FIELD EXPOSURE OF FROG EMBRYOS AND TADPOLES ALONG A POLLUTION GRADIENT IN THE FOX RIVER AND GREEN BAY ECOSYSTEM IN WISCONSIN, USA

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Abstract—We raised embryos and tadpoles of green frogs (Rana clamitans melanota) and leopard frogs (R. pipiens) in situ along a contamination gradient in the Fox River/Green Bay ecosystem (WI, USA). Differences in exposure were reflected by significant positive regressions between concentrations in sediment and in frogs of polychlorinated biphenyl (PCB) congeners and some heavy metals (Cd, Cr, and Pb), which could have predictive value for estimating exposure of amphibians to contaminants in this ecosystem and, perhaps, in others. On average, non-ortho-substituted coplanar PCBs made up a very small percentage (average, 0.2%) of the PCB congeners in all samples analyzed, with larger fractions accounted for by mono- and di-ortho congeners (average, 19%) and routine congeners (average, 81%). Hatchability of frog embryo batches and anuran species richness at the sites were negatively correlated with level of contamination, but tadpole growth and survival were not. Sediment and tissue levels of PCBs and metals generally were correlated with each other, confounding the association of effects on frogs with any particular contaminant. It is also plausible that high levels of un-ionized ammonia (NH₃) reduced hatching success of green frog, but not leopard frog, embryos in the field enclosures. Other environmental factors that were present but unmeasured in the field, such as ultraviolet-B radiation as well as water flow and level fluctuations, might have caused differences in hatching success at the field sites.

Keywords—Ammonia, Amphibian, Heavy metals, Polychlorinated biphenyls, Tadpoles

INTRODUCTION

The Green Bay watershed (WI, USA; Fig. 1) is polluted with heavy metals, dioxins, polychlorinated biphenyls (PCBs), and more than 100 other organic contaminants [1]. Until 1972, PCB contamination of water and sediments in this watershed was linked to the recycling of carbonless copy paper by pulp and paper mills that line the Fox River, Green Bay’s main tributary [2]. Although production of PCBs and other contaminants (e.g., DDT, dieldrin) has been banned or restricted in the United States since the 1970s, these compounds persist because of sediment contamination, slow biodegradation, atmospheric deposition, and bioaccumulation up the food web. High PCB and organochlorine concentrations are associated with lowered reproductive success in fish-eating birds in Green Bay [3,4].

The Green Bay watershed also suffers from anthropogenic wetland loss, introduced species, water-level fluctuations caused by dams and seiches, and poor water quality [5]. From the 1920s until the late 1970s, the Fox River supported few aquatic invertebrates and fish and was considered to be biologically dead [1]. Municipal and agricultural discharges contributed to high nutrient and sediment loads, which can lead to algal blooms, low dissolved oxygen levels, reduced aquatic macrophyte growth, and relatively high ammonia levels [5]. All these factors can negatively impact aquatic wildlife, including amphibians.

Amphibians in the Green Bay watershed are undoubtedly exposed to PCBs and heavy metals. Body burdens of PCBs, their possible relation to sediment contamination, and the sensitivity of amphibians to these compounds in the field are relatively unknown and are not easily predicted or approximated [6]. Two studies [7,8] found that levels of PCBs in adult leopard frogs (Rana pipiens) and green frogs (Rana clamitans melanota) were positively correlated with levels in sediments (n = 5 sites each). We have extended these studies by performing PCB congener-specific analyses on tadpoles and metamorphosed frogs raised at many sites and on the embryo batches from which they were derived. The numbering system used for PCB congeners is that of the International Union of Pure and Applied Chemistry [9]. The term coplanar is applied to PCB congeners with no substitution at the ortho position (PCBs 77, 81, 126, and 169). Many of the responses elicited by these non-ortho PCBs correlate with their binding affinity to the aryl hydrocarbon (Ah) receptor, which is believed to mediate several of the effects induced by these compounds [10]. Compounds that bind to the Ah receptor and elicit responses through this mechanism of action are considered to have “dioxin-like action,” because 2,3,7,8-tetrachlorodibenz-p-dioxin is the most potent compound known to bind to the Ah receptor [11]. Among the variety of toxic responses in animals to these compounds [11], loss of body mass and edema occur in green and leopard frogs [12]. Noncoplanar PCBs exhibit low or negligible binding affinity for the Ah receptor because of the presence of ortho chlorine substitution; those with mono- or di-ortho chlorine substitutions are derivatives of coplanar PCBs and exhibit some dioxin-like responses.

Few field studies have related heavy metals in amphibians to metals in their immediate environment [13]. Bioaccumulation (defined as net concentration in the body or a compartment as a result of the balance between uptake and elimination) will not necessarily occur for metals, because levels of some may be homeostatically controlled [13]. However, at least one field study [14] and one laboratory study [15] have

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Fig. 1. Map showing the location of study sites on the Fox River and along Green Bay (WI, USA). Areas in gray are local municipalities. For specific locations of study site, see Materials and Methods.

Materials and Methods

Study sites

We identified 11 sites along the Fox River and Green Bay with emergent vegetation and either historic or current presence of frogs, and we used these sites for field-enclosure studies in 1994 and 1995 (Fig. 1). We refer to the sites with labels (R1–R4 for the four reference sites, and C1–C7 for the seven relatively contaminated sites) rather than by their local names, which are listed below for identification. To represent low-contamination reference sites, we selected four sites northwest of the mouth of the Fox River, the major source of contaminants into Green Bay, because prevailing currents in the bay tend to run northeast of the river mouth. Two of the reference sites were along the bay (Lineville [R1] and North Sensiba [R4]), and two were wetland and pond sites inland from Green Bay (South Sensiba [R3] and Barlhausen Waterfowl Preserve [R2]). Sites with suspected or known higher levels of sediment contaminants were along the Fox River (deposit A [C1], deposit C2 [C2], and deposit X [C4]), along tributaries to the river (deposit C1 [C3] and Railroad Museum [C5]), or just west (Bay Port [C6]) or east (University of Wisconsin–Green Bay Pond [C7]) of the river mouth. Site C6 is a wetland partially filled with fly ash from a coal-burning power plant and dredge spoils from channel maintenance. We recorded all anuran species seen or heard during more than 40 visits to each of these sites in 1994 and 1995, and we conducted anuran-calling surveys during 1996 and 1997 following the protocol of the Wisconsin Frog and Toad Survey [23].

