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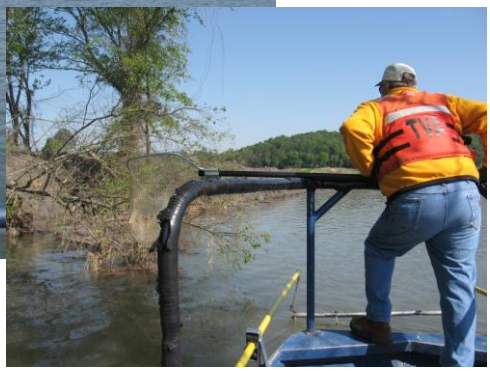
EVALUATING THE EFFECTS OF THE KINGSTON FLY ASH RELEASE ON FISH REPRODUCTION: SPRING 2009 – 2010 STUDIES



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May 2012

UT-BATTELLE

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1. INTRODUCTION

On December 22, 2008, a dike containing fly ash and bottom ash at the Tennessee Valley Authority's (TVA) Kingston Fossil Plant in East Tennessee failed and released a large quantity of ash into the adjacent Emory River. Ash deposits from the spill extended 4 miles upstream of the facility to Emory River mile 6 and downstream to Tennessee River mile 564 (~8.5 miles downstream of the confluence of the Emory River with the Clinch River, and ~4 miles downstream of the confluence of the Clinch River with the Tennessee River). A byproduct of coal combustion, fly ash contains a variety of metals and other elements which, at sufficient concentrations and in specific forms, can be harmful to biological systems.

The ecological effects of fly ash contamination on exposed fish populations depend on the magnitude and duration of exposure, with the most significant risk considered to come from elevated levels of certain metals in the ash, particularly selenium, on fish reproduction and fish early life stages (Lemly 1993; Besser and others 1996). The ovaries of adult female fish in a lake contaminated by coal ash were reported to have an increased frequency of atretic oocytes (dead or damaged immature eggs) and reductions in the overall numbers of developing oocytes (Sorensen 1988) associated with elevated body burdens of selenium. Larval fish exposed to selenium through maternal transfer of contaminants to developing eggs in either contaminated bodies of water (Lemly 1999) or in experimental laboratory exposures (Woock and others 1987, Jezierska and others 2009) have significantly increased incidences of developmental abnormalities. Contact of fertilized eggs and developing embryos to ash in water and sediments may also pose an additional risk to the early life stages of exposed fish populations through direct uptake of metals and other ash constituents (Jezierska and others 2009).

The establishment and maintenance of fish populations is intimately associated with the ability of individuals within a population to reproduce. Reproduction is thus generally considered to be the most critical life function affected by environmental contamination. 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 exposed fish populations, ORNL has undertaken a series of studies in collaboration with TVA that include: (1) a combined field study of metal bioaccumulation in ovaries and other fish tissues (Adams and others 2012) and the reproductive condition of sentinel fish species in reaches of the Emory and Clinch Rivers affected by the fly ash spill (the current report); (2) laboratory tests of the potential toxicity of fly

ash from the spill area on fish embryonic and larval development (Greeley and others 2012); (3) additional laboratory experimentation focused on the potential effects of long-term exposures to fly ash on fish survival and reproductive competence (unpublished); and (4) a combined field and laboratory study examining the *in vitro* developmental success of embryos and larvae obtained from fish exposed *in vivo* for over two years to fly ash in the Emory and Clinch Rivers (unpublished).

The current report focuses on the reproductive condition of adult female fish in reaches of the Emory and Clinch Rivers influenced by the fly ash spill at the beginning of the spring 2009 breeding season – the first breeding season immediately following the fly ash release − and during the subsequent spring 2010 breeding season. Data generated from this and related reproductive/early life stage studies provide direct input to ecological risk assessment efforts and complement and support other phases of the overall biomonitoring program associated with the fly ash spill.

2. APPROACH AND METHODS

Objectives

The primary objective of this study was to evaluate the reproductive health of female fish of sentinel fish species at Emory and Clinch River locations downstream of the fly ash spill. A further objective was to evaluate potential relationships between measured reproductive indices and the bioaccumulation of metals of reproductive concern in organs such as the liver and ovary that provide a route of maternal transfer of reproductive toxicants such as selenium to the developing eggs. Ovarian and liver bioaccumulation is also discussed and evaluated elsewhere in relation to fillet and whole body burdens of metals of concern (Adams and others 2012).

Sampling sites

Sentinel fish species were sampled by TVA personnel using boat electrofishing from five sites in the Emory and Clinch rivers (Fig. 1) at the beginning of the 2009 and 2010 reproductive seasons. Locations included upstream reference sites (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). An additional reference site in the Little Emory River (LERM 2.0) directly upstream of the spill location was added in 2010.

Study species

Largemouth bass (*Micropterus salmoides*), bluegill sunfish (*Lepomis macrochirus*), and white crappie (*Pomoxis annularis*) − important sport fish in the Clinch River/Watts Bar Reservoir system − were the initial target species for this study. However, sufficient white crappie in appropriate reproductive condition were unable to be collected from all the study sites in 2009, and this species was replaced in 2010 with the redear sunfish (*Lepomis microlophus*) which is both relatively abundant at all study sites and also the focus of a related experimental spawning study. The goal was to collect at least eight adult female fish of each species per site per season.

Sample processing

Fish were returned live to ORNL from study sites, anesthetized with MS 222, and processed as described in Adams and Fortner (2012). For the purposes of assessing reproductive condition, ovaries were removed from the body cavity and weighed to the nearest 10 mg. Representative pieces of tissue were carefully cut from the midpoint of the largest ovarian lobe, weighed to the nearest 1 mg, and placed in vials containing a half-strength solution of Karnovsky's Fixative for later analysis of ovary stage, oocyte (immature developing eggs) condition, and fecundity.

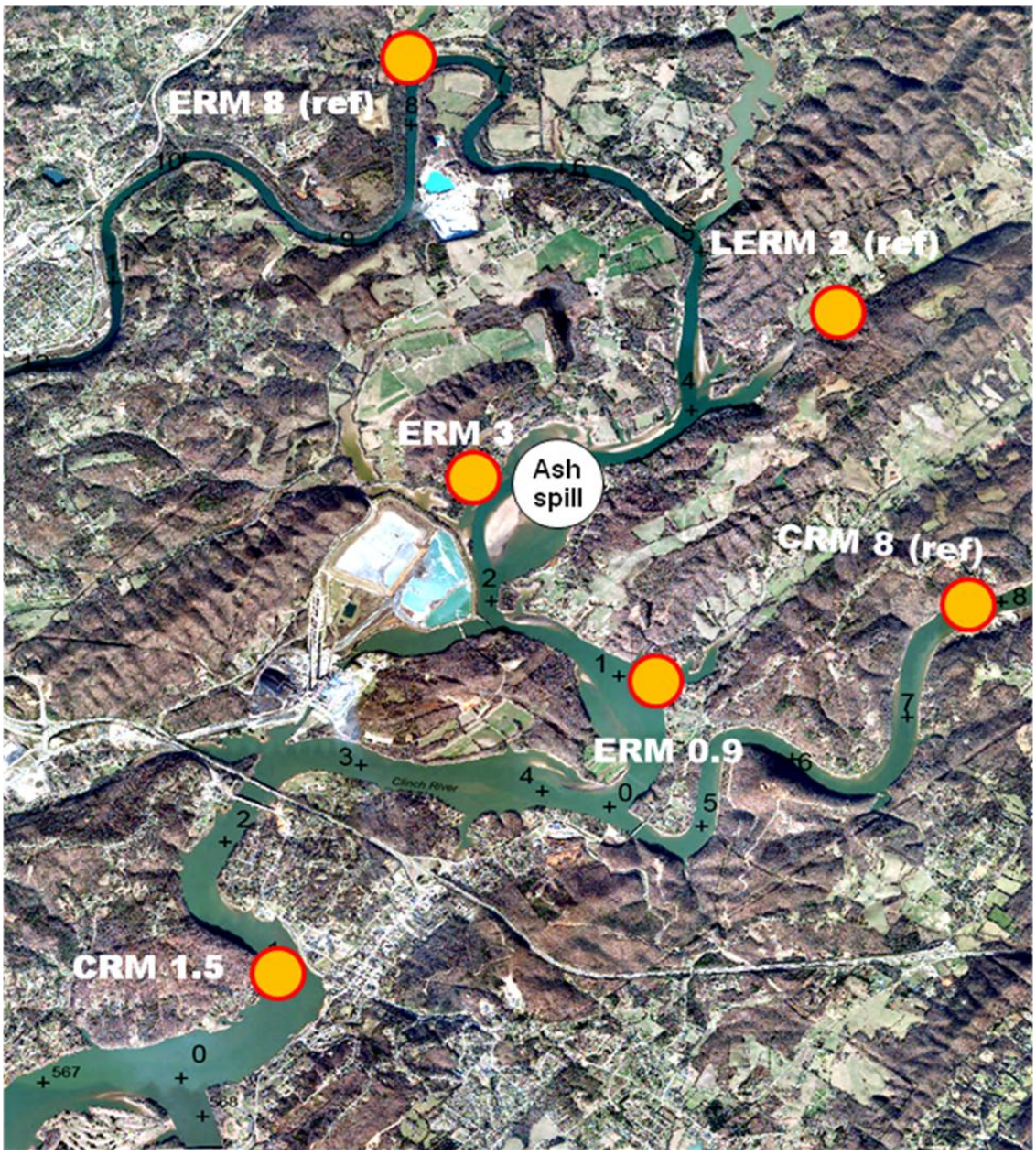

Figure 1. Map showing fish sampling locations in the Emory and Clinch Rivers. ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile (sampled only in 2010).

Additional slices of ovary tissue were placed in vials with 10% buffered formalin for subsequent histopathology analysis.

GSI as a reproductive indicator

The gonadsomatic index (GSI), or relative size of the gonad to the whole body, was calculated for each fish (see Fig. 2). The GSI is a widely-used and easily measured indicator of fish reproductive status (Nikolsky 1963). Changes in the GSI (generally downward) correlated with contaminant exposure have been observed 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. However, the GSI is best suited for use in a larger suite of reproductive indicators since it provides only limited data on actual reproductive condition (Greeley 2002).

