Frogs Respond to Commercial Formulations of the Biopesticide Bti, especially their Intestine Microbiota

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ABSTRACT. It is generally believed that *Bacillus thuringiensis* var. *israelensis* (Bti) biopesticides are harmless to non-target organisms; however, new research shows controversial results. We exposed acutely and chronically *Lithobates sylvaticus* and *Anaxyrus americanus* tadpoles until metamorphic climax to VectoBac® 200G (granules) and VectoBac® 1200L (aqueous suspension) at 300 to 20,000 ITU/L covering field relevant concentrations and higher. The data show that the exposure parameters tested did not affect significantly the survival, total length, total weight, hepatosomatic index, gonadosomatic index, the expression of genes of interest (i.e., related to xenobiotic exposure, oxidative stress, and metamorphosis) and the intestine tissue layer detachment of *L. sylvaticus* and *A. americanus* in a concentration-response pattern. In contrast, VectoBac® 200G significantly increased the median time to metamorphosis of *L. sylvaticus* tadpoles by up to 3.5 days and decreased the median by up to one day in *A. americanus*. VectoBac® 1200L significantly increased the median time to metamorphosis of *L. sylvaticus* and *A. americanus* tadpoles by up to 4.5 days. Also, the exposure to VectoBac® 200G and 1200L altered the intestine bacterial community composition in *A. americanus* at application rates recommended by the manufacturer, which led to an increase in the relative abundance of *Verrucomicrobia*, *Firmicutes*, *Bacteroidetes,* and *Actinobacteria*. Changes in the intestine microbiota might impact the fitness of individuals, including the susceptibility to parasitic infections. Our results indicate that the effect of Bti commercial products is limited; however, we recommend that Bti-spraying activities in amphibian rich ecosystems should be kept minimal until there is more conclusive research to assess if the changes in the time to metamorphosis and microbiota can lead to negative outcomes in amphibian populations, and eventually, the functioning of ecosystems.

KEYWORDS. Wood frog, American toad, *Bacillus thuringiensis* var. *israelensis*, Bti, Biopesticide, ecotoxicity.

SYNOPSIS

Bti-based pesticides modify the intestine microbiota in frogs, which should be addressed when using (or applying) Bti in aquatic ecosystems.

INTRODUCTION

Biopesticides are pesticides derived from natural materials as animals, plants, bacteria, and certain minerals.[1] As of April 2016, the U.S. EPA had 299 registered biopesticide active ingredients, and 49 active ingredients correspond to products related to microorganisms of the *Bacillus* genus.[2] Specifically, *Bacillus thuringiensis* var. *israelensis* (Bti) is a Gram-positive bacterium first isolated from soil samples of a mosquito breeding site in Israel.[3] Currently, Bti-based products are used in urban as well as rural settings to prevent and control the dissemination of diseases by mosquitos, decrease the nuisance of biting insects and increase human population comfort.[4–6]

The pesticide activity of Bti is due to the production of crystal (Cry) and cytolytic (Cyt) toxins during sporulation.[7] The Cry proteins are specific to the Lepidoptera, Coleoptera, Hymenoptera, and, Diptera orders; while, Cyt proteins are specific to the Diptera.[8] For the activation of the toxins, the target insect has to ingest the protein crystals, which are dissolved in the alkaline gut environment and the toxins are activated with midgut insect proteases.[9–12] The activated toxin can bind to specific receptors of the midgut epithelium, leading to the formation of lytic pores, septicemia and death of the mosquito larvae.[13,14]

Because of the Bti pesticide mode of action, several authors have suggested that the Bti toxic activity is specific to their target insect, and is innocuous to vertebrates and plants.[15,16] However, there are reports on the deleterious effects of Bti on non-target species[5] and the evidence on the effects of Bti on biodiversity and the food web are mixed.[17] For example, the Bti formulation Introban® induced a 100% mortality in tadpoles of *Leptodactylus latrans* (South American common frog) after 48 h of exposure at 40 mg/L.[18] Yet, the exposure to the VectoBac® WG formulation did not affect survival in *Rana temporaria* (European common frog) tadpoles but significantly induced the response of detoxification enzymatic biomarkers.[19] Furthermore, Schweizer et al. (2019) reported that the exposure to VectoBac® WG during 11 days did not significantly affect biomarkers for proteotoxicity and neurotoxicity or metabolic action in *R. temporaria* at field concentrations and higher.[20]

Amphibians are important in the food web with a unique life cycle where they spend early development in the water, and their passage to adult life is defined by metamorphosis, a process highly controlled by hormones.[21] All these aspects make amphibians susceptible to pollutants in water and thus are commonly used for toxicity testing.[22–25] Therefore, it is possible to identify potential toxic compounds using frog and toad tadpoles as bioindicators. Also, the global amphibian population is experiencing decline[26] and the alteration of their skin and intestine microbiota could be making them susceptible to infectious diseases.[27] In this study, we report on the acute and chronic toxicity and the sublethal effects of Bti on the frog *Lithobates sylvaticus* (Wood frog) and the toad *Anaxyrus americanus* (American toad). We exposed tadpoles of these two species to granular and liquid commercial formulations of Bti to test if Bti affects survival, development, time to metamorphosis, expression of genes related to xenobiotic exposure, oxidative stress and metamorphosis, intestine histology and intestine microbiota.

