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Extreme Arsenic and Antimony Uptake and Tolerance in Toad Tadpoles during Development in Highly Contaminated Wetlands

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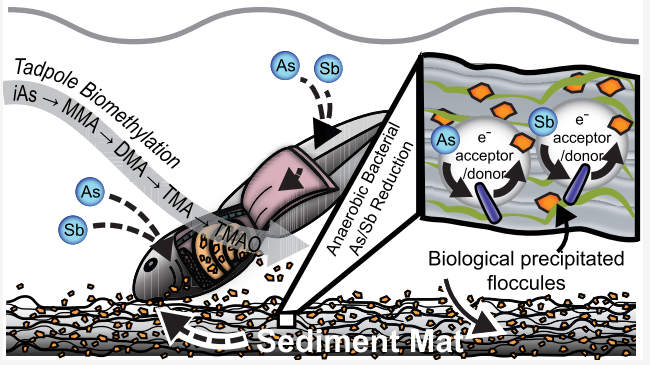
ABSTRACT: The effects of extreme concentrations of toxic metalloids, such as arsenic (As) and antimony (Sb), on larval amphibians are not well-understood. We sampled Western Toad tadpoles (*Anaxyrus boreas*) living in As- and Sb-contaminated wetlands throughout their development. Although the tadpoles completed metamorphosis, they accumulated among the highest concentrations of As and Sb ever reported for a living vertebrate (3866.9 mg/kg; 315.0 mg/kg (dry weight), respectively). Ingestion of contaminated sediment had a more important role in metalloid accumulation than aqueous exposure alone. Metalloids were initially concentrated in the gut; however, by metamorphosis, the majority were found in other tissues. These concentrations subsequently decreased with the onset of metamorphosis, yet remained quite elevated. Sublethal effects, including delayed development and reduced size at metamorphosis, were associated with elevated metalloid exposure. The presence of organic arsenicals in tadpole tissues suggests they have the ability to biomethylate inorganic As compounds. The arsenical trimethyl arsine oxide accounted for the majority of extractable organic As, with lesser amounts of monomethylarsonic acid and dimethylarsinic acid. Our findings demonstrate remarkable tolerance of toad tadpoles to extreme metalloid exposure and implicate physiological processes mediating that tolerance.



INTRODUCTION

The presence of toxic trace metals in surface waters due to industrial production, combustion, and mining activities is an important factor contributing to the decline of some amphibian populations.[1] Arsenic, lead, aluminum, cadmium, zinc, mercury, copper, and other heavy metals have been implicated in causing a variety of negative effects in amphibian species including oral deformities, delayed development, and changes in antipredator behaviors.[2−4] Anuran tadpoles, which are the larvae of frogs and toads, can acquire and accumulate toxins via respiration (i.e., across the gill), ingestion (i.e., across the intestine or gut), and absorption (i.e., across the epidermis during osmotic regulation). The multiple routes of toxin uptake are thought to make tadpoles useful bioindicators of environmental contamination.[5] However, the effects of toxic metals on tadpoles are complex, because sensitivity and tolerance vary, depending on the type of contaminant, habitat conditions, and amphibian species.[1,5−7] Furthermore, tadpoles sequester contaminants in different tissues and few studies have assessed tissue depuration during metamorphosis when biochemical and cellular changes elicit rapid morphogenesis and tissue restructuring.[8]

The metalloids arsenic (As) and antimony (Sb) are environmental contaminants of concern, leading the U.S. Environmental Protection Agency to set the maximum contaminant levels (MCL) for drinking water at <10 μg/L for As and <6 μg/L for Sb, and chronic exposure limits for aquatic life at 150 μg/L for As and a proposed value of 30 μg/L for Sb.[9] These metalloids share geochemical and toxicological properties and primarily occur in the environment as either the +5 or +3 valence state, with the latter being considered more toxic.[10,11] Arsenic and antimony are chalcophile elements and commonly co-occur in hydrothermal ore deposits as sulfide minerals, such as stibnite (Sb_2S_3) and realgar (As_4S_4). The Stibnite Mine site, located in the mountains of central Idaho in the United States, provides a unique opportunity to study ecological effects of these metalloids, as As- and Sb-rich tailing piles leach these elements into nearby headwater streams and wetlands. We previously reported on food web bioaccumulation trends from this freshwater ecosystem and measured elevated levels of As and Sb in water, sediment, and stream biota, as well as extremely

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elevated levels of As and Sb in the wetlands adjacent to tailing piles.[12] In that study, we found the highest ever reported whole-animal As and Sb concentrations in a living vertebrate, which we measured in Western Toad (*Anaxyrus boreas*) tadpoles at 3866 mg/kg and 377 mg/kg (dry weight), respectively. The primary route of uptake for metalloids in tadpoles at the site was determined to be through ingestion of contaminated sediment and microbial mats that contained amorphous As- and Sb-bearing floccules. These floccules were interpreted as biogenic precipitates derived from microbiological As and Sb cycling in the mats and sediment.[12]