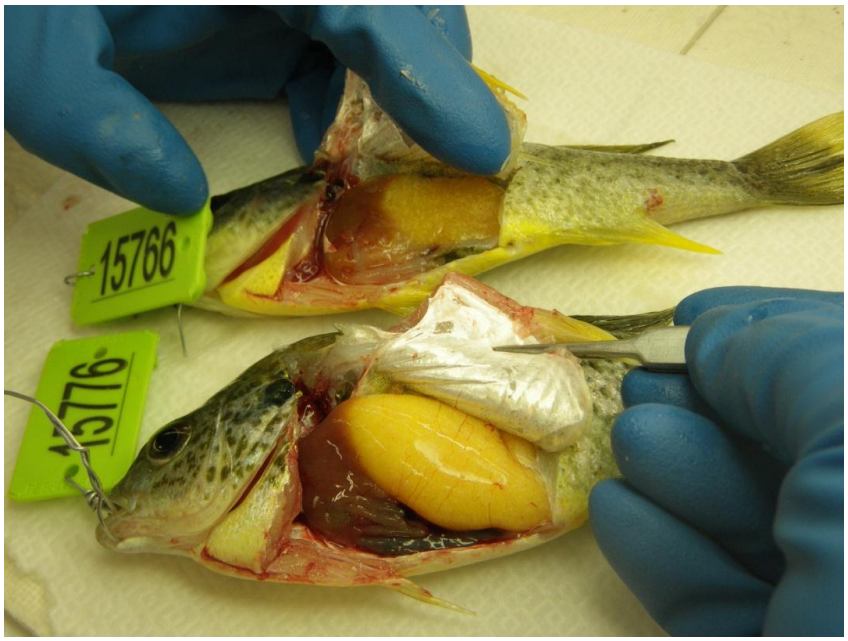

Figure 2. Comparison of redear sunfish ovaries demonstrating a fish with a high gonadosomatic index (GSI) (front) and a fish with a relatively low GSI (back).

Ovarian morphometric analyses

The reproductive condition of fish ovaries was quantitatively evaluated by sizing, staging and enumerating all oocytes above a certain threshold size contained within a weighed piece of ovary by procedures adapted from Greeley and others (1987), Lin and others (1989) and Hsiao and others (1994). Ovarian pieces fixed and stored in Karnovsksy's Fixative were soaked and washed with several changes of distilled water to remove potentially toxic residues. Each pre-weighed ovary portion was gently teased apart in buffer with the aid of a dissecting microscope and watchmaker's forceps. Both largemouth bass and sunfish ovaries contained oocytes ranging in size from <0.1 mm to 2.0 mm in diameter. Oocytes were in various stages of development, including numerous small pre-vitellogenic oocytes (oocytes that have not yet begun accumulating yolk), larger vitellogenic oocytes which are actively accumulating yolk, and more infrequently, maturing oocytes or mature unfertilized eggs. Individual oocytes of a species-specific diameter (generally 0.3 – 0.5 mm) and larger were measured to the nearest 0.1 mm with an ocular micrometer attached to the eyepiece of a dissecting microscope. The developmental stage of each oocyte was recorded, as was the presence of any atretic (dead or non-viable) oocytes and post-ovulatory follicles (tissue which surrounds the developing eggs prior to ovulation). Oocyte staging was adapted from Wallace and Selman (1981) and Greeley and others (1988). Since oocyte development is essentially uniform throughout the length and width of both largemouth bass and sunfish ovaries, results of analyses on these representative ovarian pieces were extrapolated to give the total number of oocytes of a certain size class or stage in each ovary. Measures of reproductive condition derived from this exercise include one or more estimates of fecundity, total counts of vitellogenic oocytes within an ovary, and the prevalence of parasitic lesions and oocyte atresia (oocyte death and/or reabsorption). Similar procedures were used previously to evaluate the reproductive condition of largemouth bass at Hartwell Reservoir along the GA/SC border (Greeley and others 1994) and both largemouth and bluegill sunfish in Watts Bar Reservoir, TN, (Adams and others 1999) for Remedial Investigation Feasibility Studies at the respective locations.

Fecundity determinations

The production of viable eggs is among the most sensitive indicators of contaminant exposure and effects in experimental life-cycle tests of potential toxicants (Suter and others 1987). Fecundity has been shown to be very sensitive to environmental contaminants in a number of laboratory studies (Tam and Payson 1986, Kovacs and others 1995, Carlsson and others 2000). In fish species with large single clutches of eggs, fecundity is often calculated through a volumetric approach of enumerating eggs in a subset of the entire egg mass. However, in fractional spawners such as largemouth bass and sunfish, ovulation and spawning occur rapidly and there are multiple spawns during the breeding season, so mature eggs that are present when fish are

collected may represent only a fraction of the total number of eggs produced during the course of a breeding season. Therefore, the morphometric method we employ to estimate fecundity by enumerating either preovulatory oocytes before a spawn or postovulatory follicles immediately following a spawn is the most accurate method for use in fractional spawning species.

In the current study, no attempt was made to estimate annual fecundity for sunfish, since this would require careful monitoring of egg development patterns in each species at each study site throughout relatively extended breeding seasons. To compare the reproductive potential of sunfish populations at the various study sites, batch or clutch fecundity estimates were calculated for fish collected at or just prior to the beginning of the breeding season when fish ovarian developmental status was most synchronous within and between sites. For female bass and sunfish that spawned prior to collection, freshly shed post-ovulatory follicles were counted to determine the number of eggs released in the spawn. For fish collected before their first seasonal spawn (the majority of these samples), batch fecundity estimates for sunfish were based upon the number of synchronously-developing oocytes in the leading clutch of developing oocytes destined to be released during the first spawn. As such clutches are more difficult to estimate from oocyte size-distributions in pre-spawn largemouth bass ovaries, batch fecundity for bass lacking post-ovulatory follicles were instead estimated by enumerating oocytes $\geq$ 1.3 mm in diameter (the size of oocytes typically absent from ovaries with freshly-shed follicles in post-spawn fish). An estimate of annual fecundity was also calculated for largemouth bass by enumerating oocytes $\geq$ 0.7 mm (as suggested by Kelley 1961) plus any post-ovulatory follicles present in the ovaries, although this may be an underestimation of the actual annual fecundity since at least some new vitellogenic oocytes may be developed during the breeding season.

Atresia as a reproductive indicator

An example of oocyte atresia in a sunfish ovary is shown in Figure 3. The presence of atretic oocytes, characterized by the degeneration and necrosis of developing oocytes and the subsequent infiltration by macrophages in fish ovaries, is representative of gamete quality rather than quantity and thus may be one of the better reproductive indicators of an actual pathological condition (Greeley 2002). Elevated incidences of oocyte atresia have been reported in fish populations affected by effluents from paper and pulp mills (Sandström and others 1988, Adams and others 1992); cooling water discharges (Lukšienė and Sandström 1994, Lukšienė and others 2000); acid-induced stress (McCormick and others 1987, 1989); disease (Winstead and others 1991); confinement stress (Clearwater and Pankhurst 1997); pesticides (Rastogi and Kulshretha 1990, Sukumar and Karpagaganapathy 1992); PAHs (Johnson and others 1997); mercury (Adams and others 1999); a mixture of aromatic hydrocarbons, PCBs, and other chlorinated compounds

(Johnson and others 1999); and treated sewage effluent (Jobling and others 2002). However, oocyte atresia has also been observed to occur naturally at specific times during or immediately following the breeding season of unstressed fish populations as well (Hunter and Macewicz 1985, Hsiao and others 1994), so the presence of atresia in sampled fish must be considered in light of the general reproductive condition of the fish and timing within the reproductive cycle.

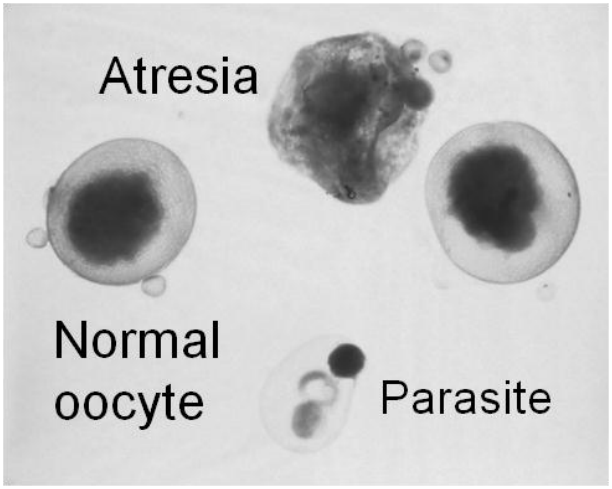

Figure 3. Examples of oocytes from a sunfish ovary illustrating normal vitellogenic oocytes (far right and far left), an atretic oocyte, and a parasitic cyst that was imbedded within the ovarian tissue. From Greeley 2002.

Ovarian histopathology

For histopathological analysis of ovaries, samples were shipped to Dr. Swee J. Teh (Toxicology Consulting, Davis, California). Lesions measured in ovary slices included: (1) pre-vitellogenic oocyte necrosis; (2) follicular vitellogenic oocyte atresia; (3) macrophage aggregates; and (4) parasitic infections of follicular and pre-vitellogenic oocytes. The severity of lesions in ovaries were scored based on a qualitative scale of 0 = no lesions present, 1 = mild lesions, 2 = moderate lesions, and 3 = severe lesions, with a score of 2 or 3 being considered a significant lesion. A composite lesion score for each species at each site was calculated as the average sum of lesion scores for each organ for that species and site. These results are presented along with other histopathological analyses in Adams and Fortner (2012), but are also included herein for discussion purposes.

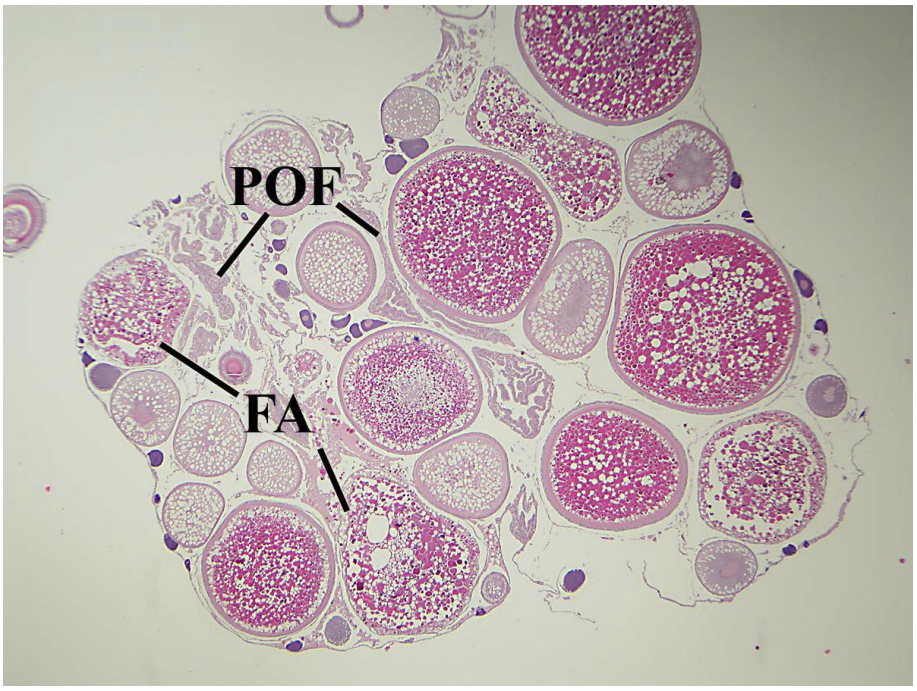

Figure 4. Photomicrograph showing follicular atresia (FA) and postovulatory follicles (POF) in largemouth bass collected from ERM 3.0 in the spring of 2009. The presence of POF, previtellogenic (arrowheads), and vitellogenic (arrows) oocytes is due to the largemouth bass being a multiple spawner. Histological preparation and interpretation by Dr. Swee J. Teh, Toxicology Consulting, Davis, California.

