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INFLUENCE OF LARVAL PERIOD ON RESPONSES OF OVERWINTERING GREEN FROG (*RANA CLAMITANS*) LARVAE EXPOSED TO CONTAMINATED SEDIMENTS

JOEL W. SNODGRASS,*† WILLIAM A. HOPKINS,‡ BRIAN P. JACKSON,‡ JENNIFER A. BAIONNO,‡ and JEFFROY BROUGHTON†
†Department of Biological Sciences, Towson University, Towson, Maryland 21252, USA
‡University of Georgia, Savannah River Ecology Laboratory, Aiken, South Carolina 29802, USA

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Abstract—Pond-breeding amphibians exhibit large intra- and interspecific differences in the duration of the aquatic larval phase. In contaminated aquatic environments, a prolonged larval phase means prolonged exposure to pollutants and, potentially, more severe toxic effects. In the laboratory, we tested this hypothesis by exposing green frog larvae (*Rana clamitans*) to commercial clean sand (control), sediment from an abandoned surface mine (mine), or sediment contaminated with coal combustion waste (CCW). By collecting eggs late in the breeding season, we obligated larvae to overwinter and spend a protracted amount of time exposed to contaminated sediments. The experiment was continued until all larvae either successfully completed metamorphosis or died (301 d). Larvae exposed to mine sediments accumulated significant levels of Pb and Zn, whereas larvae exposed to CCW-contaminated sediment accumulated significant levels of As, Se, Sr, and V. Larvae exposed to mine sediments suffered sublethal effects in the form of reduced growth and size at metamorphosis, but the proportion of larvae successfully completing metamorphosis (93%) was the same for both control and mine treatments. In contrast, larvae exposed to CCW-contaminated sediment suffered greatly reduced survival (13%) compared to both control and mine treatments. Moreover, among larvae in the CCW treatment, the majority of mortality occurred during the latter part the overwintering period (after day 205), corresponding to the onset of metamorphosis in the controls. Our results suggest that the length of the larval period may be one of many life-history or ecological characteristics that influence the sensitivity of aquatic breeding amphibians to environmental pollutants.

Keywords—Amphibians Frogs Metals Metalloids Larval development

INTRODUCTION

Overwintering is an inherently stressful period in the life history of many amphibian species. In North America, larvae of some anuran species (specifically Ranids [1]) are plastic, with the tendency to overwinter varying based on date of oviposition and environmental conditions. As a generalization, eggs of Ranids with long larval periods that are deposited late during the breeding season in moderate northern latitudes are more likely to develop into larvae that overwinter than are conspecifics deposited earlier [1,2]. As with most life-history strategies, the ‘‘decision’’ to overwinter is a trade-off. The overwintering environment experienced by larvae typically is characterized by cooler temperatures and reduced food resources (relative to warmer months), resulting in temperature-dependent reductions in metabolism and an increased reliance on stored energy to fuel metabolism. However, the longer larval periods of overwintering larvae also afford the benefit of metamorphosing in the spring, when terrestrial resources typically are most abundant, and at larger body sizes compared to nonoverwintering conspecifics [2,3].

Recently, it was suggested that the long larval period characteristic of overwintering species might result in enhanced sensitivity to human perturbations of aquatic habitats. Specifically, Snodgrass et al. [4] hypothesized that species with long larval periods might be more sensitive to the effects of environmental contaminants than closely related species with shorter larval periods. Their hypothesis was based on experimental results that demonstrated larvae of green frogs (*Rana clamitans*), a species with a protracted and variable larval period (70 d to greater than one year [5]), were more sensitive to exposure to contaminated sediments than were larvae of wood frogs (*R. sylvatica*), a species with a shorter larval period (~40–90 d [6]). Whereas larvae of *R. sylvatica* experienced reductions in growth after exposure to contaminants, *R. clamitans* larvae experienced reduced growth, developmental rate, and metamorphic success compared to unexposed conspecifics. Reduction in metamorphic success among *R. clamitans* was the combined result of reduced survival and high incidence of arrested development, a trait that is characteristic of larvae destined to overwinter. The poor body condition of *R. clamitans* experiencing arrested development led Snodgrass et al. [4] to hypothesize that overwintering larvae eventually would either die during the stressful overwintering period or emerge in poor condition during the spring, but that study did not track development for the full overwintering period.