Other studies on As and Sb concentrations and their effects on amphibians are limited. To our knowledge, no studies have investigated the effects of high (i.e., concentrations greater than MCL values) Sb concentrations on tadpole growth rates or development, the effects when elevated levels of As and Sb co-occur in the same wetland, or how these metalloids partition among tadpole body parts. Tadpoles are a strictly aquatic life stage found in most anurans and, as such, they are at risk of exposure to contaminants in aquatic environments, because they respire through gills and have specialized mouthparts to feed on benthic biofilm composed of macrophytes, algae, plankton, microbes, and fungi intermixed with inorganic sediments.[13] Thus, to better understand anuran tadpole tolerance to extreme environmental As and Sb concentrations in a field setting, we investigated the following:

(1) What factors, including tadpole developmental stage, water temperature, and sediment and water metalloid concentrations, influence As and Sb concentrations in tadpole tissues?
(2) How are As and Sb, and different As chemical species, partitioned among the animal's gut (gastrointestinal tissue with any remaining ingested material), body tissues without the gut, and tail tissues?
(3) How might metalloid concentrations influence tadpole growth rates and developmental timing?

METHODS AND MATERIALS

Study Area. The Stibnite Mine is located within the Payette National Forest in central Idaho and is highly contaminated with arsenic and antimony from mining activities related to the extraction of gold, antimony, and tungsten ore since 1931. Extensive mine tailing piles covering ~6 km^2 were deposited in and along wetlands and streambanks, providing a source of arsenic and antimony to aquatic habitats.[12]

Western Toads breed in some wetlands that have formed within the mine area, including seasonally flooded impoundments, small, permanent, spring-fed ponds, and semipermanent, spring-fed sites adjacent to the tailings area. We selected three sites for this study that had similar habitat characteristics: each site was ~200 m^2 in area by 1 m in depth at the peak of water storage and each contained submergent macrophytes, filamentous algae, or photosynthetic microbial mats within the upper few millimeters of the sediment surface. Arsenic and antimony concentrations in water, sediment, primary producers, and tadpoles at sites HS, CP, and OP were previously quantified; detailed descriptions of methods and results were provided in 2016 in the work of Dovick et al.[12] For the present study, we quantified As and Sb concentrations in water and sediment samples collected during the middle of our sampling schedule (day 185), using the protocol described in the Dovick et al. work[12] and in tadpole samples collected on each site visit using protocols described below. To account for the known effects of water temperature on tadpole growth and development,[14] including interactions with contaminants,[15] we deployed a water temperature data logger (32K StowAway Tidbit −5 °C to 37 °C logger, Onset Computer Corp., Bourne, MA, USA) in each site at a shoreline used by tadpoles. We placed a solar radiation shield over each logger to prevent direct sunlight from reaching the device through the shallow (ca. 10 cm) water. Temperatures were recorded hourly from the first through final visits at each site. We calculated the cumulative degree-hours (DEGHR, defined as the sum of hourly Celsius temperature values) received at each site from initial deployment to the time of each visit. Temperature loggers were moved periodically, to keep them submerged as water levels receded in each pond.

Collection of Tadpoles. We collected toad tadpoles from June 2012 to August 2012, during five visits spanning their development from hatchlings through metamorphosis as defined by Gosner.[16] Visits were made on days 158, 172, 185, 200, and 207 of the calendar year. On each visit, we randomly selected and captured a minimum of three individuals and a maximum of six individuals at each site using dip nets, for a total of 72 individuals. We measured the size as the length from snout to vent (SVL), using calipers, and quantified the Gosner developmental stage of each tadpole. Gosner stages range from 1−46 (stages: 1−20 = embryo, 21−24 = hatchling, 25−41 = larva, and 42−46 = metamorphosing).[16] We began collecting tadpoles late in the hatchling stage (Gosner stage 23) after they began feeding actively on the sediment and microbial mat. Following capture, individuals were held individually for 48 h in plastic bottles containing 1 L of room temperature water from their site of origin to allow time for defecation to eliminate their intestinal contents.[17] We then euthanized each tadpole following approved protocols (IACUC Permit Nos. 692-AC11-013 and 692-AC12-014) and placed each in a new, sealable plastic bag at −20 °C for later laboratory preparation and analyses.

Sample Preparation. In the laboratory, we triple-rinsed thawed tadpoles with Milli-Q water (resistivity of 18.2 MΩ cm) to remove environmental debris and sediment. Excess water was removed with lint-free tissues and the whole-animal specimens were weighed to determine the initial wet mass (given in grams). We dissected a subset of tadpoles for analysis of metalloid partitioning among tissues using a dissecting stereoscope. Using sterile dissecting tools, we carefully separated the gastrointestinal tract (which included the foregut, midgut, hind gut, and any residual gut contents that remained following our purging procedure) from the body. We then separated the rest of the body and the tail. We hereafter refer to the animal's gastrointestinal tissue with any ingested material remaining after the purging process as "gut", body tissues including internal organs but without the gut and tail as "body", and tissues caudal to the vent as "tail". All whole-animal and dissected tadpoles were freeze-dried using a Labconco FreeZone 4.5L freeze-drying system for 48 h and immediately weighed to determine the dry mass of the sample.

