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Abstract. Coal fly ash and effluent from coal ash settling basins negatively affects metabolism and reproduction in a variety of organisms, including a number of fish species. Some species, most notably the eastern mosquitofish (*Gambusia holbrooki*), are known to maintain viable populations in areas contaminated by coal ash. While eastern mosquitofish are present in these systems, their degree of tolerance to coal ash has not been investigated using sublethal metrics of exposure. It is possible that eastern mosquitofish persist in habitats affected by coal ash, but experience significant costs such as changes in metabolism and fecundity. Thus, we investigated the effects of coal ash on standard metabolic rate and reproduction of eastern mosquitofish inhabiting a coal-ash contaminated settling basin. Standard metabolic rates of mosquitofish from the ash contaminated site and a reference site (mean O_2 consumption = 0.286 mL/g · h ± 0.007 and 0.291 ± 0.008 mL/g · h, respectively) were not significantly different. Despite elevated contaminant concentrations in ash basin females (selenium, arsenic, copper, and cadmium) and their offspring (selenium), brood sizes and offspring viability did not differ between clutches collected from ash basin and reference site females. Our data provide further evidence of the high degree of tolerance of eastern mosquitofish to exposure to aquatic coal ash disposal generated by power plants. However, the basis for such tolerance to ash remains unclear. Further investigations are required to determine whether such tolerance is a result of species-specific characteristics or population characteristics due to local adaptation.

Species can vary significantly in their sensitivity to environmental contaminants with negative effects ranging from death due to acute exposure to sublethal changes associated with chronic exposure. A number of fish species and other organisms have been documented to be sensitive to coal ash, a byproduct of coal combustion at electricity generation plants, and wastewater runoff from coal ash settling basins (Cherry *et al.* 1976, 1984; Hopkins *et al.* 1999, 2000a, 2000b; Lemly 1985; Lohner *et al.* 2001a, 2001b, 2001c; Rowe *et al.* 1998). Of 20 fish species inhabiting Belews Lake, North Carolina prior to selenium (Se) contamination from ash basin drain water, 16 were extirpated from the lake apparently due to reproductive failure associated with the contamination (Lemly 1985, 1997). Eastern mosquitofish (*Gambusia holbrooki*) were one of four species (including common carp, *Cyprinus carpio;* fathead minnows, *Pimephales promelas;* and black bullheads, *Ictalurus melas*) that remained in Belews Lake after Se-contamination and was the only species that appeared to maintain a reproductively viable population (Lemly 1985). In other systems associated with power plants, eastern mosquitofish persist in ash settling basins (Cherry *et al.* 1976; Hopkins *et al.* 1999) and sunfish remain in streams that receive fly ash pond runoff with seemingly few detectable negative effects (Lohner *et al.* 2001a, 2001b, 2001c).

While eastern mosquitofish have been noted to be tolerant of exposure to coal ash (Cherry *et al.* 1976) and drainwater associated with coal ash basins (Lemly 1985, 1997), they may still incur physiological costs associated with exposure to these waste products. In the past, the tolerance of mosquitofish to coal ash and coal ash effluent has been inferred by presence/absence surveys of adults (Cherry *et al.* 1976; Lemly 1997) and juveniles (Lemly 1997). Potential negative effects of coal ash on eastern mosquitofish have not been assessed using sublethal metrics of exposure. Changes in standard metabolic rate (SMR) and reproductive characteristics are common responses found in other species exposed to ash and may be useful metrics of exposure for mosquitofish. A variety of organisms (i.e. bullfrogs, *Rana catesbeiana;* banded water snakes, *Nerodia fasciata;* and crayfish, *Procambarus acutus*) exposed to conditions in ash basins located on the Savannah River Site (SRS), Aiken, South Carolina, exhibit increased SMRs, an indirect estimate of an organism's maintenance costs (Rowe *et al.* 1998, 2001; Hopkins *et al.* 1999). Presumably an increase in SMR results from energetically costly processes associated with combating contaminant exposure (*e.g.*, cellular repair mechanisms, elimination of contaminants). In addition, previous field surveys (*e.g.*, Belews Lake) have documented a high frequency of teratogenic effects in mosquitofish exposed to coal ash effluent (Lemly 1997). However, no studies have directly investigated SMR or fecundity (as measured by brood size and offspring viability) in mosquitofish exposed to coal ash.

