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Species-specific responses of developing anurans to coal combustion wastes

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Abstract

Field surveys and field experiments have previously documented adverse effects of solid byproducts from coal incineration (coal combustion wastes (CCW)) on larval amphibians inhabiting aquatic habitats. However, a definitive link between CCW-exposure and developmental abnormalities has not been established because no studies have addressed the direct effects of prolonged exposure to CCW on larval amphibian development under controlled laboratory conditions. In the laboratory we exposed green frog (*Rana clamitans*) and wood frog (*Rana sylvatica*) larvae to either clean sand or CCW-contaminated sediment to investigate the direct effects of CCW exposure on trace element accumulation, growth, developmental rate, malformations, survival, and metamorphic success. While both species accumulated significant ($P < 0.05$) concentrations of at least six trace elements (As, Cd, Fe, Se, Sr, and V), effects of exposure to CCW varied between species, with *R. clamitans* larvae experiencing more severe effects including a 26% reduction in survival and a 45% reduction in metamorphic success. Furthermore, exposure to CCW decreased growth and developmental rates among larvae of both species that successfully completed metamorphosis. Larval period duration was increased by 10 and 11%, and size at metamorphosis was decreased by 10 and 39% in *R. clamitans* and *R. sylvatica* exposed to CCW, respectively. Rates of malformations were ≤4%, and were not dependent on species or sediment treatment. Our results confirm the direct effects of CCW on aquatic amphibian larvae suggested by previous field studies, and indicate that considerable variation may exist in sensitivity among species exposed to CCW. These findings have important implications for the management of CCW since >50 million t are discharged annually to surface impoundments in the US, which are often used by breeding amphibians.
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1. Introduction

Aquatic disposal of slurried wastes from coal combustion has recently been implicated as a potential threat to the health of amphibian populations.

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Approximately 57 million t of coal combustion wastes (CCW), which contain high concentrations of trace elements including As, Cd, and Se, are discharged into aquatic settling basins in the United States each year (Rowe et al., 2002). Field surveys in one such disposal system located adjacent to the Savannah River in SC, USA, revealed that at least 14 species of anurans utilize the 86 ha site during their respective breeding seasons (Hopkins and Rowe, personal observation). Bullfrog (*Rana catesbeiana*) larvae sampled from the system accumulate high concentrations of trace elements and exhibit developmental abnormalities, increased energy expenditures, and altered swimming performance (Hopkins et al., 2000a,b; Raimondo et al., 1998; Rowe et al., 1996, 1998; Snodgrass et al., 2003). Moreover, transplantations of eggs of the southern toad (*Bufo terrestris*) to the contaminated system suggest that reduced reproductive success or complete reproductive failure may result from amphibians being attracted to CCW-contaminated breeding sites (Rowe et al., 2001).

Although previous field studies provided compelling evidence that CCW adversely affects developing amphibians, clear linkages between CCW and observed effects have been complicated because field surveys are correlative and field experiments are often confounded by site-specific environmental variables (e.g. temperature, food resources, etc.). Laboratory studies, although environmentally unrealistic compared to field studies, can definitively establish cause–effect linkages between contaminants and biological responses. By allowing continuous observations of growth and development throughout the larval period, laboratory studies also provide insight into the responses of individuals that may serve as mechanisms for changes observed at the population-level. To date, only one study has examined the effects of CCW on developing anurans. Birge (1978) demonstrated that leachate from CCW was toxic to amphibian eggs, but the exposures were short (1–2 days) and confounded by low pH (<5.0). Thus, further laboratory experiments are needed to definitively establish a connection between CCW exposure and toxic effects in developing amphibians.

We conducted laboratory studies to evaluate how two species of anurans, green frogs (*Rana clamitans*) and wood frogs (*Rana sylvatica*), with different patterns of larval development respond to CCW. Whereas, *R. sylvatica* has a restricted larval period of ∼40–90 days (Martof, 1970), the larval period of *R. clamitans* is longer and more variable, ranging from ∼70 days to >1 year (Martof, 1956). Based on previous observations from the field, we hypothesized that exposure to CCW would decrease larval growth, developmental rate, and survival, as well as the size and number of successful metamorphs. Thus, our experiments provide insight into the potential mechanism responsible for reproductive failures observed in field studies. Secondly, we hypothesized that *R. clamitans* would experience more severe effects from CCW-exposure due to their relatively protracted larval period compared to *R. sylvatica*. Our second hypothesis has important implications for revealing life history traits that may form the mechanistic basis behind species-specific differences in sensitivity to contaminants.

