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Arsenic(+3) and DNA methyltransferases and arsenic speciation in tadpole and frog life stages of Western Clawed frogs (Silurana tropicalis) exposed to arsenate‡

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‡ This paper is dedicated to Bill Cullen on the occasion of his 80th birthday

Abstract

Western clawed frog (Silurana tropicalis) embryos were exposed to control, low (nominally 0.5 mg/L) and high (nominally 1 mg/L) arsenate (AsV) culture water concentrations to investigate the effects of arsenic (As) on different life stages, namely tadpole (Nieuwkoop and Faber stage 56, NF56) and frog stages (NF66). The effects were assessed by measuring arsenic (+3) and DNA methyltransferases (AS3MT and DNMT1), as well as As speciation in the tissues. The As content in frog tissues increased with water As concentration. The As species observed by high performance liquid chromatography – inductively coupled plasma mass spectrometry (HPLC-ICPMS) were mostly inorganic, dimethylarsinic acid (DMA) and trimethylarsine oxide (TMAO). With solid state x-ray absorption near edge structure (XANES) analysis, arsenobetaine /tetramethylarsonium ion were also seen. AS3MT levels decreased upon low As exposure in NF56, rising again to control levels at the high As exposure. In NF66 tissues, on the other hand, AS3MT decreased only with NF66 high As exposure. DNMT1 increased with exposure, and this was statistically significant only for the high As exposure at both life stages. Thus these enzymes seem to be affected by the As exposure. Methylation of As to form monomethylarsonate (MMA), DMA and TMAO in the frogs appeared to be inversely related to AS3MT levels. A possible interpretation of this finding is that when AS3MT is higher, excretion of MMA+DMA+TMAO is more efficient, leaving lower concentrations in the tissues, with the opposite effect (less excretion) when AS3MT is lower; alternatively, other enzymes or linked genes may affect the methylation of As.

Introduction

Arsenic (As) is recognized as a poisonous substance but its toxicity depends largely on its chemical form, or speciation. Generally organoarsenic species, those that contain As-carbon bonds, are considered to be less toxic than inorganic As (such as arsenate AsV and arsenite AsIII), although notable exceptions have been reported in recent years. Examples of these include trivalent methyl and dimethylarsenic III species, and thiolated dimethylarsenicV, which are found in some tests to be more toxic than AsIII. Arsenobetaine (AB, (CH3)3AsCH2COO−) is essentially non-toxic but other organoarsenicals demonstrate
a measurable toxicity; even arsenosugars, which exhibit minimal in vitro toxicity, can be converted in biological systems to DMA(V), which exhibits some toxicity and possible links to cancer in animals.

Because of their differing toxicities, biotransformations of inorganic As species to organoarsenicals are an important aspect of As cycling in the environment. Methylation of inorganic As, for example, to dimethylarsinate (DMA(V), ((CH₃)₂As(O)O⁻) in higher organisms may be responsible for both detoxification via excretion, and activation of toxic action since the toxic trivalent methylarsenic species are also formed. Methylation of As is commonly thought to occur via the Challenger pathway, a series of reducing and oxidative methylation steps. The oxidative methylation of As is catalyzed by the enzyme As (+3 oxidation state) methyltransferase (AS3MT), with S-adenosylmethionine (SAM) as a source of methyl groups; SAM also serves the same purpose in DNA methylation. Therefore it is not a surprise that As exposure and administration interfere with DNA methylation; and it has been further demonstrated that As over-exposure could trigger epigenetic-related carcinogenesis. As such, it has been proposed that one mechanism behind the As-dependent disruption of DNA methylation is the competition for SAM between two methylation events.

Furthermore, As metabolism is able to alter the activity of DNA methyltransferase (DNMT), which is directly responsible for DNA methylation. The extra consumption of SAM from As methylation yields excessive accumulation of S-adenosylhomocysteine (SAH), which has been demonstrated to be inhibitory to DNMT activity. Among the three major DNMTs (DNMT1, 3A and 3B), DNMT1 plays central roles in keeping epigenomic integrity by maintaining genomic DNA methylation patterns during DNA replication. Previous studies have proposed As-dependent DNMT1 responses in mammalian systems, indicating potential consequences on DNA methylation profiles from As exposure.

Therefore, the investigation of As methylation and its impact on DNA methylation machineries, via the AS3MT and DNMT1 enzymes, along with As species, in an organism may be a complementary approach in assessing the effect of inorganic As. Others have found a possible association of superior As methylation capacities with the hepatocytes of some mammalian species compared to others. However, factors other than the presence of AS3MT may contribute to this characteristic. Methylation and AS3MT expression may in some cases be associated with enhanced susceptibility of cell lines to acute toxic effects of inorganic As. At the same time, the absence of AS3MT in knockout mice was associated with an accumulation of inorganic As in tissues, and reduced overall urinary clearance of As, compared with wild-type mice. The aforementioned studies showed primarily that the presence or absence of AS3MT significantly affects methylation processes and toxicity of As; however, the implications of AS3MT levels are not as clear. For example, they appear to be largely independent of As-administered dose in most parts of the mouse brain.

The organism of interest in the current study is the Western clawed frog (Silurana tropicalis). Frogs are generally useful indicators of environmental quality because adults and larvae live in both aquatic and terrestrial environments. Frogs have been found to accumulate As in the mg/kg range both in tadpoles and adults at contaminated sites, as well as in laboratory-reared tadpoles. Inorganic As has long been known to be toxic to frogs, but frogs nevertheless appear to be able to live in waters contaminated with high levels of As, and with no apparent ill effect when body burdens are
as high as 4 mg/kg wet weight. The present study therefore aims to add to the fairly limited existing knowledge on the impact of As exposure to aquatic amphibians, in the context of As speciation and related key enzyme expression patterns.

While previous studies on As in frogs have utilized frog species that occur naturally in North American and European sites, we target *S. tropicalis*, because of its well-characterized genome and developmental record and its use as a model system in epigenetic research. These features have led to the present investigation of the impact of As on DNA methylation behaviours, and to additional studies probing the effects of As on gene and protein expression (manuscripts in preparation).

