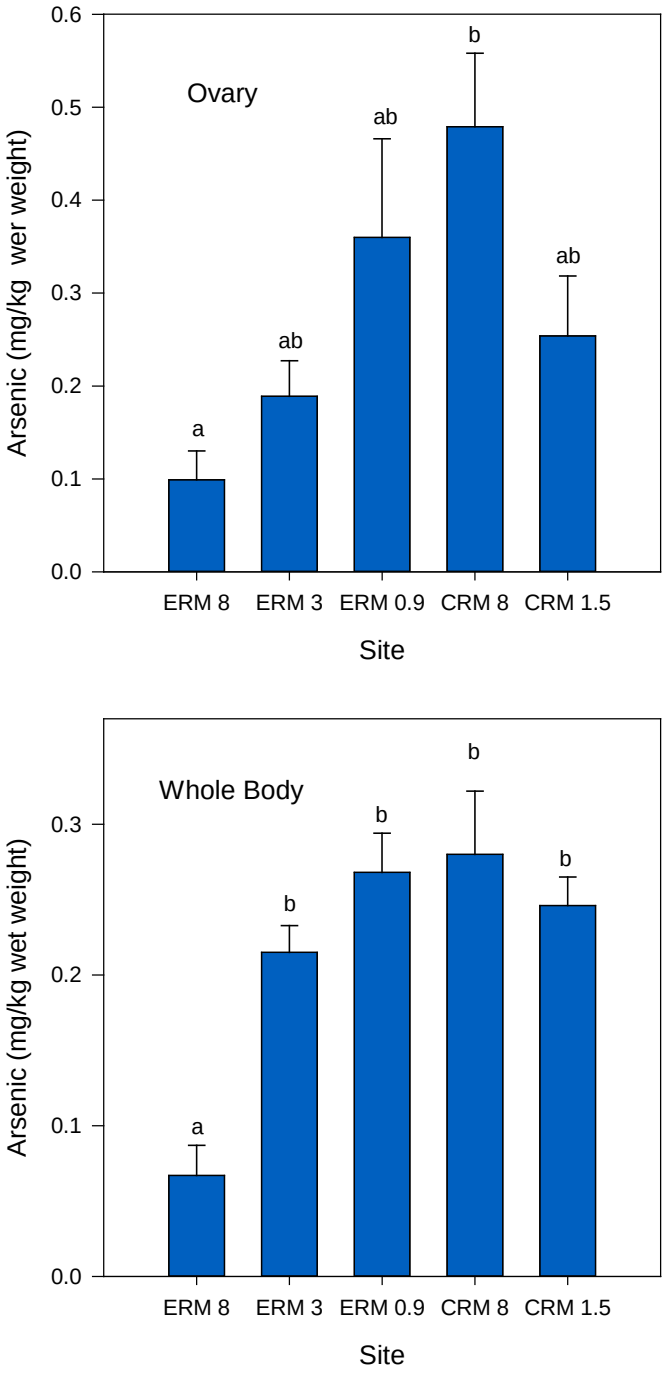

Figure 6. Concentrations of total arsenic in whole bodies and ovaries of parental female redear sunfish supplying eggs for the *in vitro* spawning study. Presented as means ± standard errors. Similar letters above error bars indicate the absence of significant differences between means at $\alpha = 0.05$.

