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EFFECTS OF COAL PARTICLES IN AQUATIC SEDIMENTS ON ORGAN TISSUES OF RAINBOW MUSSELS *VILLOSA IRIS* (UNIONIDAE)

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ABSTRACT Two laboratory studies were conducted to determine the effects of coal particles in aquatic sediments on survival and organ tissues of rainbow mussels *Villosa iris* (Lea, 1929). First, mussel survival was assessed using treatments comprised of sand substrates with different percentages of pulverized coal, including 0%, 10%, 25%, and 50%. At the end of this 7-wk pilot experiment, there were no significant differences in survival of *V. iris* among substrate treatments. Second, effects of coal particles in substrate on organ tissues of *V. iris*, including gills, digestive glands, kidneys, and gonads, were assessed during a 20-wk experiment. Two sand substrates, containing 0% coal (control) and 50% coal (treatment), were tested. Organ tissues of five mussels from each of the treatment and control tanks were collected at 8, 16, and 20 wk. Sublethal alterations in organ tissues of coal-exposed mussels were observed. Fractions of gill filaments without cilia and digestive cells of digestive glands with condensed cytoplasm were significantly greater in coal-exposed mussels compared with those from the control. Females from the coal treatment showed significantly higher fractions of acini containing atretic, resorbing oocytes than the control females. Significantly higher fractions of lipofuscin, an insoluble lipid peroxidation byproduct that can be related to contaminant exposure, in kidney diverticula of the coal-exposed mussels suggested that unidentified contaminants were present in the water. Further study of the effects of these contaminants on freshwater mussels are warranted given the co-occurrence of declining mussel populations and coal mining and processing operations in Appalachian watersheds.

KEY WORDS: coal, histology, freshwater mussels, *Villosa iris*

INTRODUCTION

Historically, the Clinch and Powell Rivers in Virginia and Tennessee have been strongholds for numerous populations of freshwater mussel species, with ~45 species still extant (Dennis 1981, Ahlstedt & Tuberville 1997, Ahlstedt et al. 2005). Between 1979 and 2004, mean mussel densities (mussels/m^2) at four long-term monitoring sites in the Powell River declined by 63%, and 12 species are nearing extirpation (Dennis 1981, Ahlstedt et al. 2005). Declines of mussel populations in these rivers have been attributed to human activities, including coal mining and processing operations (Ahlstedt et al. 2005). There are an estimated 287 active discharges from these operations in Appalachian watersheds (USEPA 2002).

Stressors associated with discharges from coal mine sites and processing plants in the Clinch and Powell Rivers include sediment, coal particles (commonly referred to as fines), and acidic runoff (e.g., in Ely Creek, a coal-mined tributary of the Powell River in Tennessee, pH ranged from 2.5 to 2.9) (USEPA 2002). Several contaminants are present in coal, and elevated trace metal and ionic concentrations have been documented downstream of coal mines, stockpiles, mine fills, settling ponds, and power plant fly-ash containments (Lemly 2004, Yudovich & Ketris 2005a, 2005b, Merricks et al. 2007). Water quality stressors in coal-mined watersheds negatively affect lotic biota. In the Clinch and Powell River subwatersheds, mining intensity has been associated with changes in the structure of macroinvertebrate and fish communities that are indicative of environmental stress (Diamond et al. 2002, Locke et al. 2006). In other basins, organism-level studies using Asian clams, *Corbicula fluminea* (Müller, 1774), have demonstrated altered growth rates and elevated tissue concentrations of glutathione and trace elements downstream of effluents from coal processing facilities and coal-fired power plants (Kennedy et al. 2003, Peltier et al. 2009); however, the sublethal effects of coal mining–related stressors on freshwater mussel organ tissues have yet to be studied.

Histopathological effects of organic and inorganic contaminants (e.g., hydrocarbons, polychlorinated biphenyls, and metals) have been observed in bivalve gills, digestive glands, kidneys, and gonads (Bayne et al. 1981, Seiler & Morse 1988, Domouhtsidou & Dimitriadis 2000, Au 2004). In the digestive gland, digestion, nutrient absorption, production of digestive enzymes, energy substrate storage, and contaminant detoxification occur (Owen 1970, Lobo-da-Cunha 1999, 2000, Petrović et al. 2001). Epithelia of digestive diverticula include secretory (basophilic) and digestive cells, and digestive cells have shown cytoplasmic vacuolation, epithelial desquamation, atrophy, and necrosis due to contaminant exposure [e.g., oil-derived aromatic hydrocarbons, metals, polychlorinated biphenyls (PCB), and chlorinated pesticides] (Lowe et al. 1981, Au 2004, Usheva et al. 2006, Sabatini et al. 2011). Gills of bivalves can be sites of contaminant uptake, and observed cellular alterations due to contaminant exposure include fusion of gill filaments, loss of cilia, inflammation, necrosis, epithelial sloughing, and increased mucus production due to proliferation of secretory cells (Domouhtsidou & Dimitriadis 2000, Lajtner et al. 2003, Gómez-Mendikute et al. 2005, Supanopas et al. 2005).