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The goal of this study was to investigate the degree of tolerance of eastern mosquitofish to coal ash in a population that resides within an ash basin system on the SRS. The first portion of our investigation compared SMRs of mosquitofish from the ash basin to those of reference site individuals. In addition, we compared reproductive parameters (number of offspring and offspring viability) among mosquitofish from the ash basin and a reference site. While mosquitofish appear to be tolerant of ash basin conditions they may incur sublethal costs. Thus, we hypothesized that SMR and reproductive characteristics would differ between sites.

Materials and Methods

Mosquitofish Natural History

Eastern mosquitofish are a small (35–60 mm), live-bearing topminnow (Poeciliidae) that are distributed throughout the United States. They naturally occur in coastal plain drainages from Mobile Bay, Alabama eastward to New Jersey (Wooten *et al.* 1988) and have also been introduced by humans to many places in the United States and throughout the world (Courtenay and Meffe 1989), oftentimes in an attempt to control mosquitoes. They occur in both lentic (lakes, ponds, etc.) and lotic (streams) habitats and in fresh and brackish water (Meffe 1991). Mosquitofish are omnivorous and feed both on live organisms (worms, insects, etc.) and periphyton (Jenkins and Burkhead 1994). Numerous toxicological studies including research investigating the effects of estrogenic contaminants (Angus *et al.* 2002), mercury (Mulvey *et al.* 1995; Hopkins *et al.* 2003), and other contaminants (Vinson *et al.* 1963) have been conducted on mosquitofish.

Site Descriptions

Eastern mosquitofish were collected from two sites: the D-Area ash basin system (ASH; contaminated site) and Risher Pond (REF; uncontaminated reference site). The ASH system consists of a receiving ditch, a 15-ha primary settling basin, a 6-ha secondary settling basin and a 2-ha swamp. Coal ash is mixed with water at the D-Area coal-fired power plant and pumped into the receiving ditch. Water flows from the receiving ditch through the rest of the ASH system and ultimately drains into Beaver Dam Creek, a tributary of the Savannah River. For this study mosquitofish were collected from the primary and secondary ash basins (pH and conductivity [mean $\pm$ SE]: 8.36 $\pm$ 0.10 and 323 $\pm$ 0.2 μS/cm, respectively). The design of the ASH system prevents immigration of individuals from other mosquitofish populations into the ASH system; the basins are elevated above the flood plain with the sole input of water being rainwater and the water-ash slurry from the power plant. In addition, the outfall from the basins is a vertical standpipe that prevents colonization from downstream sources. Numerous studies have documented trace element concentrations at D-area and the responses of organisms inhabiting the site (Rowe *et al.* 2002). The REF site is an abandoned (greater 50 years) farm pond (1.1 ha, 2.5 m maximum depth; pH and conductivity (mean $\pm$ SE): 6.33 $\pm$ 0.11 and 28 $\pm$ 0.2 μS/cm respectively), and is located approximately 4.1 km from the ash basins. At least 12 previous toxicological and life history studies have used Risher Pond as a reference site because it is located in a forested area, has not been subject to human encroachment within the watershed for greater than 50 years, and has no known history of contamination, exposure to elevated levels of any toxicant, nor any history of input of coal ash (*e.g.*, Hopkins *et al.* 1999; Lee *et al.* 1992; Meffe 1991; Meffe and Snelson 1993).

Data Collection

In June and July 1999 male eastern mosquitofish were collected using dipnets from the ASH and REF sites (n = 26 and 20, respectively) for measurements of SMR. For a description of sediment trace element concentrations at the contaminated site in the summer of 1999, see Hopkins *et al.* 2002. Fish were transported in aerated five-gallon buckets to the lab (approximately half hour travel time). Standard metabolic rates at 25°C of male mosquitofish (24 h postabsorptive; *i.e.*, held for 24 h without food) were measured for 24 h using a computer-controlled, indirect, closed-circuit respirometer (Micro-Oxymax, Columbus Instruments, Columbus, OH, USA). Respirometry procedures follow those outlined in Hopkins *et al.* 2000b, 2002. During each trial one respirometry chamber contained only water (blank), while another contained an 8.4-V battery standard that consumes a known amount of oxygen per minute (Procell Zinc Air medical Battery, DA146, Duracell, Bethel, CT, USA) for quality-control purposes. Body wet masses of male mosquitofish were measured to the nearest 0.001 g after SMR data was collected.

In July 1999, gravid female eastern mosquitofish were collected using dipnets from ASH and REF sites and transported to the lab in aerated coolers (approximately half hour travel time). Female fish were fed Tetramin fish food ad libitum upon arrival in the lab. After a ten-day acclimation period in twenty, 38-L aquaria, gravid ASH and REF females (n = 75/site) were transferred into individual brood chambers contained within 200-L tanks (three tanks per site; 25 brood chambers per tank; maintained at 28°C). Brood chambers were constructed from 1-L plastic containers that were suspended in the water with a ring of flotation material. To prevent cannibalism, small openings were made in the base of the brood chamber to allow neonates to escape into an attached bag.