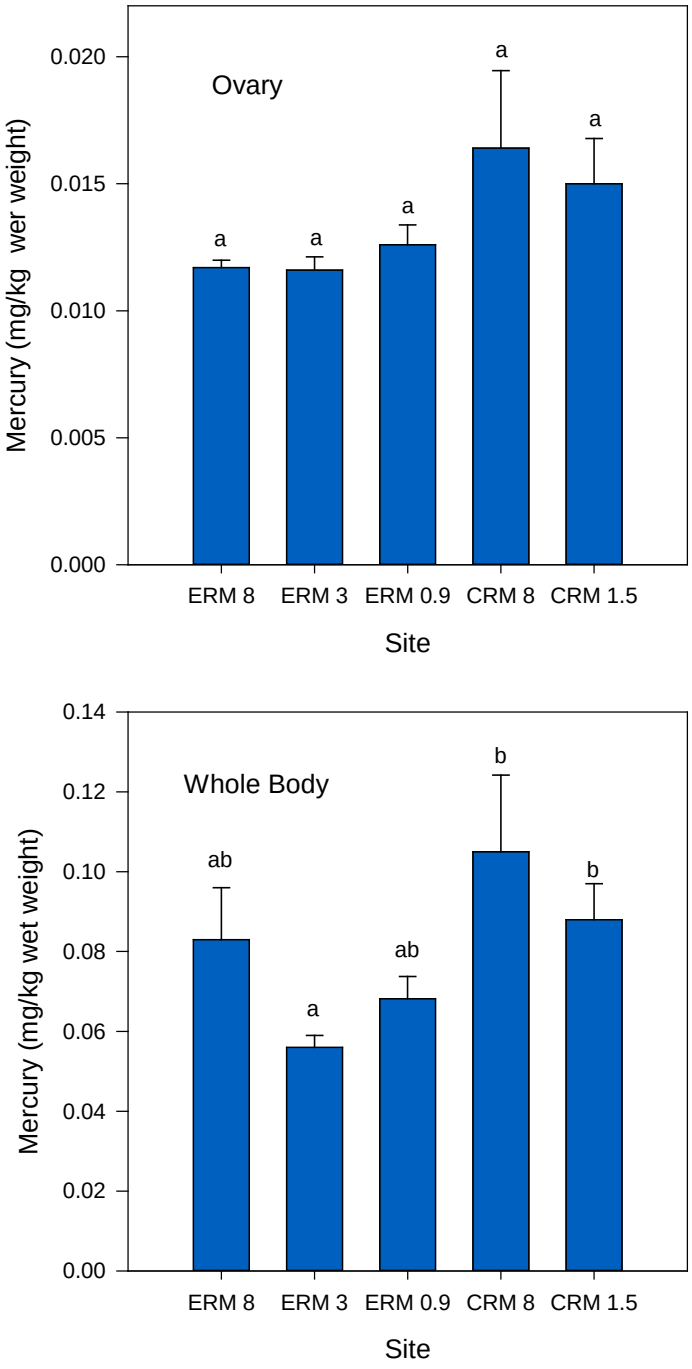

Figure 7. Concentrations of total mercury in whole bodies and ovaries of parental female redear sunfish supplying eggs for the *in vitro* spawning study. Presented as means ± standard errors. Similar letters above error bars indicate the absence of significant differences between means at $\alpha = 0.05$.