Although the bivalve kidney is responsible for ultrafiltration of hemolymph and ion exchange, another notable function of the organ regarding contaminant elimination is excretion of lipofuscin (ceriod) granules by exocytosis (Doyle et al. 1978, Seiler & Morse 1988, Dietz et al. 2000, Fahrner & Haszprunar 2002, Seehafer & Pearce 2006). The occurrence of intracellular

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lipofuscin granules can be a means for compartmentalizing contaminants by combining them with insoluble lipid peroxidation byproducts (Lomovasky et al. 2002, Riveros et al. 2002, Dimitriadis et al. 2004, Seehafer & Pearce 2006). The abundance of intracellular lipofuscin granules has been related to the degree of contaminant exposure (e.g., metals, PCB, and polycyclic aromatic hydrocarbons) (Riveros et al. 2002, Kagley et al. 2003, Usheva et al. 2006).

In adult unionid mussels, gametes develop through the mitotic–meiotic pathway from germ cells within acini (Henley et al. 2007). The gametogenic cycle in bivalves includes development and maturation of gametes, spawning, and subsequent resorption of residual atretic gametes (Kennedy & Battle 1964, Pipe 1987, Dorange & Le Pennec 1989, Barber 1996). Although postspawning resorption of oocytes is a normal element of the oogenic cycle, untimely resorption of oocytes has been observed due to stress and exposure to contaminants (Bayne & Thompson 1970, Bayne et al. 1981, Tay et al. 2003).

The objective of this study was to assess the effects of coal fines in sediments (substrate) on rainbow mussels *Villosa iris* (Lea, 1829). A preliminary pilot experiment was conducted to assess mussel survival in various mixtures of sand–coal substrates. Results were used to select the coal-sand substrate mixture used in a subsequent experiment to determine the effects of coal fines on organ tissues of *V. iris*, including gonads, gills, digestive glands, and kidneys. The rainbow mussels *V. iris* were used because they naturally occur in coal-impacted streams in Virginia and Tennessee, and hatchery-reared mussels were available in sufficient numbers for the experiments.

MATERIALS AND METHODS

Initial Survival Experiment

An initial small pilot study lasting 7 wk was conducted in 2009 to determine the effects of various sand–coal substrate mixtures on survival of *Villosa iris*. Forty hatchery-reared *V. iris*, ~14 mo of age (mean length ± SD = 19.1 ± 1.9 mm), were obtained from the Aquatic Wildlife Conservation Center (AWCC), Virginia Department of Game and Inland Fisheries, Marion, VA. These mussels were originally cultured at White Sulfur Springs National Fish Hatchery, White Sulfur Springs, WV, and transferred to AWCC for rearing. Ten mussels were held in each of four 37-l closed recirculating aquaculture systems housed at the Freshwater Mollusk Conservation Center (FMCC), Department of Fish and Wildlife Conservation, VA Tech, Blacksburg, VA. Each of the systems consisted of a water pump, sump, and round holding container, and well water from FMCC was used during the experiments. Well water was delivered from the sumps to the top of the holding containers to create circular flows. One each of the four tanks (n = one per substrate mixture) contained different sand-coal substrate mixtures, including: 100% sand (control), 90% sand and 10% by volume pulverized coal, 75% sand and 25% coal, and 50% sand and 50% coal. These treatments were termed "0%", "10%", "25%", and "50%", as defined by the percentage of coal fines in tank substrates. Commercial play sand was purchased, sieved (less than or equal to 1.4 mm), and thoroughly washed before placement in the containers. Coal was obtained from the coal-fired Virginia Tech Electrical Plant (Blacksburg, VA), pulverized, sieved (less than or equal to 425 μm), and thoroughly washed until the rinse water ran clear. The coal (1.4% sulfur by dry weight) originated from Sidney Mine, KY (Massey Energy Corporation, Richmond, VA).

To determine the size of coal particles (less than or equal to 425 μm) to use in the substrate mixtures, substrate from a site on the Clinch River with heavy deposition of coal fines was analyzed. Substrate from a site located at Clinch River kilometer 478.8 (river mile 297.5, Russell County, VA) was collected, dried, and coal particulates were measured for size (μm) using a stage micrometer and a dissecting microscope. The dominant size class for coal particles (77%) was less than or equal to 425 μm.

Temperature and water quality parameters, including turbidity (nephelometric turbidity units, NTU, 2100P Turbidimeter, Hach Company, Loveland, CO), dissolved oxygen (mg O_2/l), conductivity (μS/cm), and pH, were measured approximately every three days during the first 2 wk in all tanks with a YSI Professional Plus hand-held meter (YSI, Incorporated, Yellow Springs, OH) that was calibrated before use. After the first 2 wk, water quality parameters were measured once per week. Over the course of the experiment, mussels were fed daily with a commercial algae mix (Shellfish Diet 1800, Reed Mariculture, Incorporated, Campbell, CA). Mussel mortalities within tanks were recorded, and statistical differences among survival of mussels in treatments (10%, 25%, and 50% coal fines in substrate) and the control (0%) tanks ($n = 4$) were determined by survival analysis using LIFETEST (SAS Institute, Incorporated, Cary, NC). Water quality data were log-transformed and analyzed for differences among the treatments and the control using GLM in Minitab 16 (Minitab Incorporated, College Station, PA).