2. Methods

2.1. Experimental

We exposed larval *R. clamitans* and *R. sylvatica* to CCW-contaminated sediment in randomized block experiments following methods established for benthic feeding fish (Hopkins, 2001; Hopkins et al., 2000a,b, 2002, 2003). Exposures of larval *R. clamitans* and *R. sylvatica* took place from 2 July 2001 to 13 January 2002, and 22 April 2002 to 31 May 2002, respectively. We used commercially available clean sand as a control and obtained weathered CCW from the D-area disposal system on the Savannah River Site, South Carolina. Both of these sediments have exceptionally low organic content (<4%; Hopkins, 2001) and allow us to control food resources in our experiments. The experiments consisted of five (*R. clamitans*) or four (*R. sylvatica*) blocks of 2 l plastic bins, each block containing three replicates of each sediment treatment ($n = 15$ and 12 per sediment treatment for *R. clamitans* and *R. sylvatica*, respectively). Each 2 l bin contained 1.5 l of aged tap water, approximately 1 cm of either clean or CCW-contaminated sediment, and a sponge to allow metamorphosing larvae to leave the water. We placed bins for each experiment on a laboratory bench receiving indirect sunlight through windows so that animals experienced natural photoperiods.

From an uncontaminated wetland in Baltimore County, Maryland, USA, we collected and returned to the laboratory three clutches of *R. clamitans* and *R. sylvatica* eggs on 21 June 2001 and 12 April 2002, respectively. Upon return to the laboratory, we placed egg clutches in separate 6 l aerated plastic bins filled with aged tap water where they were allowed to hatch (Gosner stage 25; Gosner, 1960). At Gosner stage 25, larvae from all three clutches were placed into a common bin of aged tap water to allow clutches to mix. One larva was then arbitrarily selected for placement into each 2 l bin.

During each experiment we changed 1/2 of the water volume and measured temperature and pH in each experimental bin weekly; temperature ($\bar{x} = 24.3\,^{\circ}$C) and pH ($\bar{x} = 7.6$) did not vary significantly ($P > 0.23$) among treatments or experiments. We fed developing larvae ground Tetramin fish food every other day. In both experiments larvae initially received 10 mg per feeding and we increased feeding levels by 5 mg every 1–3 feedings to accommodate larval growth. Adjustments in feeding levels resulted in final feeding levels of 490 mg and 75 mg for the *R. clamitans* and *R. sylvatica* experiments, respectively. Developing larvae remained in the bins until they had completed 2/3 resorption of their tail (Gosner stage 45–46), at which time we placed metamorphosing individuals in ∼600 ml cups with perforated tops and moistened paper towels, where they were held until they completed metamorphosis (i.e., complete tail resorption). Metamorphs were euthenized and frozen at $-40\,^{\circ}$C within 10 h of complete tail resorption. We terminated the *R. clamitans* experiment after 195 days of exposure, when it was apparent that the remaining larvae would not metamorphose until the following spring, if at all. Larval *R. clamitans* alive at Day 195 were euthenized and frozen at $-40\,^{\circ}$C. We terminated the *R. sylvatica* experiment when all larvae had either completed metamorphosis or died.

2.2. Trace element analyses

We determined trace element concentrations in whole bodies of all individuals successfully completing metamorphosis and larvae still alive at the end of the experiments. To document exposure conditions we randomly selected and analyzed trace element (As, Cd, Cu, Fe, Ni, Se, Sr, V and Zn) concentrations in sediments from five contaminated and five control bins at the end of each experiment. Additionally, we collected and analyzed water samples from the randomly selected bins in the *R. sylvatica* experiment.

Water, sediment, and tissue samples were prepared for trace element analysis as follows. We filtered water samples (0.45 μm filter) before acidification with 70% nitric acid. Prior to being digested and analyzed for trace element concentrations we dried sediment samples and lyophilized tissue samples. For sediment samples we digested 150 mg, and for tissue samples ∼75–150 mg. We added nitric acid (2.5 or 5.0 ml, tissue and sediment, respectively) to samples before digestion in a microwave (CEM Corp., Matthews, NC) with heating steps of 60, 60, 70, and 80% microwave power for 10, 10, 15, and 20 min, respectively. Following HNO_3 digestion we added 1.0 ml of H_2O_2 to the samples and microwaved at the same power and duration as the HNO_3 digestion. After digestion we brought samples to a final volume of 10 or 25 ml (tissue and sediment, respectively) with *d*-deionized water.

We performed trace element analysis by ICP–MS (Perkin Elmer, Norwalk, CT) on samples diluted 1:1, and 1:10 with *d*-deionized water (tissue and sediment, respectively). We calibrated the instrument daily using calibration standards covering a range of 1–500 μg/l prepared by serial dilution of NIST traceable primary standards. For quality control purposes, we included certified reference material (Tort 2; NRC, Ottawa, Canada) and blanks in the digestion and analysis procedure. Mean percent recoveries for trace elements in tissue reference material ranged from 84.5 to 107.7%. Coefficients of variation of percent recoveries from tissue reference material replicated among digestion sets ranged from 7.0 to 18.9%.

2.3. Biological responses

We compared developmental rate, size, and survival of control larvae to larvae exposed to CCW. Larvae were inspected every 1–2 days. During inspections we noted the occurrence of malformations, larval deaths and the time at which larvae reached the complete hind limb stage (Gosner stage 39–40), complete front limb stage (Gosner stage 42), and complete metamorphosis. We quantified development rates as the number of days to reach each of these discrete developmental stages. To investigate relationships between the fate of larvae and their growth during early development, we weighed all surviving larvae to the nearest 0.001 g on Days 50 and 20 of the *R. clamitans* and *R. sylvatica* experiments, respectively, when all larvae were in the very early stages of hind limb development (Gosner stage 31–36). We also weighed larvae when they reached the complete hind limb stage, complete front limb stage and after completing metamorphosis, or at the end of the experiment if they had not completed metamorphosis (in the case of *R. clamitans*).