Mosquitofish offspring size is determined during vitellogenesis (before fertilization) and gestation is approximately four weeks (Krumholz 1948). To ensure that offspring size was established by field conditions, only broods of females that gave birth within the first three weeks of captivity were used. Once born, offspring were removed from the brood chamber and immediately inspected for dead or deformed individuals. Females and their offspring were then killed with an overdose of MS-222 and frozen.

After approximately three weeks, fish were thawed and females were dissected and inspected for cannibalized offspring (no cannibalized offspring were detected). Female standard lengths (to the nearest 0.01 mm) were measured using calipers and masses (to the nearest 0.001 g) were obtained using a Sartorius balance. Females and offspring were then freeze-dried and the dry masses of females and offspring (to the nearest 0.001 g) were obtained. A subset of females (n = 5 per site) and their offspring (n = 25–42) were analyzed for trace elements. To attain a sample mass large enough for trace element analysis, offspring in each brood were pooled into a single sample.

Response variables compared between ASH and REF fish were: (1) SMR of male mosquitofish, (2) brood size of female mosquitofish, (3) percent of live offspring at parturition, and (4) trace element concentrations in females and offspring.

Trace Element Analysis

Trace element content of lyophilized tissue samples was determined using inductively coupled plasma mass spectroscopy (ICP-MS) (PerkinElmer SCIEX ELAN 6000, PerkinElmer, LLC, Norwalk, CT, USA). Samples were digested in a microwave (CEM Corporation, Matthews, NC, USA) with HNO_3 and H_2O_2 in high-pressure teflon PTFE (polytetrafluoroethylene) vessels and analyzed for Se, arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn). For additional details concerning digestions and analysis by ICP-MS, see Hopkins *et al.* 2000b. Analysis of samples included certified reference materials (TORT-2, lobster hepatopancreas; National Research Council of Canada, Ottawa, Ontario). Mean percent recoveries of trace elements in the certified reference materials were 83.4–101.4% and blanks for all elements were below detection. For mean detection limits for all trace elements see Table 3.

Statistical Analyses

Standard metabolic rate data was analyzed in the following manner. Prior to statistical comparisons, the highest 50% of SMR values for each individual were excluded from the dataset. The upper 50% of measurements were excluded to ensure that periods of activity were not included in the estimation of SMR (Rowe *et al.* 1998) because SMR is a measure of an organism's metabolic rate at rest. The mean of the remaining 50% of measurements was then used as an estimate of SMR for each individual. Standard metabolic rates ($\log_{10}$ transformed) were compared between ASH and REF fish using ANCOVA with wet mass as a covariate (Packard and Boardman 1987).

Fecundity data was analyzed in the following manner. Brood sizes ($\log_{10}$ transformed) of ASH and REF mosquitofish were compared using ANCOVA with female standard length as a covariate. Offspring viability was compared using a Kruskal-Wallis test (Zar 1996). Kruskal-Wallis tests were also used to compare trace element concentrations in eastern mosquitofish from the two sites. One half the element quantitation limit (EQL) of the ICP-MS was assigned to samples with trace element concentrations below EQLs to allow their incorporation in nonparametric analysis (Hopkins *et al.* 2001). Because the comparisons of trace element concentrations are not independent of one another, a sequential Bonferroni correction was utilized to maintain the experiment wide error rate at $p \leq 0.05$ (Sokal and Rohlf 1995). All statistical analyses were performed using SAS statistical software (SAS, Cary, NC, USA).

Results

Standard metabolic rates of ASH and REF male eastern mosquitofish were affected by wet mass ($F_{1,43} = 102.35$, $p < 0.001$) but were not significantly different ($F_{1,43} = 0.00$, $p = 0.954$; Table 1).