The present study was designed to determine how a species with a long larval period (*R. clamitans*) responds to exposure to sediments containing complex mixtures of metals and metalloids. To manipulate the larval period, we collected eggs of *R. clamitans* late in the breeding season, when resultant larvae typically are obligated to overwinter in the aquatic habitat [2]. We exposed overwintering larvae in the laboratory to sediments from two metal- and metalloid-contaminated sites for the full 301-d larval period, and we examined contaminant bioaccumulation and effects on early larval growth, development, metamorphic traits, and survival. In addition, we compared the present results to those of our previous study [4] to draw inferences regarding the relationship between larval period and successful recruitment to the terrestrial environment.

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* To whom correspondence may be addressed (jsnodgrass@towson.edu).

MATERIALS AND METHODS

We exposed *R. clamitans* larvae to sediments from two contaminated sites: A coal combustion waste settling basin (CCW) downstream from a power plant located on the Savannah River Site (SC, USA) and an abandoned surface mine (mine) at Oregon Ridge Park in Baltimore County (MD, USA). Both sites are used extensively by pond-breeding amphibians; at least 19 and 8 species of amphibians use the CCW and mine sites, respectively. The CCW sediments contain high concentrations of a number of trace elements, including As, Cd, and Se, and they have been associated with sublethal and lethal effects among anuran larvae in both field and laboratory studies (see, e.g., [4,7]). A detailed site description and review of studies at the power plant are provided in Rowe et al. [7]. The surface mine is a depression (depth, ~10 m) that creates an ephemeral wetland in the bottom of a mine pit. During wet periods, approximately 250 m^2 of water, with a maximum depth of approximately 1 m, can be found in the abandoned mine pit. Iron mining operations were conducted for approximately 25 years at the site and ended in early 1900. Between approximately 1900 and 1962, lands surrounding the mine pit were under agriculture use. Since the establishment of Oregon Ridge Park by Baltimore County in 1962, forests have been allowed to recover in most of the surrounding areas.

Laboratory exposures

We exposed larval *R. clamitans* to CCW, mine, and control sediments in a randomized block design following the protocols used in previous experiments [4]. Briefly, we used commercially available clean sand as a control. Larvae were housed individually in 2-L plastic bins containing 1.5 L of aged tap water and approximately 1 cm of control, CCW, or mine sediment. Bins were arranged in 15 blocks of three bins (one of each sediment treatment, $n = 15$ for each treatment) on three shelves in the aquatic research laboratory at Towson University (Towson, MD, USA). On a weekly basis, we adjusted light-dark cycles to mimic sunrise and sunset times in Baltimore (MD, USA). We changed half the water volume and recorded both temperature and pH on a weekly basis throughout the experiment. Mean temperature (23.2°C) did not differ significantly among sediment treatments (analysis of variance [ANOVA], $p > 0.813$). Although pH was significantly (ANOVA, $p = 0.009$) lower in bins containing mine sediments compared to the other two sediment treatments, all treatments had circumneutral pH ($\bar{x} = 7.01$, 7.75, and 7.80 for mine, control, and CCW treatments, respectively).