Installation of field enclosures

We made field enclosures (height, 102 cm; diameter, 61 cm) of stainless-steel rods formed into a circle at the top, with six equally spaced rods vertically connected to a stainless-steel metal skirt (height, 10 cm; 20 gauge) at the bottom (tapered 4 cm relative to the top) [18]. We sewed nylon mesh (mesh size, 500 μm; Nitex; SEFAR America, Briarcliff Manor, NY, USA) into sleeves to fit around the enclosures, which were held in place at the top with nylon cable ties and at the bottom with a polypropylene strap and clip (13 mm wide; Band-It, Idex, Denver, CO, USA). We pounded enclosures into the sediment and held them in place using elastic cords fastened to a tripod arrangement of stakes around each enclosure. We placed enclosures at study sites between 2 and 8 m from shore and accessed them by walking out in chest waders. Enclosures were open at the top (covered with chicken wire to deter predators) and at the bottom, allowing tadpoles access to sediments, and we used dip nets to remove predators in enclosures before adding embryos.

Embryo collection, distribution, and monitoring

We collected, from sites thought to have relatively low contamination, embryos to distribute into enclosures at all the field sites, keeping embryos from individual embryo masses in separate enclosures. Portions of the embryo masses or just-hatched tadpoles were frozen for contaminant analysis. On June 11, 1994, we collected two green frog embryo masses from the University of Wisconsin–Green Bay Cofrin Arboretum Prairie Pond, a site that is not connected to either the river or the bay and at which no pesticides have been applied since it was constructed approximately 25 years earlier, near what were once agricultural fields. On April 24, 1995, we collected two leopard frog embryo masses from site R2, one
of the reference sites. In 1995, we also collected leopard frog embryos from site C, one of the relatively contaminated sites, but only for contaminant analysis. We transported embryos to a laboratory at the University of Wisconsin-Green Bay in water from the collection sites, staged them [24], and separated them into groups to be placed within enclosures. Within 24 h, we distributed embryos (stages 7–12) from individual masses to separate field-site enclosures (n = 70 embryos/enclosure in 1994 and 100 in 1995). The number of enclosures at each site varied from two (one for each clutch) to eight (seven for each clutch), depending on the likelihood that some enclosures might fail (see below). We initially placed embryos into enclosures within smaller enclosures (for details, see [25]) inside the larger enclosures to protect the embryos or tadpoles from wave action and to ensure accurate counts. We made embryo cups from the bottoms of 1-L soda bottles with 500-μm nylon mesh at the base to allow water flow. The embryo cups were kept buoyant by corks affixed to their sides, which held the embryos approximately 4 cm below the water surface, the depth at which leopard frog egg masses are commonly laid in the wild [26]. The distance of the embryo cups above the sediment varied but generally ranged from 0.12 to 0.75 m.

At each enclosure, we recorded the proportion of embryos that hatched. After hatching at site R2, we manipulated tadpole density (n = 80, 60, 40, and 20 from each batch per enclosure) to evaluate its impact on tadpole growth rate. We visited field sites after hatch twice per week to provide supplemental food to tadpoles in case natural food was insufficient [27,28] so that differences in growth or metamorphosis at this or other sites might not be attributed to food effects [28]. We initially supplied 5 g of food, consisting of boiled Romaine lettuce blended into a puree with 10% of a 3:1 mixture of Amazon Smythe Superior Nutrition Rabbit Food (Chilton, WI, USA) and TetraMin Flake Food for tropical fish (TetraSales, Blacksburg, VA, USA), but we gradually increased the supplement to 60 g/enclosure by September. Animals were removed from enclosures when they metamorphosed or after 16 to 18 weeks in September, when the weather began to cool. Until that time, we measured total lengths of a subset of tadpoles collected randomly with a dip net once per week. Growth rate (mm/d) was calculated for each enclosure, but these determinations terminated once we began removing metamorphosed individuals from an enclosure. We documented the proportion of tadpoles that survived and the mean stage [24] or mean time to metamorphosis of tadpoles in each enclosure. All tadpoles and just-metamorphosed frogs collected from enclosures were brought to the laboratory to be measured (body mass, snout-vent length, or total length) and killed (overdose of 3-amino-benzoic acid ethyl ester; 0.05% solution for tadpoles, pitting for metamorphosed frogs; Sigma Chemical, St. Louis, MO, USA). The liver was removed for a study of mixed-function oxidase enzyme activity, and the remainder of the carcass was frozen until contaminant analysis. Each year after hatch, some enclosures were lost because of a variety of factors: Loose netting, water levels rising above the tops of the enclosures or falling to critically low levels, or the enclosure washing away because of flooding. Thus, whereas hatch success data were available for all sites, data regarding tadpole survival, growth, and residues were available only for six of nine sites in 1994 (C4, C5, C7, R1, R3, and R4; sites C1, C3, and C6 were lost) and seven of eight sites in 1995 (C1, C4, C5, C6, C7, R2, and R3; site C2 was lost). Two enclosures that failed within two weeks of hatch (one at site C4 in 1994 and one at site C1 in 1995) were restocked (with the same genetic stock in 1995 but a different one in 1994), so those tadpoles spent 86 to 88% of the development time in enclosures compared to tadpoles in other enclosures.

Characterization of sediments and water quality

For this and past studies [7], we used sediment PCB and heavy-metal concentrations, which are associated [1], as an index to the relative level of contamination at the sites. In 1994 and 1995, we collected sediment samples (depth, top 5 cm) from around the edge of the enclosures that we installed at the field sites, although sediment PCB data were from other sources for sites C1 and C3 [2] and site R1 (K.A. Palmer and H.J. Harris, University of Wisconsin, Green Bay, WI, USA, personal communication; 1993 data). Analytical costs were prohibitively high to measure contaminants in sediments annually. However, PCB sediment levels decline slowly, with a rate constant of 0.16 year⁻¹ [2], making it unlikely that 10- to 100-fold differences in contaminant levels among the sites would dramatically change over the two-year study. Levels of PCBs and heavy metals were not measured in water samples, but these levels usually are positively correlated with levels in underlying sediment [29,30].