Determination of Metalloid Content by ICP-OES. We analyzed tissue samples for total As and Sb concentration using a Varian VISTA-MPX inductively coupled plasma optical emission spectrometry (ICP-OES) system. We prepared samples through hot plate acid digestion, using 9 mL of a 16 M HNO_3 reflux (trace metal grade) and 3 mL of hydrochloric acid (trace metal grade)[18,19] and reconstituted with 10 mL of

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2% HNO_3 Milli-Q water. Samples were filtered using a 0.45 μm nylon filter and stored in falcon snap tubes prior to analysis. ICP-OES instruments were calibrated daily with National Institute of Standards and Technology (NIST) traceable standards. A second-source NIST-traceable standard was analyzed after every 10th sample to verify accuracy within 10% standard error. A deionized water blank was also run after every 10th sample to monitor baseline drift. Three individuals from each site per visit were analyzed for whole-animal concentrations (n = 43 individuals after two samples were lost during preparation) and 19 additional individuals, collected on days 185−207 across all sites (2−3 individuals per site per visit), were analyzed for tissue-specific concentrations (n = 57 tissue samples [3 tissues × 19 individuals]). Prior to our visit on day 185, tadpoles were too small to accurately dissect.

Determination of As Species by IC-ICP-DRC-MS. To investigate potential metalloid tolerance mechanisms, we conducted an ad hoc As speciation analysis, as described in the work of Alberti et al.[20] on a small subset of 10 freeze-dried tadpoles. We analyzed both whole-animal (n = 6; two individuals from each visit on days 172, 200, and 207) and tissue-specific samples (n = 9 tissue samples, derived from two individuals from each visit on days 200 and 207 [only one of four individuals had a tail at this point; consequently, there were 9 samples rather than 12]). Tadpoles from day 158 were omitted because we had not yet decided to conduct this analysis and those from day 185 were omitted because of a sample collection oversight by field personnel. Similar analysis was not conducted for Sb because reliable speciation methods had not been developed. Each sample was weighed and homogenized by grinding with a mortar and pestle prior to extraction. Samples were combined with 10 mL of a water:methanol (1:1) mixture in a 15 mL centrifuge tube, shaken, and placed in an ultrasonic bath for 20 min. We then centrifuged each sample for 10 min at 3700 rpm and decanted fluid into a 50-mL tube. This process was repeated three times for a total decanted volume of 30 mL. Samples were then allowed to evaporate to dryness at 45 °C. All samples were reconstituted with Milli-Q water to 10 mL and filtered through a 0.45 μm nylon syringe filter (Fisher Co.) prior to analysis of inorganic and organic species of As via ion chromatography−inductively coupled plasma−dynamic reaction cell−mass spectrometry (IC-ICP-DRC-MS).

All prepared samples were analyzed by Brooks Rand Laboratories, where each sample was preceded by a minimum of a five-point calibration curve spanning the entire concentration range of interest. Calibration curves, associated with each species of interest, were standardized by linear regression resulting in a response factor. An instrument blank was used to correct and account for any operational biases. Prior to sample analysis, all calibration curves were verified using second-source standards, which were identified as initial calibration verification standards (ICV). Ongoing instrument performance was identified by the analysis of continuing calibration verification standards (CCV) and continuing calibration blanks (CCB) at a minimal interval of every 10 analytical runs. Quality control analyses indicated a mean (across all analytes) recovery rate of 96.15% of certified reference material, and RPD values for duplicate samples ranged from 0.1 to 1.8.

We report tadpole As speciation values normalized to whole-animal sample dry mass. The total As recovered from speciation analysis (average whole-animal total As = 50.4 ± 5.5 mg/kg) was comparable to values recovered using the standard nonspeciation approach on similar aged individuals captured at the same sampling location (average whole-animal total As = 57.4 ± 14.6 mg/kg).

Data Analysis. We used nonparametric multiplicative regression (NPMR) (HyperNiche 2.30 software[21]) to model the influence of potentially multiple, interacting predictor variables on univariate responses, including whole-animal As or Sb (n = 43 individuals), tissue-specific As or Sb (n = 57 tissue samples), and tadpole developmental stage, SVL, or mass (n = 43 tadpoles, the same 43 individuals used for whole-animal As and Sb). This approach allowed us to assess which predictor variables (tadpole developmental stage, water temperature, and sediment and water metalloid concentrations) were important, after accounting for the influence of the other predictors in the model. Potential predictor variables were not retained in final models when they did not improve model fit by at least 5% over the best model with one fewer predictor variable. Using NPMR also allowed us to fit nonlinear, nonparametric curves to the data which, after preliminary inspection, appeared to exhibit curvilinear patterns. For each analysis, we report xR^2, which is a "leave-one-out" cross-validated measure of model fit that is analogous to, but more conservative than, the traditional R^2-value.[22] We also report a p-value derived from Monte Carlo randomizations testing the null hypothesis that the fit of the best model is no better than what could be obtained by chance using the same number of predictor variables in 100 free-search iterations with randomly shuffled response values. Finally, we report N^*, which represents the average number of data points contributing to each estimate on the modeled response surface.[22] This test statistic provides an estimate of model robustness. Bootstrap resampling (resampling each dataset with replacement 1000 times) was used to assess the stability of models when different input data were used and to create 95% and 5% variability bands for figures, indicating the range of values that new, unsampled individuals would have 90% of the time for any given combination of predictor variables. Arsenic speciation results were not analyzed using NPMR due to small sample sizes. Instead, we simply compare mean concentrations (± standard error, SE) and percent of As recovered (±SE) for each As species in whole-animal samples and in gut versus body and tail tissue to demonstrate the potential for biomethylation in these tadpoles.