2.4. Statistical analyses

We used a series of MANOVAs to compare trace element concentrations in sediment, water and tissues between treatments. To normalize the data we $\log_{10}$-transformed trace element values before analyses. In the sediment MANOVA model we included experiment and its interaction with sediment type to investigate potential differences in exposure conditions in the two experiments. As a large number (80%) of As and Se values were below detection limits (BDL) in control sand, we did not include these elements in the MANOVA of sediment concentrations. In the MANOVA of water concentrations we only included the main effect of sediment type because no water samples were collected for the *R. clamitans* experiment. As Cd concentrations were BDL in the majority of water samples from control bins, we did not include this element in the MANOVA of water concentrations. In the MANOVA of trace element concentrations in metamorphs we included sediment type, species, and their interaction as effects. As some larvae did not complete metamorphosis before the end of the *R. clamitans* experiment, we conducted a fourth MANOVA of trace element concentrations in this species, which included sediment type, stage (larvae versus metamorphs), and their interaction as effects in the analysis.

For those larvae completing metamorphosis, we used a mixed model approach to repeated measures analysis (PROC MIXED; SAS Institute, Carey, North Carolina) to compare days to stage and weight at stage between sediment treatments. For comparison of days to stage and size at stage we modeled individual larvae as a random effect and the covariance matrix as having compound symmetry; in both cases, a null model likelihood test indicated a significant ($P < 0.001$) model including the covariance matrix parameters. For comparison of size at stage between sediment treatments we included days to stage as a covariate. We included all interaction terms in the initial models, but dropped non-significant ($P > 0.10$) interactions from the final models. We used a *t*-test with corrections for heterogeneity of variance to compare mean final weights of *R. clamitans* larvae surviving but not completing metamorphosis by the end of the experiment (hereafter, referred to as arrested development) between the CCW and control treatments; no such test was conducted for the *R. sylvatica* experiment because all *R. sylvatica* completed metamorphosis or died by the end of the study. All data were tested for normality (Ryan–Joiner test), and when needed, $\log_{10}$-transformations were made before analyses.

To compare survival, percent completing metamorphosis, and the occurrence of malformations between treatments within experiments, and between species in the two experiments, we used a series of Fisher's exact tests because of low expected cells counts (i.e., <5 in 20% or more of the cells). Survivors included larvae that experienced developmental arrest as well as those successfully completing metamorphosis. Within the *R. clamitans* experiment, ANOVA was used to test for a difference in size at Day 50 of the experiment between larvae that ultimately experienced arrested development and those completing metamorphosis; this analysis was not conducted for *R. sylvatica* larvae because none experienced arrested development. One CCW-exposed *R. clamitans* larvae jumped from its bin during a water change and subsequently died; this individual was excluded from all statistical analyses.

3. Results

3.1. Exposure conditions

Concentrations of Cd, Cu, Fe, Ni, Sr, V and Zn were significantly ($F_{7,10} = 454.69$; $P < 0.001$ for the main effect of sediment type in the MANOVA model) higher in CCW-contaminated sediment when compared to clean sand (Table 1). Although As and Se concentrations in sediments were not compared statistically, both elements were orders of magnitude higher

Table 1
Trace element concentrations in sediment (ppm dry mass) and water (ppb) sampled from experiments on *R. clamitans* and *R. sylvatica* exposed to coal combustion wastes (CCW) or sediments from a reference site (control)

Element	Experiment on *R. clamitans*		Experiment on *R. sylvatica*			
	Sediments		Sediments		Water	
	Control	CCW	Control	CCW	Control	CCW
As	BDL	87.93 ± 11.92	0.66 ± 0.13	110.52 ± 2.00	0.92 ± 0.13	34.39 ± 13.20
Cd	0.18 ± 0.01	1.15 ± 0.17	0.08 ± 0.01	1.63 ± 0.05	BDL	BDL
Cu	2.63 ± 0.44	74.88 ± 8.97	2.22 ± 0.34	79.36 ± 1.21	10.04 ± 0.76	6.95 ± 1.51
Fe	1812.00 ± 141.00	37776.00 ± 4409.00	1203.00 ± 161.00	27060.00 ± 1651.00	65.28 ± 6.30	92.68 ± 4.17
Ni	4.80 ± 0.98	71.04 ± 9.87	0.58 ± 0.15	43.03 ± 1.76	0.65 ± 0.10	1.01 ± 0.06
Se	BDL	8.43 ± 1.14	BDL	9.00 ± 0.58	0.88 ± 0.16	44.03 ± 12.46
Sr	2.52 ± 0.13	330.00 ± 54.54	3.30 ± 0.35	292.00 ± 6.57	104.67 ± 13.11	424.70 ± 29.38
V	4.45 ± 0.38	112.35 ± 17.74	5.18 ± 0.67	78.93 ± 1.45	1.38 ± 0.18	24.81 ± 6.51
Zn	7.25 ± 0.88	46.36 ± 5.15	1.86 ± 0.35	156.24 ± 9.33	36.16 ± 10.34	70.60 ± 35.27