We characterized water quality at the field sites on a weekly or biweekly basis by measuring water temperature, dissolved oxygen, pH, hardness, acidity, carbon dioxide, ammonia–nitrogen, nitrite, and chloride using a Hach Test Kit FF-2 (Hach, Ames, IA, USA). Un-ionized ammonia (NH₄) concentration was calculated using measured ammonia–nitrogen, temperature, and pH following [31].

Contaminant analyses in frogs and sediments

Sample preparation. Embryo masses, tadpoles, and metamorphs sans livers were frozen in hexane-washed aluminum foil, ground in dry ice, pooled per site, and sublimed gradually in a −20°C freezer. Pooled samples always contained tissues from the same life stage of one species except in 1994, when the pool from site C5 contained tadpoles and metamorphs of green frogs. Each pooled tissue sample was divided into two subsamples for separate analyses of metals (>10 g of tissue) and of PCBs and other organochlorines (⅔ g for embryos, ⅓ g for tadpoles and metamorphs). Sediment samples were pooled by site, and subsamples were distributed for organic, inorganic, and percentage total organic carbon analyses.

Analysis of sediment samples. Sediment samples were analyzed for total Aroclor, p,p'-dichlorodiphenyldichloroethylene (DDE), and dieldrin at the Wisconsin State Laboratory of Hygiene (WSLH) [32]. Total levels of Aroclor (a commercial mixture of PCBs) were determined using packed-column chromatography. Minimum detection limit (MDL) was 0.05 mg/kg dry matter. Metals were analyzed at WSLH [33] by inductively coupled plasma–mass spectroscopy (ICPMS; for Cd, Cr, Cu, and Pb) after acid digestion. However, Hg was analyzed by atomic absorption spectrophotometry using the cold-vapor technique, and arsenic was analyzed using graphite- furnace atomic absorption spectrophotometry. Minimum detection limits ranged from 0.03 to 0.80 mg/kg dry matter, depending on the metal. Sediment samples from C3 and R1 were analyzed for metals using ICPMS at the University of Wisconsin Soil and Plant Analysis Laboratory.

Analyses of tissue samples. Analyses of specific congeners of PCBs as well as other organochlorine compounds were performed by the Geochemical and Environmental Research
Group at Texas A&M University (College Station, TX, USA). Samples were analyzed (procedures 9720, 9724, 9807, and 9810) using high-resolution gas chromatography/mass spectrometry. One aliquot was analyzed for non-ortho PCB congeners (PCBs 77, 81, 126, and 169) and 10 mono- and di-ortho congeners (PCBs 105, 114, 118, 123, 156, 157, 167, 170, 180, and 189; for details, see [34]), and another aliquot was analyzed for 87 single or coeluting PCB congeners (routine congeners; for details, see [35]). Duplicate, spiked, and procedural blank analyses were run with each set of samples. Surrogate (PCB 103, PCB 198, and 4,4'-dibromoocofluorobiphenyl) recoveries for samples and quality-control samples ranged from 61 to 83%, and recoveries in matrix-spiked duplicates averaged 95% for PCB congeners. Minimum detection limits ranged from 0.002 to 0.80 ng/g wet mass, depending on the congener and amount of sample analyzed. In 1994, frog tissues were analyzed for metals by ICPMS at WSLH as described previously. In 1995, frog tissues were analyzed for metals by ICPMS at the University of Wisconsin Soil and Plant Analysis Laboratory (MDL, 0.001–0.03 μg/g, depending on the metal except for subsamples analyzed for Hg at WSLH as described previously).

**Statistical analyses**

**Characterization of field sites.** Sediment samples with non-detectable levels of PCBs or metals were assigned a value of half the MDL for the particular analysis, and all sediment values were log_{10}-transformed to control heteroscedasticity. The initial classification of sites as relatively uncontaminated reference sites or relatively contaminated sites was validated by measures of sediment PCBs below the MDL at all four of the former and none of the latter sites (see Results). Furthermore, we validated the distinction by another analysis that also took into account sediment metal levels as follows: To evaluate the correlation of contaminants with one another by the method of least-squares linear regression, relative contaminant levels for each contaminant at each site (Z scores) were first calculated. This normalized each log_{10} contaminant value (X) to its respective mean and standard deviation (SD) across all 11 sites: Z = (X - mean)/SD [36]. Discriminant analysis of sediment contaminant Z scores [36] was then used to describe the patterns of variation in total contaminants across the sites and to validate the use of the summed Z score for each site as an overall index of its sediment contamination. The summed Z scores of reference and contaminated sites were compared by t test (t value presented with degrees of freedom subscripted). Summed Z scores were also used in some subsequent analyses as an overall index of sediment contamination. Water-quality variables at reference versus relatively contaminated sites were compared by t test. The relationship of anuran species richness at the sites to sediment contamination was tested by least-squares linear regression of species number at each site versus summed Z score at each site.

**Analyses of hatch, survival, growth, and development.** The basic unit in these analyses was a batch of embryos or tadpoles, which could be coded according to its species origin (green or leopard frog) and its particular clutch within each species (clutch 1 or 2). These batches were variously exposed to different levels of contamination, which were included in statistical analyses as either a single factor (reference vs relatively contaminated) or as a continuous variable or covariate (summed Z score).

Hatchability (proportion of embryos that hatched) in each of the field enclosures was calculated as number of tadpoles/ (number of unhatched embryos remaining + number of tadpoles). Thus, any embryos lost from cups or small enclosures because of, for example, wave action were excluded. All statistical analyses were performed on the logit of hatchability (ln [hatchability + 0.01/(1 - hatchability + 0.01)]) rather than the hatchabilities themselves, because proportions tend not to be normally distributed. The logit hatchabilities of 55 embryo batches were compared in an analysis of variance (ANOVA; general linear model in [36]) with three factors (site, species, and clutch nested within species) and also in an analysis of covariance (ANCOVA) with species as a factor and summed Z scores as the covariate. In these and other analyses of variance, F values are presented with their respective degrees of freedom subscripted, and interactions between factors and covariates were removed if they were not significant (α > 0.05). Logit transformation and similar kinds of ANOVA and ANCOVA were also used to analyze the proportion of tadpoles that survived, although clutch was excluded as a factor for lack of sample size. Because studies with anurans have shown that density can affect rate of growth and rate of metamorphosis (probably through its effect on rate of growth) [27,28,37], we included along with summed Z score the tadpole density or growth rate as covariates in multiple-regression analyses of snout-vent length and time to metamorphosis, respectively.