RESULTS AND DISCUSSION

Metalloid Concentrations through Development. Tadpoles accumulated extreme amounts of whole-animal As and Sb, with mean (among individuals from a given site and day; ± SE) As values ranging from 106.5 ± 5.83 mg/kg to 3099.1 ± 389.4 mg/kg (dry weight) and mean Sb concentrations ranging from 5.31 ± 0.13 mg/kg to 286.6 ± 14.2 mg/kg (dry weight). These values are similar to those obtained previously at our sites.[12] However, previous studies of tadpoles elsewhere in contaminated freshwater environments reported whole-animal As values that ranged from below the detection limit up to 62 mg/kg (dry weight) when As water values were up to 1000 μg/L and the sediment As value was 60 μg/g.[17,23−25] One study also reported whole-animal Sb values of 174 mg/kg (dry weight), where the Sb water concentration was 381 μg/L and sediment Sb concentration was 777 μg/g.[25] Tadpoles at our sites were living with 2 orders of magnitude more As and 1.6 times as much Sb in their bodies than has been reported elsewhere.

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Moreover, we found substantial differences in metalloid concentrations, depending on the development stage and environmental metalloid exposure. Tadpole whole-animal As was best predicted by an interaction between cumulative DEGHR and sediment As concentration (xR^2 = 0.89; p < 0.001, N^* = 4.3; see Figure 1a). The water As concentration,

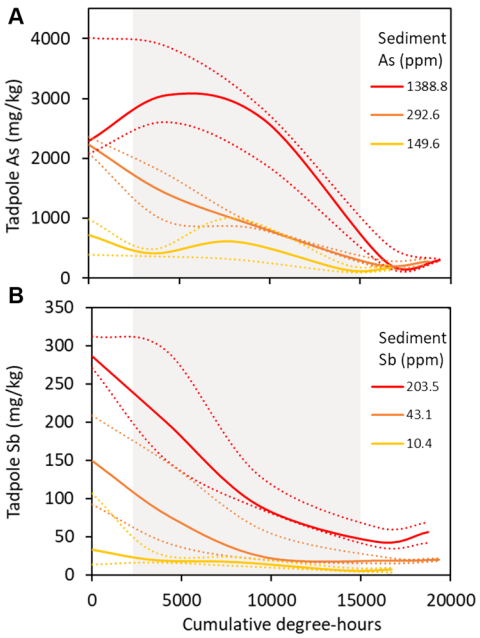

Figure 1. NPMR modeled relationships between water temperature exposure (cumulative DEGHR, x-axes), sediment metalloid concentrations (colored line series, with high, moderate, and low values shown), and tadpole whole-animal (a) As and (b) Sb concentrations (y-axes). Dashed lines represent 95% and 5% variability bands. Gray shaded areas indicate when the majority of tadpoles were in the larval stage (Gosner stages 28−39). Unshaded samples earlier in each time series mostly consisted of late-hatchlings (Gosner stages 23−26), and unshaded samples later in each time series consisted of animals going through metamorphosis (Gosner stages 41−46).

site of origin, and day of the year did not improve the explanatory power and were not included in the final model. Similarly, whole-animal Sb was best predicted by an interaction between cumulative DEGHR and sediment Sb concentration (xR^2 = 0.81; p < 0.001, N^* = 4.0; see Figure 1b). The water Sb concentration, site of origin, and day of the year also did not improve the explanatory power in this model. Others have found that tadpoles raised in laboratory settings under varying concentrations of As^V (50−1000 μg/L) accumulate dissolved As in proportion to the concentration in water.[26,27] However, our tadpoles were not only exposed to dissolved As and Sb in water, but also to As and Sb associated with ingested mixtures of benthic sediment and biofilms. Our results, along with those from other field-based studies,[17,24,28,29] suggest that the intake of sediment during grazing has a greater effect on total tadpole metalloid concentration than does aqueous metalloid concentration. Our high values and close association with sediment concentrations could be attributed to ingested material that remained in the intestinal tract. However, our analysis of tissues, excluding the gut (see below), corroborate the findings of high values and greater correlation between sediment and tissue metalloid concentrations than that observed between water and tissue.

Generally, metalloid concentrations were highest during the late-hatchling and early larval stages (<2500 DEGHR; day 158 with Gosner stages 23−26) and larval stages (2,500−10,000 DEGHR; days 172 and 185 with Gosner stages 28−39), before declining and then leveling off during the metamorphosis stage (15,000−19,000 DEGHR; days 200 and 207 with Gosner stages 41−46; see Figure 1). The mean whole-animal As concentration decreased by 73%−87% (depending on the site) between the first and final sampling events. Similarly, the mean whole-animal Sb also declined by 73%−87% (depending on the site) between the first and final sampling events. However, the developmental stage in which these metalloids decreased varied, depending on the relative abundance of the two metalloids in the tadpoles' environments. Tadpoles exposed to relatively high As and Sb exhibited a concurrent decrease in the two metalloids throughout the development (see blue points in Figure 2). However, those exposed to greater As, relative to Sb,