in CCW-contaminated sediment compared to clean sand. While differences between CCW-contaminated sediment and clean sand varied significantly ($F_{7,10} = 41.85$; $P < 0.001$ for the interaction term in the MANOVA model) between experiments, there was no tendency for element concentrations to be higher in one experiment than the other. In the *R. clamitans* experiment concentrations of Fe, Ni and V in CCW-contaminated sediment were higher than in the *R. sylvatica* experiment, while the opposite was true for concentrations of Cd and Zn. In water from the *R. sylvatica* experiment, As, Fe, Ni, Se, Sr and V were significantly ($F_{8,1} > 100{,}000$; $P < 0.001$) higher in the bins containing CCW-sediment when compared to controls. However, concentrations of trace elements in water were orders of magnitude lower than in sediment (Table 1).

3.2. Trace element tissue concentrations

Exposure to CCW-contaminated sediment resulted in significant ($F_{9,13} = 581.21$; $P < 0.001$ for the main effect of sediment treatment in the MANOVA model) accumulation of As, Cd, Fe, Se, Sr and V by metamorphs of both *R. clamitans* and *R. sylvatica* (Table 2). However, for some elements the degree of difference between control and exposed metamorphs varied significantly between species ($F_{9,13} = 4.46$; $P = 0.007$ for the interaction term in the model). Exposure of larvae to CCW-contaminated sediment

Table 2
Whole body trace element concentrations (ppm dry mass) in *R. clamitans* larvae and metamorphs and in *R. sylvatica* metamorphs following exposure to either coal combustion wastes (CCW) or control sediments

Element	*R. clamitans* larvae		*R. clamitans* metamorphs		*R. sylvatica* metamorphs	
	Control	CCW	Control	CCW	Control	CCW
As	0.45 ± 0.13	9.90 ± 1.02	0.15 ± 0.01	8.28 ± 0.56	0.19 ± 0.01	13.58 ± 2.62
Cd	1.11 ± 0.27	1.58 ± 0.19	0.54 ± 0.07	0.79 ± 0.20	0.20 ± 0.01	0.28 ± 0.02
Cu	66.47 ± 29.80	67.88 ± 26.36	49.61 ± 8.11	37.26 ± 9.68	15.62 ± 1.20	17.04 ± 1.84
Fe	2283.98 ± 981.87	3785.74 ± 1018.56	209.02 ± 7.35	244.38 ± 24.18	104.32 ± 3.50	263.08 ± 86.17
Ni	3.16 ± 1.00	4.89 ± 1.06	1.06 ± 0.04	2.75 ± 1.65	1.36 ± 0.09	1.62 ± 0.15
Se	1.14 ± 0.14	10.06 ± 0.39	1.44 ± 0.08	20.88 ± 3.43	1.53 ± 0.05	18.92 ± 1.75
Sr	14.26 ± 6.14	60.29 ± 9.46	12.53 ± 0.74	41.84 ± 10.29	10.69 ± 0.44	38.19 ± 4.120
V	3.62 ± 1.41	11.76 ± 1.03	0.34 ± 0.04	4.94 ± 0.38	0.44 ± 0.02	3.99 ± 0.56
Zn	109.69 ± 15.73	153.64 ± 48.67	166.35 ± 10.13	141.46 ± 15.77	117.64 ± 5.51	154.28 ± 16.21

Data are presented as mean ± S.E.

resulted in higher accumulation of V in *R. clamitans* metamorphs when compared to *R. sylvatica* metamorphs (difference between control and treatment of 4.60 and 3.55 ppm for each species, respectively), while the opposite occurred for Fe (difference between control and treatment of 35 and 159 ppm for each species, respectively). Additionally, regardless of sediment treatment, Cd and Cu concentrations were higher among *R. clamitans* metamorphs, while As concentrations were higher in *R. sylvatica* metamorphs. There was little difference in concentrations of Ni and Zn between species or sediment treatments (Table 2).

Larval *R. clamitans* experiencing developmental arrest had significantly ($F_{9,10} = 58.33$; $P < 0.001$ for the main effect of stage in the MANOVA model) higher concentrations of As, Cd, Fe, Ni and V in their tissues compared to conspecifics that completed metamorphosis (Table 2). Furthermore, differences between CCW-exposed and control individuals in concentrations of As and V were greater for larvae experiencing developmental arrest than for metamorphs ($F_{9,10} = 5.81$; $P = 0.005$ for the sediment by stage interaction term in the MANOVA model). In contrast, among CCW-exposed *R. clamitans* Se concentrations were greater than two times higher in metamorphs compared to larvae experiencing arrested development and Sr concentrations were similar among developmental stages. Finally, Cd, Fe and Ni concentrations in larvae experiencing developmental arrest were similar among sediment treatments, but were 2 (Cd and Ni) to ~12 (Fe) times higher in tissues of *R. clamitans* metamorphs compared to larvae (Table 2).