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, a one-way Analysis of Variance (ANOVA) was 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 was 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 reproductive parameters and arsenic and selenium measured in livers and ovaries were examined by Pearson Product Moment Correlation. In all cases, the significance level for rejecting the hypothesis of equal means between sites was set at $\alpha = 0.05$.

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3. RESULTS AND DISCUSSION

Bluegill sunfish

Results of reproductive analyses conducted on female bluegill sunfish sampled at the beginning of the 2009 and 2010 breeding seasons are shown in Tables 1 − 2 and Figures 5 − 7. In the spring of 2009, mean GSIs were significantly lower in female bluegill from the spill site (ERM 3.0) than in fish collected from the upstream reference site at ERM 8.0 (Fig. 5), although GSIs at sites farther downstream at ERM 0.9 and CRM 1.5 remained similar to those at the reference site. The abundance of vitellogenic oocytes and the batch fecundity, normalized for fish size, also tended to be lower at ERM 3.0 than at either the upstream reference site or sites farther downstream of the spill (Fig. 6), although the reductions did not have statistical significance. The incidences of histopathological lesions in bluegill ovaries from the site were also greater than at the reference site or farther downstream sites (Fig. 7). Oocyte atresia was relatively uncommon at all sites sampled in 2009, including at ERM 3.0 (Fig. 5), and so it is doubtful that direct toxicity to the developing oocytes was a factor in either the lower GSIs and batch fecundity or the decreased abundance of vitellogenic oocytes at this location. Vitellogenic oocytes in ovaries from ERM 3.0 were relatively smaller and less developed than at the other study sites, suggesting a delay in oocyte development in the ovaries of fish from this location.

By the spring of 2010, bluegill GSIs were similar at both the spill site and the upstream Emory River reference site, while GSIs at sites further downstream were actually significantly higher than at the reference site (Fig. 5). The abundance of vitellogenic oocytes and the batch fecundity of bluegill did not differ significantly among the study sites in 2010 (Fig. 6). Oocyte atresia was noticeably more common in bluegill ovaries at all sites sampled in 2010, and tended to be somewhat more prevalent at both the downstream sites and reference sites at LERM 2.0 and CRM 8.0 than at the Emory River reference site (Fig. 5). Incidences of histopathological lesions in sampled ovaries were relatively fewer at both the spill site and study sites downstream of the spill than at the three upstream reference locations (Fig. 7).

Considered together, these results suggest that the reproductive condition of female bluegill sunfish was adversely affected at the spill site during the 2009 breeding season immediately following the fly ash spill, with a delay in ovarian development more likely due to habitat alterations and food chain disruptions than to toxic effects. However, by the spring of 2010 the reproductive condition of female bluegill at the spill site and other study sites downstream of the spill did not differ in an ecologically-significant manner from fish at the upstream reference sites.

Table 1. Reproductive indices measured in bluegill sunfish sampled at the beginning of the spring 2009 breeding season from reaches of the Emory River and the Clinch River. Includes fish from fly ash-affected river reaches (ERM 3.0, ERM 0.9, and CRM 1.5) as well as upstream reference sites (ERM 8.0 and CRM 8.0)[a]

Site[a]	Year		Fish Length (cm)	Fish Weight (g)	Ovary Weight (g)	GSI	Eggs/Oocytes per Fish			Eggs/Oocytes per gram of Fish Weight		
							Atresia	Vitellogenic	Batch fecundity	Atresia	Vitellogenic	Batch fecundity
ERM 8.0	2009	*N*[b]	7	7	7	7	7	7	7	7	7	7
		Mean	14.6	54.2	3.93	7.39	25	8363	5315	0.40	157.03	95.33
		SEM	0.3	2.5	0.52	1.08	16	1243	1402	0.27	26.27	24.47
ERM 3.0	2009	*N*	11	11	11	11	11	11	11	11	11	11
		Mean	14.0	46.8	1.94	4.05	35	4447	3599	0.67	94.74	76.01
		SEM	0.2	2.4	0.27	0.53	22	755	540	0.41	17.18	12.11
ERM 0.9	2009	*N*	12	12	12	12	12	12	12	12	12	12
		Mean	13.7	45.8	3.86	8.48	168	8092	5404	2.97	188.64	127.25
		SEM	0.4	3.7	0.36	0.50	103	930	560	1.58	20.88	17.32
CRM 8.0	2009	*N*	11	11	11	11	11	11	11	11	11	11
		Mean	13.5	45.4	5.79	12.68	118	8714	4750	2.59	190.51	107.15
		SEM	0.3	3.0	0.63	0.76	48	831	473	0.97	9.26	8.93

Table 1. (continued)

Site[a]	Year		Fish Length (cm)	Fish Weight (g)	Ovary Weight (g)	GSI	Eggs/Oocytes per Fish			Eggs/Oocytes per gram of Fish Weight		
							Atresia	Vitellogenic	Batch fecundity	Atresia	Vitellogenic	Batch fecundity
CRM 1.5	2009	*N*	12	12	12	12	12	12	12	12	12	12
		Mean	14.2	47.3	3.06	6.43	148	7370	5551	2.62	151.41	113.48
		SEM	0.4	3.5	0.34	0.46	112	1176	894	1.72	15.17	11.25
LERM 2.0	2009	*N*										
		Mean					Site not sampled in 2009					
		SEM										

[a] ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile
[b] N = number of fish in sample
[c] SEM = Standard error of the mean

Table 2. Reproductive indices measured in bluegill sunfish sampled at the beginning of the spring 2010 breeding season from reaches of the Emory River, Clinch River, and Little Emory River. Includes fish from fly ash-affected river reaches (ERM 3.0, ERM 0.9, and CRM 1.5) as well as upstream reference sites (ERM 8.0, CRM 8.0, and LERM 2.0)[a]

Site[a]	Year		Fish Length (cm)	Fish Weight (g)	Ovary Weight (g)	GSI	Eggs/Oocytes per Fish			Eggs/Oocytes per gram of Fish Weight		
							Atresia	Vitellogenic	Batch fecundity	Atresia	Vitellogenic	Batch fecundity
ERM 8.0	2010	*N*[b]	7	7	7	7	7	7	7	7	7	7
		Mean	14.6	53.2	3.54	6.52	193	7331	5557	4.16	136.30	106.10
		SEM	0.5	5.3	0.56	0.43	112	1073	478	2.61	9.01	5.31
ERM 3.0	2010	*N*	11	11	11	11	11	11	11	11	11	11
		Mean	14.4	55.9	4.81	8.72	462	10288	6573	8.43	186.02	123.75
		SEM	0.5	5.2	0.42	0.54	275	1111	816	5.27	15.38	16.55
ERM 0.9	2010	*N*	9	9	9	9	9	9	9	9	9	9
		Mean	14.2	52.1	5.35	10.12	864	9346	6740	17.04	178.96	129.75
		SEM	0.3	3.1	0.58	0.57	471	829	672	9.84	9.16	9.60
CRM 8.0	2010	*N*	9	9	9	9	9	9	9	9	9	9
		Mean	14.0	51.8	6.09	11.82	1540	9046	5600	29.81	175.08	108.05
		SEM	0.3	3.4	0.41	0.52	738	1151	799	14.05	21.14	15.01

Table 2. (continued)

Site[a]	Year		Fish Length (cm)	Fish Weight (g)	Ovary Weight (g)	GSI	Eggs/Oocytes per Fish			Eggs/Oocytes per gram of Fish Weight		
							Atresia	Vitellogenic	Batch fecundity	Atresia	Vitellogenic	Batch fecundity
CRM 1.5	2010	*N*	11	11	11	11	11	11	11	11	11	11
		Mean	12.8	36.8	3.37	9.34	364	7209	4332	11.84	197.55	115.48
		SEM	0.5	3.4	0.33	0.64	177	793	517	5.70	14.12	8.55
LERM 2.0	2010	*N*	10	10	10	10	10	10	10	10	10	10
		Mean	14.2	48.3	3.80	8.15	748	7862	4889	14.23	168.46	101.28
		SEM	0.4	2.9	0.34	0.83	621	651	587	12.01	17.42	10.05

[a] ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile
[b] N = number of fish in sample
[c] SEM = Standard error of the mean