Brood sizes of female eastern mosquitofish were similar between ASH and REF individuals ($F_{1,70} = 3.32$, $p = 0.073$; Table 2). Offspring viability at birth was not significantly different between sites ($Pr >$ Chi-Square $= 0.9905$). Brood characteristics (percent viable offspring and number of offspring) and trace element concentrations in females and offspring are summarized in Tables 2 and 3, respectively. In general, ASH female mosquitofish exhibited elevated concentrations of several potentially toxic trace elements. As, Cu, Se, and Cd concentrations were significantly higher in ASH females compared to REF females (Se: $\chi_{[1]}^2 = 6.818$, $p = 0.009$; As: $\chi_{[1]}^2 = 6.818$, $p = 0.009$; Cd: $\chi_{[1]}^2 = 6.860$, $p = 0.008$; Cu: $\chi_{[1]}^2 = 6.818$, $p = 0.009$), while Pb and Zn were not different in ASH females compared to REF females (Pb: $\chi_{[1]}^2 = 0.884$, $p = 0.347$; Zn: $\chi_{[1]}^2 = 0.098$, $p = 0.754$) (Table 3). Since female mosquitofish were allowed to depurate under uncontaminated conditions until parturition, trace element concentrations in female fish underestimate concentrations of fish in the field. Se was the only element found to be significantly higher in ASH offspring compared to REF offspring (Se: $\chi_{[1]}^2 = 6.818$, $p = 0.009$; As: $\chi_{[1]}^2 = 2.151$, $p = 0.143$; Cd: $\chi_{[1]}^2 = 0.535$, $p = 0.465$; Cu: $\chi_{[1]}^2 = 2.455$, $p = 0.117$; Pb: $\chi_{[1]}^2 = 0.011$, $p = 0.917$; Zn: $\chi_{[1]}^2 = 0.011$, $p = 0.917$) (See Table 3). It is likely that trace element concentrations measured in offspring more closely approximate trace element concentrations in offspring from the field since mosquitofish are lecithotrophic (i.e., embryos are nourished by yolk deposited prior to fertilization and require little post-fertilization contribution from the mother) (Constantz 1989).

Table 1. Masses, standard metabolic rates at 25°C, and mass adjusted standard metabolic rates of ASH and REF male mosquitofish

	ASH	REF
n	26	20
Mean Mass (g)	0.184 (0.008)	0.156 (0.006)
Mean VO_2 (mL O_2/h)	0.053 (0.003)	0.045 (0.002)
Mean VO_2 (mL O_2/g · h)	0.286 (0.007)	0.291 (0.008)

Data are means ± 1 SE.

Discussion

We found no difference in SMR of male mosquitofish from the REF site and ASH system. The lack of effect on SMR contrasts previous work (Rowe *et al.* 1998, 2001; Hopkins *et al.* 1999) in which other diverse organisms (bullfrogs, banded water snakes, and crayfish) collected from the ASH system exhibit SMRs (mL/g · h) approximately 23–32% higher than conspecifics from reference sites. Given that SMR is an indirect measure of an organism's maintenance costs, the elevated SMRs of other ASH organisms suggested that they incurred energetic costs, perhaps as a result of energy required to facilitate toxicant elimination or repair toxicant-induced cellular damage (Calow 1991). Eastern mosquitofish sampled from the confined ASH population do not appear to incur such energetic costs.

Despite significantly elevated concentrations of trace elements in ASH females and significant maternal transfer of Se to their offspring, there was no evidence that maternal body burdens or maternal transfer negatively affected mosquitofish reproduction; number of offspring and offspring viability of ASH mosquitofish did not differ from REF mosquitofish. A number of studies have documented negative effects of coal ash or exposure to elements commonly found in coal ash (particularly Se) on fish reproduction (Cumbie and Van Horn 1978; Saiki and Ogle 1995; Lemly 1997). However, our study is the first to directly examine brood size and offspring viability in ash-exposed mosquitofish.

Se concentrations in ASH females and offspring (11.85 ± 0.85 and 15.87 ± 1.91 μg/g dry mass, respectively) were in excess or approximated the concentrations necessary to cause mortality or reproductive failure in a variety of other fish species. Juvenile rainbow trout (*Oncorhynchus mykiss*), juvenile chinook salmon (*Oncorhynchus tshawytscha*), and juvenile bluegill (*Lepomis macrochirus*) all exhibit mortality at whole body concentrations of 5–6 μg/g Se dry mass (Hilton *et al.* 1980; Hamilton *et al.* 1990; US Fish and Wildlife Service 1990). Schultz and Hermanutz (1990) documented reproductive failure in fathead minnows when their embryos contained whole body Se concentrations of 16 μg/g dry mass. Hermanutz *et al.* (1992) and Coyle *et al.* (1993) showed reproductive

Table 2. Summary reproduction data for ASH and REF female mosquitofish and ASH and REF offspring

Site	Number of Females	Female Length (mm)	Brood Size	Total # Offspring	% Viable Offpring at Birth
ASH	35	32.73 (1.13)	12.82 (1.44)	411	99.8 (0.5)
REF	38	35.11 (1.11)	13.31 (1.64)	476	99.2 (0.1)

Data are means ± 1 SE.