3.3. Malformations

Malformations occurred among *R. clamitans* larvae, but not among *R. sylvatica* larvae. However, only two *R. clamitans* exhibited malformations and there was no dependence of malformation occurrence on sediment treatment ($P > 0.999$) or species ($P = 0.495$). One control and one CCW-exposed *R. clamitans* developed scoliosis on the 43rd and 56th days of the experiment, respectively. Both larvae completed metamorphosis, but the resulting metamorphs exhibited reduced femur lengths and subluxation of the femur at the pelvic girdle in one (control) or both (CCW-exposed) legs.

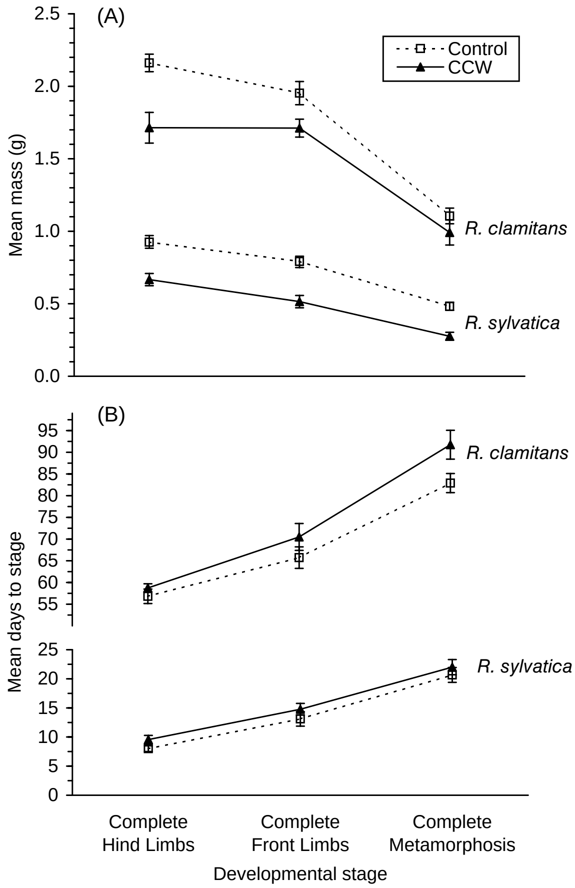

Fig. 1. Mean mass at (A) stage and (B) days to stage of *R. clamitans* and *R. sylvatica* larvae exposed to either clean sand (control) or CCW-contaminated sediment (CCW). Error bars are ±1S.E.

3.4. Size

Among those larvae that completed metamorphosis before the end of the experiments, exposure to CCW-contaminated sediment reduced both *R. clamitans* and *R. sylvatica* size at discrete developmental stages (Fig. 1). There was a significant main effect of sediment type on both *R. clamitans* ($F_{1,13} = 7.27$; $P = 0.035$) and *R. sylvatica* ($F_{1,18} = 6.66$; $P < 0.001$) larval mass at stage. However, for *R. clamitans* there was also a significant dependence of differences in mass on stage and days to stage ($F_{1,13} = 5.91$; $P = 0.023$ and $F_{1,13} = 4.38$; $P = 0.024$ for the interaction between sediment treatment and stage and

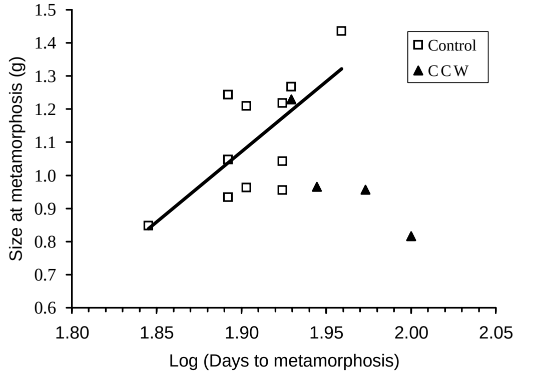

Fig. 2. Relationship between days to metamorphosis and size at metamorphosis for *R. clamitans* larvae exposed to either clean sand (control) or CCW-contaminated sediment (CCW). The line is a simple linear regression for controls ($y = 4.24x - 6.98$; $R^2 = 0.4786$; $P = 0.018$). No line is given for the CCW-exposed metamorphs because the relationship was not significant ($P = 0.113$).

sediment treatment and days to stage, respectively; Fig. 1). Overall, differences in mean mass of control and CCW-exposed *R. clamitans* larvae decreased from the complete hind limb stage through metamorphosis. Additionally, *R. clamitans* larvae exposed to CCW contaminated sediments showed a negative relationship between days to stage and size at stage ($r = -0.97$, -0.64 and -0.89 for complete hind limbs, complete front limbs and metamorphs, respectively), while larvae exposed to clean sand showed a positive relationship ($r = 0.01$, 0.58 and 0.69 for complete hind limbs, complete front limbs and metamorphs, respectively; Fig. 2). Among *R. clamitans* larvae that experienced arrested development, large differences in size developed between CCW-exposed and control treatments by the end of the experiment. On Day 195, when the *R. clamitans* experiment was terminated, CCW-exposed larvae that underwent arrested development were only 52% of the mean size of control larvae ($P = 0.006$; Fig. 3).