Histological Effects Experiment

A total of 160 *Villosa iris* (23.0 ± 1.6 mm), ~16 mo of age, were obtained from the AWCC. Again, mussels were originally cultured at White Sulfur Springs National Fish Hatchery and transferred to AWCC for rearing. Twenty of the mussels were randomly assigned to each of four treatment and four control 37-l closed recirculating aquaculture tanks as described previously. The treatment tanks each held 20 mussels in 50% sand and 50% coal fines (by volume), and control tanks each held 20 mussels in only sand. Coal and sand were processed prior to use as previously described. Water quality was measured once in every 2 wk, and mussels were fed daily as previously described.

The experiment was conducted over 20 wk at FMCC in the spring of 2010. Organs of five mussels were collected from each of the treatment and control replicates on day 56 (wk 8), day 108 (wk 16), and day 139 (wk 20) of the exposures, with a total of 15 mussels each collected for organ tissues from the four treatment and four control tanks during the experiment (120 total mussels). Tissue samples from gonads, gills, digestive glands, and kidneys were excised and fixed in Bouin's fixative. Tissues were processed for paraffin embedding using routine histological methods (Bancroft & Gamble 2002). Two sections (~5 μm) were cut with a rotary microtome from ~50% of the tissue block depths; one section was stained with hematoxylin (Gill-2) and eosin-Y for evaluations of gonad, gill, and digestive gland tissues, and the other section was stained with Perls' Prussian blue for elaboration of lipofuscin in kidney tissues (Blazer et al. 1987).

Histological evaluations determined fractions of gonads with reproductive acini containing mature and/or developing

TABLE 1.

Means (±SE) for water quality parameters measured during the initial survival and histological studies.

	Initial Survival Study†					Histological Study‡				
% Coal	Conductivity (μS/cm)	pH	DO (mg O_2/l)	°C	Turbidity§ (NTU)	Conductivity (μS/cm)	pH	DO (mg O_2/l)	°C	Turbidity‡ (NTU)
0	598.1 (19.1)	8.8 (0.03)	8.2 (0.15)	19.6 (0.3)	0.9 (0.1)	798.8 (2.4)	9.0* (0.01)	7.2 (0.26)	19.8 (0.7)	0.7 (0.1)
10	571.2 (19.6)	8.8 (0.03)	8.4 (0.14)	19.7 (0.3)	1.9 (0.2)	–	–	–	–	–
25	575.7 (16.4)	8.8 (0.03)	8.2 (0.16)	19.8 (0.3)	2.5 (0.3)	–	–	–	–	–
50	566.0 (20.0)	8.8 (0.03)	8.2 (0.17)	19.9 (0.4)	21.9 (3.0)	774.3 (26.2)	8.9 (0.04)	7.2 (0.29)	19.8 (0.7)	30.1 (4.9)

* Treatment significantly different from control ($P < 0.05$). Note that conductivity was higher during the histological study than in the survival study due to normal temporal variation in the well water used during the studies.

†Parameters measured approximately every three days during first 2 wk, thereafter every week (n = one tank per substrate).

‡ Parameters measured approximately every 2 wk ($n = 4$ tanks per substrate).

§ Three replicates per tank. DO, dissolved oxygen; dash, substrate percentages not included in histological study.

gametes, acini containing atretic, resorbing gametes, gill filaments without cilia, kidney diverticula cells containing lipofuscin, and digestive cells of digestive glands (termed "digestive cells" hereafter) with condensation of cytoplasm. Description and justification of "condensation of cytoplasm" will be subsequently provided with results.

Histological sections were evaluated by light microscopy using point counting (Chalkey 1943). All tissues were microscopically examined using a 40× objective, and a 100× oil objective was used when needed for clarification. Six dots were placed on one of the ocular lenses of the microscope, and the stage of the microscope was randomly positioned in two dimensions. Tissues were assessed for the histological dependent variables by recording whether the types of tissues described for the variables visually occurred under the ocular dots (zero = absence and one = presence), with each observation relating to a dot regarded a datum. Fifty observations were recorded for each variable from each histological section for statistical analyses. Evaluations were blind with regard to the origin of the sections (treatment or control).

In the course of the gametogenic evaluations, it was evident that gonad sections from some mussels did not contain mature, developing, or resorbing gametes in any stage of oogenesis, spermatogenesis, spermiogenesis, or resorption. Since gender of these mussels could not be determined, their gender was classified in the data as "indeterminate."

Histological data were analyzed using a generalized linear mixed model for binomial data with SAS GLIMMIX at the Laboratory for Interdisciplinary Statistical Analysis, Department of Statistics, VA Tech, Blacksburg, VA. Within the mixed models, treatment status was a fixed factor, sampling time was a repeated factor, and tank (sampling unit) was assigned as a random factor. The combined effect of these variables with gender (fixed factor) also was tested. Interactions among treatment status, sampling event, and gender were tested and sequentially removed from final models if nonsignificant. Overdispersion of final models was corrected using a residual term. When the effect of a factor was statistically significant, a Tukey–Kramer *posthoc* multiple comparisons test was used to compare least squares means among factor levels. Statistical analyses of fractions gill filament termini without cilia, kidney diverticula cells containing lipofuscin, and digestive cells with condensation of cytoplasm included data from tissues of female, male, and indeterminate genders. We conducted two statistical analyses of data from fractions of acini containing mature and/or developing gametes and acini containing resorbing gametes, including analyses of data from all genders (female, male, and indeterminate) and data from only female and male mussels alone.