MATERIAL AND METHODS

Chemicals

Two Bti formulations produced by Valent BioSciences Corporation (Illinois, USA), VectoBac® 200G in the granule form (200 International Toxic Units (ITU)/mg) and VectoBac® 1200L in aqueous suspension form (1200 ITU/mL) were used (gifts from GDG Environnement, Quebec, Canada).[28,29] The spore count of Bti products does not reflect their pesticide potency;[30] therefore their potency activity is measured using the mosquito *Aedes aegypti* (4th instar larvae of the Bora Bora strain) as test insect and a lyophilized pure culture of *B. thuringiensis* H-14 (strain 1884) as reference material. The reference material is also called Institut Pasteur Standard 1982 (IPS 82) and the results of the bioassay are expressed in ITU.[30,31] These VectoBac® products are biological pesticides of the Bti HD-14, strain AM65-52. The manufacturer does not provide information on other ingredients of the formulations. For exposures, to ensure water purity, a Ringer's solution was prepared with distilled water.[32] Additional details of the chemicals are included in the material and methods of the supplementary document.

Biopesticide Potency Verification

Ochlerotatus sp. mosquito larvae (n= 15) at third instar were placed in 6-L glass tanks filled with water to verify the potency of the two biopesticide formulations. To cover a variety of realistic environmental scenarios, five different concentrations were calculated based on the manufacturer's guidelines. The suggested application rate of VectoBac® 200G is 3–10 kg/ha, while VectoBac® 1200L is recommended at a rate of 0.25–1 L/ha. Higher rates are recommended for deep very cold water, and/or polluted water, and when late 3rd and 4th instar larvae predominate.[28,29] The concentrations selected correspond to the minimum recommended application rate (1×min), 2× the minimal recommended application rate (2×min), the maximal recommended application rate (1×max), 2× the maximal recommended application rate (2×max), and 10× the maximal recommended application rate (10×max) (Table 1). Based on the calculations in Table 1, the concentrations selected for the exposure for VectoBac® 200G were 2.5, 5, 10, 20, and 100 mg/L and for Vectobac® 1200L were 0.25, 0.5, 1, 2.5 and 10 µL/L. The ITU/L concentrations overlap (Table 1) allowing the observation of potential effects due to additives in the formulations. The application of the products was on the water surface without mixing to mimic Bti application in treated ponds. Every 24 h, the mosquito larvae were observed and dead larvae were removed. The exposure was performed in a controlled light/temperature room with a 15:9 light:dark cycle. The water temperature was 16 ± 1 °C.

Table 1. Concentrations of VectoBac® 200G and 1200L used during the exposure experiments based on the manufacturer's recommendations for minimum (min) and maximum (max) application rates.

Formulation	Suggested application rate	ITU/m^2	Nominal concentration	ITU/L	Used nominal concentration	ITU/L
VectoBac® 200G	1 × min = 3 kg/ha	60,000	2.3 mg/L	460	2.5 mg/L	500
	2 × min = 6 kg/ha	120,000	4.6 mg/L	920	5 mg/L	1,000
	1 × max = 10 kg/ha	200,000	7.7 mg/L	1,540	10 mg/L	2,000
	2 × max = 20 kg/ha	400,000	15.4 mg/L	3,080	20 mg/L	4,000
	10 × max = 100 kg/ha	2,000,000	76.7 mg/L	15,340	100 mg/L	20,000
VectoBac® 1200L	1 × min = 0.25 L/ha	30,000	0.19 µL/L	240	0.25 µL/L	300
	2 × min = 0.5 L/ha	60,000	0.38 µL/L	480	0.5 µL/L	600

	1 × max = 1 L/ha	120,000	0.77 µL/L	960	1 µL/L	1,200
	2 × max = 2 L/ha	240,000	1.53 µL/L	1,920	2.5 µL/L	3,000
	10 × max = 10 L/ha	1,200,000	7.67 µL/L	9,600	10 µL/L	12,000

The aquarium surface = 0.046 m^2. The volume of water used per tank = 6 L.

Viable Cells Count

Bti's cell concentration was determined using the plate count method to validate the application rates and to understand if there was bacterial growth in tanks over time. For this, tanks without tadpoles were spiked with the different tested Bti concentrations (Table 1). The experiment was performed in a controlled light/temperature room with a 15:9 light:dark cycle. The water temperature was 16 ± 1 °C. Water samples were taken after 1, 24, 48 and 72 h after inoculation. The results are expressed in colony-forming units per millilitre of water in the tank (CFU/mL). Further methodology details are included in the material and methods section of the supplementary document.