When all batches are analyzed together, as in the analysis of hatchability described above, it is possible in principle to discriminate significant variation caused by level of contamination, clutch within a species, or species. Note, however, that species and year are perfectly confounded, because green frogs were studied in 1994 and leopard frogs in 1995. Even so, the ability to make these distinctions often was not powerfully realized because of the pattern of loss of enclosures that resulted in the nonuniform distribution of species or particular clutches over all sites. Nonetheless, when sample size is sufficient, retaining the coding of embryo or tadpole batches according to original-source clutch recognizes, somewhat, the nonindependence of the analytical units (i.e., it reduces degrees of freedom), and coding blocks for so-called clutch effects, if they exist. As for species differences, in some cases we checked conclusions by analyzing species separately.

**Analyses of residues in frogs.** The log_{10} levels in either frog lipid (for PCBs) or frog dry mass (for metals) were compared in ANCOVA with species as a factor and log_{10} sediment level as the covariate. Organochlorine pesticide levels were tested for correlation with PCBs or relative sediment contamination index by least-squares linear regression. Comparisons between species were made using the t test.

Numerical data are presented as the mean ± standard error (n = number of sites, batches of embryos, or individuals, as specified). Statistical significance was accepted for α < 0.05. One-tailed tests were used for a priori predictions.

**RESULTS**

**Characterization of field sites**

Sites preselected as relatively uncontaminated reference sites had the lowest levels of PCBs and heavy metals in sediment relative to those at all other sites (Table 1). Sediment organochlorine pesticide (e.g., dieldrin, p,p'-DDE) levels were less than the MDL at all sites and so are not presented. Relative contaminant levels for total PCBs and metals at each site (Z scores) were significantly correlated with each other (r > 0.72,
Table 1. Habitat and sediment characteristics (% total organic carbon based on dry mass [TOC]) and contaminant concentrations (mg/kg dry mass) at field sites located along the Fox River and Green Bay (WI, USA)*

<table>
<thead>
<tr>
<th></th>
<th>Preselected reference sites</th>
<th>Preselected relatively contaminated sites</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>R2</td>
<td>R4</td>
</tr>
<tr>
<td>Habitat type</td>
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<td>38°13'</td>
</tr>
<tr>
<td>Longitude 88° W</td>
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<td>60°37'</td>
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<tr>
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</tr>
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<td>Mercury</td>
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<tr>
<td>Contamination index</td>
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<td>-6.86</td>
</tr>
</tbody>
</table>

* Amphibian presence is based on original surveys (1993), observations made during maintenance of enclosures (>40 visits/site in 1994–1995), and anuran calling survey data (1996–1997). The letters indicate the species and developmental stages that were observed and are in parentheses: A = calling males and adults; B = bullfrog (Rana catesbeiana); C = chorus frog (Pseudacris triseriata); E = embryo masses; G = green frog; L = leopard frog; S = spring peeper (Pseudacris crucifer); T = American toad (Bufo americanus); T (in parentheses) = tadpoles; W = wood frog (Rana sylvatica). Concentration preceded by < signifies the sample was below the minimum detection limit represented by the number. In a few cases, designated by —, specific analyses were not performed.

From Kevin A. Palmer and Hallett J. Harris, University of Wisconsin, Green Bay, Wisconsin, USA (personal communication, 1993 data).

From Steuer et al. [2].

Sum of Z scores for each contaminant. The more positive the value, the higher the relative combined contamination.
Field exposure of frogs along a pollution gradient

$p < 0.05$). Thus, sites with relatively high levels of PCBs also tended to have relatively high levels of metals. Discriminant analysis of the seven standardized contaminant levels for each site indicated that these sites could be classified adequately by a single principal component that accounted for 87.5% of the variation. Component loadings for this first principal component were essentially unity for each of the contaminants (range, 0.891−0.972). Considering this, we, as a simple quantitative index to relative contamination among the sites, simply added together the $Z$ scores for each contaminant to calculate a summed $Z$ score for the site, an overall index of its sediment contamination (Table 1, bottom row). Reference sites had a significantly lower mean summed $Z$ score than the more contaminated sites ($t_8 = 4.4, p = 0.002$).

The sites also differed in some water-quality characteristics (tabulated results can be found in [18,19]). For example, during the embryo stage in 1994, mean NH$_4$ concentration tended to be lower at reference sites (0.006 ± 0.001 mg/L, $n = 3$) than at contaminated sites (0.224 ± 0.092 mg/L, $n = 6, t_1 = 2, p = 0.09$), although not in 1995 ($t_8 = 0.9, p > 0.3$). The NH$_4$ level was higher in 1994 (range of means at nine sites, 0.004−0.516 mg/L) than in 1995 (0.002−0.052 mg/L, $n = 8$), because water temperatures were higher in June 1994 (when green frogs laid eggs) than in April 1995 (when leopard frogs laid eggs). Higher temperatures cause more total ammonia in solution to be found in this more toxic form than as the less toxic ammonium (NH$_4$) [31]. Water pH was not acidic at any of the sites (pH ≈7) and was higher at reference (pH 8.5 ± 0.2, $n = 6$ sites) compared to more contaminated sites (pH 7.1 ± 0.1, $n = 3$) in 1994 ($t_5 = 5.1, p = 0.001$) but not in 1995 ($t_6 = 1.2, p > 0.2$). No other water-quality measures differed significantly between reference and more contaminated sites in either year.