Survival of mussels held in the four replicates of the treatment (50% coal fines) and the control (0%) were compared with SAS LIFETEST with replicates designated with the GROUP option in the STRATA statement. Water quality data were statistically analyzed as previously described with Minitab.

RESULTS

Initial Survival Experiment

Water Quality

Conductivity, pH, dissolved oxygen, and temperature in all substrate mixtures did not differ significantly over the course of the initial survival experiment ($P \geq 0.15$, Table 1). Turbidities (NTU) of the 0%, 10%, and 25% coal substrate treatments were not significantly different; however, turbidity of the 50% coal treatment was significantly greater than turbidities of the other treatments ($P < 0.001$, Table 1). The SE of turbidity data from the 50% coal tank (±3.0 NTU) was higher than SE of data from the 0%, 10%, and 25% tanks (±0.1, 0.2, and 0.3, respectively) (Table 1).

Survival

Survival of mussels held in the 0%, 10%, 25%, and 50% coal substrates during the seven-wk pilot study were not significantly different throughout the experiment ($\chi^2 = 4.18$, $df = 3$, $P = 0.243$). Three of the 40 mussels died during the experiment. One mussel from the 50% coal-fines treatment died during wk 2 of the experiment, and one mussel each from the 25% and 50% treatments died during wk 6. No control (0% coal-fines in substrate) mussels died during the experiment.

Substrate Selection

Due to the lack of mortality of mussels in the coal-sand substrate mixtures during the initial survival experiment and

TABLE 2.

Means (±SE) for fractions of gill filament termini without cilia (FGC), digestive gland diverticula cells containing condensed cytoplasm (FDGCC), kidney diverticula cells containing lipofuscin (FKL), acini containing mature and/or developing gametes (FAMD), and acini containing resorbing gametes (FAR).

Time exposed	Dependent variables†									
	FGC		FDGCC		FKL		FAMD		FAR	
	T	C	T	C	T	C	T	C	T	C
56 days (8 wk)	0.38* (0.17)	0.15 (0.03)	0.63* (0.11)	0.16 (0.04)	0.47* (0.08)	0.14 (0.01)	1.00 (0.00)	1.00 (0.00)	0.37* (0.12)	0.04 (0.01)
108 days (16 wk)	0.51* (0.14)	0.11 (0.03)	0.44* (0.09)	0.18 (0.03)	0.65* (0.06)	0.19 (0.01)	0.89 (0.06)	1.00 (0.00)	0.18* (0.08)	0.07 (0.05)
139 days (20 wk)	0.51* (0.06)	0.11 (0.06)	0.63 (0.07)	0.45 (0.10)	0.52* (0.10)	0.27 (0.08)	1.00 (0.00)	1.00 (0.00)	0.30* (0.14)	0.13 (0.13)

* Treatment significantly different from the control ($P < 0.05$).

† Mussels held in 0% coal (control, C) and 50% coal (treatment, T) substrates (n = four replicate tanks for T and C, with five mussels sampled from each tank on sample days).

significantly greater turbidity in the 50% coal treatment, the 50% coal/sand substrate was selected for use in the subsequent histological study. Selection of the 50% sand–coal mixture also was justified by the findings of Wolcott (1990), who found coal depositional zones at mussel survey sites in the Powell River that contained ~50% coal fines.

Histological Effects Experiment

Water Quality

Conductivity did not vary over the course of the histological study ($P = 0.27$, Table 1). Dissolved oxygen in the treatment and control replicates did not differ significantly ($P = 0.76$), and changes in dissolved oxygen in the treatment and control over the course of the experiment were related to temperature ($r = -0.74$, $P < 0.001$). The pH values of the control replicates were significantly higher than in those of the treatment replicates ($P = 0.006$, Table 1), but the difference between means was very small (±0.1, Table 1). Mean turbidity (NTU) of treatment replicates were significantly greater than in those of the sand control ($P < 0.001$, Table 1).

Survival

Survival of mussels held in the zero (sand control) and 50% (treatment) coal substrates did not differ significantly throughout the experiment ($\chi^2 = 5.34$, $df = 6$, $P = 0.501$). Six of the 160 mussels died during the experiment. One mussel from a control tank died during wk 15, and two and three mussels from separate treatment tanks died during wk 15 and 18, respectively. The mortality of the treatment mussels indicates that, if the 20-wk experiment were extended, mussel survival could have become significantly lower in the coal treatment than in the control.

Histological Evaluations

Exposure to coal fines led to histologically detectable changes in mussel tissues.

Gill.

Exposure to coal particles negatively affected abundances of cilia of gill filaments. Fractions of gill filaments without cilia were significantly higher in the coal-exposed mussels compared with control mussels throughout the experiment ($P = 0.0002$, Table 2, compare Fig. 1A, B). There was no significant effect of sample event ($P = 0.96$) or gender ($P = 0.15$) on data of this variable (Tables 2 and 3). During the evaluations, edema, desquamation, fusion, and hyperplasia of gill filament tissues also were observed in some of the coal-exposed mussels (Fig. 1C, D).