Frog Egg Collection

Several fertilized egg masses of *L. sylvaticus* (Wood frog) and *A. americanus* (American toad) were collected from wetlands not treated with Bti, nor with agriculture or industrial inputs to minimize the possibilities of a pre-exposure and to increase the genetic diversity of the individuals. Larval development for both species was classified according to the Gosner staging (GS) system.[33] At collection time, *L. sylvaticus* and *A. americanus* individuals were GS 10 and 5, respectively. The egg masses were transported to the INRS facilities (Quebec City, Quebec, Canada) in aerated plastic coolers filled with water from the pond. The egg masses were later moved to aerated glass tanks containing a mixture of reconstituted water solution and pond water (2:1) and housed in an environmentally controlled room (16 ± 1 °C and 15:9 h light:dark). Further egg collection details are included in the material and methods section of the supplementary document.

Exposures and Sample Collection

All the bioassays were performed following the guidelines of the Canadian Council on Animal Care and approved by the Centre national de biologie expérimentale at the INRS. The exposures started when the tadpoles reached GS 25, which marks the beginning of feeding of external sources and they can actively swim.[33] Tadpoles were fed twice a day with lyophilized spirulina and krill (SERA MICRON). Each glass tank contained 50 individuals in 6 L of water. Control tanks were run in quadruplicate, while the treatment tanks were run in duplicates. The exposure was performed in a controlled light/temperature room. The room conditions followed the conditions published by Environment and Climate Change Canada for Quebec City for the spring of 2019 aiming to match tadpoles' metamorphosis timeline (Table S1). The temperature varied between 15 and 21 °C, and the daily light exposure ranged from 14 to 16 h. The acute test was performed for 48 h and the mortality was registered daily.

The chronic exposure continued until the tadpoles reached GS 42. At GS 42, metamorphosis starts with changes in the mouth, then forelimbs appear marking the metamorphic climax.[33] During the exposure, every 24 h, the survival was recorded, and dead tadpoles were removed. Every three days water quality was assessed and 50% of the exposure solution (with waste) was replaced with Ringer's solution and followed by Bti application to maintain the exposure levels. To monitor the development of the tadpoles, once a week five tadpoles were randomly selected to assess the length and the stage of development. When individuals reached GS 42, the tadpoles were anesthetized with a solution of 0.1 g/L tricaine methanesulfonate (MS-222; Sigma-Aldrich), weighed, and decapitated. The intestine was collected for histopathology (n = 7) or microbiota (n = 3), and the tail (n = 10) and liver (17-20) tissues were collected for gene expression analysis. The hepatosomatic index (HSI) and the gonadosomatic index (GSI) were calculated using Equations S1 and S2. In aquatic organisms, the HSI and the GSI are commonly used as indicators of contaminant exposure and gonadal health and maturation, respectively.[34] Additional sample collection details are included in the material and methods section of the supplementary document.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from the liver and tail using TRIzol™ reagent and phase maker tubes (Thermo Fisher Scientific) as described in the manufacturer's protocol and adding 1 µL of 20 µg/µL glycogen (Thermo Fisher Scientific) to increase the RNA yield. The RNA integrity was assessed by the presence of two defined bands on an agarose gel.[35] The top band represents the 28S ribosomal RNA subunit (rRNA) and the second band represents the 18S rRNA.[36] However, smearing was observed in the liver samples of *L. sylvaticus*, and therefore, not included in the gene expression analysis to avoid false results. Total complementary DNA (cDNA) was prepared using Maxima™ H Minus cDNA Synthesis Master Mix with dsDNase (Thermo Fisher Scientific) as described in the manufacturer's protocol. The cDNA samples were synthesized in parallel in a Mastercycler® nexus gradient (Eppendorf) and stored at -20 °C. Further details of extraction and cDNA synthesis are included in the Material and Methods section of the supplementary document.

Real-Time Quantitative Polymerase Chain Reaction

In this study, we targeted the expression of genes related to xenobiotic exposure and oxidative stress, i.e. cytochrome P450 1A (*cyp1a*),[37] superoxide dismutase (*sod*),[38] glutathione peroxidase (*gpx*),[38] glutathione-disulfide reductase (*gsr*)[39] and metamorphosis, i.e. thyroid receptor α (*trα*),[40] and thyroid receptor β (*trβ*).[40] We used the expression of ribosomal protein L8 (*rpl8*) and ornithine decarboxylase (*odc*) as reference genes. Previous research has shown that *cyp1a* in the tail in other aquatic organisms is a robust indicator of exposure to contaminants,[41] and its relative gene expression level is similar in the liver and tail after exposure.[41] The genomes of *A. americanus* and *L. sylvaticus* are not yet available, thus, degenerate polymerase chain reaction (PCR) primers were designed based on conserved regions of known sequences from other frog species (Table S2).