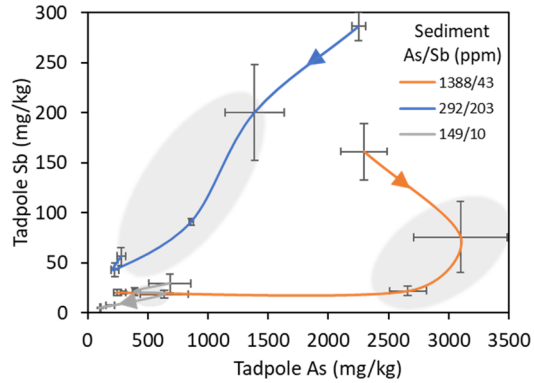

Figure 2. Mean ± SE tadpole metalloid concentrations through time for tadpoles exposed to different sediment metalloid concentrations (colored series with mean sediment As and Sb concentrations shown). Arrowheads point toward the final sampling event at metamorphosis; gray shaded areas indicate when the majority of tadpoles were in the larval stage (Gosner stages 28−39); unshaded samples earlier in each time series mostly consisted of late hatchlings (Gosner stages 23−26); and unshaded samples later in each time series consisted of animals going through metamorphosis (Gosner stages 41−46). Sites included are HS, CP, and OP.

exhibited a decrease in Sb concentrations throughout the first three sampling events (days 158, 172, and 185), while As concentrations remained unchanged or slightly increased during this time (see the orange and gray points in Figure 2). Substantial reductions in As did not occur until the last two visits (days 200 and 207) at these locations. Our findings suggest that (1) there are pronounced changes in tissue metalloid concentrations as tadpole feeding behavior and morphology change and (2) physiological mechanisms may result in differential depuration of metalloids through time when individuals are exposed to more than one contaminant. These conclusions are supported by a similar study that found when gray treefrogs (*Hyla versicolor*) were exposed to a single contaminant, such as selenium or vanadium, they exhibited contaminant-specific depuration patterns through development, with tadpole selenium concentrations remaining fairly constant (possibly because, at low tissue concentrations, selenium is an essential micronutrient that the body may retain), whereas vanadium concentrations peaked pre-metamorphosis, before declining post-metamorphosis.[30]

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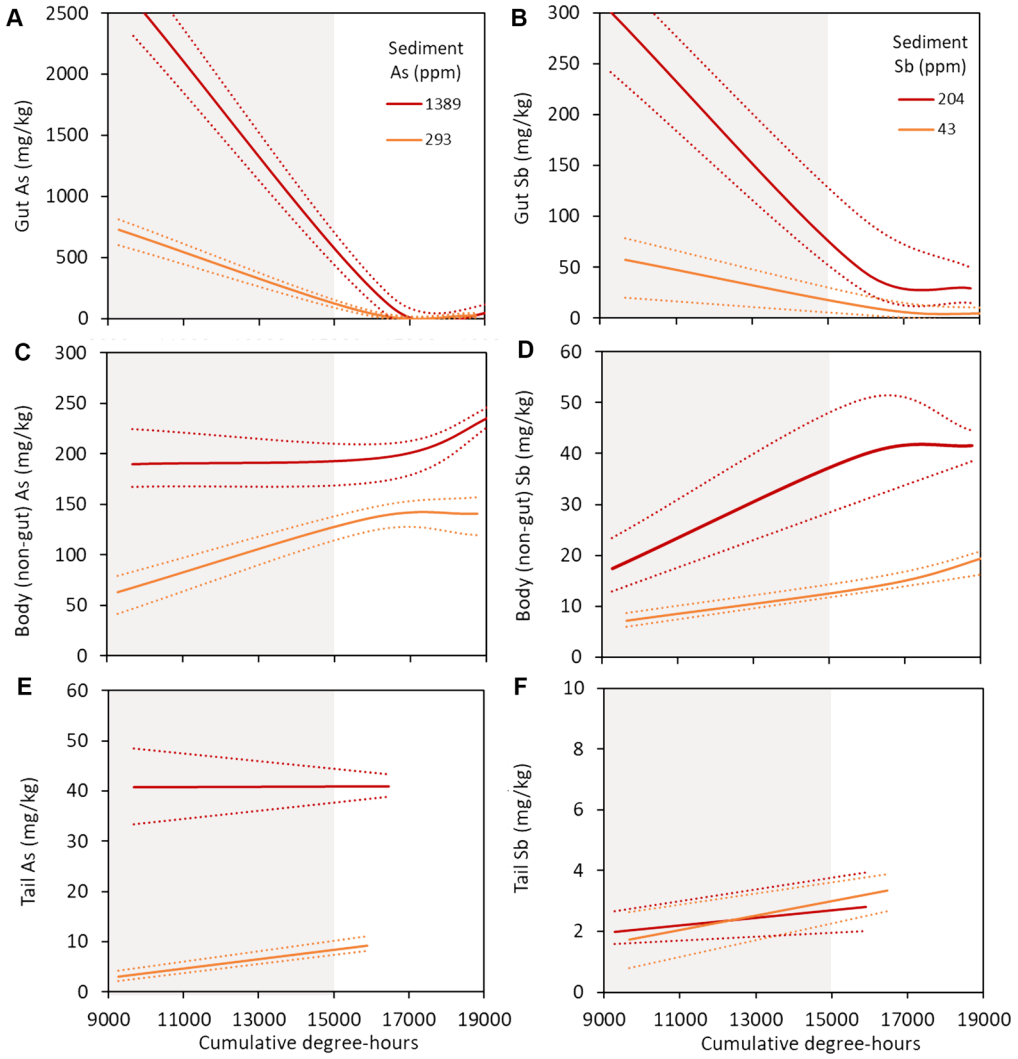