Digestive Gland.

During microscopical evaluations, lesions were frequently observed in digestive glands of coal-exposed mussels collected throughout the experiment. The lesions only occurred in digestive cells of diverticula, as opposed to basophilic cells, and were characterized by cytoplasm condensed to finely granulated, coalesced foci that were separated from cell membranes by unstained halos (compare Fig. 1E, F). The fine cytoplasmic granules did not appear to be contained in vacuoles, nor were membranes of vacuoles apparent in the halos. Areas within digestive glands of some coal-exposed mussels contained digestive diverticula that were in the disintegration stage of necrosis (Fig. 2A), and these necrotic areas were frequently contiguous with areas that contained diverticula with condensed cytoplasm in digestive cells (Fig. 2B). Therefore, it is possible that cytoplasmic condensation in digestive cells is a prenecrotic condition that portends necrotic tissue disintegration. For purposes of this manuscript, we summarized this condition with the descriptive phrases ''condensation of cytoplasm'' and ''cytoplasmic condensation'' (R. Smolowitz, Aquatic Diagnostic Laboratory, Department of Biology and Marine Biology, Roger Williams University, Bristol, RI and J. Robertson, Department of Biomedical Sciences and Pathology, VA-Maryland Regional College of Veterinary Medicine, VA Tech, Blacksburg, VA, personal communications). As described in Materials and Methods, we consequently collected data for fractions of digestive cells with condensation of cytoplasm as the dependent variable concerning impacts to digestive glands.

Fractions of digestive cells with cytoplasmic condensation were significantly different between treatment and control mussels ($P = 0.0004$) and sample dates ($P = 0.047$). Fractions of cytoplasmic condensation were higher in coal-exposed mussels than in control mussels on the first (day 56) and second sample (day 108) dates ($P = 0.0008$ and $P = 0.04$, respectively) (Table 2); however, fractions of digestive cells with condensation of cytoplasm in treatment and control mussels were not

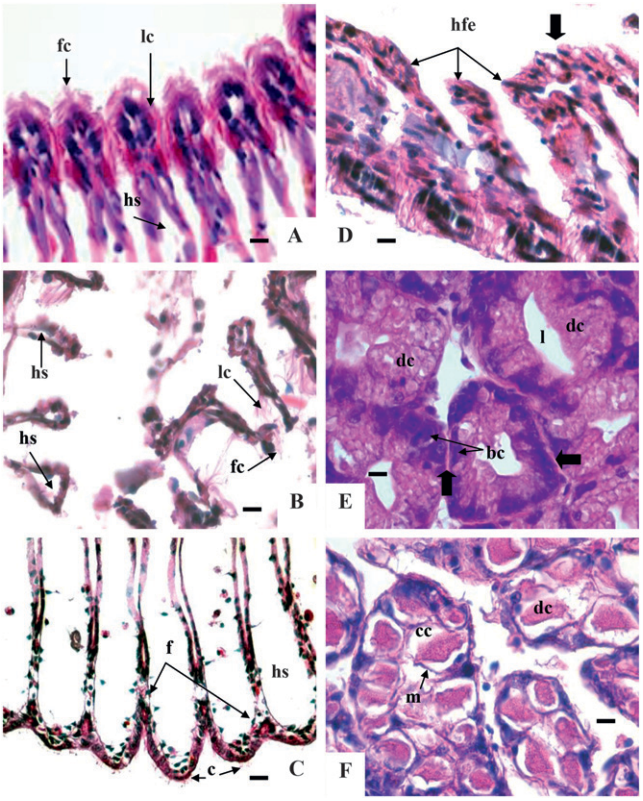

Figure 1. Gill and digestive gland tissues (H & E stain). (A) Normal gill filaments from sand control with frontal (fc) and lateral (lc) cilia, and hemocoelic spaces (hs) (scale bar, 2 μm). (B) Gill filaments from coal treatment with necrotic epithelia of filaments, scarcity and absence or frontal (fc) and lateral cilia (lc), and decrease or absence of hemocoelic spaces (hs) (scale bar, 2 μm). (C) Gill filaments from coal treatment showing fusion (f) and scarcity of cilia (c) of filaments. Note expansion of hemocoelic spaces (hs) associated with edema of filaments (scale bar, 5 μm). (D) Gill filaments from coal treatment showing hyperplasia of filament epithelia (hfe), fusion of filaments (bold arrow), and absence of frontal and lateral cilia (scale bar, 2 μm). (E) Normal digestive diverticula from sand control containing digestive (dc) and basophilic (bc) cells, lumina (l) within diverticula, and basement membranes (bold arrows) of diverticula (scale bar, 2 μm). (F) Digestive diverticula from coal treatment showing condensation of cytoplasm (cc) in digestive cells (dc), with separation of cytoplasm from cell membranes (m) and absence of lumina in diverticula (scale bar, 2 μm).

different at the third sample date (day 139) ($P = 0.19$); this result indicates that there may have been an effect of captivity in digestive glands of the control mussels that began after the second sample date (day 108) of the experiment. There was no significant difference in cytoplasmic condensation between female and male mussels throughout the experiment ($P = 0.45$, Table 3). Desquamation of digestive cells was common in the coal-exposed mussels, with sloughed cells apparent in lumina of diverticula (Fig. 2C). Desquamation was so pervasive in some of the coal-exposed mussels that the ducts of their digestive glands were filled with sloughed cell debris (Fig. 2D).