Although we heard anurans calling at all sites, we were unable to observe tadpoles or adults at 5 of 11 sites. Observations made during repeated visits ($n > 40$ per site) to install and maintain enclosures in 1994 and 1995 revealed seven anuran species (listed from most common to least common over all sites): Northern leopard frog, green frog, American toad (Bufo americanus), wood frog (R. sylvatica), spring peeper (Pseudacris crucifer), western chorus frog (P. triseriata), and bullfrog (R. catesbeiana). Calling surveys in 1996 and 1997 ($n ≥ 3$ per site each year) added no new species. Generally, higher contamination was associated with lower anuran species richness, and the index of sediment contamination was negatively associated with the number of anuran species recorded at the sites ($F_{1,9} = 6.2, p = 0.03$) (Table 1).

### Hatch, survival, growth, and development

Hatchability of embryo batches placed in enclosures ranged from 0 to 100% and averaged 87% ± 3% ($n = 55$). The single instance of 0% hatchability in one enclosure at C6 in 1994 did not correspond with any particular disturbance event, such as rising or falling water level. The logit proportion of embryos that hatched differed significantly among sites ($F_{6,41} = 2.35, p < 0.027$) but not among clutches nested either within species ($F_{2,41} = 2.53, p = 0.092$) or between species ($F_{1,41} = 1.67, p > 0.2$) (Fig 2A). In ANCOVA, logit hatch success was negatively correlated with the index of sediment contamination (covariate, $F_{1,42} = 14.1, p < 0.001$) (Fig 2A), with no significant difference being found between species (factor $F_{1,42} = 0.1, p > 0.7$). When species were tested separately, these negative correlations were significant for both green frogs in 1994 ($F_{1,24} = 5.7, p = 0.024$) and leopard frogs in 1995 ($F_{1,27} = 14.9, p < 0.001$).

Because some enclosures were lost each year, analyses of survival and other amphibian parameters were only possible for a subset of enclosures at the sites (8 of 26 in 1994, 21 of 28 in 1995). The pattern of enclosure loss in relation to the site contamination gradient was spotty, resulting in low power to detect significant variation caused by relative contamination. Using an ANCOVA, logit survival was not significantly correlated with the index of sediment contamination ($F_{1,26} = 0.1, p > 0.7$) (Fig 2B), but it differed significantly between species ($F_{1,26} = 9.4, p = 0.005$). At the four sites where the species were directly comparable (C4, C5, C7, and R3), survival was significantly lower in green frogs (55% ± 7%, $n = 7$ enclosures) than in leopard frogs (80% ± 9%, $n = 10$; $F_{1,14} = 5.1, p = 0.04$). In 1994, only 4% of the green frogs ($n = 3$ at C5 and 4 at C7) metamorphosed by September 26, when the remaining tadpoles at the enclosures were collected (mean mass, 3.34 ± 0.38 g; $n = 7$ enclosures). In 1995, 51% of the leopard frogs metamorphosed (range among enclosures, 1–100%) by late September (mass, 1.37 ± 0.26 g; $n = 21$ enclosures). At site R2, where we manipulated leopard frog density to evaluate its impact on tadpole growth and development, growth rate (mm/ d) was inversely related to the number of tadpoles in the enclosures ($F_{1,6} = 8.2, p = 0.028$), and time to metamorphosis was inversely related to growth rate ($F_{1,6} = 46.6, p < 0.001$). Accordingly, when we analyzed growth and development over all sites for possible effects of relative contaminant exposure, we included these factors as covariates along with the summed $Z$ score in a multiple regression. For both leopard and green frogs, when analyzed separately, final size was not significantly related to relative sediment contamination index ($t_{18} = -0.5, p > 0.6$ and $t_{18} = -1.9, p = 0.13$, respectively) but was significantly inversely related to tadpole density ($t_{18} = -3.0, p = 0.007$ and $t_{19} = -4.9, p = 0.008$, respectively) (Fig 3). Time to metamorphosis of leopard frogs was not significantly related
limited sampling of embryos or newly hatched tadpoles, because PCB levels were higher for both species in those batches collected at more contaminated sites (Table 2). More complete results of the congener-specific analyses are presented in the Appendix [SETAC Supplemental Data Archive, Item ETC-24-04-001; http://etc.alienpress.com] for young of both species raised at one of the more contaminated sites, C4, as well as for embryos collected at C6 and R2.

Levels of several heavy metals in green frog tadpoles and metamorphosed leopard frogs also were positively associated with their respective levels in the sediments at the sites where the tadpoles were raised (Fig. 5). Statistical correlations with sediment levels were significant for Cd, Pb, and Cr (Fig. 5), and in none of these cases were significant differences found between the species (ANCOVA, $p > 0.3$), although we had low power to detect a difference. The correlation coefficients for As, Cu, and Hg ranged from +0.49 to +0.56 ($p > 0.05$).

Green frog tadpoles had significantly higher levels of DDT and its metabolites than did leopard frog metamorphs (18.2 ± 2.6 ng/g wet mass [$n = 5$ pools] vs 5.4 ± 1.1 ng/g wet mass [$n = 7$]; $t_{0.05} = 5.5$, $p < 0.001$), but no significant correlations were found between the levels of these organochlorines and either log$_{10}$ total-tissue PCBs in frogs ($F_{1,10} = 0.9$, $p > 0.3$) or relative sediment contamination index at the sites ($F_{1,10} < 0.01$, $p > 0.9$). Dieldrin levels averaged 0.78 ± 0.46 ng/g wet mass in green frog tadpoles ($n = 5$ pools) but were undetectable in all the leopard frog metamorph pools except at site C5 (1.19 ng/g wet mass). No significant differences were found between green and leopard frogs in either total chlordanes (1.70 ± 0.21 ng/g wet mass [$n = 5$] vs 2.21 mg/g wet mass ± 1.1 [$n = 5$]; $t_{0.05} = 0.5$, $p > 0.6$) or hexachlorobenzene (0.33 ± 0.10 mg/g wet mass vs 0.21 ± 0.10 ng/g wet mass; $t_{0.05} = 0.9$, $p > 0.4$). A number of other pesticides that were tested were detected inconsistently in the samples at relatively low levels and so are not presented.

For comparison with other studies, data regarding levels of PCBs and metals in the present study’s frogs can be expressed to other bases of normalization using the following conversion factors: Metamorphosed leopard frogs, 0.01 ± 0.002 g lipid/g wet mass and 0.176 ± 0.015 g dry mass/g wet mass ($n = 7$ pools, 1 for each site); green frog tadpoles, 0.021 ± 0.003 g lipid/g wet mass and 0.176 ± 0.015 g dry mass/g wet mass ($n = 5$); leopard frog embryos, 0.11 ± 0.01 g lipid/g wet mass and 0.097 ± 0.001 g dry mass/g wet mass ($n = 2$ batches); and green frog embryos, 0.058 ± 0.015 g lipid/g wet mass ($n = 2$).