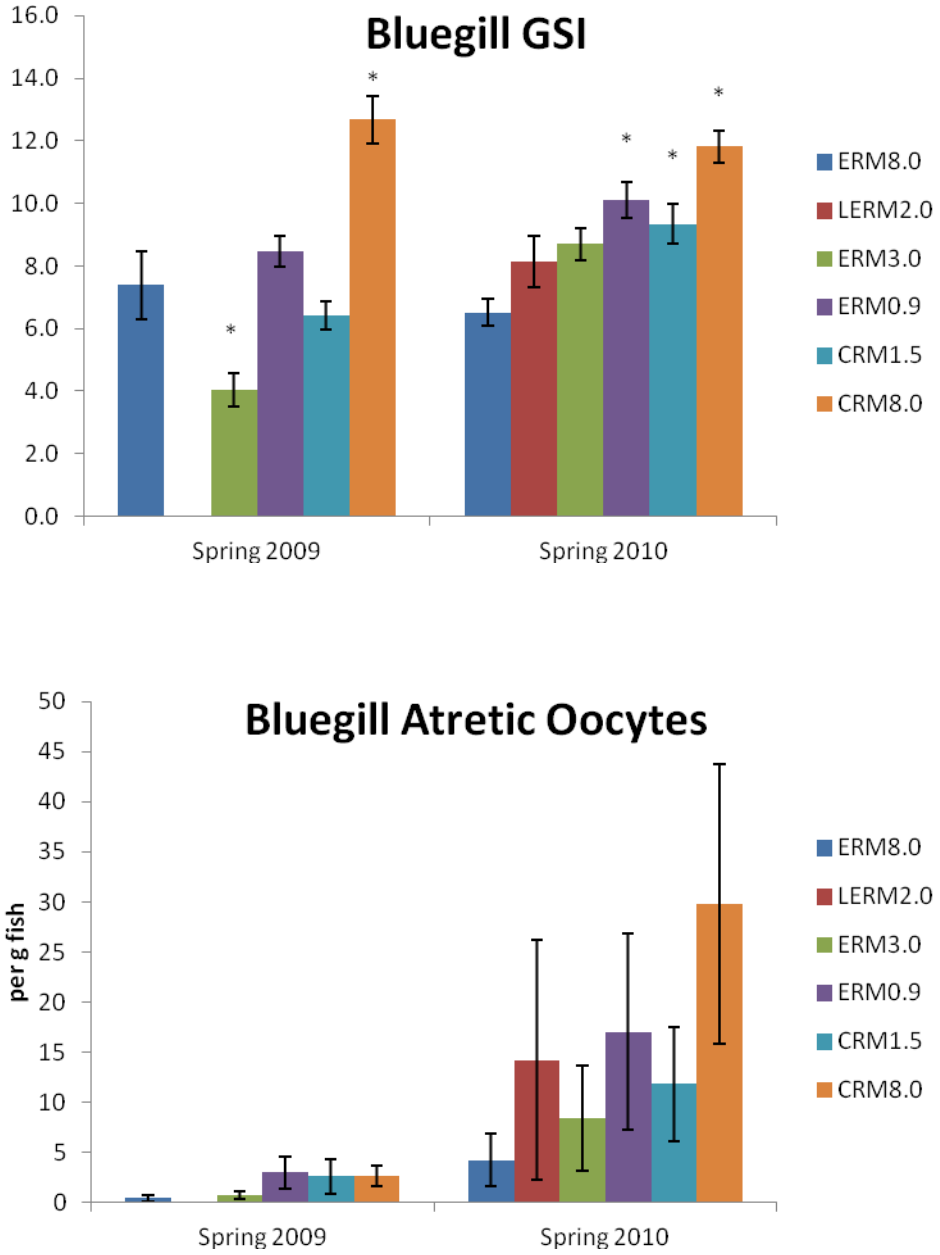

Figure 5. Comparison of select reproductive indices measured in bluegill sunfish sampled from sites within the Emory and Clinch River systems at the beginning of the 2009 and 2010 breeding seasons. GSI = gonadosomatic index; ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile (sampled only in 2010). Asterisks indicate a statistical difference from the primary upstream reference site (ERM 8.0) at $\alpha = 0.05$.

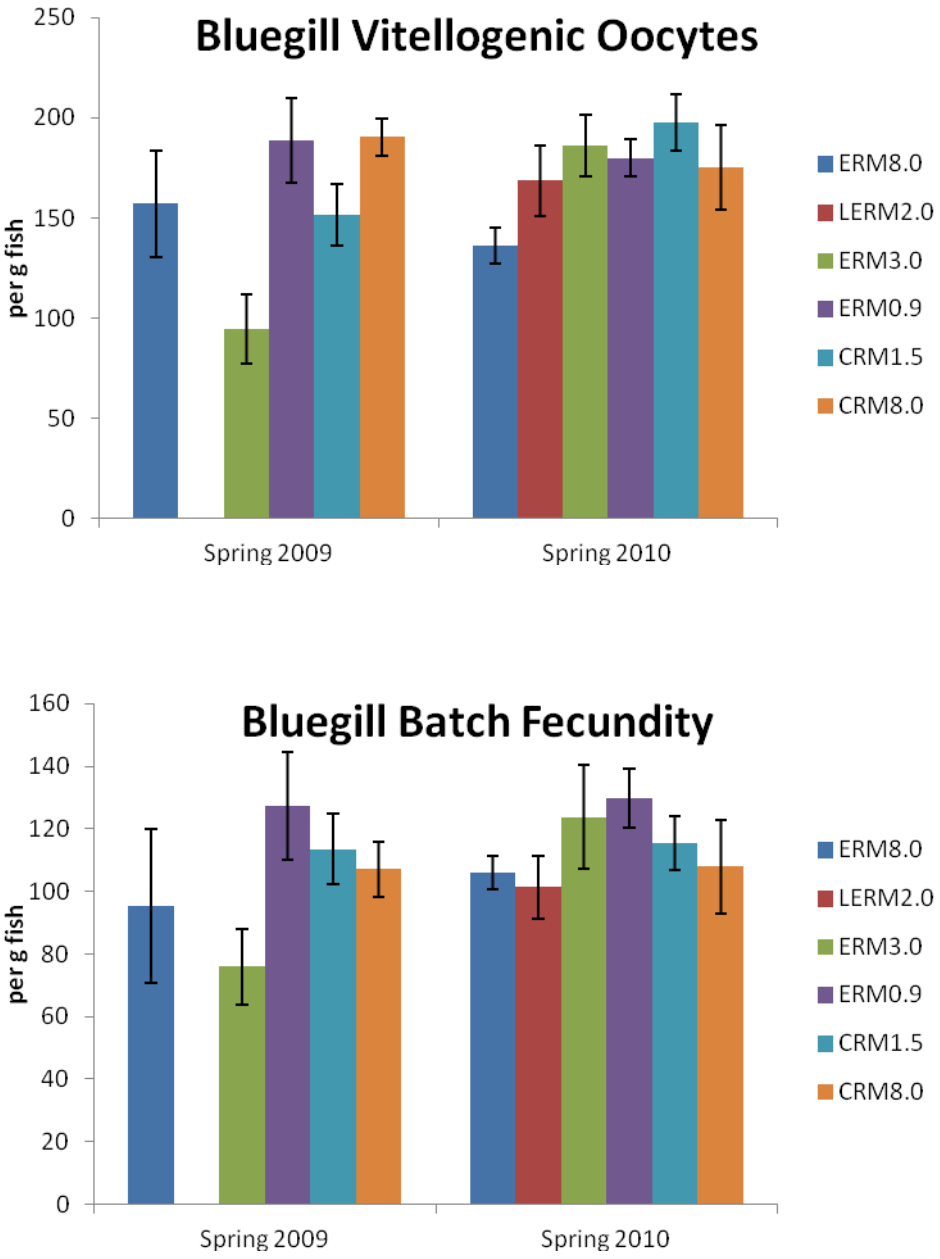

Figure 6. Comparison of select reproductive indices measured in bluegill sunfish sampled from locations within the Emory and Clinch River systems at the beginning of the 2009 and 2010 breeding seasons. ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile (sampled only in 2010). Asterisks indicate a statistical difference from the primary upstream reference site (ERM 8.0) at $\alpha = 0.05$.

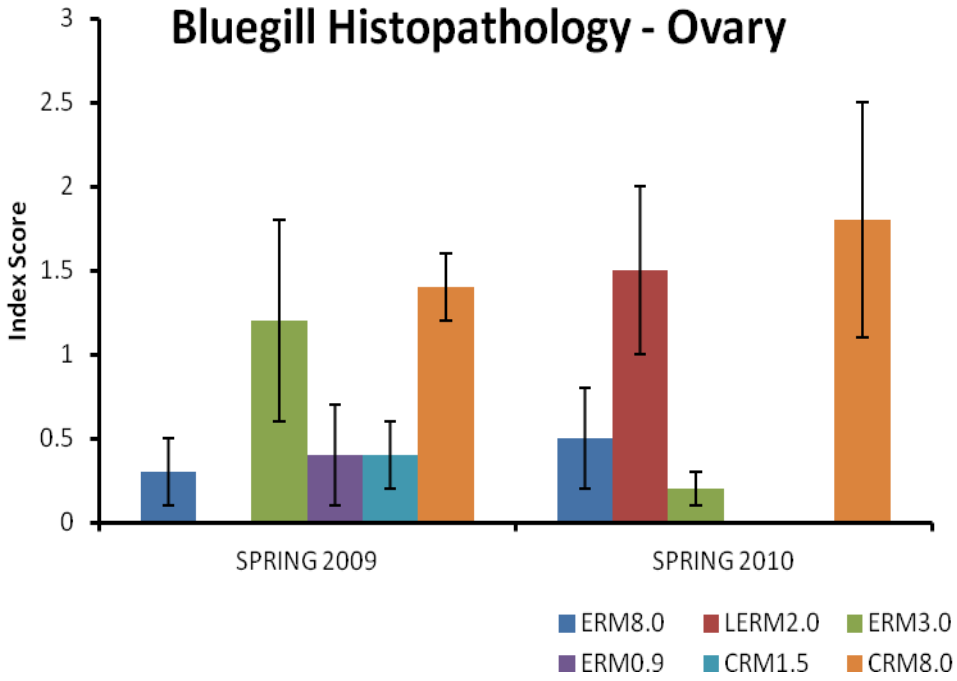

Figure 7. Spatial pattern of the incidences of ovarian histopathological lesions measured in bluegill sunfish sampled from locations within the Emory and Clinch River systems at the beginning of the 2009 and 2010 breeding seasons. ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile (sampled only in 2010). Asterisks indicate statistical difference from the primary upstream reference site (ERM 8.0) at $\alpha = 0.05$. Figure adapted from Adams and Fortner (2012).

Largemouth bass

Results of reproductive analyses conducted on female largemouth bass sampled at the beginning of the 2009 and 2010 breeding seasons are shown in Tables 3 − 4 and Figures 8 − 11. In the spring of 2009, largemouth bass GSIs were significantly lower at CRM 1.5 and also tended to be lower at the spill site than at either ERM 0.9 or the upstream reference sites (Table 3 and Fig. 8). A similar pattern was observed in the site-to-site variation in batch fecundity (Figs. 9), although in this case the observed differences between sites were not statistically significant. The total number of vitellogenic oocytes (Fig. 9) and the estimated annual fecundity (Fig. 10) were both markedly lower at the other sites as compared to ERM 8.0, with annual fecundity significantly lower at both Clinch River sites (including the upstream reference site at CRM 8.0). Oocyte atresia was observed at all sites but there were no significant differences between sites due to the relatively high variability of this reproductive parameter within each site. Incidences of histopathological lesions were not significantly greater at fly ash-affected sites than at the ERM 8.0 reference site (Fig. 11).

In the spring of 2010, largemouth bass GSIs were similar among all study sites (Table 4 and Fig. 8). The abundance of vitellogenic oocytes, the batch fecundity (Fig 9), and the estimated annual fecundity (Fig. 10), normalized for fish size, were actually higher at other study sites than at the ERM 8.0 reference site, in certain cases with statistical significance. Oocyte atresia was again observed at all sites in the spring of 2010 including the ERM 8.0 reference site (Fig. 8), but with no statistically significant differences noted due to relatively high variability within each site. As in 2009, the incidences of histopathological lesions were not significantly greater at fly ash-affected sites than at the ERM 8.0 reference site (Fig. 11).