Kidney.

Fractions of kidney diverticula cells containing lipofuscin in the coal-exposed mussels were significantly higher than in mussels from the sand control on all three sample dates ($P < 0.0001$, Table 2, compare Fig. 2E, F), and there were no effects of sampling event ($P = 0.17$) or gender ($P = 0.62$) (Table 3).

Gonad.

Reproductive acini of almost all female and male mussels from the treatment and control replicates contained mature and/or developing gametes throughout the entire experiment (Tables 2 and 3, Fig. 3A, B); variation in these data was so low that statistical analyses could not be performed. During the first and third tissue sampling events, most of the data were identical (almost all acini contained mature and/or developing gametes regardless of gender status). Lack of variation also was evident in data of fractions of atretic, resorbing gametes in males; only two male mussels (both collected from the coal treatment) contained resorbing gametes in their acini (Table 3, Fig. 3C). Eight indeterminate mussels were observed in the treatment replicates, and 11 were observed in the control replicates (Table 3). Because of the lack of variation in data from evaluations of fractions of acini containing mature and/or developing gametes and acini containing resorbing gametes, SAS GLIMMIX did not produce convergence of models during statistical analyses of data from these variables for datasets that included all genders (female, male, and indeterminates), females versus males, and males alone. Fractions of acini containing resorbing oocytes were significantly higher in coal-exposed females than in control females ($P = 0.03$), and these fractions did not significantly vary by sample date ($P = 0.48$) (Table 3, Fig. 3D).

Other Observation.

Since it was reflective of mussel condition, we note that severe necrosis of vesicular connective tissues was occasionally observed in the coal-exposed mussels, but was not observed in the control mussels (compare Fig. 3E, F).

DISCUSSION

Major organs evaluated during this experiment were affected by presence of coal particles in substrate. Gill lesions observed in tissue sections from the coal-exposed mussels included necrosis, loss of gill cilia, edema, and fusion of filaments. Abundances of lipofuscin in kidney diverticula of the coal-exposed mussels were higher than in kidneys of the control mussels. The majority of females held in coal substrate showed resorption of oocytes. In the most extreme cases, some of the coal-exposed mussels showed severe atrophy of vesicular connective tissues. Digestive cells of digestive diverticula of the coal-exposed mussels showed condensation of cytoplasm, and severe sloughing of digestive gland epithelial tissues was apparent in some of the coal-exposed mussels.

The observed frequencies of tissue pathologies support the hypothesis that reproductive and physiological functions were negatively impacted in mussels exposed to coal in substrate. Although the water quality data obtained during this study do not provide a causative explanation for resorption of oocytes in the coal-exposed females, coal in substrate was associated with increased oocyte resorption. Oocyte resorption can be related to stress due to dietary deficiency and chemical exposure (Bayne & Thompson 1970, Bayne et al. 1981, Tay et al. 2003). The biological significance of increased oocyte resorption is reduced gamete production. In the natural

TABLE 3.

Gender means (±SE) of fractions of gill filament termini without cilia (FGC), digestive gland diverticula cells containing condensed cytoplasm (FDGCC), kidney diverticula cells containing lipofuscin (FKL), acini containing mature and/or developing gametes (FAMD), and acini containing resorbing gametes (FAR)†.

Time exposed	Gender	*n*		Dependent variables									
				FGC*		FDGCC*		FKL*		FAMD		FAR	
		T	C	T	C	T	C	T	C	T	C	T	C
56 days (8 wk)	♀	8	7	0.25 (0.05)	0.17 (0.04)	0.51 (0.06)	0.19 (0.06)	0.46 (0.12)	0.11 (0.0)	1.00 (0.00)	1.00 (0.00)	0.70 (0.07)	0.11 (0.04)
	♂	8	10	0.47 (0.15)	0.16 (0.04)	0.72 (0.10)	0.16 (0.04)	0.39 (0.09)	0.16 (0.02)	1.00 (0.00)	1.00 (0.00)	0.03 (0.03)	0.00 (0.00)
	I‡	4	3	0.49 (0.17)	0.09 (0.01)	0.68 (0.11)	0.11 (0.03)	0.59 (0.16)	0.13 (0.07)	1.00 (0.00)	1.00 (0.00)	0.50 (0.29)	0.00 (0.00)
108 days (16 wk)	♀	7	12	0.43 (0.10)	0.12 (0.05)	0.32 (0.08)	0.15 (0.04)	0.73 (0.05)	0.17 (0.03)	0.85 (0.14)	1.00 (0.00)	0.38 (0.18)	0.12 (0.09)
	♂	13	5	0.55 (0.10)	0.05 (0.02)	0.50 (0.06)	0.30 (0.07)	0.64 (0.05)	0.21 (0.04)	0.92 (0.08)	1.00 (0.00)	0.08 (0.08)	0.00 (0.00)
	I‡	0	3	–	0.17 (0.08)	–	0.14 (0.12)	–	0.19 (0.03)	–	1.00 (0.00)	–	0.00 (0.00)
139 days (20 wk)	♀	6	7	0.55 (0.06)	0.11 (0.05)	0.64 (0.07)	0.46 (0.06)	0.52 (0.16)	0.34 (0.11)	1.00 (0.00)	1.00 (0.00)	0.77 (0.11)	0.33 (0.21)
	♂	10	7	0.47 (0.09)	0.12 (0.07)	0.63 (0.06)	0.37 (0.10)	0.54 (0.13)	0.25 (0.12)	1.00 (0.00)	1.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	I‡	4	5§	0.58 (0.20)	0.08 (0.05)	0.61 (0.12)	0.61 (0.14)	0.29 (0.20)	0.23 (0.12)	–	1.00 (0.00)	–	0.00 (0.00)