Real-time quantitative polymerase chain reaction (RT-qPCR) with SYBR green dye technology was used to validate relative gene expression. Gene-specific primers based on the *L. sylvaticus* and *A. americanus* sequences obtained in this study were developed using Primer-BLAST and synthesized by Sigma Aldrich (Table S3). The primers for *trα*, *trβ*, and *rpl8* for *L. sylvaticus* were previously reported.[42] The Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) and CFX96 Real-time PCR Detection System (Bio-Rad®) were used to amplify and detect the transcripts of interest. The thermal cycling parameters were as suggested by the manufacturer. The efficiency of all RT-qPCR reactions was 95.5 ± 5.5% and the coefficient of determination (R^2) was ≥ 0.990 (0.994 ± 0.003). Data were analyzed using the Bio-Rad CFX Manager Software (Bio-Rad). The relative standard curve method was used to calculate relative mRNA abundance between samples. The signal was normalized using the reference genes *rpl8* and *odc* and then presented as fold change of gene expression from replicates (n = 5–10; assayed in duplicate) for each group. Further details are included in the supplementary document.

Histopathology

To observe the potential impact of the Bti-based pesticides on the amphibian's intestine, the intestine tissues (n = 7) were immediately fixed in a 3.7% formalin solution and sent to the tissue engineering platform of the Centre Hospitalier of the Laval University-Enfant Jesus Hospital (Quebec, Canada). The tissues were observed for the presence of infiltration in the connective tissue and dilation of blood vessels under the intestine epithelium. The intestinal tube wall is formed by three distinct layers: the mucosa, the submucosa, and the muscularis.[18,20] The intestine samples from both species were classified according to the level of detachment between these tissue layers. In class I, these three layers are attached and the cells of the mucosa have a cylindrical shape. For class II, the submucosa detaches slightly from the muscularis and there is a slight dilation of the blood vessels. Detachment and dilation are more pronounced in class III. The degree of severity was determined considering the magnitude of the histological effect (i.e., the amount and complexity of the effect compared to the control). All samples were screened blindly. Further histopathology details are included in the Materials and Methods section of the supplementary document.

Metabarcoding and Bioinformatics

The genomic DNA (gDNA) and the total RNA of the intestine microbiota of *L. sylvaticus* and *A. americanus* were extracted (n = 2-3) using the kit ZymoBIOMICS^{TM} DNA/RNA Miniprep Kit (Zymo Research). The cDNA was synthesized from the RNA samples using the iScriptTM gDNA Clear cDNA Synthesis Kit (Bio-Rad). We used the 16S ribosomal RNA (16S rRNA) gene sequence for the taxonomic identification of the intestine microbiota of *L. sylvaticus* and *A. americanus* exposed to the Bti products. The 16S rRNA gene is composed of variable and conserved regions,[43] and the sequence of the hypervariable regions such as the V3-V4 is commonly used in metabarcoding studies of bacterial community composition.[44]

The amplification of the 16S rRNA gene, equimolar pooling and sequencing were performed at the Genomic Analysis Platform of the Institut de Biologie Intégrative et des Systèmes (IBIS, Université Laval, Quebec, Canada). The amplification of the 16S V3-V4 hypervariable regions was performed using the universal primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC)[45] using a two-step double-indexing PCR approach specifically designed for Illumina instruments following the manufacturer's protocol. The barcoded amplicons were pooled in equimolar concentration for sequencing on a MiSEQ Illumina sequencer. A quality control of the sequences was included to remove the sequence of the primers using the Cutadapt 2.7 tool.[46] The sequence analysis was performed using the IBIS computational infrastructure and the Dada2 algorithm.[47] The taxonomy was assigned using the SILVA database (v.138).[48] The taxonomic identity of the intestine microbiota was visualized in stacked bar charts using ggplot2 based on a subsampled sequence dataset of 26,159 sequences per sample that corresponds to the median number of sequences across all samples (transform_sample_counts function in Phyloseq). The presence of *B. thuringiensis* var. *israelensis* sequences was performed by selecting the amplicon sequence variants (ASV) sequences which taxonomy at the family and genus level corresponded to that of Bti. The corresponding sequences were then compared to that of Bti (taxID: 1430) using the Megablast tool and the nr/nt nucleotide collection. Only the ASVs with a percentage similarity greater than 98% were retained (9 ASVs). Data can be accessed at http://www.ncbi.nlm.nih.gov/bioproject/720921. Further metabarcoding details are included in the supplementary document.