On July 23, 2002, we collected three *R. clamitans* egg masses from an uncontaminated wetland in Baltimore County and transported them to the laboratory. Field observations suggest that *R. clamitans* larvae developing from eggs deposited after July 20, 2002, in Maryland and Virginia (USA) are obligated to overwinter [2]. We housed eggs in 26.5-L plastic bins containing aerated, aged tap water until they hatched (Gosner stage 25 [8]). On July 30, 2002, we placed all larvae of Gosner stage 25 into a common bin and allowed them to mix thoroughly before arbitrarily selecting 45 individuals for assignment to individual 2-L treatment bins. During the experiment, we fed larvae ground Tetramin® (Blacksburg, VA, USA) fish food every other day. Larvae initially received 10 mg of food per feeding, and we increased food levels by 5 mg every one to three feedings to accommodate larval growth. The final feeding level was 520 mg. We checked bins daily and noted any malformations. To measure growth during early development, we weighed larvae to the nearest milligram on days 61, 128, and 162. Measures ceased at day 162, because some individuals began to show signs of front-limb development. We weighed larvae by blotting excess water from them while holding them in a dipnet and then transferring them to a preweighed beaker of water from their bin. Our weighing procedure kept us from having to directly handle or anesthetize larvae. Because recently hatched larvae are delicate, we did not obtain initial masses on experimental subjects. Instead, we estimated mean initial mass of larvae entering the experiment by blotting dry 20 additional larvae (not assigned to bins) and weighing them to the nearest milligram. Late in development, we placed a small piece of floating sponge into bins to allow metamorphs to leave the water. Upon front-limb emergence, we weighed individuals to the nearest milligram and placed them in 600-ml plastic cups with perforated lids and moistened paper towels until they completed tail resorption (Gosner stage 46). On completion of metamorphosis, individuals were weighed, killed, and stored at −40°C for future element analyses.

Element analyses

To document exposure conditions, we analyzed element (As, Cd, Cu, Fe, Ni, Pb, Se, Sr, V, and Zn) concentrations in sediments from five randomly selected exposure bins at the end of the experiment. Water samples were not analyzed, although our previous results with similar systems and sediments [4,9,10] suggest that dissolved concentrations of elements of toxicological concern likely were less than 0.5 ppm in water from treatments containing CCW sediments. To determine which elements were bioaccumulated, we determined element concentrations in whole bodies of all individuals that completed metamorphosis successfully.

Sediment and tissue samples were prepared for element analysis as follows: Before being digested and analyzed for element concentrations, we dried sediment samples and lyophilized tissues. We digested approximately 75 to 150 mg of tissue and sediment by first adding trace metal–grade nitric acid (HNO_3, 2.5–5.0 ml) to samples before digestion in a microwave oven (MDS 2000; CEM, Matthews, NC, USA), 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 hydrogen peroxide (H_2O_2) to the samples and microwaved them at the same power and duration as the HNO_3 digestion. After digestion, we brought samples to a final volume of 10 ml with deionized water.

We performed element analysis by inductively coupled plasma mass spectrometry (Perkin-Elmer, Norwalk, CT, USA) on samples diluted 1:10 with deionized water. We calibrated the instrument daily using calibration standards covering a range of 1 to 500 μg/L prepared by serial dilution of National Institute of Standards and Technology (Gaithersburg, MD, USA) traceable primary standards. For quality control, we included certified reference material (Tort 2; National Research Council of Canada, Ottawa, ON, Canada) and blanks in the digestion and analysis procedure. Mean percentage recoveries for elements in tissue reference material ranged from 94 to 114% with the exception of Ni and Pb, which averaged 127 and 79%, respectively. Coefficients of variation for percentage recoveries from tissue reference material replicated among digestion sets ranged from 4 to 12% with the exception of Ni and Pb, which averaged 54 and 24%, respectively.

Table 1. Element concentrations in sediment (μg/g dry mass) sampled from experimental chambers used to expose *Rana clamitans* to various sediment types[a]

	Sediment treatment		
Element	Clean sand	Coal combustion waste	Mine
As	BDL[b]	107.66 ± 3.69	4.66 ± 0.19
Cd	BDL	0.19 ± 0.02	0.84 ± 0.05
Cu	2.40 ± 0.32	72.79 ± 2.71	57.04 ± 1.57
Fe	630 ± 64	20,655 ± 1387	24,270 ± 901
Ni	0.28 ± 0.08	34.68 ± 1.54	27.97 ± 0.72
Pb	0.87 ± 0.18	24.54 ± 0.63	85.72 ± 2.12
Se	BDL	9.75 ± 0.54	3.76 ± 0.21
Sr	3.53 ± 0.62	246.28 ± 10.98	44.95 ± 4.24
V	2.25 ± 0.34	69.23 ± 1.70	73.49 ± 3.30
Zn	5.21 ± 1.20	47.20 ± 3.60	591.85 ± 23.97

[a] Data are presented as the means ± standard error (n = 5). Mean detection limits for As, Cd, and Se were 0.31, 0.06, and 0.38 μg/g dry mass, respectively.