Considered together, these results do not provide definitive evidence that the fly ash spill had ecologically significant adverse impacts on the reproductive condition of female largemouth bass during either the 2009 or 2010 breeding seasons. There were some downward trends and even some statistically significant differences among several reproductive parameters measured in fish from the upstream reference site at ERM 8.0 during the spring of 2009 as compared with downstream sites at ERM 3.0 and CRM 1.5 in particular. However, values of these reproductive parameters at the downstream sites were similar in both years to those measured in fish from the Clinch River reference site, and also to values measured at these same locations in 2010. In fact, most of the observed statistical differences among sites in both years appeared to be due primarily to relatively high year-to-year variability in the values of reproductive parameters measured in bass from the ERM 8.0 reference site.

Table 3. Reproductive indices measured in largemouth bass sampled at the beginning of the Spring 2009 breeding season from reaches of the Emory River and the Clinch River. Includes fish from fly ash-affected river reaches (ERM 3.0, ERM 0.9, and CRM 1.5) as well as upstream reference sites (ERM 8.0 and CRM 8.0)[a]

Site[a]	Year		Fish Length (cm)	Fish Weight (g)	Ovary Weight (g)	GSI	Eggs/Oocytes per Fish				Eggs/Oocytes per gram of Fish Weight			
							Atresia	Vitellogenic	Batch fecundity	Annual fecundity	Atresia	Vitellogenic	Batch fecundity	Annual fecundity
ERM 8.0	2009	*N*[b]	11	11	11	11	11	11	11	11	11	11	11	11
		Mean	43.7	1269.6	81.68	6.56	4302	133152	31636	124897	5.39	96.12	21.44	89.39
		SEM	1.7	143.3	10.36	0.71	3077	46218	12235	39935	4.43	22.71	6.88	20.11
ERM 3.0	2009	*N*	7	7	7	7	7	7	7	7	7	7	7	7
		Mean	44.6	1337.3	57.61	4.34	12573	82066	17297	77079	8.63	66.25	13.72	63.80
		SEM	2.2	203.0	11.76	0.70	4896.9	13481	9581	18459	2.78	10.00	7.32	14.96
ERM 0.9	2009	*N*	11	11	11	11	11	11	11	11	11	11	11	11
		Mean	43.8	1320.1	84.61	6.47	4802	84740	28145	94247	4.18	63.71	22.23	72.31
		SEM	1.6	145.2	10.03	0.41	1927	14109	4296	12497	2.17	7.03	3.79	5.72
CRM 8.0	2009	*N*	7	7	7	7	7	7	7	7	7	7	7	7
		Mean	36.5	665.4	35.24	5.30	6742	38971	6751	32696	9.65	57.60	9.85	47.73
		SEM	1.0	71.5	5.63	0.63	2874	5843	2860	6432	3.81	4.28	3.65	6.04

Table 3. (continued)

Site[a]	Year		Fish Length (cm)	Fish Weight (g)	Ovary Weight (g)	GSI	Eggs/Oocytes per Fish				Eggs/Oocytes per gram of Fish Weight			
							Atresia	Vitellogenic	Batch fecundity	Annual fecundity	Atresia	Vitellogenic	Batch fecundity	Annual fecundity
CRM 1.5	2009	*N*	7	7	7	7	7	7	7	7	7	7	7	7
		Mean	39.8	901.7	31.21	3.61	1185	38930	13078	43340	1.26	45.47	15.82	50.88
		SEM	1.1	92.8	2.87	0.36	627	4951	1646	4691	0.62	6.37	2.90	6.63
LERM 2.0	2009	*N*												
		Mean						Site not sampled in 2009						
		SEM												

[a] ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile
[b] N = number of fish in sample
[c] SEM = Standard error of the mean

Table 4. Reproductive indices measured in largemouth bass sampled at the beginning of the Spring 2010 breeding season from reaches of the Emory River and the Clinch River. Includes fish from fly ash-affected river reaches (ERM 3.0, ERM 0.9, and CRM 1.5) as well as upstream reference sites (ERM 8.0, CRM 8.0, and LERM 2.0)[a]

Site[a]	Year		Fish Length (cm)	Fish Weight (g)	Ovary Weight (g)	GSI	Eggs/Oocytes per Fish				Eggs/Oocytes per gram of Fish Weight			
							Atresia	Vitellogenic	Batch fecundity	Annual fecundity	Atresia	Vitellogenic	Batch fecundity	Annual fecundity
ERM 8.0	2010	*N*[b]	7	7	7	7	7	7	7	7	7	7	7	7
		Mean	40.8	982.1	37.52	3.94	5190	34954	4281	28496	6.99	34.79	4.09	28.44
		SEM	1.9	152.3	6.86	0.56	3809	9614	1482	6642	5.04	6.40	1.26	4.09
ERM 3.0	2010	*N*	8	8	8	8	8	8	8	8	8	8	8	8
		Mean	46.4	1587.2	70.87	4.54	414	77090	16020	75227	0.26	48.21	9.95	46.47
		SEM	1.2	154.9	9.65	0.46	239	13538	3110	14375	0.12	6.41	1.42	6.31
ERM 0.9	2010	*N*	8	8	8	8	8	8	8	8	8	8	8	8
		Mean	43.8	1336.0	64.11	4.76	2557	71578	23758	68741	2.10	53.07	17.46	50.31
		SEM	2.5	257.1	12.56	0.39	1006	14509	5552	14333	0.72	3.33	3.01	3.76
CRM 8.0	2010	*N*	8	8	8	8	8	8	8	8	8	8	8	8
		Mean	43.6	1386.0	77.47	5.72	3746	79830	19460	78991	2.26	58.70	13.67	57.60
		SEM	1.9	215.4	11.06	0.46	1930	12113	3506	11745	0.82	4.44	2.09	3.80

Table 4. (continued)

Site[a]	Year		Fish Length (cm)	Fish Weight (g)	Ovary Weight (g)	GSI	Eggs/Oocytes per Fish				Eggs/Oocytes per gram of Fish Weight			
							Atresia	Vitellogenic	Batch fecundity	Annual fecundity	Atresia	Vitellogenic	Batch fecundity	Annual fecundity
CRM 1.5	2010	*N*	7	7	7	7	7	7	7	7	7	7	7	7
		Mean	42.8	1295.2	60.63	4.77	2467	86631	19273	78218	1.53	67.59	15.13	56.62
		SEM	3.5	358.2	17.46	0.49	1423	25257	6727	25939	0.72	7.54	3.75	5.62
LERM 2.0	2010	*N*	8	8	8	8	8	8	8	8	8	8	8	8
		Mean	43.0	1206.0	49.60	3.88	1385	68839	12276	64001	0.81	54.00	10.77	49.39
		SEM	2.1	194.6	13.06	0.50	1146	18299	1707	17123	0.64	6.94	1.39	6.21

[a] ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile
[b] N = number of fish in sample

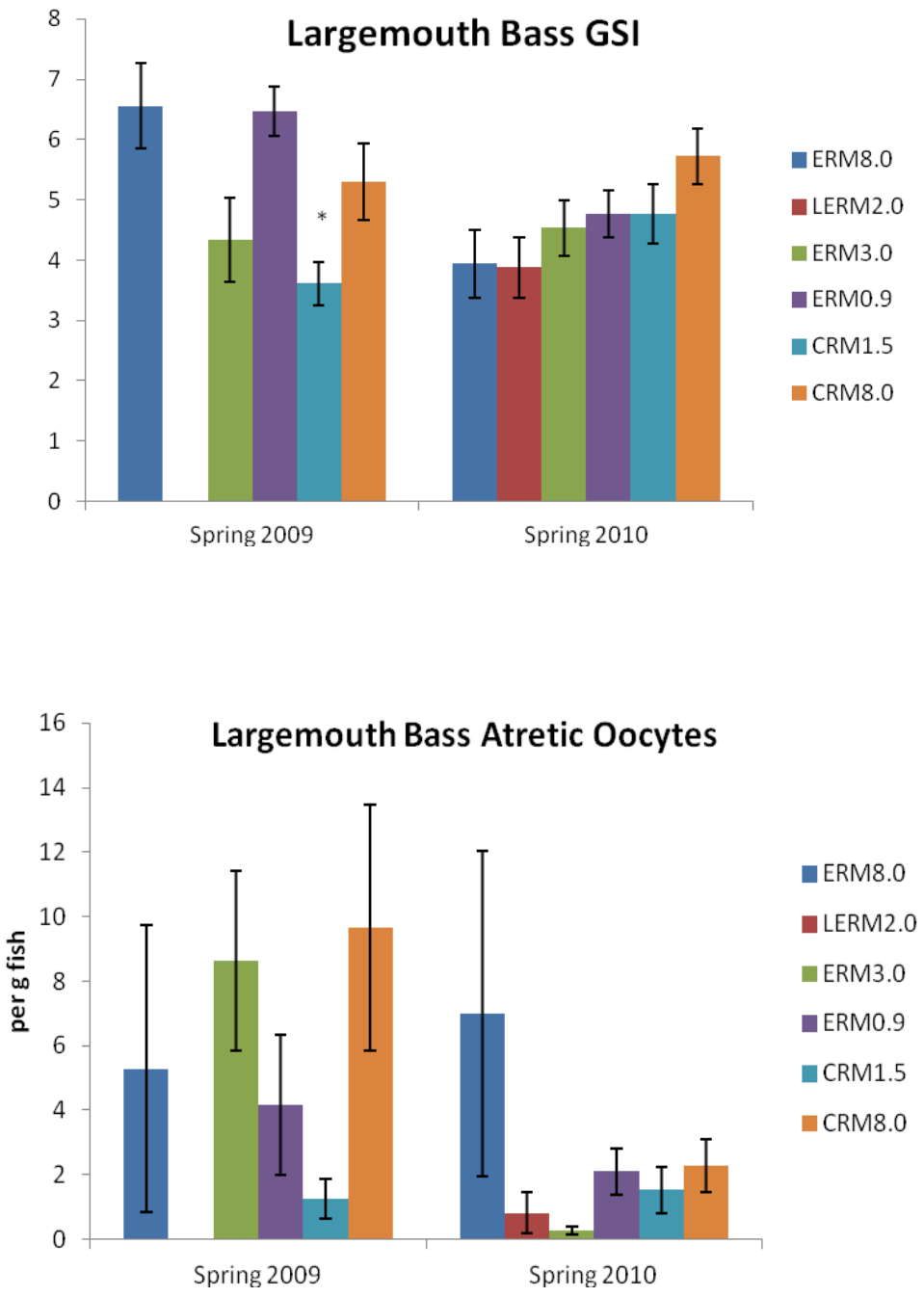

Figure 8. Comparison of select reproductive indices measured in largemouth bass sampled from locations within the Emory and Clinch River systems at the beginning of the 2009 and 2010 breeding seasons. ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile (sampled only in 2010). Asterisks indicate statistical difference from the primary upstream reference site (ERM 8.0) at $\alpha = 0.05$.