In this study on the effects of inorganic As on *S. tropicalis*, we report the measurement of As content and species in tadpoles at Nieuwkoop and Faber stage 56 (NF56), corresponding to approximately 7 weeks of age (when limbs first appear), and NF66, corresponding to around 15 weeks of age (immediately after metamorphosis). In previous studies, one of the major organoarsenicals measured was the tetramethylarsonium ion (Tetra, (CH$_3$)$_4$As$^+$), found in tissues of laboratory-reared frogs (*Rana* sp.) up to a tadpole stage just before metamorphosis, as well as in adult frogs collected from an As-contaminated site. Tetra is generally considered non-toxic in marine and terrestrial animals, although the halide salts have been found to have higher acute toxicity in mammalian living systems than some other organoarsenicals. Tetra is fairly common in the marine environment, found in clams, marine polychaetes, sea anemones, and gulls, but in the terrestrial environment is limited primarily to the afore-mentioned frogs along with rare occurrences in mushrooms and some terrestrial plants. One of our hypotheses is that Tetra will also be formed in *S. tropicalis*, as it is in other frogs. We also hypothesize that the measurement of AS3MT and DNMT1 protein levels will help to explain the observances (if any) of methylated species in the different life stages of *S. tropicalis*, and these trends may yield information about the effect of inorganic As on this organism.

**Materials and Methods**

**Materials**

For the animal exposure experiments, a sodium arsenate dibasic heptahydrate solution (As$^V$, ≥98.0%, Sigma-Aldrich, Oakville, ON, CA) was used. Other chemicals used included 70% nitric acid (reagent grade, Fisher), 30% hydrogen peroxide (in water, reagent grade, Caledon Laboratory), pyridine (99% purity, Sigma), formic acid (reagent grade, Fisher), ammonium nitrate (99.999% purity, Aldrich), and nitric acid for ICP-MS analysis (Optima grade, Fisher). Internal standards were rhodium and indium (1000 mg/L, SCP Science). As standards were obtained from two suppliers where possible (one for calibration, the other for quality control checks) as follows: As$^{III}$ as 1000 mg/L solutions prepared from arsenic trioxide (Inorganic Ventures and Fluka); As$^V$ as 1000 mg/L solutions prepared from arsenic pentoxide (Inorganic Ventures) and As metal (AccuStandard, 1000 mg/L); DMA as cacodylic acid (Fluka and City Chemical LLC); monomethylarsonate, MMA, as monosodium acid methane arsonate sesquihydrate (two lots from Chem Service); arsenobetaine, AB (Wako and Argus); arsenocholine, AC, as AC-bromide (Wako and Argus); tetramethylarsonium ion, Tetra, as tetramethylarsonium iodide (Wako); and trimethylarsine oxide, TMAO (Argus). Arsenosugars used as standards were extracted from brown algae (*Fucus*).
vesiculosus) collected in Nova Scotia, Canada: the extraction method followed the steps described by Madson et al. Certified reference material BCR 627 (Tunafish, Institute for Reference Materials and Measurements, Belgium) was used.

Animals and Exposures

S. tropicalis were bred as described previously. Briefly, two pairs of mature frogs were injected with 12.5 U human chorionic gonadotropin (hCG) prior to mating and kept in a pH 5.8 – 6.0 water environment for 24 h after injection, followed by a booster injection of 200 U hCG to each frog. Fertilized eggs were then collected and maintained according to the Standard Guide for Conducting the Frog Embryo Teratogenesis Assay: Xenopus (FETAX). Collected eggs were treated with 2% w/v L-cysteine solution (pH 8.1) to remove their jelly coat. Eggs were then washed with FETAX solution (10 mM NaCl, 1 mM NaHCO₃, 0.4 mM KCl, 0.1 mM CaCl₂, 0.3 mM CaSO₄,•2H₂O and 6 mM MgSO₄ in dechlorinated tap water) three times before the next step.

Eggs were collected, grouped into 50 per glass jar and kept in FETAX solution with 0.4 ppm gentamycin sulphate. Developmental stages of the animals were determined according to the Nieuwkoop and Faber (NF) developmental table. Exposure to As started at the embryonic stage NF12 (approximately 8 h post fertilization). From NF12 to NF46 (approximately 72 h post fertilization), water was changed every 24 h, and then animals were transferred into 8-L tanks and water changes occurred twice a week. Experimental groups were exposed to nominal concentrations of 0.5 (low) or 1 (high) mg/L As⁴ and an As-free control group was maintained in the same way. These corresponded to measured water concentrations of 0.0058 ± 0.0005 mg/L (control), 0.33 ± 0.07 (low) and 0.83 ± 0.12 mg/L (high); measurements are means of two samples, one collected at 72 h (3 d) and one at 168 h (7 d) at the same time that the FETAX solution was changed. Animals were fed twice a day so as to provide an ad libitum supply; tadpoles were fed a high protein powdered food (Sera Micron®; AniDis, St. Laurent, QC, Canada, containing spirulina, krill and shrimps) and after arms emerged (stage NF58), they were fed crushed adult food pellets (Nasco, California, USA, containing fish, meat and soybean meal, grains, yeast, egg, whey and vitamins). For both control and experimental animals, samples were collected at stages NF56 (premetamorphic tadpoles, corresponding to approximately 7 weeks of age and when limbs first appear), and NF66 (metamorphic frogs, corresponding to around 15 weeks of age, immediately after metamorphosis). Animals were sacrificed for tissue collection upon reaching the targeted stages. NF66 animals were dissected to remove brain, liver, gonads, stomach, intestines and kidneys, leaving the remaining tissue as the carcass. All tissues were immediately frozen upon sampling and stored at -80 °C until analysis for AS3MT. Prior to As analysis subsamples were freeze-dried to constant weight and then ground by hand in a ceramic pestle and mortar. All animal experiments were conducted following the guidelines from Canadian Council of Animal Care and exposure protocols were approved by Queen’s University Animal Care Committee.

AS3MT and DNMT1 Analysis

Protein extraction. Samples of frozen NF56 tadpoles (whole body) and NF66 frog tissues (carcass) were homogenized using a sonicator at a ratio of 1:2.5 (m/v) in a pre-chilled homogenization buffer (20 mM
Hepes, pH 7.4, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na$_2$VO$_4$, 10 mM β-glycerophosphate) with 1mM phenylmethylsulfonyl fluoride and 1% protease inhibitor (Bioshop Canada Inc. Canada) added immediately to the mixture before homogenization. Homogenates were then centrifuged at 11,000 x g for 15 min at 4 °C. Supernatants were collected as total soluble protein extracts. Protein concentrations were then determined by using a Bradford assay and then adjusted to 5 µg/µl and 10 µg/µl for stages of NF56 and NF66, respectively. Aliquots of the protein samples were mixed 1:1 with diluted 4X Laemmli sample buffer (BioRad, USA; with 10% v/v β-mercaptoethanol) to reach the working concentrations of 2.5 µg/µl (NF56) and 5 µg/µl (NF66). The protein extracts were boiled to eliminate any quaternary structures (in preparation for immunoblotting) and stored at -40 °C for future use.