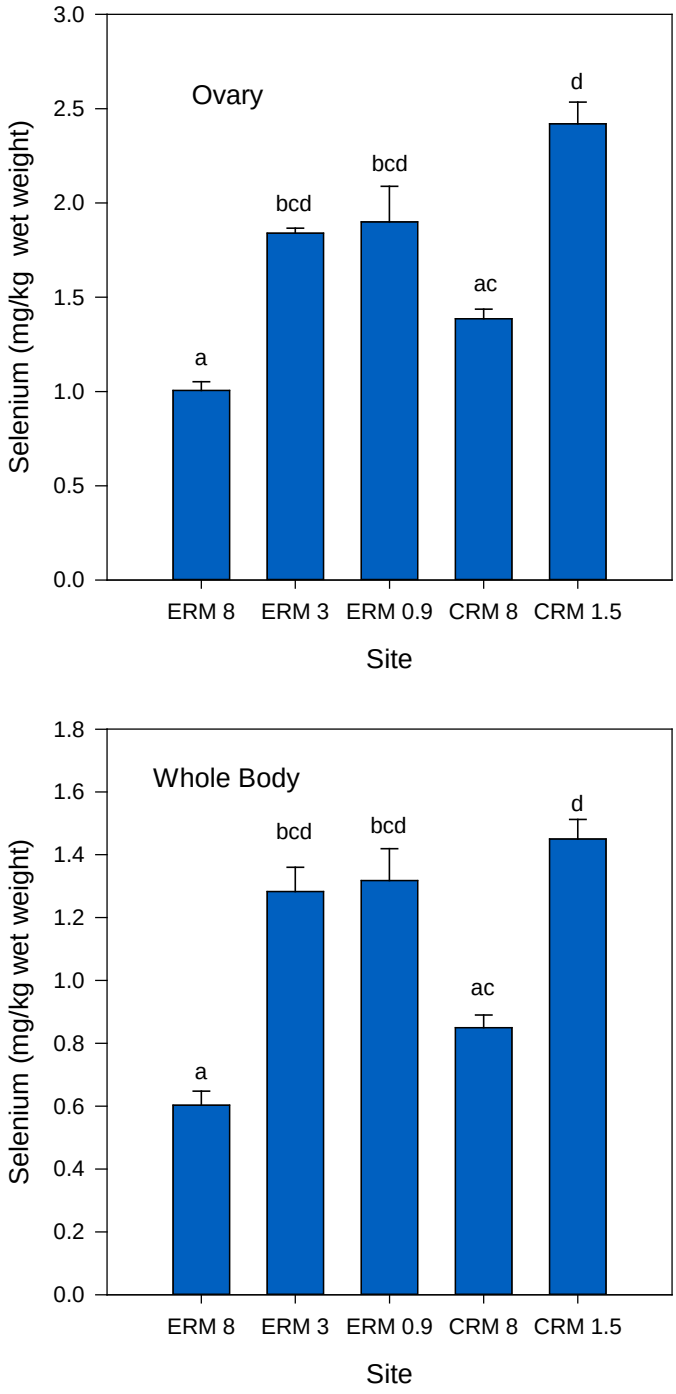

Figure 8. Concentrations of total selenium in whole bodies and ovaries of female redear sunfish supplying eggs for the *in vitro* spawning study. Presented as means ± standard errors. Similar letters above error bars indicate the absence of significant differences between means at $\alpha = 0.05$.

In general, the results of correlation analyses between the embryo-larval survival results of the redear sunfish *in vitro* spawning trials and the metal analyses conducted on ovaries and whole bodies of the parental fish were not unexpected (Table 3). No significant correlations were observed between metal concentrations and embryo-larval survival results, with the exception of a highly significant inverse relationship between arsenic concentrations in ovaries and embryo-larval survival in site-water. A similar correlation was not observed between ovary arsenic concentrations and embryo-larval survival in the corresponding reference water group. A closer examination of the results indicated that the apparent correlation between arsenic concentrations in ovaries and embryo-larval survival in site-water was due solely to poor survival in the embryos derived from a single fish, and only in site-water and not the associated reference water trial (Fig. 9). The correlation was lost when this outlier point was removed from the statistical analysis (Table 4).

Also during the spring of 2011, a total of 10 clutches of bluegill sunfish eggs were successfully spawned *in vitro* and fertilized and cultured in the laboratory (Table 5). No significant differences in embryo-larval survival were noted between trials with fish from ERM 3.0 and the other sites combined. *In vitro* spawning efforts with bluegill sunfish were significantly hampered by an inability to collect sufficient female bluegill sunfish in the correct reproductive condition from most of the study sites. In addition, the relatively small body sizes of the female bluegill sunfish that were collected for this study appeared to make them highly sensitive to the effects of electroshocking, so that even short-term survival of these fish in the laboratory for *in vitro* spawning purposes was very limited.

Table 3. Pearson Product Moment Correlation Analyses for embryo-larval survival in site water and reference water versus total arsenic, mercury, and selenium concentrations (mg/kg wet wt) in whole bodies and ovaries of parental female redear sunfish from sites in the Emory River and Clinch River used for *in vitro* spawning trials in the spring of 2011. Presented as the correlation coefficient[a]/*p* value[b]/number of samples[c]. Asterisks indicate a significant correlation between survival and metal/metalloid concentrations at $p < 0.05$. Note that larvae with obvious developmental abnormalities at the end of each test were considered dead for test purposes.