[b] BDL = below detection limit.

Statistical analyses

We used a series of three multivariate ANOVAs to compare element concentrations in sediment and tissues among treatments. To normalize the data, we $\log_{10}$-transformed element values before analyses. Because As, Cd, and Se were less than the limit of detection in control sediment, we compared concentrations of these three elements between CCW and mine sediments only; we compared concentrations of the remaining seven elements among all three sediment types. Element concentrations of metamorphs from all three sediment types were compared in a single multivariate model.

To compare growth rates over the first 162 d of the experiment, we used a mixed-model approach to repeated-measures ANOVA, with sediment type and time as independent variables and wet mass as the dependent variable. We used the proc mixed procedure of SAS (SAS Institute, Cary, NC, USA) with larva included as a random effect and unconstrained covariance structure; examination of fit statistics indicated that compared to other models (compound symmetry and variance components alone), a model including unconstrained covariance structure provided the best fit and was significantly better than a model including no covariance parameters (null model likelihood ratio test, $\chi^2 = 95.39$, $p < 0.001$). We $\log_{10}$-transformed wet mass data to meet the normality assumptions of the model.

To compare days to metamorphosis and size at metamorphosis among sediment types, we used a mixed-model approach to ANOVA and analysis of covariance, respectively, as generally outlined above. For analysis of size at metamorphosis, we included days to metamorphosis as a covariate in the mixed model and the interactions of days to metamorphosis with sediment treatment. Because a Ryan–Joiner test indicated that sizes at metamorphosis were normally distributed, we did not transform this variable before analysis. However, days to metamorphosis was $\log_{10}$-transformed to meet the normality and linearity (in the case of analysis of covariance) assumptions of the models.

To compare the final percentage survival among sediment treatments within this experiment, we used an exact likelihood ratio chi-square test, because 50% of our contingency table cells had expected frequencies of less than five. We also used exact likelihood ratio chi-square tests to compare percentage survival of larvae exposed to CCW sediments between the present experiment and our previous experiment, which was terminated before the end of the larval period (195 d) [4]. When comparing survival among experiments, we conducted two tests: One comparing survival to 195 d in each experiment, and one comparing overall survival to the end of each experiment (i.e., 195 and 301 d).

RESULTS

Exposure conditions

Concentrations of all 10 elements were higher in CCW and mine sediments compared to controls ($F_{14,14} = 239.27$; $p < 0.001$) (Table 1), but the two contaminated sediment types differed significantly from one another in elemental composition ($F_{3,6} = 5{,}255.82$, $p < 0.001$). Concentrations of As, Sr, and Se were 20-, 5-, and 2-fold higher in CCW sediments compared to mine sediments (Table 1). In contrast, concentrations of Cd, Pb, and Zn in mine sediments were 3- to 10-fold the concentrations found in CCW sediments and up to 100-fold the levels found in control sediments. Concentrations of Cu, Fe, Ni, and V were comparable in CCW and mine sediments but 25- to 100-fold the concentrations found in control sand (Table 1).

Bioaccumulation

With the exception of Cd, larval exposure to the three sediment types resulted in significant differences ($F_{20,36} = 110.68$, $p < 0.001$) in element concentrations in metamorphs. Larvae exposed to sediments from the mine accumulated higher concentrations of Fe, Ni, Pb, and Zn compared to individuals reared on the other sediment types (Table 2). Of these, concentrations of Zn and Pb were most notable in metamorphs emerging from the mine treatment, reaching 2- to 13-fold the concentrations of controls. Concentrations of As, Se, Sr, and V were 2- to 35-fold higher in metamorphs from the CCW treatment compared to metamorphs from the control and mine treatments (Table 2). Two elements, Cu and Zn, were lower in metamorphs raised on mine sediments (Cu) and CCW sediments (Zn) compared to controls.