3.5. Developmental rate

The response of larval developmental rates to CCW-contaminated sediment exposure also varied by species. While *R. sylvatica* larvae that competed metamorphosis took longer on average to reach specific developmental stages when exposed to CCW-contaminated sediments, there was no significant ($F_{1,18} = 1.88$; $P = 0.188$) effect of exposure on days to specific stages (Fig. 1). In contrast, differences between control and CCW-exposed *R. clamitans* larvae were marginally significant ($F_{1,13} = 4.52$; $P = 0.053$), and the difference between mean days to stage between control and exposed *R. clamitans* larvae increased from the complete hind limb stage through metamorphosis (Fig. 1).

3.6. Survival

Among *R. clamitans* larvae, exposure to CCW also reduced survival ($P = 0.035$) and the percent of larvae successfully completing metamorphosis ($P = 0.018$) before the end of the experiment (Fig. 4). Among *R. clamitans* larvae that died the average time-to-death was 78 days (range 46–105 days), and four out of

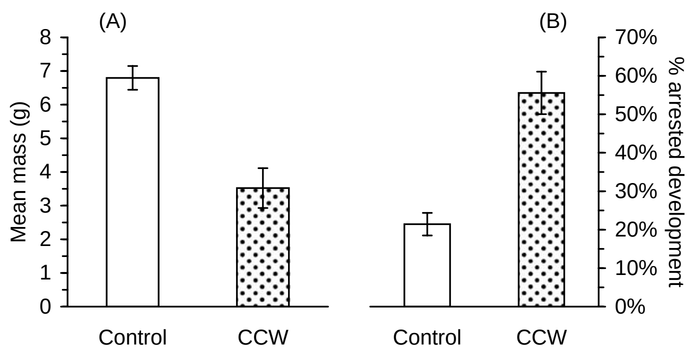

Fig. 3. (A) Mean mass of *Rana clamitans* larvae that experienced arrested development when exposed to clean sand or CCW-contaminated sediment, and (B) percent of surviving larvae that experienced arrested development. Error bar are ±1S.E.

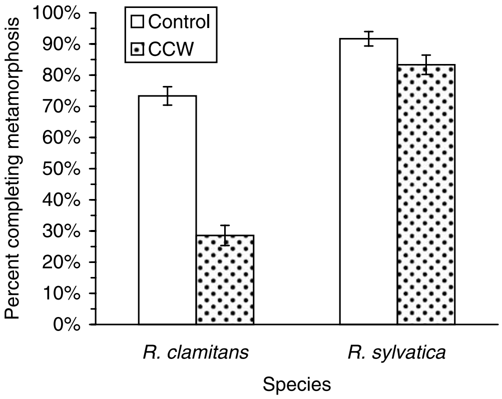

Fig. 4. Percent of *R. clamitans* and *R. sylvatica* larvae exposed to clean sand (control) or CCW-contaminated sediment (CCW) that completed metamorphosis before the end of a 195- or 39-day exposure period, respectively. Error bars are ±1S.E.

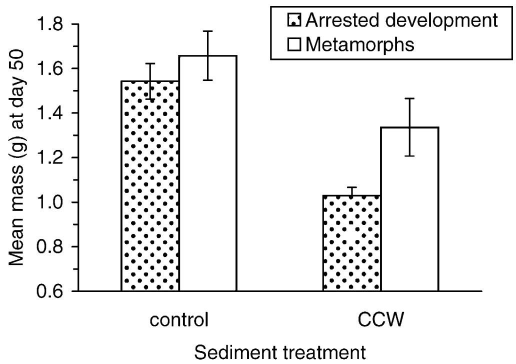

Fig. 5. Mean mass of *R. clamitans* larvae after 50 days of exposure to clean sand or CCW-contaminated sediments. Larvae experiencing arrested development survived but did not complete metamorphosis before the end of the 195-day exposure period, while metamorphs completed metamorphosis before the end of the exposure period. Error bars are ±1S.E.

five completed hind limb development before dying. Larval *R. clamitans* that completed metamorphosis before the end of the experiment did so during a discrete period between the 70th (10 September 2002) and 100th (10 October 2002) day of the experiment. In contrast, most *R. sylvatica* larvae completed metamorphosis in both the control and CCW-exposed groups, and there was no significant dependence of survival ($P > 0.999$) or percent completing metamorphosis ($P > 0.999$) on sediment treatment. There was a significant ($P = 0.021$) difference in percent of CCW-exposed larvae completing metamorphosis between *R. clamitans* and *R. sylvatica*. Finally, among *R. clamitans*, those CCW-exposed individuals that experienced arrested development were significantly ($P = 0.039$) smaller at Day 50 of the experiment than those CCW-exposed individuals that completed metamorphosis (Fig. 5).