Table 3. Trace element concentrations in ASH ($n = 5$) and REF ($n = 5$) females and associated offspring (μg/g dry mass)[a]

	Se	As	Cd	Cu	Pb	Zn
Females						
ASH	11.85 (0.85)*	1.73 (0.20)*	0.79 (0.06)*	8.35 (0.49)*	0.41 (0.06)	188.81 (10.88)
REF	0.61 (0.24)[b]	0.23 (0.03)	BDL	2.59 (0.23)	0.75 (0.22)	185.44 (14.09)
Offspring						
ASH	15.87 (1.91)*	BDL	BDL	7.66 (1.45)	10.73 (3.48)	179.89 (6.86)
REF	BDL	BDL	BDL	5.66 (0.20)	9.86 (1.89)	178.89 (3.60)

[a] Values are expressed as means ± SE when three or more of the samples for a particular element were above the detection limit of the ICPMS.
[b] Denotes cases in which one or two values for that particular element were below detection limit (BDL) and 0.5 EQL (element quantitation limit) was used to calculate a trace element concentration for that sample. Detection limits (μg/g dry mass) for each element are as follows: Se, 0.481; As, 0.032; Cd, 0.089; Cu, 0.078; Pb, 0.010; Zn, 0.463.
* Denotes statistically significant differences after sequential Bonferroni correction to maintain an experiment wide error rate at $p \leq 0.05$.

failure in female bluegills at whole body Se concentrations ranging from 12–35 μg/g dry mass.

Other studies have shown that Se can affect reproduction in mosquitofish, but these reproductive anomalies arose in association with Se concentrations much higher than those documented in females in the current study. For example, Saiki and Ogle (1995) reported Se concentrations over 100 μg/g (dry mass) in female western mosquitofish (*Gambusia affinis*) from an area contaminated with agricultural drainwater. Female mosquitofish from the contaminated site experienced decreased reproductive success when compared to reference site females; broods of Se-exposed females contained fewer offspring and had a higher percentage of stillborn offspring compared to reference site females (Saiki and Ogle 1995). Thus, it appears that Se concentrations in excess of those documented in ASH mosquitofish are needed to negatively affect brood size and offspring viability.

Our investigation corroborates other studies that found that eastern mosquitofish are tolerant of elevated concentrations of trace elements found in coal ash, particularly Se (Cherry *et al.* 1976; Cumbie and Van Horn 1978; Lemly 1985). The fact that eastern mosquitofish are consistently the sole fish species found to inhabit the D-area ash basins (Cherry *et al.* 1976; Hopkins *et al.* 1999) and the only species to maintain a reproductively viable population immediately after ash basin effluent discharge (Lemly 1985) is a testament to their ability to survive adverse conditions.

Further studies are needed to assess whether the ability of eastern mosquitofish to persist in sites contaminated by coal ash is a result of species-specific characteristics or population characteristics due to local adaptation in populations of eastern mosquitofish chronically exposed to ash. Unlike piscivorous fishes that may be exposed to high contaminant concentrations as a result of biomagnification, mosquitofish occupy a lower trophic level and feed on items in the water column or graze from the surface of plants or other objects (Jenkins and Burkhead 1994) which may contain lower contaminant concentrations relative to prey items of piscivorous fishes. In addition, mosquitofish spend much of their time near the edges and surfaces of ponds, rather than near the sediments where contaminant concentrations encountered by benthic fishes are high (Hopkins 2001). Eastern mosquitofish may also survive in ash-contaminated sites because they possess proximate physiological mechanisms such as cellular repair mechanisms, stress proteins (Pedersen *et al.* 1997; Wheelock *et al.* 1998), or contaminant excretion and biotransformation pathways (Huckle and Millburn 1990) that may confer tolerance to elevated trace elements found in coal ash. An alternative explanation for the tolerance of eastern mosquitofish to ash-contaminated sites is that local mosquitofish populations possess adaptations (an ultimate, population-level response) resulting from selective pressures from exposure to coal ash. The basins at the D-area power plant have been in operation since 1978 and D-area eastern mosquitofish have been exposed to coal ash contamination for approximately 24 years. At two to three generations per year (Stearns 1983), eastern mosquitofish may have been exposed to coal ash for as many as 72 generations; a time span sufficient for selection to result in measurable changes of life history parameters (Stockwell and Weeks 1999). Lab and field studies such as reciprocal transplant feeding experiments and common garden experiments need to be conducted in order to assess how mosquitofish are able to maintain a viable population at D-area.

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