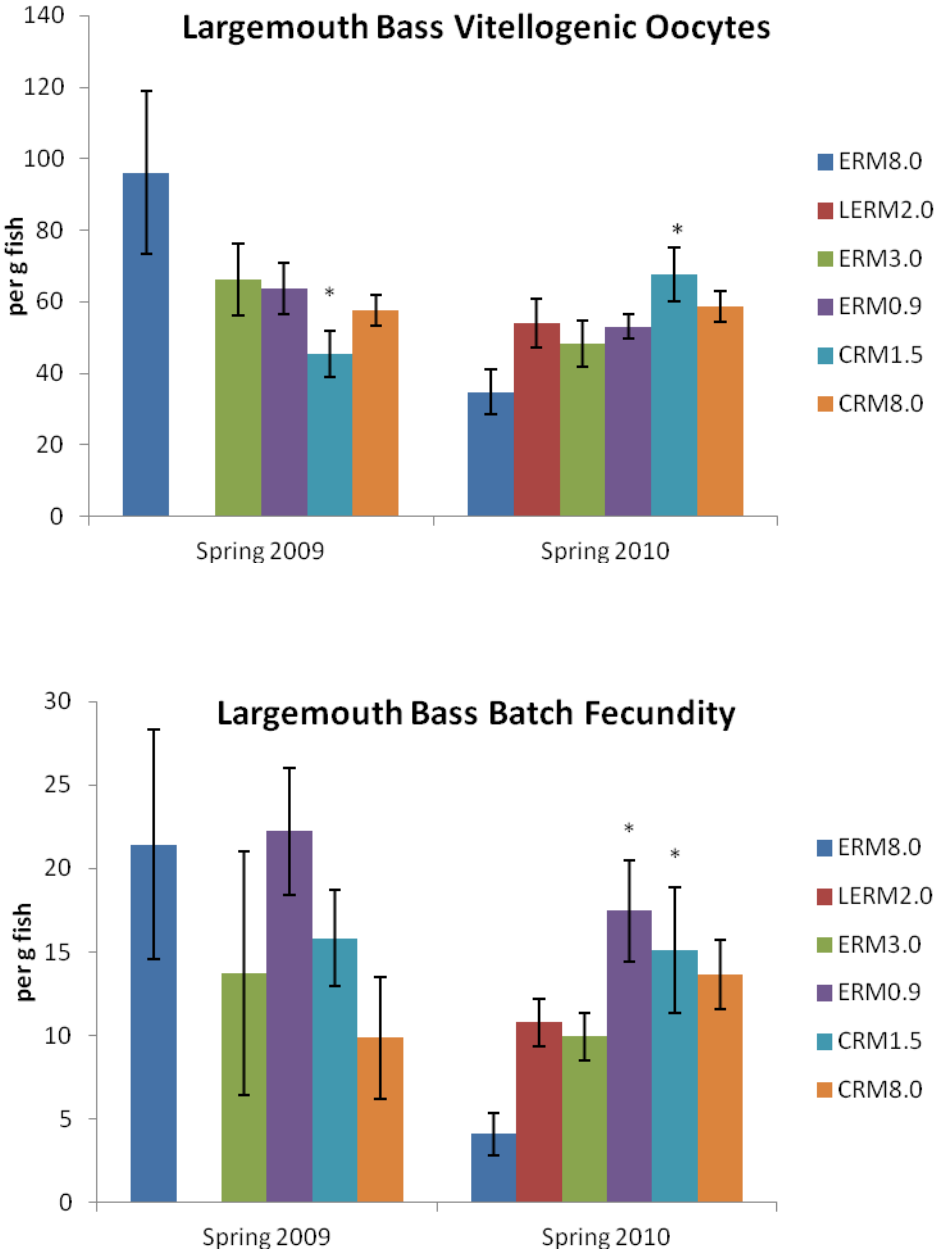

Figure 9. Comparison of select reproductive indices measured in largemouth bass sampled from locations within the Emory and Clinch River systems at the beginning of the 2009 and 2010 breeding seasons. ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile (sampled only in 2010). Asterisks indicate statistical difference from the primary upstream reference site (ERM 8.0) at $\alpha = 0.05$.

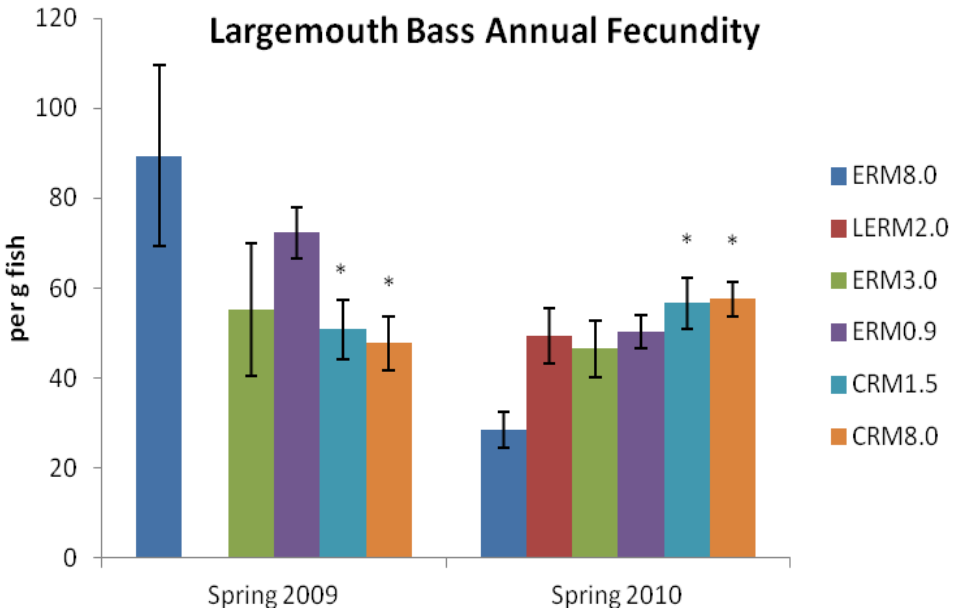

Figure 10. Comparison of select reproductive indices measured in largemouth bass sampled from locations within the Emory and Clinch River systems at the beginning of the 2009 and 2010 breeding seasons. ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile (sampled only in 2010). Asterisks indicate statistical difference from the primary upstream reference site (ERM 8.0) at $\alpha = 0.05$.

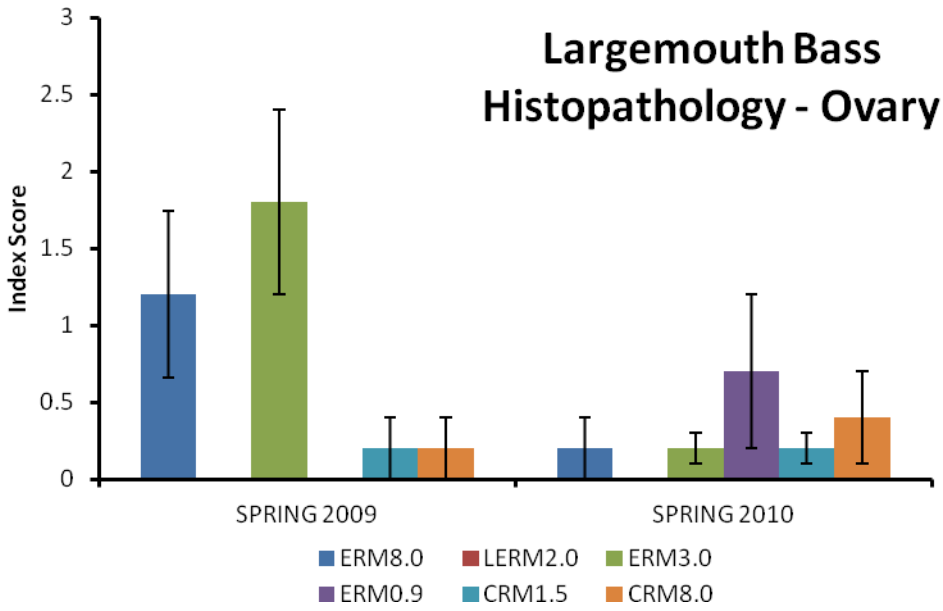

Figure 11. Spatial pattern of the incidences of ovarian histopathological lesions measured in largemouth bass sampled from locations within the Emory and Clinch River systems at the beginning of the 2009 and 2010 breeding seasons. ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile (sampled only in 2010). Asterisks indicate statistical difference from the primary upstream reference site (ERM 8.0) at $\alpha = 0.05$.
Figure adapted from Adams and Fortner (2012).

Redear sunfish

Summary results of reproductive analyses conducted on female redear sunfish sampled at the beginning of the 2010 breeding season are shown in Table 5 and Figures 12 − 14. In the spring of 2010, none of the reproductive parameters measured in redear GSIs differed with statistical significance between the upstream reference site at ERM 8.0 and the other study sites (Table 5 and Fig. 12 - 14). Considered together, these results do not provide definitive evidence that the fly ash spill had ecologically significant adverse impacts on the reproductive condition of female redear sunfish at the beginning of the 2010 breeding season.

However, parallel bioaccumulation studies conducted on these same fish showed that redear sunfish ovaries and livers (an organ critically important to the formation of vitellogenic oocytes) from study sites at or downstream of the fly ash spill demonstrate distinct spatial patterns of increased selenium and arsenic as compared with upstream reference sites (Adams and Brandt 2012). Furthermore, concentrations of selenium are much higher in the ovaries and livers of redear sunfish than in the tissues of bluegill sunfish, largemouth bass, or white crappie sampled from these same locations. Similarly, arsenic concentrations in redear ovaries and livers are also relatively higher than in other species, particularly at ERM 0.9 and CRM 1.5. When reconsidered along with the tissue metal data, the non-statistically significant tendencies towards increased oocyte atresia at both ERM 0.9 and CRM 1.5 and towards a greater incidence of histopathological lesions in ovaries from CRM 1.5, although not definitive, could be warning signs of incipient metal toxicity to reproductive processes in this species at sites downstream of the spill site.