Biological responses

We observed no developmental malformations in any sediment treatment, but growth rates were affected by exposure to contaminated sediments. Growth differed significantly ($F_{2,39} = 43.47$, $p < 0.001$) among sediment treatments over the first

Table 2. Whole-body element concentrations (μg/g dry mass) in metamorphic *Rana clamitans* exposed to various sediment types[a]

	Sediment treatment		
Element	Clean sand	Coal combustion waste	Mine
As	0.28 ± 0.04	9.94 ± 1.69	0.37 ± 0.03
Cd	0.59 ± 0.08	0.41 ± 0.21	0.58 ± 0.07
Cu	44.11 ± 5.84	58.30 ± 16.84	18.24 ± 2.74
Fe	303.8 ± 20.3	305.1 ± 44.8	379.0 ± 18.4
Ni	1.92 ± 0.11	1.83 ± 0.28	2.30 ± 0.10
Pb	0.37 ± 0.03	0.16 ± 0.07	5.07 ± 0.36
Se	1.45 ± 0.06	21.34 ± 4.28	2.51 ± 0.10
Sr	21.37 ± 1.15	46.46 ± 13.56	22.33 ± 1.22
V	0.30 ± 0.01	5.16 ± 2.59	1.28 ± 0.12
Zn	253.1 ± 11.7	145.0 ± 7.8	470.4 ± 32.3

[a] Data are presented as the mean ± standard error. Note that standard errors are large for animals exposed to coal combustion waste, because $n = 2$ compared with 13 and 14 for clean sand and mine sediments, respectively.

162 d of the experiment, but differences were dependent on time ($F_{4,39} = 537.23$, $p < 0.001$) (Fig. 1). By day 162 of the experiment, control larvae were, on average, 28 and 129% larger than larvae exposed to mine and CCW sediments, respectively.

Exposure to contaminated sediments also influenced developmental rates of *R. clamitans* larvae. Mean days to metamorphosis was significantly ($F_{2,27} = 13.81$, $p < 0.001$) affected by sediment treatment. On average, larvae exposed to mine and CCW sediments took 22 and 98 d longer, respectively, to complete metamorphosis than did larvae exposed to control sediments (controls: $\bar{x} = 201.3$, standard error [SE] = 6.1; mine: $\bar{x} = 222.6$, SE = 7.4; CCW: $\bar{x} = 298.5$, SE = 6.6). Moreover, the effects of days to metamorphosis on size at metamorphosis was dependent on sediment treatment ($F_{2,27} = 4.04$, $p = 0.031$ for the sediment by days-to-stage interaction in the mixed model). Metamorphs emerging from control sediment had a greater increase in body size per unit time than metamorphs exposed to sediments from the mine (Fig. 2). Because only two of the larvae exposed to CCW sediment completed metamorphosis, the relationship between days to metamorphosis and size at metamorphosis for these larvae remains unclear.

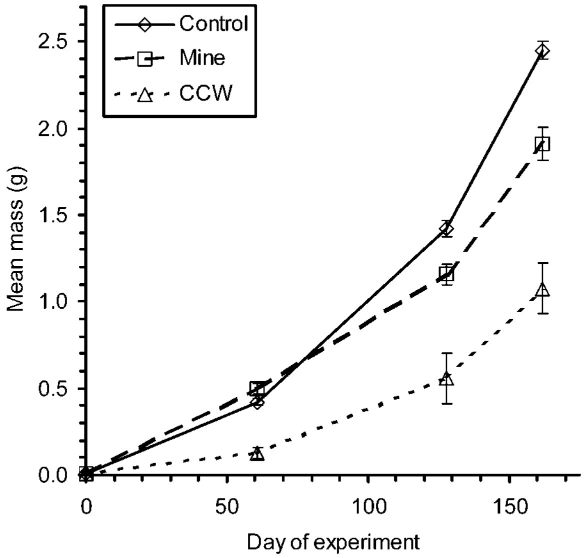

Fig. 1. Change in mean mass with time of *Rana clamitans* larvae exposed to commercial clean sand (control), sediment from an abandoned surface mine (mine) in Baltimore County (MD, USA), or sediment from a coal combustion waste settling basin (CCW) at the Savannah River Site (SC, USA). Error bars are ±1 standard error. All larvae are between Gosner stages 25 and 40.