Figure 3. NPMR modeled relationships between water temperature exposure (cumulative DEGHR, *x*-axes), sediment metalloid concentrations (colored line series, with high and moderate values shown), and metalloid concentrations (*y*-axes) in (A, B) tadpole gut, (C and D) body excluding the gut, and (E and F) tail tissue. Dashed lines represent 95% and 5% variability bands. Gray shaded areas indicate when the majority of tadpoles were in the larval stage (Gosner stages 28−39). Unshaded samples later in each time series consisted of animals going through metamorphosis (Gosner stages 41−46). Hatchling stage tadpoles were too small to be reliably dissected; consequently, they are not included here. Almost all of the tadpoles had almost resorbed their tails by the final two sampling events; consequently, there are no values for tail tissue after 16 000 DEGHR.

Tissue-Specific Metalloid Concentrations and Arsenic Speciation Analysis. Arsenic was partitioned differently among tadpole tissues and the relative proportions of As burden changed through the developmental process. Arsenic partitioning was best predicted by an interaction between cumulative DEGHR, sediment As concentration, and specific tissue (xR^2 = 0.96; p < 0.001, N^* = 4.5; see Figure 3). The water As concentration, site of origin, and day of the year did not improve the explanatory power and were not included in the final model. Antimony partitioning was best predicted by an interaction between cumulative DEGHR and sediment Sb concentration and specific tissue (xR^2 = 0.81; p < 0.001, N^* = 4.0; see Figure 3). The water Sb concentration, site of origin, and day of the year did not improve the explanatory power.

Generally, metalloid accumulation transitioned (or potentially transferred) from the intestinal tract to the body during tadpole development. Tail tissue had comparatively low metalloid accumulation and changed little throughout development. Arsenic and antimony in specific tissues varied together through time, with tissue concentrations of each metalloid depending on the sediment metalloid concentrations to which tadpoles were exposed (see Figure 3). In larval stage tadpoles, As and Sb located in the gut accounted for 90%−94% of As and 84%−93% of Sb in the total whole-animal concentrations. During this stage, the body contained 5%−8% (As) and 5%−11% (Sb), with the remaining 1% (As) and 2%−5% (Sb) located in the tail. These findings are in agreement with several previous studies that reported the intestinal tract as being the primary location of metalloid concentration in tadpoles, because of the ingestion of contaminated sediment.[12,17,24,28]

During the metamorphosis stage, sediment and microbial mat material in the intestinal tract was negligible, because the animals had ceased eating when their oral anatomy and digestive tract began to change.[31] During this phase, almost all tadpoles had resorbed their tails and the gut accounted for only 18%−19% of As and 19%−41% of Sb, with metalloids mainly located in the body (As, 81%−82%; Sb, 59%−81%) with its internal organs including the liver. This change in feeding behavior, and the lack of visible sediment in the gut at the metamorphosis stage during our dissections, explains the

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substantial decrease in whole-animal concentration of As and Sb. However, even following the pronounced decrease in metalloid concentrations after the completion of metamorphosis, these individuals retained As and Sb concentrations greater than those that have been reported for tadpoles in other contaminated systems. Furthermore, we found that metalloid concentrations in nongut tissues continued to increase through the end of metamorphosis, even while whole-animal values decreased due to absence of gut material (or due to the loss of tissue mass through metamorphosis, resulting in a concentration of contaminants in the remaining tissue). This evidence for tissue transference, particularly in tissues developing *de novo* after depuration, was observed in metamorphosing frog tadpoles that had been exposed to dissolved ^{75}Se (selenite) for 7 days.[8] These findings have important food web implications, since the consumption of tadpoles by higher trophic level species could introduce metalloid contaminants into terrestrial or other aquatic systems, regardless of whether tadpoles contain contaminated sediment in their gut.[32]

Although the sample size for our post hoc As speciation analysis was limited to 10 individuals, there were patterns suggesting As biomethylation in developing tadpoles. Across all samples, the inorganic forms As^{V} and As^{III} combined, averaged (±SE) 19.03 ± 0.93 mg/kg, which accounted for 65% ± 0.02% of recovered As. Organic forms, which consisted of monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and trimethyl arsine oxide (TMAO), averaged a combined 10.79 ± 0.95 mg/kg, which accounted for 35.0% ± 0.02% of the As recovered across samples. Arsenobetaine (AsB) levels were below the instrument detection limit (<0.0032 mg/kg) in all samples. As^{V} was more prevalent than As^{III} (As^{V} average = 16.49 ± 0.87 mg/kg [54.5% ± 0.02% of As recovered] and As^{III} average = 2.55 ± 0.19 mg/kg [10.4% ± 0.01%]). TMAO was, by far, the most prevalent organic form detected (average = 8.67 ± 0.76 mg/kg) and accounted for 27.5% ± 0.01% of recovered As. DMA and MMA each averaged <1.28 ± 0.16 mg/kg and each accounted for <5.1% ± 0.01% of recovered As.