Table 5. Reproductive indices measured in redear sunfish sampled at the beginning of the Spring 2010 breeding season from reaches of the Emory River, Clinch River, and Little Emory River. Includes fish from fly ash-affected river reaches (ERM 3.0, ERM 0.9, and CRM 1.5) as well as upstream reference sites (ERM 8.0, CRM 8.0, and LERM 2.0)[a]

Site[a]	Year		Fish Length (cm)	Fish Weight (g)	Ovary Weight (g)	GSI	Eggs/Oocytes per Fish			Eggs/Oocytes per gram of Fish Weight		
							Atresia	Vitellogenic	Batch fecundity	Atresia	Vitellogenic	Batch fecundity
ERM 8.0	2010	*N*[b]	13	13	13	13	13	13	13	13	13	13
		Mean	18.3	94.1	7.18	7.46	81	12290	8341	0.94	129.56	88.71
		SEM	0.4	5.1	1.09	0.96	31	1440	819	0.36	11.66	7.42
ERM 3.0	2010	*N*	8	8	8	8	8	8	8	8	8	8
		Mean	19.8	118.0	9.04	7.99	71	16008	11805	0.64	141.45	103.96
		SEM	0.6	10.2	1.09	1.01	48	1512	879	0.42	15.76	9.71
ERM 0.9	2010	*N*	10	10	10	10	10	10	10	10	10	10
		Mean	19.0	107.3	8.38	7.94	232	11873	9747	2.26	102.14	89.87
		SEM	0.7	8.6	1.15	0.93	89	1757	1378	0.79	17.43	8.37
CRM 8.0	2010	*N*	7	7	7	7	7	7	7	7	7	7
		Mean	17.9	103.6	9.91	9.63	38	13535	9445	0.46	134.19	93.42
		SEM	0.4	11.0	1.14	0.60	38	1143	766	0.46	11.09	6.82

Table 5. (continued)

Site[a]	Year		Fish Length (cm)	Fish Weight (g)	Ovary Weight (g)	GSI	Eggs/Oocytes per Fish			Eggs/Oocytes per gram of Fish Weight		
							Atresia	Vitellogenic	Batch fecundity	Atresia	Vitellogenic	Batch fecundity
CRM 1.5	2010	*N*	9	9	9	9	9	9	9	9	9	9
		Mean	20.3	134.5	7.24	5.13	228	12426	11304	1.82	92.17	84.27
		SEM	0.9	17.6	2.40	1.17	117	2505	2000	0.95	9.96	9.37
LERM 2.0	2010	*N*	12	12	12	12	12	12	12	12	12	12
		Mean	17.2	73.2	3.70	5.12	60	6433	4850	0.95	88.51	68.23
		SEM	0.4	4.0	0.64	0.84	37	490	603	0.63	8.95	8.86

[a] ERM = Emory River Mile; CRM = Clinch River Mile; LER = Little Emory River Mile
[b] N = number of fish in sample
[c] SEM = Standard error of the mean

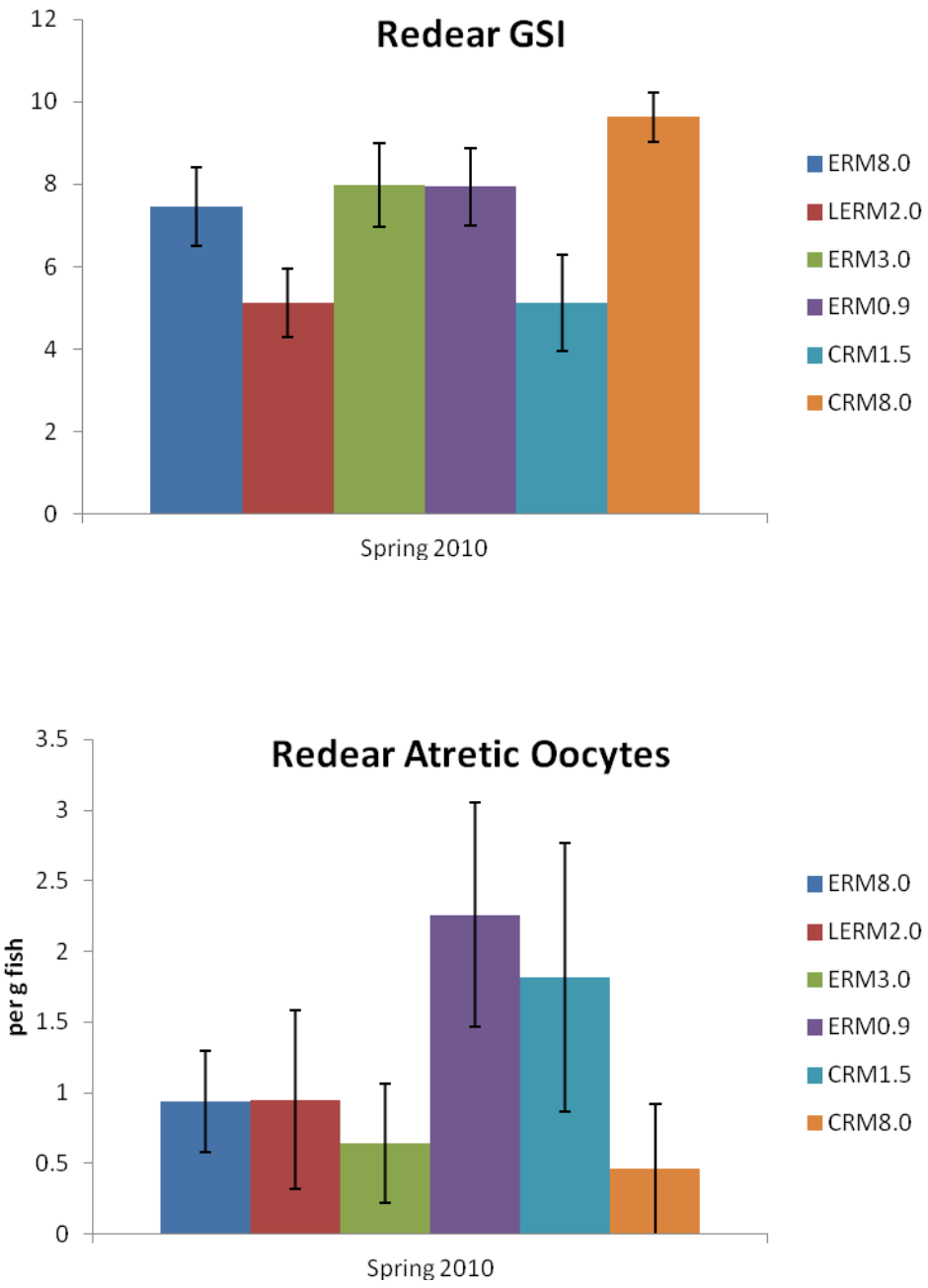

Figure 12. Comparison of select reproductive indices measured in redear sunfish sampled from locations within the Emory and Clinch River systems at the beginning of the 2010 breeding season. ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile (sampled only in 2010). Asterisks indicate statistical difference from the primary upstream reference site (ERM 8.0) at $\alpha = 0.05$.

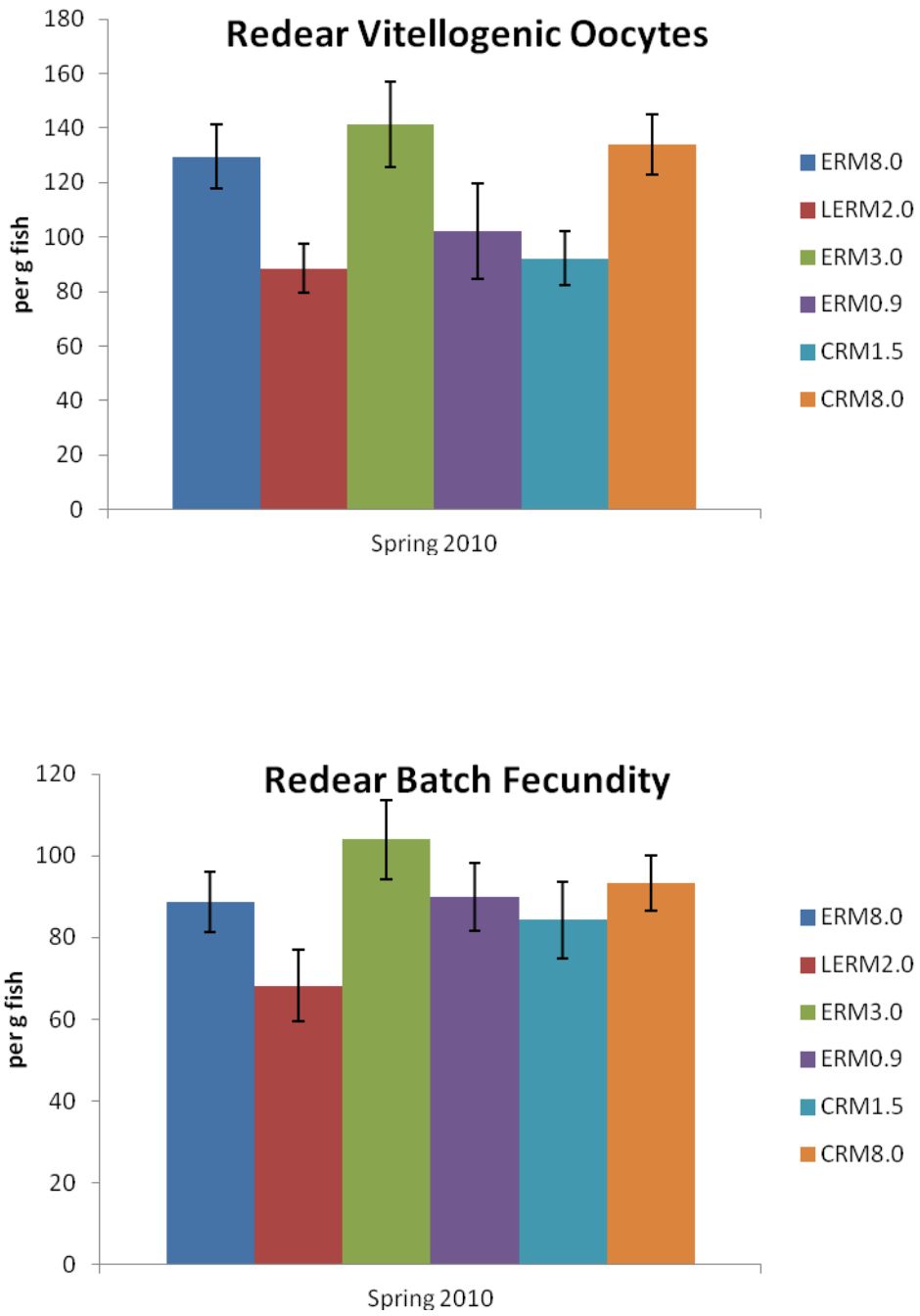

Figure 13. Comparison of select reproductive indices measured in redear sunfish sampled from locations within the Emory and Clinch River systems at the beginning of the 2010 breeding season. ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile (sampled only in 2010). Asterisks indicate statistical difference from the primary upstream reference site (ERM 8.0) at $\alpha = 0.05$.