**Immunoblotting.** Depending on the proteins of interest, 8% and 10% SDS polyacrylamide resolving gels were prepared (8% or 10% v/v acrylamide, 1 M Tris pH 8.8, 0.1% SDS, 0.1% APS, 0.1% TEMED) with a 5% stacking gel (5% v/v acrylamide, 1.5 M Tris pH 6.8, 0.1% SDS, 0.1% APS, 0.1% TEMED). Aliquots of protein samples containing 20 – 40 µg protein were loaded into the gel wells and separated at 180 V for 45 min in a Tris/Glycine/SDS buffer (BioRad, USA). After electrophoresis, proteins were electroblotted onto a PVDF membrane by using a Trans-Blot Turbo system with the associated Trans-Blot Turbo RTA Mini PVDF transfer kit (BioRad, USA). Membranes were then blocked with 2.5% w/v skim milk in TBST (20 mM Tris base, 150 mM NaCl, 0.05% Tween 20) for 45 min, followed by appropriate TBST wash steps. Protein blots were then incubated with primary antibody solutions (specific to the proteins of interest, 1:1000 dilution of the stock antibody with TBST) overnight at 4 °C on a rocking platform. The next day membranes were washed several times with TBST before probed with a HRP-linked secondary antibody corresponding to the primary antibodies (anti-rabbit IgG or immunoglobulin G, Cat#: APA002P, anti-goat IgG, Cat#APA004P, BioShop Canada Inc., Canada) (1:12,000 v/v dilution in TBST) for up to 45 min. After incubation with secondary antibodies and wash with TBST, membranes were visualized with enhanced chemiluminescence (Luminal and peroxide solution) using the BioRad ChemiDoc XRS+ Molecular Imager system.

Primary antibodies used in the current study were specific for AS3MT (Abnova, Taiwan) and DNMT1 (Santa Cruz Biotechnology).

**Data analysis.** The intensity of the immunoblot bands was quantified and standardized against the rest of the same sample lane on the membrane stained by Coomassie blue, by using the ImageLab software (BioRad, USA). Data were then analyzed by 1-way analysis of variance (ANOVA) by a Tukey test using Sigmaplot 12; a statistically significant difference was accepted with p<0.05.

**Analysis of As Content**

**Digestion for total As.** Residues of extraction and original samples of food were digested prior to arsenic analysis. Approximately 0.1 g of dry sample was weighed in a test tube, or the entire extraction residue was used; 2 mL of 10% (v/v) nitric acid was added, the samples were digested at 50 °C for 30 min, and finally diluted in 10 mL of 2% (v/v) nitric acid for ICP-MS analysis.

**Water extraction for As speciation.** Approximately 0.1 g of dry sample was weighed in a test tube and 10 mL of double deionised water (DDW) was added. The extraction method (60 °C with shaking 4 h,
followed by 10 min sonication and 15 min centrifugation at 4000 rpm) had been optimized for marine fish and algae samples\(^49\).

**ICP-MS for total As.** All digested samples and extracts were diluted with 2% nitric acid to ensure that As concentrations were within the calibration limits. Calibration standards of 1, 5, 10, 25, 50, 100, 250, and 500 µg/L of As as As\(^{V}\) were used for total As analysis. The instrument used was an ICP-MS DRC II from Perkin Elmer (Perkin Elmer, Massachusetts, USA). The nebulizer is concentric-type, the flow of argon was 0.87 L/min, the RF power was 1300 W, and the lens voltage was 6 V. The analysis mode used was peak hopping, the dwell time was 100 ms, and the instrument read 10 sweeps and three replicates per sample. The masses monitored were \(^{75}\)As with \(^{115}\)In and \(^{103}\)Rh as internal standards to control for signal stability. For quality control, two standard solutions of 5 and 50 µg/L As were analyzed every 10 samples, and results were accepted only if recovery was in an acceptable range (i.e., 90-110%). The limit of detection of the instrument was calculated as 0.5 µg/L. Total As in frog tissues was calculated as [Total As]\(_{\text{extract}}\) + [Total As]\(_{\text{residue}}\). All samples were analyzed in triplicate and relative standard deviations (RSD = 100% × standard deviation/average) averaged 6% (3-12%) in extracts and 6% (3-27%) for total As, indicating acceptable precision. BCR627 tunafish (certified value of 4.8 ± 0.2 mg/kg) was analyzed in triplicate together with the frog tissues. The sum of extracted and residual As was 4.4 ± 0.2 mg/kg, which was within 10% of the certified value and considered acceptable. Detection limits were 0.1 mg/kg for As extraction and 0.05 mg/kg for residue digestion and method blanks (n = 3) were less than the detection limits. Data were analyzed using XLSTAT 2014.1.10, using 1-way ANOVA (NF56 data) and 2-way ANOVA (NF66 data) by a Tukey test, and substituting random numbers below the detection limit for non-detect data (control NF66).

**HPLC-ICP-MS for As speciation analysis.** Water extracts were analyzed by HPLC-ICP-MS for As speciation, with anion and cation exchange columns. The chromatographic system consisted of a Perkin-Elmer pump (Flexar LC pump, Perkin Elmer, Massachusetts, USA). A PRP-X100 anion exchange column (Hamilton, 4.6 x 250 mm, 10 µm) with matching guard column was used with anion mobile phases (A) 4 mM ammonium nitrate and (B) 60 mM ammonium nitrate in DDW, both adjusted to pH 8.65 with ammonia. An elution gradient was used, developed by Watts et al.\(^50\) 100% A 0-2 min; 100% B 3-6.5 min; 100% A 7.5-10.75 min; 100% B 11-13 min; 100% A 13.25-15 min, with linear changes between mobile phase compositions and a flow rate of 1 ml/min. A Chrompack cation exchange column (Varian, 4.6 x 150 mm) with a PRP-X200 (Hamilton) guard column was used with cation mobile phase 20 mM pyridinium formate and 0.05 M tetramethylammonium formate at pH 2.7, at a flow rate of 1.1 ml/min.