Data Processing and Statistical Analysis

To test if the survival curves were significantly different, the data of the chronic exposure were analyzed using a Mantel-Cox test in GraphPad Prism 8.[49] Analysis of outliers was performed using the ROUT method (Q = 1%) in GraphPad Prism 8.[50] To assess the effect of the exposure on the size development, linear regression and an analysis of covariance (ANCOVA) was performed on GraphPad Prism 8. To assess data normality and homogeneity of variance, Shapiro–Wilk's test and Levene's test were performed, respectively. Data that failed the normality and/or the equal variance tests were transformed (log10). One-way ANOVA and post hoc Student–Newman–Keuls

(SNK) analyses were performed on normally distributed data. Non-parametric Kruskal–Wallis tests were performed on data that did not pass normality and/or equal variances tests followed by SNK for equal sample size or Dunn's method for unequal sample size. The proportion of different levels of detachment per treatment was analyzed by a $\chi 2$ test. The significance level was set at $\alpha = 0.05$. One-way ANOVA analyzes were performed using Sigma Plot 12.0. The RcolorBrewer pallet was used to assign colorblind-friendly colours to the graphs.[51]

The software R and R Studio as the interface were used to perform intestine bacterial diversity, statistical and graphs analyses with the PhyloSeq, Vegan and ggplot2 packages respectively.[52–56] The intestine microbial community compositional patterns were visualized by Non-metric Multidimensional Scaling (nMDS) and Horn distance was used as a measure of beta-diversity. The differences between bacterial assemblages were tested using permutational multivariate analysis of variance (PERMANOVA) with the Adonis function and 999 permutations in the vegan R package.[57] The difference in the bacterial assemblies of the controls between the two species was performed using the betadisper function in R. The correlation between the DNA and RNA compositions was carried out using Procrustes tests for each species.[58]

RESULTS AND DISCUSSION

Biopesticide Potency and Application Rate Verification

We tested the pesticide properties of both VectoBac® 200G and VectoBac® 1200L using a 72 h exposure test with *Ochlerotatus* sp. mosquito larvae. The product VectoBac® 200G was lethally toxic to *Ochlerotatus* sp. mosquito larvae in all of the tested concentrations after 24 h (Fig. S1 A–C). Similarly, VectoBac® 1200L was lethally toxic after 24 h to all the mosquito larvae at a concentration of 0.5 µL/L and higher (Fig. S1 D–F). These results indicate that the biopesticide products herein tested have pesticide properties at the recommended application rate and above (Table 1 and Fig. S1).

To confirm the different application levels used in this study, we measured the Bti concentration (CFU/mL) after 1 h and compared the results between treatments (Fig. 1 A–B). For a better understanding of the results, the concentration is expressed relative to the concentration obtained in the 2.5 mg/L and the 0.25 µL/L treatment with VectoBac® 200G and VectoBac® 1200L, respectively. The results indicate that the application was effective and significantly different between treatments except for the 2.5 and 5 mg/L treatment with VectoBac® 200G (Fig. 1 A) and the 0.25 and 0.5 µL/L treatment with VectoBac® 1200L (Fig. 1 B), which could be due to a pipetting error during the biopesticide application.

Moreover, to know if the Bti concentration was stable during 72 h (equivalent to water change frequency), we measured the Bti concentration at 1, 24, 48 and 72 h after application. For a better understanding of the results, the concentration is expressed relative to the results at 1 h after application. With VectoBac® 200G, the Bti concentration was stable after 24 h. However, the Bti concentration significantly increased after 48 h in the 2.5 and 100 mg/L treatments by up to 219% (Fig. 1 C). With VectoBac® 1200L, the Bti concentration was stable during the first 48 h. However, the concentrations significantly increased after 72 h in four out of the five treatments by up to 262% (Fig. 1 D). VectoBac® 200G is in a granule form and VectoBac® 1200L is in a viscous liquid form, thus the products potentially released more Bti spores into the water through time. *B. thuringiensis* is ubiquitous in aquatic environments,[59] and specifically, Bti was isolated from soil samples of a mosquito breeding site.[3] Bti can persist in cold water and its spores can remain in leaf litter.[60,61] Such conditions are similar to amphibian breeding ponds and they and other non-target species could be potentially exposed to Bti products. Our data suggest that Bti from VectoBac®

200G and VectoBac® 1200L could persist in the environment and its concentration could potentially increase over time.