	Whole Body			Ovary		
Test Result	Arsenic	Hg	Selenium	Arsenic	Hg	Selenium
Embryo-larval survival (%)	-0.169	0.148	0.239	-0.424*	0.124	0.266
in *Site Water* (revised)	0.304	0.370	0.144	0.007	0.451	0.102
	39	39	39	39	39	39
Embryo-larval survival (%)	0.034	0.011	0.039	0.022	-0.061	0.053
in *Reference Water*	0.837	0.945	0.812	0.893	0.711	0.748
	39	39	39	39	39	39

[a] Positive correlation coefficients and *P* values below 0.05 tend to increase together; negative correlation coefficients and *P* values below 0.05 suggest an inverse relationship between variables

[b] *P* values greater than 0.050 indicate there is no significant relationship between the two variables

[c] These analyses only include fish for which both carcass and ovary metal/metalloid analyses were available

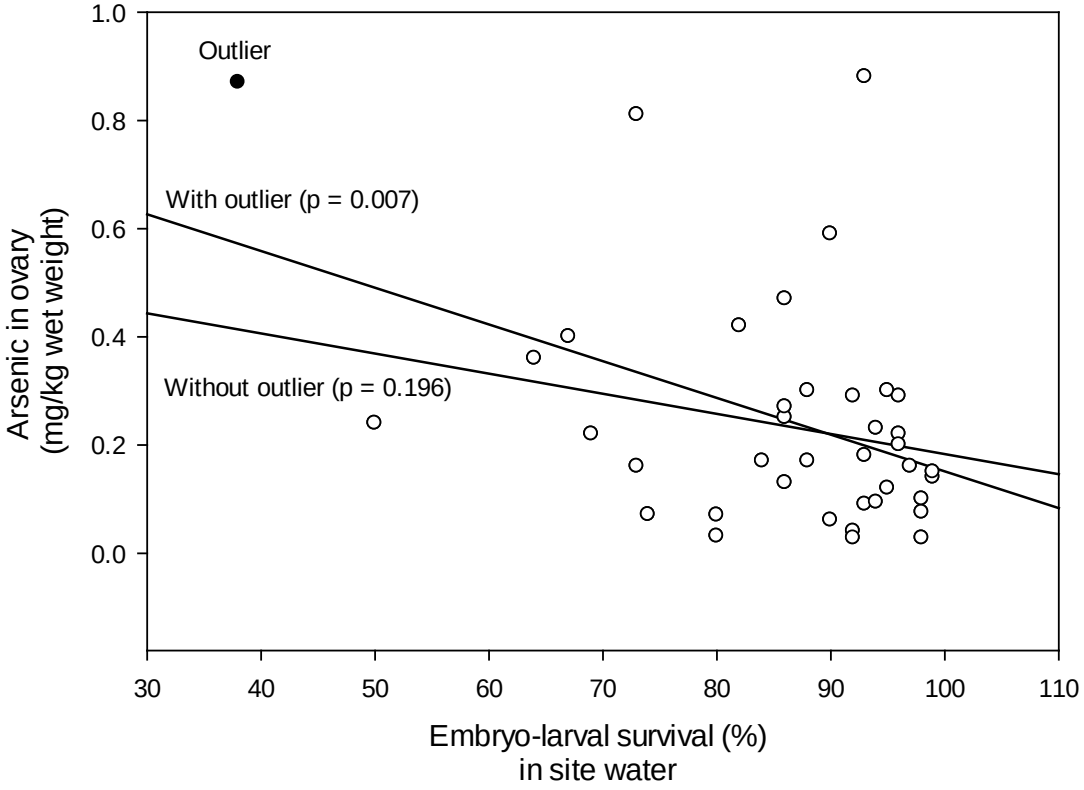

Figure 9. Comparison of regression lines and associated *p*-values − with or without inclusion of an apparent outlier − for the relationship between embryo-larval survival at the conclusion of *in vitro* spawning trials and arsenic in the ovaries of female redear sunfish supplying eggs for the study.