Standard mixtures (5, 10, 25, 50, 100 and 250 µg/L) of DMA, AB, TMAO, AC and Tetra were used for cation exchange, and of As\(^{V}\), As\(^{III}\), DMA, MMA, and AB for anion exchange. Arsenosugars were identified by matching retention times to sugars known to be present in algae extracts\(^49\) and arsenosugar concentrations were calculated by using the curve calibration of the As compound standard analysed that was closest in retention time to the arsenosugar compound. The software PeakFit (Seasolve Software Inc. 2008) was used to measure the concentration of each compound identified. The compounds that could be separated and quantified using the anion exchange column were As\(^{III}\), MMA, DMA, and As\(^{V}\), as well as the arsenosugars 2 (phosphate sugar), 3 (sulfonate sugar), and 4 (sulfate sugar), and the compounds that could be separated and quantified using the cation exchange column.
were AB, TMAO, AC, and Tetra, as well as arenosugar 1 (glycerol sugar). The column recovery (100% × sum of species/extracted total As) averaged 103% (87-110%). Method blanks (n = 3) were below detection limits (0.5 μg/L and 1 μg/L using cation and anion exchange, respectively).

BCR627 (tunafish) was analyzed in triplicate together with the samples, and results are shown in Table 1 and are considered acceptable. All samples were analyzed in triplicate and RSDs ranged from 2 to 35% for As species (except for one TMAO measurement with RSD of 87% for inexplicable reasons), with a mean RSD of 15%, indicating acceptable precision.

X-ray absorption near edge structure (XANES) analysis - XANES spectra were collected at the bending magnet beamline of the Pacific Northwest Consortium Collaborative Access Team (PNC-CAT), Sector 20 at the Advanced Photon Source (APS), Argonne National Laboratory. XANES spectra of the As Kα-edge (11686 eV) were recorded in fluorescence mode by using a solid state Ge detector (Canberra model GL0055PS) while monitoring incident and transmitted intensities in N₂ filled transmission ionization chambers. Freeze-dried NF56 whole body, and NF66 liver and carcass samples were ground and packed in an aluminum sample holder, and held between two layers of Kapton™ tape and kept at 173K by using a liquid N₂ cryostat (Model 22 CTI Cryodyne Refrigerator System, Janis). The Si(111) double crystal monochromator was calibrated using the first inflection point of the gold LIII absorption edge (11919.7 eV) and a reference gold foil was measured simultaneously with samples. XANES spectra were fit within -20 to +30 eV to E₀ using Athena software. The standard spectra used for fitting had been measured as frozen aqueous dissolved species previously by our group (21), and included As³⁺, MMA, MMA, TMAO, Tetra, and AB, and As⁵⁺.

Results and Discussion

Total As Accumulation. Tadpoles (NF56) and frogs (NF66) of S. tropicalis were raised and collected in water amended with As in two nominal concentrations (low As exposure, 0.5 mg/L, and high As exposure, 1 mg/L; corresponding to measured water concentrations of 0.33 ± 0.07 and 0.83 ± 0.12 mg/L, with the “As-free” control containing 0.0058 ± 0.0005 mg/L). The 1 mg/L As⁵⁺ concentration was expected to be nonlethal because no mortality was observed in Lithobates (Rana) pipiens tadpoles exposed to As⁵⁺ concentrations at this concentration, and only 24% mortality was observed when the exposure was 5 mg/L of As³⁺.³¹ Both low and high concentrations were also chosen to be sufficiently high to induce effects, based on the observed inhibition of Xenopus laevis tail development linked to endocrine disruption at As³⁺ concentrations from 0.008 to 0.5 mg/L.⁵¹ The concentrations were also chosen to be representative of concentrations previously found in water samples from As-contaminated environments (0.010-3.4 mg/L varying seasonally).²⁹

The total As concentrations in S. tropicalis tissues, summarized in Table 2, show an increase in tissue concentration with water As concentration. The tissues contained from 0.25 mg/kg dry weight (DW) (control, no As added) to 10.1 mg/kg DW of As for NF56, and As below the detection limit to 9.6 mg/kg DW for NF66 tissues. The increases with water As concentration were statistically significant for both NF56 (1-way ANOVA) and NF66 (2-way ANOVA) life stages (p < 0.0001).
The overall highest DW concentrations are seen in the NF56 whole tadpole and NF66 intestines. The NF66 liver, stomach and carcass concentrations are similar within exposures (2-3 mg/kg DW for low exposure and 3.3-4.4 mg/kg DW for high exposure) (see Table 2 for statistical differences), but the intestinal contents are approximately double those of the other tissues, and statistically different (2-way ANOVA, p < 0.0001).

Fresh weight (FW) concentrations are also provided to allow comparisons to the animals’ aqueous environments, and for comparison with other studies. Comparing the FW stomach contents with the dosing concentration in the NF66 animals reveals that the FW concentrations are slightly higher than the dosing concentrations (water concentrations: high 0.83 ± 0.12; low 0.33 ± 0.07), and carcass concentrations are the lowest. NF56 concentrations, when converted to FW, are similar to the dosing concentrations, indicating that the concentrations are not increased in the organism. When FW food concentrations were estimated (assuming that the dry food becomes saturated with As-containing water) for NF56 to be approximately 0.41 (low) and 0.89 mg/kg FW (high) (assuming 95% moisture in the food), the tissue concentrations again verify that As is not increased in the tadpole bodies. A similar estimation of As concentrations in NF66 food gives 0.35 (low) and 0.83 mg/kg FW (high) (assuming 64% moisture in the food, similar to the stomach contents), which are lower than liver, carcass and stomach concentrations. The intestine concentrations were 4 to 6 times higher than the food concentrations in the exposed animals, which may suggest that excretion via the intestinal route is taking place.

**Speciation of As.** Individual As compounds were measured in tissue extracts of NF56 and NF66, using a combination of anion and cation exchange HPLC with ICPMS detection, which allowed the quantification of inorganic As (only As\(^{V}\) was observed), MMA, DMA, TMAO, Tetra, and AB in the frog tissues (Table 3). The major compounds detected were TMAO in NF56 tadpoles, DMA in NF66 liver and carcass, and As\(^{V}\) in NF66 stomach and intestines (Table 3, Figure 1). The predominance of As\(^{V}\) in stomach and intestine samples suggests that the stomach and intestine contents reflect the presence of As\(^{V}\) in the growth medium. The extraction efficiency was highest in the stomach (approximately 75%, Table 3), again supporting the notion that the As present in the stomach consists of soluble As from the water. Otherwise the extraction efficiencies, ranging from 39 to 66%, are similar to those found previously for frogs\(^{29,28,30}\).