**DISCUSSION**

**Hypothesis 1: Correlation between contaminants in frogs and in local sediment**

Based on other laboratory and field studies [7,8,15], we predicted—and found—that levels of both PCBs and metals in the frogs correlate positively with levels in the sediments at the sites where they were raised. Although we did not have as large a sample size as we desired, statistical correlations were significant for all classes of PCB congeners (non-ortho, mono- and di-ortho, and routine PCBs in tissues vs total PCBs in sediment) and for three of the metals (Cd, Cr, and Pb). For the other three metals (As, Cu, and Hg), the correlation coefficients were positive and may have been nonsignificant because of low power.

The regressions between PCBs in sediment and frogs could have predictive value for estimating exposure in this ecosystem.
Table 2. Contaminant concentrations in tadpoles, newly metamorphosed frogs, and embryos of leopard and green frogs from sites located in the Green Bay and Fox River watershed (WI, USA)

<table>
<thead>
<tr>
<th>Site</th>
<th>Species</th>
<th>Year</th>
<th>Stage</th>
<th>Polychlorinated biphenyls (PCBs) (ng/g wet mass)</th>
<th>Heavy metals (µg/g dry mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total(^a) Routine(^b) Non-ortho(^c) Mono- and di-ortho(^d) %(^e) Lipid</td>
<td>%(^e) Water</td>
</tr>
<tr>
<td>Preselected reference sites</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>Leopard frog</td>
<td>1995</td>
<td>Frog</td>
<td>21.6  14.3  0.11  7.20  1.2  81.7</td>
<td>0.15  0.04  0.26  7.77  0.61  0.28</td>
</tr>
<tr>
<td>R2</td>
<td>Leopard frog</td>
<td>1995</td>
<td>Embryo</td>
<td>6.3   3.5   0.06  2.80  1.2  90.2</td>
<td>0.00  0.00  0.24  16.40 0.20  0.03</td>
</tr>
<tr>
<td>R3</td>
<td>Green frog</td>
<td>1994</td>
<td>Tadpole</td>
<td>25.9  21.3 ND(^f)  4.67  1.9 85.0</td>
<td>0.20  0.05  0.70  6.87 ND  0.11</td>
</tr>
<tr>
<td>R3</td>
<td>Leopard frog</td>
<td>1995</td>
<td>Frog</td>
<td>3.3   2.9   0.004 0.31  0.3  89.2</td>
<td>—    —     —     —    —    —</td>
</tr>
<tr>
<td>R4</td>
<td>Green frog</td>
<td>1994</td>
<td>Tadpole</td>
<td>27.0  22.3  0.18  4.68  2.9 84.0</td>
<td>ND   0.05  0.50  4.03 ND  0.05</td>
</tr>
<tr>
<td>UWGB(^b) Prairie Pond</td>
<td>Green frog</td>
<td>1994</td>
<td>Newly hatched Tadpoles</td>
<td>54.7  54.7</td>
<td>—</td>
</tr>
<tr>
<td>Contaminated sites</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>Leopard frog</td>
<td>1995</td>
<td>Frog</td>
<td>568.8 502.0 0.26 66.17 0.8 81.9</td>
<td>1.71  0.10  0.64 44.20 1.44 0.86</td>
</tr>
<tr>
<td>C4</td>
<td>Green frog</td>
<td>1994</td>
<td>Tadpole</td>
<td>311.9 278.9 0.69 32.25 2.0 84.0</td>
<td>—    —     —     —    —    —</td>
</tr>
<tr>
<td>C4</td>
<td>Leopard frog</td>
<td>1995</td>
<td>Frog</td>
<td>158.0 129.1 0.15 28.76 0.8 81.6</td>
<td>0.29  0.08  0.77 11.90 0.89 0.40</td>
</tr>
<tr>
<td>C5</td>
<td>Green frog</td>
<td>1994</td>
<td>Tadpole</td>
<td>171.1 144.2 0.63 26.23 1.4 82.0</td>
<td>—    —     —     —    —    —</td>
</tr>
<tr>
<td>C6</td>
<td>Leopard frog</td>
<td>1995</td>
<td>Frog</td>
<td>281.6 259.6 0.19 21.87 1.8 77.6</td>
<td>0.32  0.02  0.74 26.65 0.33 1.20</td>
</tr>
<tr>
<td>C6</td>
<td>Leopard frog</td>
<td>1995</td>
<td>Embryo</td>
<td>180.8 148.1 ND  32.79 1.6 81.9</td>
<td>0.83  0.08  0.87 22.09 0.24 0.91</td>
</tr>
<tr>
<td>C6</td>
<td>Leopard frog</td>
<td>1995</td>
<td>Embryo</td>
<td>105.4 85.3 0.10 20.00 1.0 90.4</td>
<td>—    —     —     —    —    —</td>
</tr>
<tr>
<td>C7</td>
<td>Green frog</td>
<td>1994</td>
<td>Tadpole</td>
<td>30.0  24.1  0.08 5.81 2.3 83.0</td>
<td>ND   0.05  0.50  5.23 ND  0.12</td>
</tr>
<tr>
<td>C7</td>
<td>Leopard frog</td>
<td>1995</td>
<td>Frog</td>
<td>14.9  9.9   0.06 4.99 0.6 82.0</td>
<td>3.20  0.11  1.14 58.16 0.53 2.10</td>
</tr>
<tr>
<td>C7</td>
<td>Green frog</td>
<td>1993</td>
<td>Embryo</td>
<td>114.5 88.8 ND  25.70 6.8</td>
<td>—    —     —     —    —    —</td>
</tr>
</tbody>
</table>