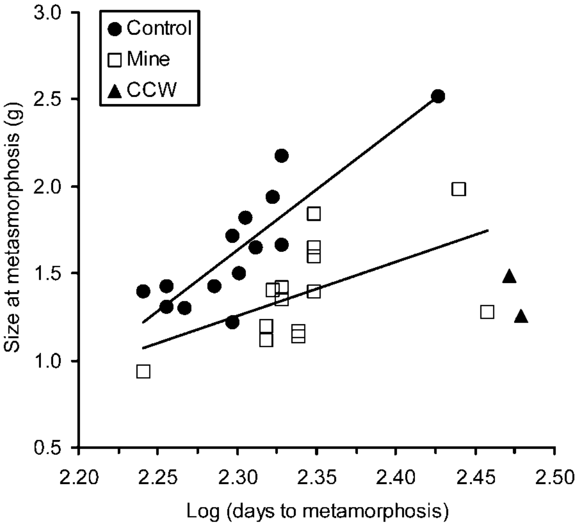

Fig. 2. Relationship between days-to-metamorphosis and size-at-metamorphosis for *Rana clamitans* larvae exposed to commercial clean sand (control), sediment from an abandoned surface mine (mine) in Baltimore County (MD, USA), or sediment from a coal combustion waste settling bin (CCW) at the Savannah River Site (SC, USA). Lines and equation are simple linear regression for clean sand (top) and mine sediments (bottom). In both cases the relationships are significant ($p < 0.041$). No line was fit for CCW sediments because only two larvae completed metamorphosis from this treatment.

All larvae in the experiment either completed metamorphosis (i.e., survived to termination of the experiment) or died. Survival was dependent on sediment type ($p < 0.001$). Survival among larvae exposed to control and mine sediments was 93%, but larvae exposed to CCW sediment had a survival rate of 13%. In comparison to our previous work [4], survival of CCW exposed larvae over the first 195 d of both experiments was similar ($p = 0.700$) (Fig. 3). In contrast, survival of CCW exposed larvae to the end of the current experiment (301 d) was significantly lower ($p = 0.008$) than that observed in the previous experiment (195 d). The percentage of CCW exposed larvae completing metamorphosis in this experiment was reduced by more than 50% compared to the previous experiment (13% vs 28%) (Fig. 4).

DISCUSSION

Overwintering larvae of *R. clamitans* exposed to contaminated sediments accumulated significant concentrations of elements in their tissues and experienced reductions in growth and developmental rate compared to controls, but the effects of CCW sediment were more severe than those resulting from exposure to sediments from the abandoned mine. Exposure to CCW sediment caused significant mortality, resulting in a reduction of the number of individuals that ultimately metamorphosed. Most mortality occurred during the latter half of the experiment, supporting our prediction that individuals experiencing arrested development during the overwintering period would not metamorphose. Our results complement previous laboratory [4] and field [9–13] studies that suggested