Tetra was detected in the majority of tissues at both stages (5-20% of extracted As), indicating some similarity in As metabolism between *S. tropicalis* and previously studied frogs, although Tetra generally occurred in higher proportions in those studies\(^{29,28,30}\) and was in some cases the major extracted compound\(^{30}\). The absence of Tetra in the food supports the hypothesis made by others\(^{30}\) that the frogs or associated organisms are biosynthesizing this compounds. An example of associated organisms might be the microbial consortium found in the intestines; some Tetra was detected in the high-exposure NF66 intestine sample, although this may have been present as a consequence of biliary excretion as well.

AB was also detected in NF66 liver and carcass tissues (and in trace, unquantified amounts in NF56); its presence in food suggests that the frogs might be eating this compound and to some extent retaining it.
An intriguing finding is the predominance of TMAO in NF56 tadpoles, but not in NF66 tissues (TMAO occurs, but DMA becomes predominant). TMAO was not observed at all in laboratory-reared frogs (Rana sp) but was present in trace to low amounts in field-collected Rana sp and Bufo americanus. Moriarty et al. hypothesized that this difference may be attributable to the presence of TMAO in food available to field-collected frogs, but not laboratory-reared frogs. In the present study TMAO is available in frog food and not detectable in tadpole food, but it is the tadpoles that contain predominantly TMAO. Overall, MMA, DMA, and TMAO were found in frog tissues at concentrations above those in food (BAFs ranged from 2.9 to 17) indicating that these species were present most likely as a result of metabolism associated with the frogs. This was observed even in the stomach contents of NF66 frogs, in which DMA was detected at concentrations 4 to 6 times higher than the food concentration. These findings suggest that the methylated compounds are present at least partially as a consequence of biomethylation.

XANES analysis of unextracted original tissues allowed the determination of all the As species; this could only be done for high As concentration tissues, which were dried to maximize the As concentrations. The XANES linear combination fitting could not reliably distinguish DMA from TMAO, and AB from Tetra, and therefore these compounds are shown together in Figure 1. A comparison of the results (Figure 1) suggests that the unextracted and unidentified As was most likely inorganic As. Large discrepancies (> 50% difference) in the proportions of the organoarsenicals, however, indicates that in some cases, they remained unextracted (Figure 1). Specifically, HPLC-ICPMS of the NF56-high exposure sample revealed only 3% of the total As as Tetra (with no AB), whereas 28% of the total As in the sample was attributed to AB/Tetra using XANES. Similar large differences were seen for AB/Tetra for NF66 carcass samples: 8% by HPLC compared with 24% by XANES for the low exposure sample, and 7% by HPLC-ICPMS compared with 34% by XANES for the high exposure sample.

The XANES results thus reveal that a substantial proportion of the total As occurs as AB/Tetra in most of the analyzed samples (17-34%) with only one sample, the whole body NF56 tadpole from the low exposure experiment, containing less at 5%. AB/Tetra proportions and concentrations (ww) in the frog tissues increased with water As concentration at both developmental stages and the highest amounts were seen in NF66 frog treated with the high concentration of As (1 mg/L). This may indicate a concentration-dependent response to As, but the results could not be examined statistically because only one replicate of each sample was analyzed by XANES.

**AS3MT protein levels.** AS3MT serves a key enzyme in the methylation dependent As metabolism by facilitating the addition of a methyl group to trivalent As metabolites. Therefore, in our attempt to explore the biological and molecular mechanisms behind any differences of As speciation resulting from exposure and development, we measured the relative protein level of AS3MT between control and two exposure conditions (nominal 0.5 mg/L, low As exposure; and 1 mg/L, high As exposure) for both NF56 and 66 developmental stages as well as between two NF stages upon both exposure levels.

As shown in Figure 2, in NF56 tadpoles, the low As exposure led to a significant decrease in AS3MT to 30 ± 9% of the control level but AS3MT was similar to the control level at the high As exposure. For the NF66 carcass, no appreciable change (from control animals) was detected after low As exposure, but
AS3MT decreased significantly to 51 ± 8% of the control value at the high As exposure (Figure 2). The change of AS3MT in NF56 upon As exposure suggests that the capability of As methylation has been established at the tadpole stage, before the completion of metamorphosis. The fact that decreased AS3MT protein level was only observed in NF56 tadpoles but not NF66 frogs upon low As exposure indicates that frogs in early developmental stages might be more sensitive to environmental change. Higher As exposure brought the AS3MT level back to the control level, suggesting that NF56 animals have established the As metabolic system to cope with As in the environment. On the other hand, the decrease in the NF66 AS3MT level, compared with control levels, at the highest dose, suggests an inhibitory effect of As on this enzyme, thus reducing its contribution to the methylation of arsenic.

When the different developmental stages (NF56 tadpole and NF66 frog) are compared with each other (Figure 3), AS3MT was higher in NF66 compared with the NF56 tadpoles by 14 ± 2 fold in control, 80 ± 6 fold at the low As exposure, and 74 ± 12 fold in the high As exposure experiments. The universal drastic increase (up to 80-fold) of the protein level in NF66 frogs compared with the NF56 tadpoles indicates that components of As metabolism machinery is more abundant in fully metamorphosed frogs. The results also confirmed frogs accumulate significantly higher AS3MT content during the course of development upon chronic As exposure than the control groups. The 15-fold increase in AS3MT in NF66 over N56 animals under control conditions might represent the maturation of As metabolism framework, while the over 80-fold developmental increase of AS3MT in As

\[ V \] exposed groups may indicate enhanced activation of As methylation (and metabolism).

**DNMT1 protein levels.** In order to interrogate the dynamics between As oxidative methylation and DNA methylation, we assessed the protein level of DNMT1 in parallel with AS3MT. Figure 4 shows that DNMT1 levels were indistinguishable from the control animals in both NF56 and NF66 stages after low As exposure, but DNMT1 increased at both stages compared with control animals (4.2 ± 0.5 fold for NF56 and 2.2 ± 0.3 fold for NF66) upon high As exposure. Elevated DNMT1 at both developmental stages upon higher As exposure indicates a more active DNA methylation machinery in these circumstances. The similarity in the trends at both developmental stages (a potential As concentration-dependent response: increasing DNMT1 with As concentration) suggests that the regulations on DNMT1-dependent DNA methylation under the influence from As exposure appears to be fairly consistent across multiple developmental stages in Western clawed frogs. The increase in DNMT1 in the high As exposure compared with the lower As exposure was more profound for the NF56 tadpoles (4-fold) tadpoles compared with NF66 frogs (2-fold), suggesting the demand for DNA methylation is higher in tadpoles (NF56) than mature frogs (NF66) at 1 mg/kg As concentrations.