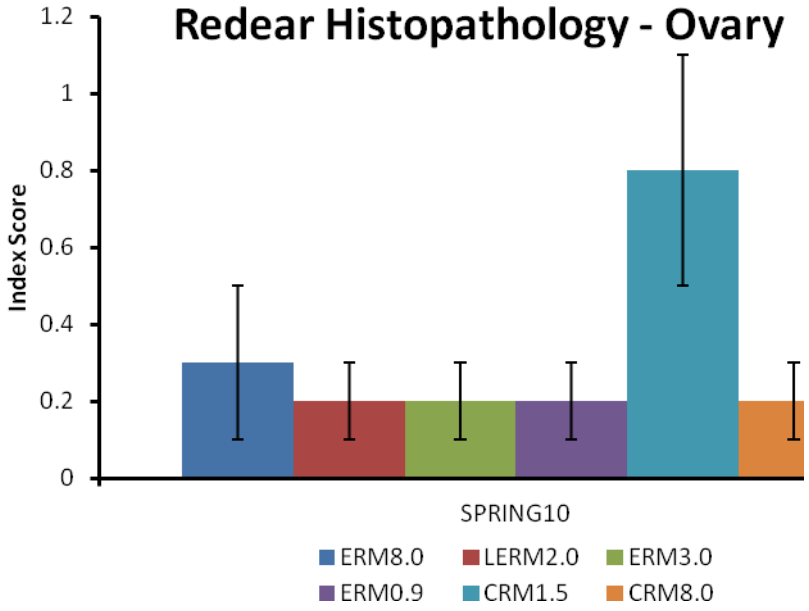

Figure 14. Spatial pattern of the incidences of ovarian histopathological lesions measured in redear sunfish sampled from locations within the Emory and Clinch River systems at the beginning of the 2010 breeding season. ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile (sampled only in 2010). Asterisks indicate statistical difference from the primary upstream reference site (ERM 8.0) at $\alpha = 0.05$. Figure adapted from Adams and Fortner (2012).

Correlations of metals with reproductive indicators

Potential relationships between metal bioaccumulation and reproduction in the three sentinel fish species were further assessed with the Pearson Product Moment Correlation procedure (Tables 6−8). Correlations were examined between selected reproductive parameters expressed per fish or normalized per gram of fish and arsenic and selenium measured in liver and ovary tissues from the same fish combined across all six study sites.

In bluegill sunfish, the only statistically significant relationships observed were positive correlations between the abundance of vitellogenic oocytes per gram of fish and both arsenic and selenium concentrations in the liver (Table 6). The liver is the site of production of the yolk proteins that constitute most the mass of the oocyte during the vitellogenic stage of development (Greeley 2002). However, there is not a ready explanation for why elevated liver arsenic and selenium would be causally associated with an increase in the abundance of vitellogenic oocytes in the ovary. Since the statistical relationship is based on relatively few samples as a result of the very small liver sizes in most female bluegill sunfish, study, this result may simply be due to the small sample sizes involved in the statistical analysis.

In largemouth bass, no correlations were noted between liver and ovary arsenic and selenium and any of the measured reproductive parameters when data from both years were combined prior to analysis (Table 7). It should be noted that when data for the two different years were analyzed individually (not shown), there were some significant correlations that did not persist when the data sets from the two sampling years were combined.

In redear sunfish, a correlation was also observed between the abundance of vitellogenic oocytes per gram of fish and liver selenium (Table 8). However, in this species the parameters were negatively rather than positively correlated, which could be interpreted as selenium bioaccumulation perhaps being causally related to a decrease in vitellogenesis by the liver. Although such an explanation appears more logical than the positive correlation observed in bluegill sunfish, similar caution must be taken in interpreting the results of this statistical analysis. The data are currently relatively limited, with a total of only 17 redear sunfish across the six study sites combined being subjected to both metal and reproductive analyses, and represents only a single year of sampling. As was seen with largemouth bass, correlations observed in one year of sampling may not persist when analytical data from additional years are added into the statistical analysis.

Table 6. Pearson Product Moment Correlation Analysis for selected reproductive parameters and arsenic and selenium (mg/kg dry wt) in livers and ovaries of female bluegill sunfish sampled in the Spring of 2009 and 2010 from sites in the Emory River and Clinch River. Presented as the number of samples/*P* value[a]/correlation coefficient[b]. Shaded area indicates significant correlations.

	Liver		Ovary	
Parameter	Arsenic	Selenium	Arsenic	Selenium
GSI	15	15	47	47
	0.135	0.092	0.227	0.568
	0.404	0.450	-0.180	0.086
Atresia	15	15	47	47
	0.596	0.617	0.321	0.993
	-0.149	0.141	-0.148	0.001
Vitellogenic oocytes	15	15	47	47
	0.323	0.221	0.309	0.560
	0.274	0.336	0.152	0.087
Batch fecundity	15	15	47	47
	0.853	0.785	0.177	0.561
	0.052	0.077	0.200	-0.087
Atresia/ g fish	15	15	47	47
	0.573	0.653	0.266	0.998
	-0.158	0.126	-0.166	-0.0003
Vitellogenic oocytes /g fish	15	15	47	47
	0.009	0.047	0.239	0.763
	0.647	0.519	0.175	0.045
Batch fecundity/g fish	15	15	47	47
	0.233	0.541	0.093	0.358
	0.328	0.172	0.248	-0.137

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

Table 7. Pearson Product Moment Correlation Analysis for selected reproductive parameters and arsenic and selenium (mg/kg dry wt) in livers and ovaries of female largemouth bass sampled in the Spring of 2009 and 2010 from sites in the Emory River and Clinch River. Presented as the number of samples/*P* value[a]/correlation coefficient[b]. Shaded area indicates significant correlations.

	Liver		Ovary	
Parameter	Arsenic	Selenium	Arsenic	Selenium
GSI	45	45	56	56
	0.205	0.092	0.842	0.697
	-0.193	-0.254	-0.027	-0.053
Atresia	45	45	56	56
	0.363	0.595	0.262	0.506
	-0.139	-0.082	-0.152	-0.091
Vitellogenic oocytes	45	45	56	56
	0.560	0.336	0.117	0.715
	-0.0893	0.147	-0.212	0.050
Batch fecundity	45	45	56	56
	0.927	0.650	0.364	0.544
	0.014	0.070	-0.124	0.083
Atresia/ g fish	45	45	56	56
	0.896	0.504	0.454	0.231
	-0.020	-0.102	-0.102	-0.162
Vitellogenic oocytes /g fish	45	45	56	56
	0.782	0.649	0.334	0.351
	-0.042	-0.070	-0.132	-0.127
Batch fecundity/g fish	45	45	56	56
	0.641	0.455	0.858	0.710
	0.072	-0.117	0.025	-0.051

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

Table 8. Pearson Product Moment Correlation Analysis for selected reproductive parameters and arsenic and selenium (mg/kg dry wt) in livers and ovaries of female redear sunfish sampled in the Spring of 2010 from sites in the Emory River and Clinch River. Presented as the number of samples/*P* value[a]/correlation coefficient[b]. Shaded area indicates significant correlations.

	Liver		Ovary	
Parameter	Arsenic	Selenium	Arsenic	Selenium
GSI	17	17	29	29
	0.777	0.341	0.772	0.277
	0.074	-0.246	0.056	0.209
Atresia	17	17	29	29
	0.109	0.613	0.399	0.166
	0.403	0.132	0.163	0.264
Vitellogenic oocytes	17	17	29	29
	0.674	0.650	0.644	0.175
	0.110	0.119	0.090	0.259
Batch fecundity	17	17	29	29
	0.222	0.064	0.164	0.409
	0.312	0.458	0.266	0.159
Atresia/ g fish	17	17	29	29
	0.216	0.951	0.553	0.101
	0.316	0.016	0.115	0.311
Vitellogenic oocytes /g fish	17	17	29	29
	0.434	0.046	0.575	0.885
	-0.203	-0.489	-0.109	0.028
Batch fecundity/g fish	17	17	29	29
	0.793	0.845	0.815	0.578
	0.069	-0.051	0.046	0.108

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

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4. SUMMARY AND CONCLUSIONS

In summary, the results of these studies indicate that the reproductive condition of female bluegill at the fly ash spill site in the Emory River was adversely affected during the 2009 breeding season immediately following the fly ash spill. An observed delay in ovarian development in this fish species with a relatively small home range was most likely due to habitat alterations and food chain disruptions in this river reach. There was no conclusive evidence of adverse reproductive effects in female bluegill sunfish sampled from the other study sites in 2009 or from any of the sites sampled in 2010. Results of studies with largemouth bass did not provide definitive evidence that the fly ash spill had any ecologically significant adverse impacts on the reproductive condition of female bass during either the 2009 or 2010 breeding seasons. Similarly, results of reproductive analyses on redear sunfish collected at the beginning of the 2010 breeding season did not provide any conclusive evidence that the fly ash spill had ecologically significant adverse impacts on the reproductive condition of females of this fish species.

Correlations between the abundance of vitellogenic oocytes and liver arsenic and selenium in bluegill sunfish and liver selenium in redear sunfish bear further examination, but the current small sample sizes and differing direction of responses between the two species suggest caution is merited at this time in interpreting these results. However, in particular the relatively high selenium and arsenic concentrations in ovaries and livers of redear sunfish at sites downstream of the spill site (Adams and Brandt 2012) should continue to be assessed for potential effects on the reproductive competence of adult fish and the developmental competence of their offspring. Although gamete quantity or quality does not appear to be significantly impacted at any of the study sites through 2010, the observed increases in metal bioaccumulation from 2009 to 2010 in several fish species suggest that the trajectory of bioaccumulation of fly ash constituents may not yet have leveled off in fish populations affected by the spill, and thus effects may be noted when fish sampled subsequent to 2010 are analyzed.

As indicated in the introduction to this report, the 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 of selenium exposure to larval fish (Lemly 1999). Selenium in particular is thought to be capable of causing reproductive failure in severely-contaminated fish populations primarily through adverse effects on the early development of offspring from exposed parents. Therefore, although gamete quantity and quality in the studied fish populations do not appear to be significantly impacted at any of the study sites through 2010 (other than bluegill at the spill site in 2009), sufficient contaminants might still be transferred to the developing eggs and potentially be impacting the offspring. A combined field and laboratory study involving the *in vitro* spawning of redear

sunfish and the assessment of embryos and larvae derived from these procedures was conducted during the 2011 breeding season to begin to address this continuing concern; results are being analyzed and will be reported in FY2012.

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