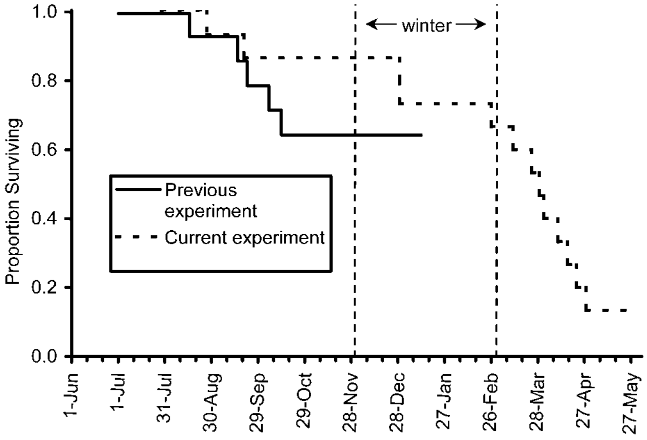

Fig. 3. Survival (%) of *Rana clamitans* larvae exposed to contaminated sediment from a coal combustion waste settling basin at the Savannah River Site (SC, USA) for 301 d during the present experiment (dashed line) or for 195 d (solid line) in a previous experiment [4]. Note that data from controls in each experiment are not shown, because survival was greater than 90% in each experiment. Where lines overlapped, they are offset on the *y*-axis by 0.01 for clarity. Winter is defined as the three months with the lowest average daily temperatures as reported for Baltimore (MD, USA) by the U.S. National Oceanographic and Atmospheric Administration.

amphibians depositing their eggs in CCW wetlands experience reduced reproductive success because of larval developmental abnormalities and mortality.

Element concentrations

In general, element accumulation patterns in *R. clamitans* agreed well with the concentrations of elements they encountered in sediments. For example, the highest concentrations of As, Se, and Sr were found in CCW sediments and in frogs reared on these sediments during larval development. Likewise, Pb and Zn were highest in mine sediments, and individuals metamorphosing from this treatment had the highest concentrations of Pb and Zn in their tissues. Cadmium, Fe, and Zn were notable exceptions to this trend. Cadmium was not accumulated by frogs in any of the sediment treatments, and Fe and Zn were accumulated most in frogs exposed to sediments with low to moderate concentrations of each element. Such accumulation patterns likely result from changes in bioavailability that arise from differences in chemical speciation or from interactions among cocontaminants that influence toxicokinetics.

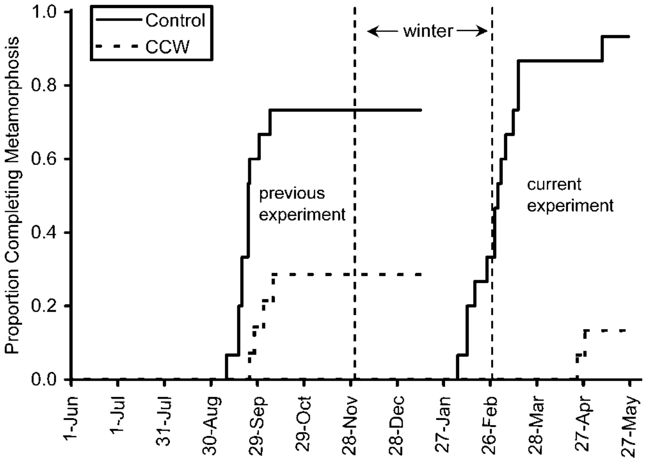

Fig. 4. Cumulative metamorphic success of *Rana clamitans* larvae exposed to commercial clean sand (control) or sediment from a coal combustion waste-settling basin (CCW) at the Savannah River Site (SC, USA). Results from the current experiment, in which all larvae overwintered, and from a previous experiment [4], in which some larvae metamorphosed before overwintering, are shown. Results from the current and previous experiments were placed on a common time scale and began on July 2, 2001, and July 30, 2002, respectively. Lines for the previous experiment end in winter, because this experiment was terminated before overwintering larvae could metamorphose.

Biological responses

Accumulation of elements was associated with decreased growth and slower development among larvae exposed to contaminated sediments. Among individuals exposed to contaminated sediments, slowed developmental rates and reductions in larval growth ultimately resulted in a 17% reduction in mean size at metamorphosis (CCW and mine sediments) and an 11% (mine sediments) to 49% (CCW sediments) increase in the length of the larval period compared to controls. For some amphibian species, decreased larval growth has implications for survival and competitive interactions [14–18], and decreased size at metamorphosis negatively influences adult growth and reproductive parameters [19–22]. Contaminant-induced delay in metamorphosis may disrupt the optimization of metamorphic timing, which could have important ecological consequences (see, e.g., [23]). Moreover, extending the larval period potentially exacerbates the effects of contaminants by increasing the duration of exposure.