Disparities of the protein expression pattern between AS3MT and DNMT1 may be a result of the competitive nature of the two methylation processes; specifically DNMT1 may compete with AS3MT for arsenic methylation.\[^{52}\] DNMT1 has been well characterized as a maintenance DNA methyltransferase, which ensures genomic DNA methylation during cell proliferation and development.\[^{17,18}\] Therefore DNMT1 plays a central role in protecting the integrity of the DNA methylation profile, which is crucial to the early development stages. As oxidative methylation competes with the DNA methylation for the methyl source but substantial methylation is required for active cell proliferation and development for the ongoing metamorphosis at NF56. Thus it is possible that methylation is favoured at this stage for
genomic DNA over As metabolism when the As toxicity is still at a tolerable level (low As exposure), and the AS3MT expression is repressed. Previous studies have proposed a similar phenomenon where low level As\(^{III}\) exposure led to an increase in DNMT1 mRNA level in a mammalian cell line.\(^{12}\)

At the high As exposure, AS3MT increased over the low exposure group, suggesting activation of As methylation events. DNMT1 also increased upon high As exposure. Thus in NF56 tadpoles, both As and DNA methylation pathways are activated when experiencing a higher As exposure, but with an emphasis on maintaining epigenetic integrity.

In NF66 frogs, AS3MT and DNMT1 only responded to the higher As exposure (with a decrease in AS3MT and an increase in DNMT1). It is possible that normal expression of DNMT1 is adequate for normal frog development without the need of slowing down As metabolism activity upon low As\(^{V}\) exposure in the mature frogs, since less active cell proliferation and differentiation rates are required in fully metamorphosed animals. The increase in DNMT1 in NF66 frogs that accompanied the decrease in AS3MT at the high As exposure indicates a more pronounced shift to DNA methylation when compared to NF56 animals.

Similar to AS3MT, DNMT1 levels were higher in later stage frogs (NF66) compared with NF56 tadpoles by 10 ± 2 fold in control, 29 ± 3 fold at the low As exposure, and 18 ± 2 fold in the high As exposure experiments (Figure 5). This is a similar trend to that seen for AS3MT, where the continuous exposure to low As\(^{V}\) level over the course of development triggers higher DNMT1 accumulation than the high As condition. However, the nearly 80-fold increase in AS3MT protein level between NF56 tadpoles and NF66 frogs upon As exposure still provides some evidence of As metabolism activation. Taken together, our data indeed demonstrate crosstalk between As and DNA methylation upon exposure to As in NF66 frogs. However, the crosstalk may also be related to the epigenetic toxicity of arsenic (i.e., leading to an increase in DNMT1 and a decrease in AS3MT) rather than to the metabolism of arsenic.\(^{53}\)

The increase in DNMT1 along with increased arsenic exposure suggests that responses in frogs are similar to those observed in previous studies of As-dependent DNMT1 responses in mammalian and human systems.\(^{19,53}\)

**Speciation of As and methylation enzymes.** AS3MT has been shown to catalyze the methylation of As to MMA, DMA, and TMAO\(^{20,21,22,54}\). We consider that the presence of MMA, DMA, and TMAO in frog tissues are a consequence, at least in part, of metabolism by the frogs, probably aided by AS3MT. The lack of bioaccumulation by the frogs overall indicates that excretion, probably via the formation of methylated species, is efficient.

The MMA+DMA+TMAO concentration changes from NF56 (whole body) to NF66 (carcass) were 0.15 mg/kg ww down to 0.12 mg/kg ww for the low As concentration, and 0.10 mg/kg ww up to 0.22 mg/kg ww for the high As concentration. These changes were much smaller than those found for AS3MT between developmental stages, where NF66 levels were much higher than NF56 levels (80-fold at the low As exposure, and 74-fold in the high As exposure experiments). Thus, AS3MT trends with MMA+DMA+TMAO were considered for each developmental stage separately. Speciation data were available only for the two As exposure points since As species could not be detected in control animals. A
negative trend was observed: higher MMA+DMA+TMAO concentrations and % MMA+DMA+TMAO occurred with lower AS3MT levels (Figure 6a). This was a surprising finding since there is at least qualitative evidence that higher AS3MT levels may be associated with enhanced methylation of As: the pituitary gland of mice had the lowest AS3MT levels and contained no methylated species at the highest administered dose in a study of mouse brain regions (although methylation was similar to that in other parts at lower doses)\textsuperscript{24}, and higher AS3MT may occur in animal hepatocytes that are faster methylators and generally contain more methylarsenic (but less total As accumulation)\textsuperscript{20}. On the other hand, AS3MT gene expression was observed to be lower in human populations that excreted higher proportions of DMA (compared with MMA and iAs) in their urine,\textsuperscript{52} a trend similar to that seen here. The nature of As methylation is complicated\textsuperscript{55}, and may rely on methylating enzymes additional to AS3MT, such as N-6 adenine-specific DNA methyltransferase 1 (N6AMT1), found by others to be capable of methylating MMA(III) to DMA\textsuperscript{56}. The lowered, but not eliminated, capacity for As methylation in AS3MT-knockout mice (mice that have been genetically altered to silence the gene for AS3MT), supports the possibility that other, not yet characterized, methyltransferases may catalyze the methylation.\textsuperscript{4,57} Additionally, genes linked to that for AS3MT may influence the methylation capacity, as has been suggested for human populations.\textsuperscript{55}

For NF66, carcass residues of MMA, DMA, and TMAO may not be indicative of the methylation taking place but rather the species that remain behind, since methylation is assumed to aid in excretion of As. That is, enhanced methylation might be occurring together with increased AS3MT levels, but it may be coincident with faster excretion rates, leaving lower tissue residues. If intestinal contents are used to estimate one excretory pathway for the frogs (i.e., the biliary excretion route), we propose that the intestinal to stomach As ratio can be used to indicate excretion; this ratio is approximately 2 for total As, and is higher at approximately 6 for the methyl species. These values must be taken in the context that we cannot discount that methyl species are present in the intestines also as a result of intestinal metabolic activity and may also be available for uptake. A slightly lower excretion ratio was observed for the higher As exposure (ratio = 5.7) commensurate with higher tissue MMA+DMA+TMAO and lower AS3MT levels, compared with the lower As exposure (ratio = 6.2). The difference in these ratios, and the limited data available, make this explanation hypothetical at best but may provide future directions for research. Urinary excretion cannot be estimated in the present study, and indeed urinary excretion of As from frogs has never been measured.