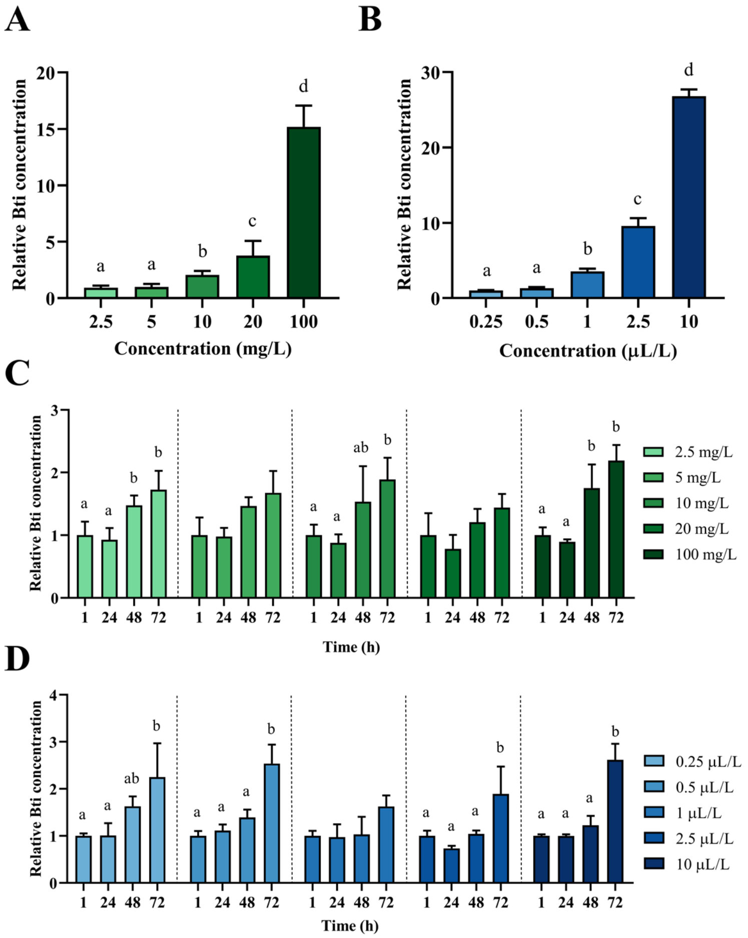

Figure 1. Confirmation of concentration levels for VectoBac® 200G (A) and 1200L (B) formulations. The values are relative to the concentration obtained in the 2.5 mg/L and the 0.25 µL/L treatment with VectoBac® 200G and VectoBac® 1200L, respectively. Analysis of the Bti concentration through time after applying VectoBac® 200G (C) and 1200L (D). The values are relative to the results at 1 h after application. Different letters indicate groups that are significantly different ($p < 0.05$).

Lethal and Sub-Lethal Effects

The acute exposure to VectoBac® 200G and 1200L did not significantly affect the survival of *L. sylvaticus* and *A. americanus* tadpoles at the tested concentrations ($p > 0.05$; Fig. S2). Similarly, the chronic exposure did not significantly affect the survival of *L. sylvaticus* (Table S4). However, VectoBac® 200G altered the survival of *A. americanus* by 20% ($p = 0.0119$) at 100 mg/L and VectoBac® 1200L by 29 and 35% at 1 and 2.5 µL/L, respectively ($p < 0.05$; Table S4). Although the chronic exposure to the Bti products significantly affected the survival of *A. americanus*; the data did not show a concentration-dependent response and the effects were observed at 1×, 2×, and 10× the maximal recommended application rate.

Our results on the lethal toxicity of the Bti products exemplify the mixed effects that have been previously published regarding the effects of commercial Bti products on amphibians. For example, the exposure to VectoBac® WG (water-dispersible granule formulation) and 12AS (aqueous suspension formulation) did not significantly affect the mortality of *R. temporaria* tadpoles after chronic exposure at 3,247–64,940 ITU/L.[19] Similarly, the exposure to VectoBac® WG for 11 days from GS 23 to 29 at 3,000–300,000 ITU/L did not alter the mortality of *R. temporaria*.[20] Both VectoBac® WG and VectoBac® 12AS contain the same Bti strain that was used in the present study (i.e., strain AM 65-52). In contrast, the product Introban® (aqueous suspension formulation) at 48,000 ITU/L led to 100% mortality on *L. latrans* GS 26–30 tadpoles after 48 h of exposure.[18] Correspondingly, Introban® has an $LC50_{48h}$ of 23,100, 12,876, and 14,244 ITU/L on *Rhinella arenarum*, *Rhinella fernandezae*, and *Physalaemus albonotatus* tadpoles GS 33, respectively.[62] Herein, we tested concentrations ranging from 300 to 20,000 ITU/L with no effects on the survival of *L. sylvaticus* and slight lethal effects on *A. americanus* (Table S4). Altogether, these data might indicate a potential sensitivity difference between amphibian species. In addition, the difference in the lethal toxicity among the different studies might be due to a different Bti strain or in the product additives. In this regard, chemical additives such as lipid emulsifying agents and nitrogen compounds could potentiate the pesticide properties of Bt products.[63] VectoBac® and Introban® do not provide the exact composition of their products. Moreover, Introban® does not provide information on the specific Bti strain in their product.

The chronic exposure to the Bti commercial products showed some effects on the total length of *L. sylvaticus* and a few effects on the total weight of *A. americanus*. However, the total length and total weight data did not show a concentration-dependent response (Table S4). Similarly, Allgeier et al. (2018) reported that VectoBac® WG and 12AS did not affect significantly the total length, total weight, and the scaled mass index of *R. temporaria* tadpoles after chronic exposure at a range between 3,247–64,940 ITU/L.[19] In addition, no significant effects were observed on the body mass of *R. temporaria* when exposed to VectoBac® WG for 11 days from GS 23 to 29 at a range between 3,000 and 300,000 ITU/L.[20] Also, our exposure spanned the start of active feeding until metamorphosis climax; therefore, we would suggest in future studies to start the exposure at earlier stages to see potential embryotoxicity from Bti or the additives.