* Effect of gender not significant ($P > 0.05$). FAMD and FAR did not converge.
† Mussels held in 0% coal (control, C) and 50% coal (treatment, T) substrates.
‡ I, indeterminate gender. Dash organ not present in histological section.
§ One section with organ tissue inadequately fixed during histological processing.

setting, reduced oocyte production could translate to reduction in subsequent recruitment. Due to funding constraints, the specific chemical contaminants (organic and inorganic) in the coal exposures were not tested, but these contaminants may have resulted in energetic stress that contributed to oocyte resorption.

Impacts on physiological functions of organs are inferred from documentation of organ pathologies. This is especially true in freshwater mussels, whose organ functions have not been adequately described. Gills, digestive glands, kidneys, and intestines of bivalves are responsible for important physiological functions, including respiration, ionic balance, digestion, water regulation, energy substrate storage, and contaminant transformations and detoxification (Seiler & Morse 1988, Dietz et al. 2000, Domouhtsidou & Dimitriadis 2000, Lobo-da-Cunha 2000, McMahon & Bogan 2001, Petrović et al. 2001, Fahrner & Haszprunar 2002). The results of this study show that the structures of the gills and digestive glands were negatively affected in mussels exposed to coal, suggesting disruption of important physiological functions and processes. Disruption of cellular processes due to destruction of parenchymal organelles during desquamation of epithelial tissues of digestive glands and gills is probable.

Depletion of stored energy reserves likely occurred in the coal-exposed mussels. Vesicular connective tissues are important in storage of glycogen, and to a lesser degree, digestive glands also store lipids and glycogen (Lobo-da-Cunha 2000, Petrović et al. 2001, Colville & Lim 2003). Glycogen in vesicular connective tissue cells is sequestered for energetic needs during gametogenesis (Lowe et al. 1982, Pipe 1985). Deteriorations of vesicular tissue cells and digestive glands probably indicate that an energy deficit existed in the coal-exposed females, which may have been linked to resorption of their gametes (Bayne & Thompson 1970, Bayne et al. 1981, Tay et al. 2003).

With the exception of turbidity and pH, water quality measurements were not significantly different between the treatment and control replicate tanks. Differences in pH were very small and were not likely biologically meaningful. Sediment exposure can cause decreased filtration rates, increased selective feeding, increased production of pseudofeces, and subsequent declines in condition in marine and freshwater bivalves (Bricelj & Malouf 1984, Ward & MacDonald 1996, Henley et al. 2000, Ellis et al. 2002). Thus, the greater turbidity in the treatment compared with control tanks may have contributed to nutritional stress in coal-exposed mussels. Higher abundances of lipofuscin in the kidney diverticula of the coal-exposed mussels was probably linked to contaminants that were not quantified during this study due to funding constraints, and these unidentified contaminants probably contributed to histopathological alterations of the tissues in coal-exposed mussels (Lomovasky et al. 2002, Riveros et al. 2002, Dimitriadis et al. 2004, Seehafer & Pearce 2006). Contaminants in coal fines include trace heavy metals, sulfate, and polycyclic aromatic hydrocarbons (Ducatman et al. 2010). Future studies concerning the effects of coal fines in substrate on freshwater mussel organs should include water quality determinations for organic and inorganic contaminants. Given the sublethal effects observed in this study, further experimentation of the effects of coal fines on freshwater mussels is warranted to ensure protection of mussel populations