Similar findings were reported for laboratory-reared tadpoles and post-metamorphic Western Clawed frogs (*Silurana* [*Xenopus*] *tropicalis*), in which As^{V} and TMAO were the predominant arsenicals in whole-animal samples.[26] The As metabolic pathway in amphibians is generally thought to consist of a series of reductive and oxidative methylation processes ending with metabolites such as TMAO and tetramethylarsonium (TMA).[33,34] Arsenic methylation is commonly assumed to be a detoxification process, because of the reduced toxicity of methylated products MMA^{V} and DMA^{V}, compared to inorganic As.[35]

Gut-only samples (n = 4) had a greater proportion of inorganic As (average = 76.7% ± 0.09% of As recovered) and a lower proportion of organic As (average = 23.3% ± 0.09% of As recovered) than four samples derived from the body (inorganic average = 59.7% ± 0.05% and organic average = 40.3% ± 0.05% of As recovered). This suggests that methylation may occur predominantly in the body and tail, most likely in the body, given that it contains the liver, where most xenobiotic detoxification occurs. Others have reported a similar distribution of gut arsenicals in a study of adult Western Clawed Frogs[26] or in whole-animal samples of Iberian Green Frog (*Pelophylax perezi*) tadpoles,[27] and they concluded that the methylated compounds were partially due to biomethylation occurring within the animal's body, or in the former study, within specific tissues. Our findings suggest that these tadpoles methylate As (and perhaps Sb), which could provide a mechanism contributing to their tolerance to extremely elevated metalloid concentrations in their environment (see the graphical abstract for a conceptual model).

Tadpole Development and Growth. Tadpole development, as measured by Gosner stage, was best predicted by an interaction between cumulative DEGHR and whole-animal As concentration (xR^2 = 0.97; p < 0.001, N^* = 5.5; see Figure 4a). The water and sediment As and Sb concentration, site of origin, and day of the year did not improve model fit. Tadpoles with the lowest whole-animal As concentration reached

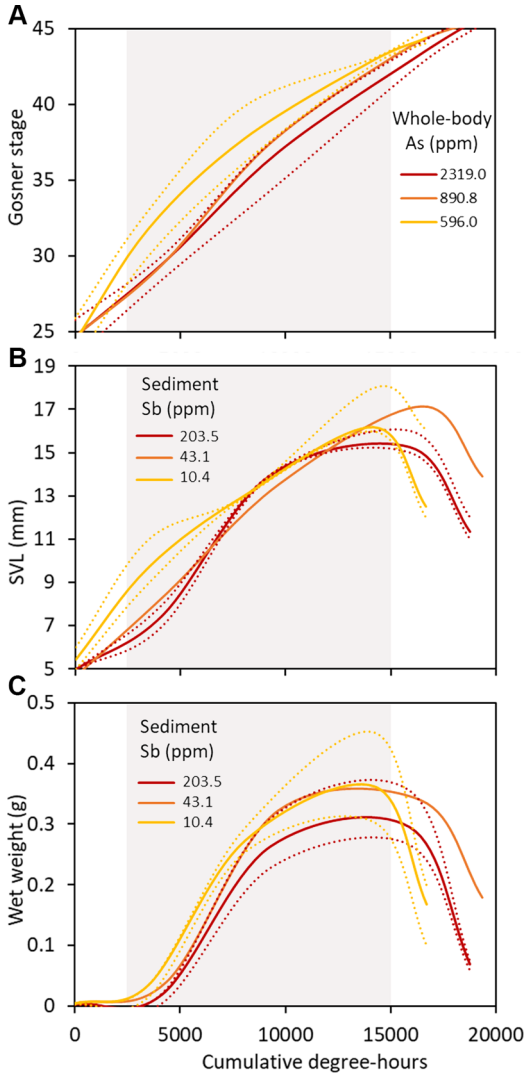

Figure 4. NPMR modeled relationships between water temperature exposure (cumulative DEGHR, x-axes), metalloid concentrations in tadpoles (whole-animal) or sediment (colored line series), and tadpole: a) Gosner stage, b) snout-to-vent length (SVL), and c) wet mass (y-axes). Dashed lines are 95% and 5% variability bands. Orange variability bands, omitted for clarity, were similar widths to those shown for yellow and red series. Gray shaded areas indicate when the majority of tadpoles were in the larval stage (Gosner stages 28−39). Unshaded samples earlier in each time series mostly consisted of late-hatchlings (Gosner stages 23−26) and unshaded samples later in each time series consisted of animals going through metamorphosis (Gosner stages 41−46).

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metamorphosis after fewer DEGHR than those with greater tissue As concentrations (see Figure 4a). The greatest effect on development rate was observed early in the larval stage after 2500−5000 DEGHR, when tadpoles with <600 ppm As were several Gosner stages more advanced than those with greater whole-animal As concentrations, although this difference remained significant for the duration of the study (see Figure 4a).

Generally, tadpole SVL increased throughout development until tail resorption began during metamorphosis. The snout-to-vent length was best predicted by cumulative DEGHR and sediment Sb concentration (xR^2 = 0.91; p < 0.001, N^* = 4.0; see Figure 4b). The water and tadpole tissue As and Sb concentration, sediment As concentration, site of origin, and day of the year did not improve model fit. Tadpoles exposed to the lowest sediment Sb levels had greater SVL during hatchling and early larval stages (500−7500 DEGHR), reached their maximum SVL after fewer DEGHR, and metamorphosed at greater final SVL than those exposed to the highest sediment Sb levels (see Figure 4b).