Another reason for the observed pattern of AS3MT c.f. (MMA+DMA+TMAO) concentrations might be that the selection of tissue for the AS3MT measurement might not have been representative of where the majority of the methylation takes place in the frog. For example in rat, liver is the primary location for methylation, accompanied by the highest mRNA and protein levels for AS3MT, although similar methylation was also found in the spleen, which contained lower protein levels\textsuperscript{58}.

The relevance of AS3MT to the presence of AB/Tetra has not been established since the formation pathways for AB and Tetra are still unknown. For example, the possibility that the fourth methyl group on Tetra is added via the Challenger pathway (and AS3MT) is questionable since a volatile intermediate (trimethylarsine) would be required\textsuperscript{1}. AB formation pathways have been discussed elsewhere\textsuperscript{59} and only the intermediates are likely to involve the stepwise methylation described by the Challenger...
mechanism. The trend of AB/Tetra with AS3MT is inconsistent between life stages (at NF56, both AS3MT and AB/Tetra increase with As concentration, but at NF66 the opposite trend is seen), possibly suggesting that AS3MT has no direct effect on AB/Tetra. On the other hand the increase in AB/Tetra with As concentration and developmental stage is similar to the trends observed for DNMT1 (Figure 6b). Both increases may be indicators of exposure and/or effect, but no relation of AB or Tetra to detoxification or adaptation to As exposure has been established yet.

Limitations of the study include the small number of dosing concentrations that could be interrogated for As speciation (only low and high As exposure; As in controls was not detectable), and the small number of samples analyzed by XANES, as well as the inability to differentiate DMA and TMAO, and AB and Tetra using this method.

Conclusions

Exposure of S. tropicalis to inorganic As appears to be associated with changes in the levels of two methylation enzymes, AS3MT and DNMT1, thus suggesting an effect on these enzymes. The As content in frog tissues increased with As exposure, but the As species profiles did not change. The results confirmed that S. tropicalis, like other frog species studied to date (Rana sp and Bufo americanus), contains Tetra, an uncommonly encountered organoarsenical with toxicity lower than inorganic As. An apparent inverse relationship of As methylation with AS3MT levels may be interpreted as resulting from more efficient excretion of methylated As species concurrent with higher AS3MT, the presence of alternative methylating enzymes, or the presence of other genes linked to that for AS3MT, affecting methylating capacity. The findings show that if amphibians are exposed to As in environmental scenarios, they may experience effects that may change their levels of methylating enzymes. Additionally their tissue As concentrations will probably increase, which may lead to increased potential risk to predators.

Acknowledgements

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References


Tables

Table 1 Arsenic (As) compounds (average ± standard deviation, n=3, mg/kg dry weight, DW) in certified reference material BCR627; see text for As compound abbreviations

<table>
<thead>
<tr>
<th>Value</th>
<th>As^V</th>
<th>DMA</th>
<th>AB</th>
<th>TMAO</th>
<th>Sum</th>
<th>[As]ext</th>
<th>CR (%)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured</td>
<td>0.19 ± 0.02</td>
<td>0.15 ± 0.01</td>
<td>3.2 ± 0.2</td>
<td>0.31 ± 0.03</td>
<td>3.7</td>
<td>3.7 ± 0.1</td>
<td>100</td>
<td>84</td>
</tr>
<tr>
<td>Certified</td>
<td>0.150 ± 0.022</td>
<td>3.90 ± 0.22</td>
<td>3.2 ± 0.2</td>
<td>0.31 ± 0.03</td>
<td>3.7</td>
<td>3.7 ± 0.1</td>
<td>100</td>
<td>84</td>
</tr>
</tbody>
</table>

^a As_{ext} = total As in extract, mg/kg DW

^b CR = column recovery = 100% × sum/[As]_{ext}

^c EE = extraction efficiency =100% × [As]_{ext}/Total DW As in sample (4.4 ± 0.2 mg/kg)
Table 2 Arsenic (As) concentrations (average ± standard deviation of three measurements) in mg/kg and % moisture in *S. tropicalis* NF56 and NF66 stage tissues.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Control</th>
<th>Low</th>
<th>High</th>
<th>% moisture</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF56 (DW)</td>
<td>0.25 ± 0.02</td>
<td>6.5 ± 0.2</td>
<td>10.1 ± 0.5</td>
<td>95</td>
</tr>
<tr>
<td>NF56 (FW)</td>
<td>0.0113 ± 0.0009 (A)</td>
<td>0.33 ± 0.01 (B)</td>
<td>0.68 ± 0.03 (C)</td>
<td></td>
</tr>
<tr>
<td>NF66 liver (DW)</td>
<td>&lt;0.05 (a)</td>
<td>2.0 ± 0.2 (b)</td>
<td>3.3 ± 0.3 (c)</td>
<td>43</td>
</tr>
<tr>
<td>NF66 liver (FW)</td>
<td>&lt;0.03</td>
<td>1.1 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>NF66 carcass (DW)</td>
<td>&lt;0.05 (a)</td>
<td>2.99 ± 0.03 (c)</td>
<td>4.4 ± 0.2 (d)</td>
<td>79</td>
</tr>
<tr>
<td>NF66 carcass (FW)</td>
<td>&lt;0.01</td>
<td>0.638 ± 0.005</td>
<td>0.94 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>NF66 stomach (DW)</td>
<td>0.26 ± 0.01 (a)</td>
<td>2.9 ± 0.1 (c)</td>
<td>4.2 ± 0.2 (d)</td>
<td>64</td>
</tr>
<tr>
<td>NF66 stomach (FW)</td>
<td>0.094 ± 0.004</td>
<td>1.05 ± 0.04</td>
<td>1.52 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>NF66 intestines (DW)</td>
<td>0.15 ± 0.04 (a)</td>
<td>6.2 ± 0.4 (e)</td>
<td>9.6 ± 0.1 (f)</td>
<td>65</td>
</tr>
<tr>
<td>NF66 intestines (FW)</td>
<td>0.05 ± 0.01</td>
<td>2.2 ± 0.1</td>
<td>3.35 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Tadpole food</td>
<td></td>
<td>2.0 ± 0.3</td>
<td></td>
<td>Dry</td>
</tr>
<tr>
<td>Frog food</td>
<td></td>
<td>0.73 ± 0.05</td>
<td></td>
<td>Dry</td>
</tr>
</tbody>
</table>

* Control, low and high concentrations all statistically different from each other (1-way ANOVA, p<0.0001)

DW = dry weight; FW = fresh weight

Different letters (A-C for NF56, and a-f for NF66) indicate statistical differences (2-way ANOVA, p<0.0001)
Table 3 Arsenic (As) compounds (average ± standard deviation, n=3, mg/kg dry weight, DW) in tadpole (NF56) whole tissues and frog (NF66) parts and food, by water extraction-HPLC-ICPMS. C = carcass; I = intestines; L = liver; S = stomach; WB = whole body; see text for As compound abbreviations. Blank spaces indicate compounds were not detectable (LOD for As\textsuperscript{V} and MMA = 0.08 mg/kg and for all others 0.04 mg/kg).