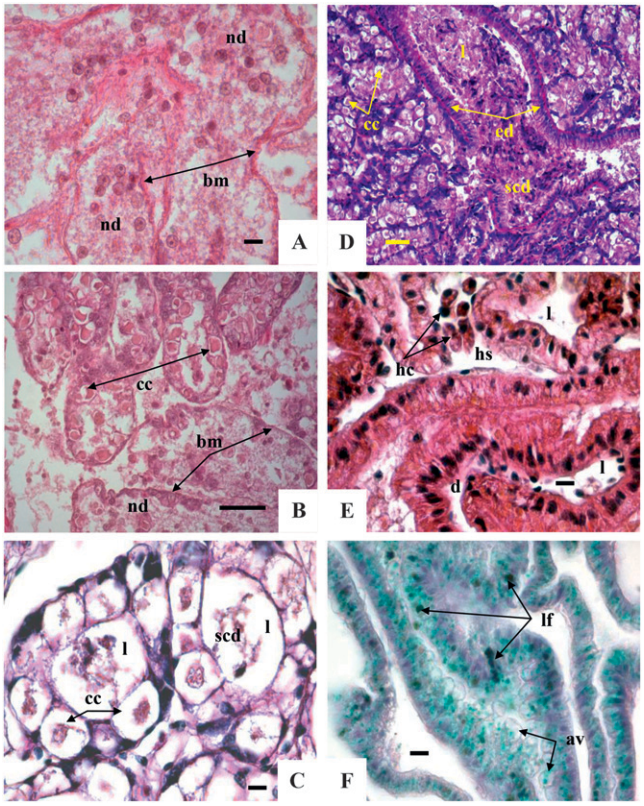

Figure 2. Digestive gland and kidney tissues. (A) Necrosis of digestive gland tissues from coal. Note remnant basement membranes (bm) of necrotic, disintegrated digestive diverticula (nd) (H & E stain; scale bar, 2 μm). (B) Two contiguous lesional regions in digestive gland from coal. Top region contains digestive diverticula with digestive cells showing condensed cytoplasm (cc) and bottom region contains necrotic disintegration of diverticula (nd) among residual basement membranes (bm) (H & E stain; scale bar, 5 μm). (C) Digestive diverticula from coal with cytoplasmic condensation (cc) in digestive cells and presence of sloughed cellular debris (scd) in lumina (l) of diverticula (H & E stain; scale bar, 2 μm). (D) Duct of digestive gland among digestive diverticula from coal. Cytoplasmic condensation (cc) in digestive cells of diverticula and presence of sloughed cellular debris (scd) in lumen (l) of duct. Note epithelium of digestive gland duct (ed) (H & E stain; scale bar, 10 μm). (E) Normal diverticula of kidney from sand control with lumina (l; urinary space) of diverticula (d) and hemocytes (hc) in hemocoelic space (hs) (H & E stain; scale bar, 2 μm). (F) Presence of lipofuscin (lf) in epithelial cells of kidney diverticula from coal treatment. Note excretion of apical vacuoles (av) containing lipofuscin (Perls' Prussian blue stain; scale bar, 2 μm).

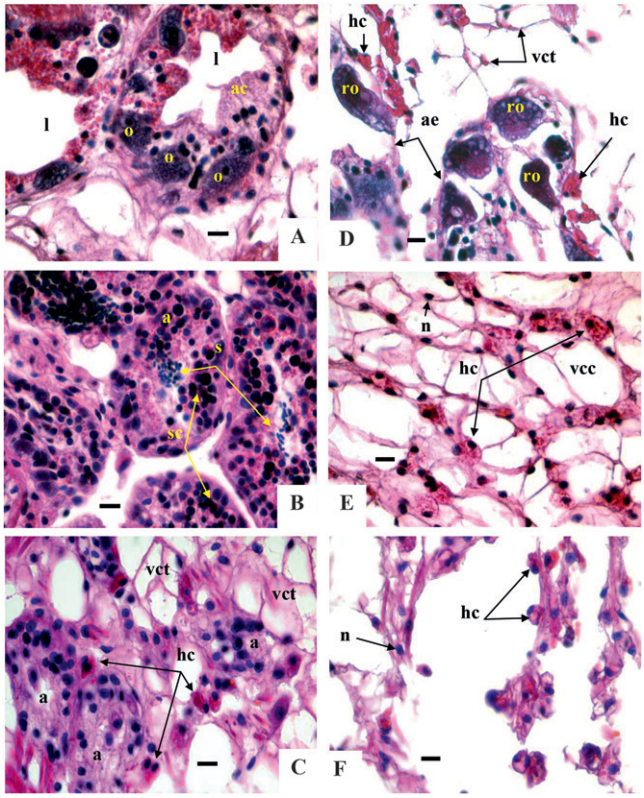

Figure 3. Reproductive acini and vesicular connective tissues (H & E stain). (A) Normal oogenic acini from sand control containing developing oocytes (o) among accessory cells (ac). Note lumina (l) within acini (scale bar, 2 μm). (B) Normal spermatogenic acini (a) from sand control containing spermatocytes (sc) and mature spermatozoa (s) in lumina of acini (scale bar, 2 μm). (C) Resorbing spermatogenic acini (a) within vesicular connective tissue (vct) from coal treatment with adherence of phagocytic hemocytes (hc) to acinar epithelia (scale bar, 2 μm). (D) Atretic, resorbing oocytes (ro) in acini within vesicular connective tissue (vct) from coal treatment. Note deteriorated and basophilic qualities of resorbing oocytes and adherence of phagocytic hemocytes (hc) to atrophic acinar epithelia (ae) (scale bar, 2 μm). (E) Normal vesicular connective tissue from sand control. Note that glycogen in peripherally nucleated (n, nucleus) vesicular connective cells (vcc) has been removed by histological processing for paraffin embedding. Hemocytes (hc) in hemocoelic spaces (scale bar, 2 μm). (F) Necrotic vesicular connective tissues from coal treatment. Note phagocytic hemocytes (hc) attached to remnant nucleated (n, nucleus) vesicular tissues (scale bar, 2 μm).

in watersheds with high densities of coal mining and processing operations.

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