\(^a\) Total PCBs is the sum of routine, non-ortho, and mono- and di-ortho congeners.
\(^b\) Routine PCBs are all congeners except the non-ortho and mono- and di-ortho congeners.
\(^c\) Non-ortho PCBs are congeners 77, 81, 126, and 169.
\(^d\) Mono- and di-ortho PCBs are congeners 105, 114, 118, 123, 156, 157, 167, 189, 170, and 180.
\(^e\) % Wet mass.
\(^f\) ND = not detected.
\(^g\) = not analyzed.
\(^h\) UWGB = University of Wisconsin Green Bay.
\(^i\) The ND was for PCB 77, which coeluted with PCB 110. Other non-ortho congeners (81, 126, and 169) were not analyzed.
\(^j\) This value includes PCBs 118, 170 (coeluted with 190), and 180. Other mono- and di-ortho congeners were not analyzed.
and, perhaps, in others. Consistent with this idea, previous studies have shown PCB sediment–frog correlations for adult leopard frogs [7] and green frogs [8]. Indeed, routine PCB congener concentrations in the metamorphosed leopard frogs from this study did not differ significantly from those in adults collected at many of the same sites [7] when concentrations were normalized either to body lipid \( F_{1,9} = 0.9, p > 0.3 \) by ANCOVA, with stage as factor and \( \log_{10} \) sediment PCB as covariate) or to wet mass \( F_{1,9} = 0.1, p > 0.7 \) (Fig. 6). In fact, PCB concentrations normalized to body lipid and to wet mass in green frog tadpoles also were not significantly different from those in the adult leopard frogs \( p > 0.6 \); the regressions for \( \log_{10} \) routine PCBs in all frog tissues as a function of \( \log_{10} \) sediment total PCBs are given in the legend of Figure 6. Although this analysis suggests there might be a rather uniform response pattern of PCBs in frogs in relation to those in local sediments independent of species or developmental stage, another study of green frogs [8] reported higher PCB levels in tadpoles than in adults for a PCB-contaminated watershed in southwestern Michigan (USA). We compared green frog tadpoles in the two watersheds after normalizing our data to dry mass, as done by Glennemeier and Begnoche [8]. No significant difference was found in tadpole PCB concentration between the two watersheds \( F_{1,4} = 5.2, p = 0.09 \) when controlling for sediment PCB (ANOVA with watershed as factor and sediment PCB as a significant covariate, \( F_{1,4} = 10.9, p = 0.029 \), although the sample size was low \( n = 5 \) pools in the present study and 2 pools in [8]). Consequently, so was the power to detect a difference.

We are not aware of comparable field studies of sediment–frog correlations for heavy metals. Gillilland et al. [38] measured metal concentrations in sediments and in green frog embryos, tadpoles, and adults in southwestern Michigan. However, most of the sites were relatively uncontaminated, and levels for many of the metals were below that study’s MDL in frog tissues. In the next section, we compare our maximum heavy-metal concentrations in frogs with some measures made in frogs at other single locations where sediment metals may or may not have been measured.

In our regressions of tissue PCB versus sediment PCB (Fig. 5), one point (C1) relies on a sediment value measured by others [2]. Our conclusions do not rely heavily on that value, because when it is omitted, the regression of total PCBs is still highly significant \( F_{1,9} = 14.1, p = 0.003 \) and the numerical values of the fitted slopes and intercepts change by only 7% and 1%, respectively. Our analysis of variation in contaminants across sites also would be stronger if we had more replication of sediment samples and tissue pools at each site, which might yield more accurate means and estimates of intrasite variability [38–40]. The \( r^2 \) value of 0.69 for adults and juveniles in Figure 6B suggests that additional research may uncover other significant factors in addition to sediment PCB levels and stage that determine bioaccumulation in frogs, such as aerial inputs from industrial wastes and fires [41,42]. Various abiotic factors, such as pH, hardness, and organic
Table 3. Contaminant levels in green frog tadpoles and leopard frog metamorphs in the Fox River/Green Bay (WI, USA) ecosystem compared with those in frogs collected in other polluted ecosystems

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Level (range) in frogs in the Fox River/Green Bay ecosystem</th>
<th>Species in other ecosystem(s)</th>
<th>Level in frogs in other ecosystem(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>As (µg/g dry mass)</td>
<td>0.15–3.22</td>
<td><em>Bufo terrestris</em></td>
<td>3.1</td>
<td>[53]</td>
</tr>
<tr>
<td>Cd (µg/g dry mass)</td>
<td>0.02–0.10</td>
<td><em>Rana catesbeiana</em></td>
<td>ND–0.31</td>
<td>[54]</td>
</tr>
<tr>
<td>Cr (µg/g dry mass)</td>
<td>0.26–1.14</td>
<td><em>Bufo terrestris</em></td>
<td>4.98</td>
<td>[53]</td>
</tr>
<tr>
<td>Cu (µg/g dry mass)</td>
<td>4.0–58.2</td>
<td>Unspecified species (tadpoles)</td>
<td>8–44</td>
<td>[55]</td>
</tr>
<tr>
<td>Hg (µg/g dry mass)</td>
<td>0.05–4.60</td>
<td><em>R. temporaria</em> and B. <em>bufo</em></td>
<td>0.12–0.76</td>
<td>[56]</td>
</tr>
<tr>
<td>Hg (µg/g wet mass)</td>
<td>0.009–0.810</td>
<td>Unspecified species (tadpoles)</td>
<td>0.325</td>
<td>[57]</td>
</tr>
<tr>
<td>Pb (µg/g dry mass)</td>
<td>0.24–1.14</td>
<td>Unspecified species (tadpoles)</td>
<td>28–1,590</td>
<td>[55]</td>
</tr>
<tr>
<td>Total PCB* (ng/g wet mass)</td>
<td>6.1–568.0</td>
<td>Various species</td>
<td>151–4,470</td>
<td>[40]</td>
</tr>
<tr>
<td>Total PCB (ng/g lipid)</td>
<td>158–71,059</td>
<td><em>R. pipiens</em> adults</td>
<td>19–1,699</td>
<td>[39, 58]</td>
</tr>
<tr>
<td>Coplanar PCB (ng/g wet mass)</td>
<td>3.13–66.40</td>
<td><em>R. ornativentris</em> and <em>R. japonica</em> adults</td>
<td>0.6</td>
<td>[39, 59]</td>
</tr>
</tbody>
</table>