4. Discussion

Anuran larvae exposed to CCW in the laboratory accumulated significant concentrations of trace elements and exhibited a variety of adverse effects, but responses differed between the two Ranid species. Adverse effects of CCW-exposure were generally worse in *R. clamitans* than in *R. sylvatica*, a finding that is consistent with our predictions based upon the duration of larval exposure to CCW. As *Rana clamitans* has a longer and more variable larval period than *R. sylvatica*, it is possible that longer exposure to CCW results in the manifestation of more pronounced adverse effects. The fact that the first CCW-exposed *R. clamitans* larva did not die until the 46th day of the experiment, well beyond the time required by *R. sylvatica* to complete metamorphosis under our experimental conditions, also supports this hypothesis. Our study is one of the few to provide a potential mechanistic explanation for differences in sensitivity of amphibians based upon life history characteristics of closely related species. However, variation in species sensitivity could also relate to a variety of other factors including inherent phylogenetic differences in physiology similar to inter-species (and even inter-population) variation described by Bridges and Semlitsch (2000).

Regardless of the underlying mechanisms behind the differences in species responses, our findings support previous field studies that suggested power plant activities threaten the health of amphibians that breed in aquatic CCW-disposal systems. More importantly, our laboratory studies establish the first definitive relationship between prolonged CCW-exposure and adverse effects in amphibians. Such findings have important implications for the management of CCW since >50 million t are discharged annually to surface impoundments in the US, which are often used by breeding amphibians.

4.1. Trace element concentrations

We focused our characterization of sediments, water, and tissues on inorganic contaminants because much previous work suggests that these constituents are the likely cause of most direct toxic effects of CCW (for review see Rowe et al., 2002). Most organic contaminants are typically volatilized during the combustion process, and the organics that remain are believed to be tightly bound to the fine ash particles making them unavailable to most organisms. However, we cannot eliminate the possibility that residual organic contaminants contribute to observed responses. Thus, biological responses should be considered a result of exposure to the complex mixture of contaminants as a whole, rather than attributing causation to individual components of CCW.

Both species exposed to CCW accumulated As, Cd, Fe, Se, Sr, and V in their tissues as larvae, and retained significant concentrations of these elements through metamorphosis. Whole body concentrations of Fe, Se, Sr, and V were similar in metamorphs of both species, but mean Cd concentrations were higher in *R. clamitans* and mean As concentrations were higher in *R. sylvatica*. Additionally, differences between concentrations of Fe in controls and CCW-exposed metamorphs were higher in *R. sylvatica* compared to *R. clamitans*, while the opposite was true for V. The simplest explanation for these species-specific differences in accumulation relates to different exposure conditions in the two experiments. However, exposure to higher sediment concentrations of an element only coincided with higher tissue burdens of that element for As and V. Species-specific differences in tissue concentrations of other elements may be related to exposure durations or phylogenetic differences in physiology (as discussed above).

Our comparisons of body burdens between larvae and metamorphs of *R. clamitans* are consistent with a recent study that examined patterns of trace element retention through metamorphosis in *R. catesbeiana* (Snodgrass et al., 2003), and support the idea that body burdens of Se and Sr accumulated during the larval period are retained through metamorphosis and can potentially be transferred to terrestrial food webs. Specifically, tissue concentrations of most elements in *R. clamitans* decreased following metamorphosis. However, concentrations of Se increased following tail resorption and Sr concentrations did not vary significantly between larvae experiencing developmental arrest and metamorphs. Because of the extensive changes in morphology and physiology of anurans during metamorphosis, many elements may be lost during reconstruction of organ systems. Remodeling of the intestines may be particularly important because some elements accumulate in the larval intestine to a greater extent than in other body parts or organs of larvae (Burger and Snodgrass, 1998, 2001). Selenium and strontium, which do not partition preferentially with the gut (Burger and Snodgrass, 1998, 2001), are retained through metamorphosis. Retention of Se and Sr through metamorphosis is likely a result of their analogous relationships with sulfur and calcium, respectively, and resultant retention within proteins and bones of the newly formed metamorph (Snodgrass et al., 2003). The fact that Se concentrations increased following metamorphosis suggests that Se was retained to a greater extent than Sr. In fact, much of the increase in Se concentrations in metamorphs can be explained by mass loss through metamorphosis, indicating very little loss of Se during morphological reorganization. On average, *R. clamitans* lost 43% of their body mass during metamorphosis and Se concentrations were 2.0 times higher in metamorphs when compared to larvae experiencing developmental arrest. Our mechanistic explanation for trace element dynamics is not only important from a toxicokinetic perspective, but also has important implications for the health of metamorphs (i.e., since some toxic elements are retained while others are eliminated) and can provide insight into the mechanisms by which contaminants are transported from aquatic to terrestrial food webs.

4.2. Biological responses

Accumulation of trace elements was associated with decreased larval growth and developmental rate. Of the individuals that eventually metamorphosed in both species, controls were larger than CCW-exposed individuals at all developmental stages. For *R. sylvatica*, the difference in body size between controls and CCW-exposed individuals was consistent across developmental stages. However, for *R. clamitans* the difference in larval body size between sediment treatments decreased as development progressed.