Tadpole mass followed a similar pattern as that which was observed for SVL, with increasing mass until the onset of metamorphosis, when individuals stopped feeding and gradually lost mass. As with SVL, tadpole wet mass was best predicted by cumulative DEGHR and sediment Sb concentration (xR^2 = 0.76; p < 0.001, N^* = 4.1; see Figure 4c). The water and tadpole tissue As and Sb concentration, sediment As concentration, site of origin, and day of the year did not improve model fit. Although they reached maximum mass after similar DEGHR, tadpoles exposed to the lowest sediment Sb contamination had greater mass during the early larval stage (2500−7500 DEGHR) and metamorphosed at greater final mass than those exposed to the highest sediment Sb contamination (Figure 4c). The difference in final mass was substantial, as tadpoles exposed to the least sediment Sb weighed 165% more than those exposed to most sediment Sb.

A critical environmental factor influencing tadpole development is water temperature, which can affect timing and rate of development, as well as tadpole size at metamorphosis.[36] Typically, cooler rearing temperatures will prolong tadpole development, but will result in larger individuals at metamorphosis. We found significant influences of increasingly elevated metalloids on tadpole growth and development in our field settings after statistically accounting for cumulative water temperature exposure. Others have reported that Iberian Green Frog tadpole developmental rate was unaffected by heavy metal exposure,[27] albeit at much lower ambient As concentrations (50−100 μg/L) than we observed. Chen et al.[23] and Bryszewska et al.[27] also reported no significant impacts on tadpole survival, overall size, tail resorption rate, or sex ratios from As exposure between 10−1000 μg/L in the Iberian Green Frog and the Northern Leopard Frog (*Lithobates pipiens*). However, tadpoles exposed to coal combustion waste, which commonly includes As as well as other heavy metals, have exhibited oral deformities, delayed development, reduced size, and changes in antipredator behaviors.[2−4,7,19,37] In our study, tadpole growth and development were influenced by increasing metalloid concentrations, but similar to the other studies, it is difficult to be certain whether this was caused directly by As, a combination of contaminants, or by other factors, such as metal-induced changes in the microbial community of the tadpole gut.[38] Delayed tadpole development and reduced tadpole mass and size also may result from other environmental factors that include low pH, high population density, and the heavy metals zinc, cadmium, and lead.[2,39,40] The pH, zinc, cadmium, and lead concentrations from our sites were not significantly elevated (see the work of Dovick et al.[12]), relative to established sediment threshold effect levels defined in the work of Smith et al.[41] and, although not quantified, population densities appeared roughly equal across sites. Our analyses suggest that elevated tissue As was correlated with development delays, whereas the ingestion of Sb-contaminated sediment may contribute to reductions in length and mass of these tadpoles. Further studies with tadpoles reared under controlled laboratory conditions exposed to aqueous Sb and Sb-contaminated food sources could provide additional insights into the sublethal effects of Sb, as well as the potential role of biomethylation in As and Sb tolerance in toad tadpoles.

Others have found that effects of metalloids on tadpoles may be mediated more strongly by maternal exposure than by direct tadpole dietary exposure.[14,42] Although we do not know the extent of maternal exposure at our sites, it is likely to have occurred, given the relatively strong breeding site fidelity of Western Toads.[43] The combined or individual effects of maternal and direct metalloid exposure on tadpole development, growth, and fitness raise the question of how populations of some amphibian species are able to persist in contaminated breeding sites. One potential mechanism is that highly contaminated breeding sites could generate strong selective pressure for adaptive tolerance mechanisms, such as biomethylation, in tadpoles and juvenile toads. At our study site, intensive mining has occurred for almost 100 years and we plan to investigate the possibility of local adaptation through a common garden study.

Together, our findings demonstrate uptake and accumulation of extremely high amounts of toxic metalloids in Western Toad tadpoles. Despite the severity of As and Sb exposure throughout development, tadpoles completed metamorphosis to juvenile toads without any observed mortality. Physiological processes such as biomethylation of As (and possibly Sb) may have contributed to the surprisingly high metalloid tolerance of toad tadpoles at this location. However, sublethal effects on tadpole growth and development were observed and these have been linked to reductions in fitness and survival post-metamorphosis.[44−46] Individuals that take longer to reach metamorphosis incur increased risk of predation and desiccation[47] and smaller post-metamorphosis individuals have reduced fitness as juveniles or adults.[46] Regardless, these tadpoles demonstrated a remarkably high tolerance to metalloid contamination, which suggests a toxicity tolerance much higher than what is commonly presumed among amphibians,[5] and this high tolerance may well reflect local adaptation to metalloid contamination. Further work is needed to determine whether metalloids in tadpole tissues influence growth, survival, reproduction and fitness of subsequent post-metamorphic life stages (i.e., carryover effects) and whether concentrated As and Sb are transferred into the terrestrial food web when these contaminated tadpoles, juveniles, or adults are consumed by higher trophic levels in both terrestrial and aquatic habitats.

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Notes

The authors declare no competing financial interest.

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