* Ash contaminated mine basin in North Carolina (USA).
* Upstream to downstream of mine tailings holding pond.
* Reference site and stream below mine tailings pond.
* Various locations including urban areas in Finland; corrected to dry mass basis assuming 4 g wet/g dry mass [60].
* Mean of four tadpoles collected around mercury mine at Idrija (Yugoslavia).
* PCB = polychlorinated biphenyl.
* Drainage ditches flowing from uranium enrichment plant in Kentucky (USA).
* Southern Ontario, Canada.
* Site in Kitakyushu (Japan) with relatively high incidence of deformed frogs.

carbon content of sediment and water, also can influence the bioaccumulation of metals and PCBs [13]. The sediment and water-quality values that we report for each of the sites (Table 1) [18] may aid others who are considering the applicability of the correlations to their systems. Larger, more homogenous datasets may yet discriminate differences between species in bioaccumulation of PCBs and metals, but in lieu of those, the regressions of contaminants in frogs versus contaminants in sediment (Figs. 5 and 6) could have predictive value for estimating exposure of amphibians to contaminants in this ecosystem and, perhaps, in others.

**Hypothesis 2: Frog life-history parameters will correlate negatively with level of contamination**

With the exception of Pb, contaminant levels in tissues of green and leopard frogs in the Fox River/Green Bay ecosystem either exceed or exceed those observed in frogs in other contaminated ecosystems (Table 3). We began our study with the a priori expectation that anurans at contaminated sites would be negatively affected by pollutants, either singly or in combination. The significant variation in hatching success among the field sites and the inverse correlations across those sites between pollutants and anuran species richness and hatching success (Fig. 2) seem to be consistent with this idea. Other studies, however, have not found differences in amphibian species richness at PCB-contaminated sites compared to reference sites [8,43].

Survivorship (Fig. 2B), growth (Fig. 3A), and rate of development (Fig. 4A) did not vary significantly with level of contamination. Much of the variation in growth correlated with density in the enclosures (Fig. 3B), which is a significant factor influencing growth rates in frogs [27,28] and one that we confirmed in our manipulation of density at site R2. Slow growth often is associated with delayed maturation at a smaller size in frogs [27,28,37], and our field results are consistent with this (Fig. 4B). The general lack of metamorphosis in green frogs is a natural feature of their life history, because they often overwinter as tadpoles [26]. It is not surprising that level of contamination had no discernible relation to variation in survival, growth, and development time in the field, because at almost all sites, with the exceptions discussed below, ambient levels of NH₃ and tissue levels of PCBs, Pb, Cd, and Zn in the frogs were lower than those associated with depression of these features in laboratory studies with ranid species [12,22,44–46; W.H. Karasov, unpublished observations].

During the field hatch study, un-ionized NH₃ was relatively high (0.2–0.5 mg/L) at some of the relatively contaminated sites (C1, C4, and C6), which were the sites where green frog hatchability was reduced (Fig. 2). In a laboratory study, we observed reduced green frog embryo survival at un-ionized NH₃ levels in excess of 0.6 mg/L [22], which is only a little higher than those observed in the field. When green frogs were exposed to a lower un-ionized NH₃ level (0.5 mg/L) in combination with PCB 126 (1 µg/L), hatchability was significantly reduced compared with that of controls [47]. Thus, it is plausible that high NH₃ depressed green frog embryo hatchability in the field especially if it was combined with another stress. This explanation cannot be applied in the case of leopard frog embryos, however, because un-ionized NH₃ levels in the field were low relative to levels that reduced hatchability in the laboratory (>1.5 mg/L [22]).

It appears that PCB levels in water in this ecosystem alone are not high enough to affect anuran embryo hatchability. Based on a sediment–water partition coefficient of 1.5 × 10⁵ µg/kg dry sediment: µg/L water [48], the total PCB concentration in water at site C1 in the Fox River, our most contaminated site, would be 0.15 µg/L of total PCBs (all congeners). Rosensheid et al. [12] and W.H. Karasov (unpublished observations) found no effects of PCBs 54, 70, 101, or 126 on green frog or leopard frog hatching success in concentrations of up to 50 µg/L, and these water concentrations were associated with tadpole PCB congener concentrations that were much higher (>1,000 ng/g wet mass) than those observed in the field tadpoles (Table 2). Birge et al. [49] measured median lethal concentrations of 3.5 µg/L or greater of Aroclors 1254 and 1242 for embryos of leopard frogs and American toads.
It thus appears that levels of PCBs in Fox River water are at least an order of magnitude too low to cause the reductions in hatchability that we observed in field enclosures. We cannot rule out so easily, however, possible effects in combination with other waterborne contaminants.

At least two environmental factors were not measured in the field that could have caused the differences in hatching success observed at the field sites. First, it is possible that the effects are related to exposure to ultraviolet (UV)-B radiation, either alone or in combination with exposure to environmental toxicants [50,51]. However, UV-B radiation may be rapidly attenuated in waters with high dissolved organic carbon, and embryos of leopard frogs appear to be less sensitive than later life stages to ambient UV-B [52]. It should be possible to test this hypothesis by measuring hatchability at relatively contaminated and uncontaminated sites in enclosures with covers that either do or do not block UV-B or other kinds of radiation [52]. Intermittent changes in water flow and/or level at the sites were never measured during the field experiments. These water-level fluctuations may have affected embryo viability by altering the diffusion distance between sediments and embryos or by stirring up sediments and increasing suspended or dissolved contaminants that reached embryos near the surface. Direct contact with contaminated sediments may adversely affect tadpole growth [39,45]. Also, sites sometimes experienced turbulent waters from current or wind, which may have adversely influenced embryo viability. We are not aware of any tests for effects of such physical disturbance on the hatchability of amphibian embryos.

In summary, tadpoles exposed to relatively high levels of PCBs and metals in sediments at contaminated sites in the Fox River/Green Bay ecosystem had high levels in their bodies relative to those at reference sites in that ecosystem and relative to those in other contaminated ecosystems. Nonetheless, no significant effects were detected in life-history characteristics, such as survival, growth, or rate of development. Although embryo hatchability was negatively correlated with level of sediment contamination, other environmental factors besides PCBs or metals contaminants might be responsible.

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