The chronic exposure to VectoBac® 1200L significantly decreased the HSI of *L. sylvaticus* at 0.25, 0.5 and 1 μL/L, however, these changes did not follow a concentration-dependent response (Table S4). The data did not show significant effects on the HSI in the other treatments. Similarly, the chronic exposure to VectoBac® 200G and 1200L did not significantly affect the GSI of *L. sylvaticus* and *A. americanus* tadpoles at the tested concentrations (Table S4). Generally, changes in the HSI would indicate an effect on the liver function and an increase of the HSI would indicate stress on the liver.[64] The sensitivity of amphibians to pesticides seems to vary among species[65,66] and could be potentially due to differences in the detoxification capacities.[67] This could explain the different results between *L. sylvaticus* and *A. americanus*.

Size and Time to Metamorphosis.

The chronic exposure to VectoBac® 200G and 1200L had more effects on the size development of *L. sylvaticus* tadpoles than *A. americanus* (Fig. S3). We did not observe a clear concentration-response relationship between the size development and the concentration of VectoBac® 200G and 1200L (Fig. S3). At the end of the chronic exposure, the size of *L. sylvaticus* and *A. americanus* tadpoles exposed to the Bti products was not significantly different from their respective control (Table S4). The exposure to VectoBac® 200G significantly increased the median (Mdn) time to metamorphosis of *L. sylvaticus* tadpoles up to 43 days (interquartile range or IQR = 43–43) at 2.5, 5, 10 and 20 mg/L (Fig. 2A). But, at a concentration of 100 mg/L, the time to metamorphosis decreased significantly and the Mdn in this treatment was 39 days (IQR = 39–39). Also, this formulation had the opposite effect on *A. americanus*, significantly decreasing the time to metamorphosis at 2.5, 5, 10 and 20 mg/L (Fig. 2B). But, at a concentration of 100 mg/L, the time to metamorphosis increased significantly and the Mdn in this treatment was 33 days (IQR = 34-27). Moreover, the exposure to VectoBac® 1200L increased significantly the time to metamorphosis of *L. sylvaticus* tadpoles in all of the tested concentrations (Fig. 2C). The *L. sylvaticus* control tadpoles reached GS 42 in a Mdn of 39.5 days (IQR = 40-39), while the exposed individuals reached metamorphosis between 42 and 48 days. Similarly, *A. americanus* control tadpoles reached metamorphosis in a Mdn of 29 days (IQR = 30–29), while the exposed individuals reached metamorphosis between 29 and 40 days (Fig. 2D). A timely metamorphosis might be crucial for survival in fast-drying ponds and could ultimately affect population survival.[68] Although the data show effects on the time to metamorphosis, we did not observe the same concentration-response pattern to the exposure of the Bti pesticides in both species (Fig. 2).

Amphibian metamorphosis is controlled by endogenous signals such as the ones from the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis.[69] This axis not only controls metamorphosis but also regulates responses to stress and other physiological processes, including sexual behaviour, immunity, digestion, and energy mobilization.[70–72] In normal conditions, glucocorticoid hormones can accelerate the larval changes that occur during metamorphosis, such as face and mouth anatomical changes, forelimbs and hind limbs development, and tail resorption.[33,73,74] Moreover, environmental conditions and external signals such as stressors and contaminants can also influence amphibian metamorphosis.[75,76] External stressors could decrease the time to metamorphosis; however, a faster metamorphosis produces smaller individuals and smaller individuals have lower survival and lower fecundity.[77–79] These effects might ultimately affect amphibian populations. Because of previous research,[77–79] we were expecting significantly smaller tadpoles in the treatments with a faster metamorphosis. However, the exposure to the Bti products did not affect the weight and total length of the tested individuals at the end of the exposure in a concentration-response pattern (Table S4). Also, our exposure spanned from the start of active feeding until metamorphosis climax; therefore, we would suggest in future studies to continue the exposure until the end of metamorphosis to observe other potential effects such as survival and weight after metamorphosis.

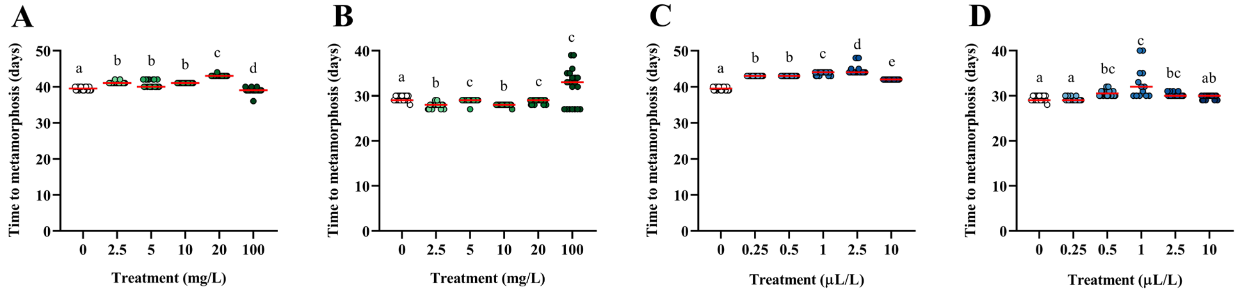

Figure 2. Effect of the chronic exposure of VectoBac® 200G on the time to metamorphosis in

tadpoles of *L. sylvaticus* (A) and *A. americanus* (B). Effect of the chronic exposure of VectoBac® 1200L on the time to metamorphosis in *L. sylvaticus* (C) and *A. americanus* (D). The red line indicates the median and the different letters indicate groups that are significantly different ($p < 0.05$).