Ultimately, the slower growth of *R. sylvatica* and *R. clamitans* exposed to CCW resulted in metamorphs that were >40 and 10% smaller than control metamorphs, respectively. On average, control larvae of both species also developed faster than CCW-exposed larvae, but the difference between treatments was minimal (<2 days at each stage) for *R. sylvatica*. In contrast, the difference in time required to attain specific developmental stages increased for CCW-exposed *R. clamitans* at advanced stages (~9 days at metamorphosis).

Although most (~90%) *R. sylvatica* successfully metamorphosed regardless of sediment treatment, some *R. clamitans* in both treatments underwent developmental arrest and did not metamorphose during the 195 day exposure period. Unlike the larval period of *R. sylvatica*, the larval period of *R. clamitans* is relatively plastic; depending on environmental conditions individuals may arrest development and over-winter in the wetland rather than emerging from the water in the fall (Martof, 1956). The percent of surviving *R. clamitans* experiencing arrested development during the study was approximately 2.5 times higher in the CCW treatment compared to controls, suggesting that CCW-exposure promoted arrested development. A possible explanation for CCW-induced changes in development may relate to the Wilbur–Collins model of amphibian development, which suggests that some amphibians must reach a minimum size in order to initiate metamorphosis (Wilbur and Collins, 1973). Once this minimum size is reached, the time and size at which an individual initiates metamorphosis is believed to be plastic and largely depends upon environmental conditions (Wilbur and Collins, 1973). Thus, environmental factors that influence the time at which this critical minimum size is attained should alter the timing of metamorphosis (Denver, 1997). Our findings for *R. clamitans* appear to support this contention; early (Day 50) in the experiment within both treatment groups, individuals that eventually underwent developmental arrest were smaller than individuals that would eventually metamorphose (Fig. 4). Exposure to CCW decreased early larval growth by 32% at Day 50 (Fig. 4), perhaps resulting in fewer individuals reaching the critical minimum size for eventual metamorphosis in the fall. Moreover, CCW-exposed larvae experiencing developmental arrest continued to grow slowly for the remainder of the study; control larvae experiencing developmental arrest were almost twice as large as CCW-exposed larvae by Day 195 (Fig. 3).

Based on previous studies of various amphibians, decreases in growth and developmental rate in CCW-exposed amphibians could have a number of adverse consequences. For some amphibian larvae, decreased growth can translate to increased vulnerability to gape-limited predators, competitive disadvantages against larger individuals, increased time to metamorphosis, and decreased size at metamorphosis (Caldwell et al., 1980; Harris, 1999; Semlitsch and Caldwell, 1982; Semlitsch and Gibbons, 1988; Semlitsch, 1990; Wilbur and Collins, 1973). Decreased developmental rate of larvae can have additional consequences including increased risk of desiccation in temporary wetlands and altered timing of recruitment to the terrestrial environment (Denver, 1997). At metamorphosis, decreased size has important implications for lifetime reproductive output for some amphibians since larger metamorphs are more likely to survive to first reproduction, achieve first reproduction faster, attain larger body size at first reproduction, and have higher adult growth rates than smaller metamorphs (Berven, 1990; Scott, 1994; Semlitsch et al., 1988; Smith, 1987).

Finally, the percentage of CCW-exposed individuals that metamorphosed during the experiments was significantly reduced compared to controls for *R. clamitans*, but not for *R. sylvatica*. This finding is particularly important since successful metamorphosis ultimately determines the number of larvae recruited from the aquatic to the terrestrial environment and, therefore, directly influences the number of individuals that can potentially contribute to population-level processes (i.e., ultimately reproduce). Rowe et al. (2001) found that 100% of *B. terrestris* offspring transplanted to a CCW-contaminated site died prior to metamorphosis. Failed recruitment by *B. terrestris* in the field study may have been due to direct toxicity of CCW, but may have also been attributable to the indirect effects of CCW on other factors such as food resource availability and competition. In contrast, our experimental design enabled us to isolate direct effects of CCW from indirect effects and suggested that for one amphibian species, *R. clamitans*, CCW is directly toxic to developing larvae. Under natural conditions, however, CCW reduces the quantity and quality of food resources (Cherry et al., 1979; Hopkins, 2001; Rowe et al., 2001), resulting in more severe toxicological effects than in laboratory sediment bioassays where ample, uncontaminated resources are supplied (Hopkins, 2001; Hopkins et al., 2000a,b, 2002, 2003). Thus, because important trophic interactions are removed in our laboratory bioassays, our results should be viewed as conservative estimates of CCW toxicity to *R. clamitans* and *R. sylvatica*.

Our results, in combination with the findings of Rowe et al. (2001), suggest that some species of anurans that breed in CCW-contaminated systems exhibit reduced reproductive success due to high mortality among developing larvae. Depending on the extent of reproductive failure, such reductions in recruitment could influence local amphibian population dynamics, as well as the ecology of nearby terrestrial habitats (Rowe et al., 2001). Future studies are needed to ascertain which amphibian species are most sensitive to CCW-exposure, and whether CCW collected from multiple aquatic disposal sites (and thus having different toxicological characteristics) exert similar effects on developing amphibians. Studies that include species with contrasting developmental characteristics (e.g., length of larval period, developmental plasticity, etc.), may be particularly important for identifying the underlying sources of variability in species sensitivity.

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