Table 4. Revised Pearson Product Moment Correlation Analyses for embryo-larval survival in site water and reference water versus arsenic, mercury, and selenium (mg/kg wet wt) measured in whole bodies and ovaries of parental female redear sunfish from sites in the Emory River and Clinch River used for *in vitro* spawning trials in the spring of 2011. Represents a revision of ovary arsenic correlation analysis of embryo-larval survival in site water to reflect removal of an identified outlier. Presented as the correlation coefficient[a]/*p* value[b]/number of samples. There were no significant correlations at $p < 0.05$. Note that larvae with obvious developmental abnormalities at the end of each test were considered dead for test purposes.

	Whole Body			Ovary		
Test Result	Arsenic	Hg	Selenium	Arsenic	Hg	Selenium
Embryo-larval survival (%)	0.085	0.090	0.157	-0.215	0.124	0.266
in *Site Water*	0.610	0.592	0.346	0.196	0.451	0.102
	38	38	38	38	38	38
Embryo-larval survival (%)	0.034	0.011	0.039	0.022	-0.061	0.053
in *Reference Water*	0.837	0.945	0.812	0.893	0.711	0.748
	39	39	39	39	39	39

[a] Positive correlation coefficients and *P* values below 0.05 tend to increase together; negative correlation coefficients and *P* values below 0.05 suggest an inverse relationship between variables

[b] *P* values greater than 0.050 indicate there is no significant relationship between the two variables

Table 5. Survival of bluegill sunfish embryos and larvae during 7-d laboratory tests following in vitro spawning of parental fish collected from study sites in the Emory and Clinch river systems (TN) during the spring 2011 breeding season.

Location[a]	Number of Spawning Trials	Embryo-Larval Survival[b] mean % ± SEM	
		Site Water	*Reference water*[c]
ERM 3.0	6	87.0 ± 3.0	81.2 ± 3.9
Other sites[d]	4	–	95.8 ± 0.5

[a] ERM = Emory River Mile; CRM = Clinch River Mile
[b] Survival in site water or reference water at end of 7-d test; larvae with abnormalities on day 7are considered dead for test purposes
[c] Sterile-filtered water from ERM 8.0
[d] Includes clutches of eggs from 2 females from ERM 8.0, 1female from CRM 8.0, and 1 female from CRM 1.5

4. SUMMARY AND CONCLUSIONS

Of contaminants enriched in coal ash, selenium in particular is known to be capable of causing reproductive failure in severely-contaminated water-sheds, primarily through adverse effects on the early development of offspring from exposed parents. As indicated in the introduction to this report, uptake by adult female fish of fly ash constituents through the food chain and subsequent maternal transfer of contaminants to the developing eggs is widely considered to be the primary route for the exposure of larval fish to the selenium component of fly ash (USEPA 2004). The metal bioaccumulation results presented in this report for redear sunfish , along with similar results from previous bioaccumulation studies conducted at the same sites (Adams and other 2012), clearly show that selenium is elevated in fish exposed to the fly ash spill as compared with fish from upstream reference sites on both the Emory and Clinch Rivers. However, selenium concentrations in the whole bodies and ovaries of the parental female redear sunfish used in these *in vitro* spawning trials continued to remain below draft aquatic life criteria (USEPA 2004).

The *in vitro* spawning results of the current study provide additional evidence that sunfish exposed in situ to ash from the Kingston fly ash release for over two years have not to date bioaccumulated selenium, other metals, or other non-metal contaminants during two years of exposure to ash from the Kingston ash release in sufficient quantities or forms to significantly impact the early development of their offspring. The average survival of redear sunfish larvae free of developmental abnormalities at the conclusion of these *in vitro* spawning trials was >70% for all study sites combined, an unexpectedly high rate of survival considering the developmental nature of these procedures as applied to these particular fish species. Of even greater significance, the average survival of redear larvae free of obvious developmental abnormalities was also >70% specifically in the cases of ERM 3.0, ERM 0.9, and CRM 1.5 − the three study sites located downstream of the fly ash spill.

It should be noted that higher concentrations of selenium approaching levels of concern have been observed in a few redear sunfish sampled from some of these same sites for bioaccumulation monitoring purposes (Teresa Mathews, ORNL, personal communication). Therefore, it is recommended that consideration be given to repeating *in vitro* spawning trials with redear sunfish in the future if levels in whole bodies or ovaries continue to rise.

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