The reduction in size at metamorphosis of contaminant-exposed frogs was particularly interesting in light of the fact that larval development also was slower for individuals exposed to contaminants. For controls, larval period was correlated positively with size at metamorphosis (Fig. 2), as might be expected in the absence of competition and ever-increasing per capita resources (see, e.g., [24]). In contrast, the strength of this relationship decreased among individuals exposed to mine sediments such that individuals accrued less mass per unit time than controls. Too few individuals exposed to CCW sediment survived to metamorphosis for us to understand the relationship between time and size at metamorphosis in this treatment, but our previous work suggests that the relationship between these life-history traits among CCW-exposed individuals is weak. Other studies also have documented the weakening of the relationship between length of the larval period and size at metamorphosis in amphibian larvae exposed to contaminants (e.g., salamanders exposed to carbaryl [25]), suggesting that this response may be common in larvae encountering anthropogenic environmental stressors and is worthy of future study.

Comparison to previous CCW research

Consistent with predictions based on previous work [4], *R. clamitans* that experienced arrested larval development during the overwintering months eventually succumb to the effects of exposure to CCW sediment. By collecting eggs 30 d later in the breeding season (compared to the date of collection by Snodgrass et al. [4]) for the present experiment, we successfully forced all larvae to overwinter and extended the time to first metamorphic events in control and CCW-exposed larvae by 149 and 211 d, respectively (Fig. 4). Whereas individuals began metamorphosing before the overwintering period in our previous study [4], most metamorphic events witnessed here occurred near the onset of spring. A comparison of survival in both experiments (Fig. 3) demonstrated that survival decreased precipitously in CCW-exposed larvae after 212 d, which is beyond the duration of the previous experiment. The result of these late-mortality events was a 50% reduction in metamorphic success in the CCW treatment compared to that in our previous study (Fig. 4).

The most parsimonious explanation for the late-larval-mortality events in the present experiment is that individuals eventually succumb to cumulative damage that results from prolonged contaminant exposure. Interestingly, the decline in survival among CCW-exposed larvae corresponded with the onset of metamorphosis among control larvae (Figs. 3 and 4) but before any visible signs of metamorphic climax (e.g., front-limb emergence) among CCW-exposed larvae. These observations suggest that physiological changes associated with the end of the overwintering period and/or the early onset of metamorphosis (e.g., changes in circulating corticosterone or thyroid hormone levels [23]) might mediate CCW-induced mortality. Because we have not observed high levels of mortality at the onset of metamorphosis among Ranids with short larval periods, including nonoverwintering *R. clamitans* [4], we suggest that mortality accompanying metamorphic onset might occur only after accumulation of long-term damage (e.g., after overwintering).

Our present results, in combination with previous findings [4], suggest that the duration of the larval period may play an important role in the sensitivity of amphibians to certain environmental contaminants. Clearly, it is impossible to make broad generalizations based on a small series of laboratory studies, especially because so many other physiological and ecological factors influence the sensitivity of species and even populations (see, e.g., [26]). However, by repeatedly examining larval development within a species under different experimental conditions, we provide evidence that the larval period is a life-history trait worthy of future evaluation in amphibian ecotoxicological studies. Also, it is worth noting that the most severe effects we observed did not arise until after 200 d of exposure, suggesting that exposures incorporating the full larval period are of much greater ecological relevance than the typically short exposures commonly used in ecotoxicological studies. Finally, it is critical to point out that our exposures likely are conservative estimates of the toxicity of contaminated sediments, because amphibians were fed ample uncontaminated resources and were maintained at relatively stable temperatures. The conservative nature of these exposure conditions is further bolstered by the accumulation patterns of larvae; *R. clamitans* metamorphs from our experiments exhibited lower body burdens of eight elements compared to field-captured metamorphs of all amphibian species collected thus far from the site of sediment collection (see, e.g., [27]). Future studies that examine the responses of amphibian larvae under conditions where resources, temperature, and other ecological parameters (e.g., competitive dynamics) fluctuate seasonally would provide further insight regarding the sensitivity of overwintering larvae.

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