<table>
<thead>
<tr>
<th>Exposure</th>
<th>As\textsuperscript{V}</th>
<th>DMA</th>
<th>MMA</th>
<th>AB</th>
<th>TMAO</th>
<th>TETRA</th>
<th>Sum</th>
<th>[As]\textsubscript{ext}\textsuperscript{a}</th>
<th>CR (%)\textsuperscript{b}</th>
<th>EE (%)\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) NF56</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low WB</td>
<td>1.3 ± 0.1</td>
<td>0.53±0.08</td>
<td></td>
<td>1.6 ± 0.2</td>
<td>0.36 ± 0.01</td>
<td>3.8</td>
<td>4.3 ± 0.2</td>
<td>88</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>High WB</td>
<td>2.3 ± 0.1</td>
<td>0.91 ± 0.02</td>
<td>0.24 ± 0.06</td>
<td>1.9 ± 0.07</td>
<td>0.29 ± 0.01</td>
<td>5.7</td>
<td>6.5 ± 0.5</td>
<td>87</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>(B) NF66</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low L</td>
<td>0.45 ± 0.06</td>
<td></td>
<td>0.09 ± 0.02</td>
<td>0.15 ± 0.01</td>
<td>0.19 ± 0.02</td>
<td>0.88</td>
<td>0.8 ± 0.1</td>
<td>106</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>High L</td>
<td>0.25 ± 0.08</td>
<td>0.9 ± 0.1</td>
<td>0.16 ± 0.04</td>
<td>0.20 ± 0.04</td>
<td>0.20 ± 0.03</td>
<td>1.7</td>
<td>1.61 ± 0.07</td>
<td>103</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Low S</td>
<td>2.1 ± 0.2</td>
<td>0.28 ± 0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High S</td>
<td>3.0 ± 0.2</td>
<td>0.43 ± 0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low I</td>
<td>2.5 ± 0.3</td>
<td>1.4 ± 0.2</td>
<td>0.34 ± 0.08</td>
<td>0.11 ± 0.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High I</td>
<td>2.6 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>0.61 ± 0.06</td>
<td>0.5 ± 0.1</td>
<td>0.15 ± 0.04</td>
<td>5.3</td>
<td>5.1 ± 0.3</td>
<td>103</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Low C</td>
<td>0.11 ± 0.04</td>
<td>0.7 ± 0.1</td>
<td>0.12 ± 0.00</td>
<td>0.20 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>1.3</td>
<td>1.16 ± 0.04</td>
<td>110</td>
<td>39</td>
<td></td>
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<tr>
<td>High C</td>
<td>0.36 ± 0.04</td>
<td>1.0 ± 0.1</td>
<td>0.18 ± 0.01</td>
<td>0.36 ± 0.05</td>
<td>0.14 ± 0.02</td>
<td>2.0</td>
<td>1.9 ± 0.1</td>
<td>108</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>(C) Food</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tadpole</td>
<td>0.22 ± 0.05</td>
<td>0.29 ± 0.05</td>
<td>0.60 ± 0.03</td>
<td></td>
<td></td>
<td></td>
<td>1.1</td>
<td>1.43 ± 0.05</td>
<td>78</td>
<td>72</td>
</tr>
<tr>
<td>Frog</td>
<td>0.07 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.035 ± 0.007</td>
<td></td>
<td></td>
<td></td>
<td>0.22</td>
<td>0.28 ± 0.01</td>
<td>77</td>
<td>38</td>
</tr>
</tbody>
</table>

\textsuperscript{a} [As]\textsubscript{ext} = total As in extract, mg/kg DW

\textsuperscript{b} CR = column recovery = 100% × sum/[As]\textsubscript{ext}

\textsuperscript{c} EE = extraction efficiency =100% × [As]\textsubscript{ext}/Total DW As in sample (from Table 2)
Fig. 1 Proportion of arsenic (As) species in tadpole (NF56) and frog (NF66) lifestages of *S. tropicalis*. X indicates XANES fitting results and other results are from water extraction-HPLC-ICP-MS. C = carcass; I = intestines; L = liver; S = stomach; WB = whole body.
Fig. 2 Effect of low (0.5 mg/L) and high (1 mg/L) dose As\textsuperscript{V} exposures on protein levels of As3MT in NF56 and NF66 \textit{S.tropicalis}. Upper portion shows the histogram with standardized relative protein levels in control, low and high As\textsuperscript{V} exposed animals; data are means ± S.E.M., \( n = 4 \) individual animals. Different letters (a, b, c) indicate statistically different results (\( p < 0.05 \)). Lower portions shows immunoblot bands from each group.
Fig. 3 Comparison of protein expression level of As3MT between NF56 and NF66 animals under control, low (0.5 mg/L) and high (1 mg/L) As\textsuperscript{V} exposure conditions. Upper portion shows the histogram with standardized relative protein levels in NF 56 and NF66 animals from control, low and high As\textsuperscript{V} exposed groups; data are means ± S.E.M., n = 4 individual animals. *Significantly different from NF56 animals under corresponding exposure conditions (p < 0.05). Lower portions shows immunoblot bands from each group.
Fig. 4 Effect of low (0.5 mg/L) and high (1 mg/L) dose As\textsuperscript{V} exposures on protein levels of DNMT1 in NF56 and NF66 \textit{S.tropicalis}. Other information as in Fig. 2.
Fig. 5 Comparison of protein expression level of DNMT1 between NF56 and NF66 animals under control, low (0.5 mg/L) and high (1 mg/L) As\textsuperscript{V} exposure conditions. Other information as in Fig. 3.
Fig. 6 Arsenic species (concentration in mg/kg wet weight on left axis, and % of total arsenic on right axis) vs enzyme intensity (relative to control for each stage of development). A. 1-3 MeAs = MMA+DMA+TMAO vs AS3MT. B. AB/Tetra vs DNMT1.