Gene Expression.

We analyzed tail tissues of *L. sylvaticus* and tail and liver tissues of *A. americanus* to understand potential xenobiotic, stress and endocrine disruption response after chronic exposure to Bti pesticides. The chronic exposure to VectoBac® 200G increased significantly the expression of *cyp1a* in the tail of *L. sylvaticus* at 2.5 to 100 mg/L compared to the control treatment (Fig. 3A). Similarly, at 10 mg/L the expression of *trβ* significantly increased (Fig. 3B). However, we did not observe a concentration-dependent response in these two genes (Fig. 3 A–B) and the expression of *sod*, *gpx*, *gsr* and *trα* was not significantly affected (Fig. S4). Moreover, the chronic exposure to VectoBac® 200G did not affect significantly the expression of the genes of interest in the tail of *A. americanus* (Fig. S5). Also, this formulation increased significantly the expression of *trα* in the liver of *A. americanus* but only at 10× the max suggested application rate (Fig. 3C) and the expression of the other genes of interest was not affected (Fig. S6).

The exposure to VectoBac® 1200L increased significantly the expression of *cyp1a* and *sod* in the tail of *L. sylvaticus* at 0.5, 1 and 10 µL/L compared to the control treatment (Fig. 3D-E). However, there is no concentration-dependent response in these two genes and the other genes of interest were not affected in the tail of *L. sylvaticus* (Fig. S7). Also, this formulation did not affect significantly the genes of interest in the tail and liver of *A. americanus* (Fig. S8–S9).

The expression of *cyp1a* significantly increased in the tail of *L. sylvaticus* exposed to VectoBac® 200G and 1200L. This gene encodes the cytochrome P450 proteins that are involved in drug metabolism and synthesis of cholesterol, steroids and other lipids.[80] The overexpression of this gene might indicate that *L. sylvaticus* is responding to the exposure to the Bti commercial products as if it was a xenobiotic. Similarly, the expression of *sod* significantly increased in the tail of *L. sylvaticus* exposed to VectoBac® 1200L. The gene *sod* expresses an isoenzyme as a response to oxidative stress and it is responsible for destroying radicals in the body by converting superoxide radicals into molecular oxygen and hydrogen peroxide.[81] Similar to our results, the literature on the effects of Bti formulations on amphibian detoxification and cellular stress is complex. For example, the exposure to VectoBac® WG for 11 days from GS 23 to 29 at a range of 3,000 to 300,000 ITU/L did not significantly change the heat shock 70kDa protein (Hsp70) levels neither among treatments nor between treatments and the controls in *R. temporaria* head-corpuses.[20] The Hsp70 is conserved and ubiquitous among cell components in eukaryotes,[82] and it is used as a biomarker of pollution and cell stress response in different toxicological studies.[83,84] Higher levels of Hsp70 could indicate proteotoxic stress, but because of their results, Schweizer et al. (2019) concluded that VectoBac® WG did not induce proteotoxic effects on *R. temporaria*. However, Lajmanovich et al. (2015) reported that the glutathione-S-transferase (GST) activity of *L. latrans* significantly increased after the exposure to Introban® at 12,000 and 24,000 ITU/L, and catalase (CAT) activity at 24,000 ITU/L. Also, the exposure to VectoBac® WG and 12AS Bti increased the GST level in *R. temporaria* larvae by up to 550%.[19] However, the stress response of *R. temporaria* tadpoles reported by Allgeier et al. (2018)[19] could be due to the high temperature conditions during the exposure and not as a response to Bti, as discussed by Schweizer et al. (2019).[19,20] The GSTs are a family of enzymes known for their ability to catalyze the conjugation of the reduced form of glutathione to electrophilic compounds for detoxification.[85] Catalase is an enzyme that neutralizes hydrogen peroxide into gaseous oxygen and water molecules and its main role is the protection against oxidative stress by reactive oxygen species.[86,87] Moreover, the expression of *trα* significantly increased in the liver of *A. americanus* exposed to VectoBac® 200G. The expression of *trβ* significantly increased in the tail of *L. sylvaticus* exposed to VectoBac® 200G. The genes *trα* and *trβ* are essential for metamorphosis in frogs and their expression increase during metamorphosis climax.[21,42] The overexpression of *trα* in *A. americanus* was not expected because in general, a lower expression of *trα* indicates a late metamorphosis.[88] However, the exposure to VectoBac® 200G increased significantly the time to metamorphosis at 100 mg/L in *A. americanus*.

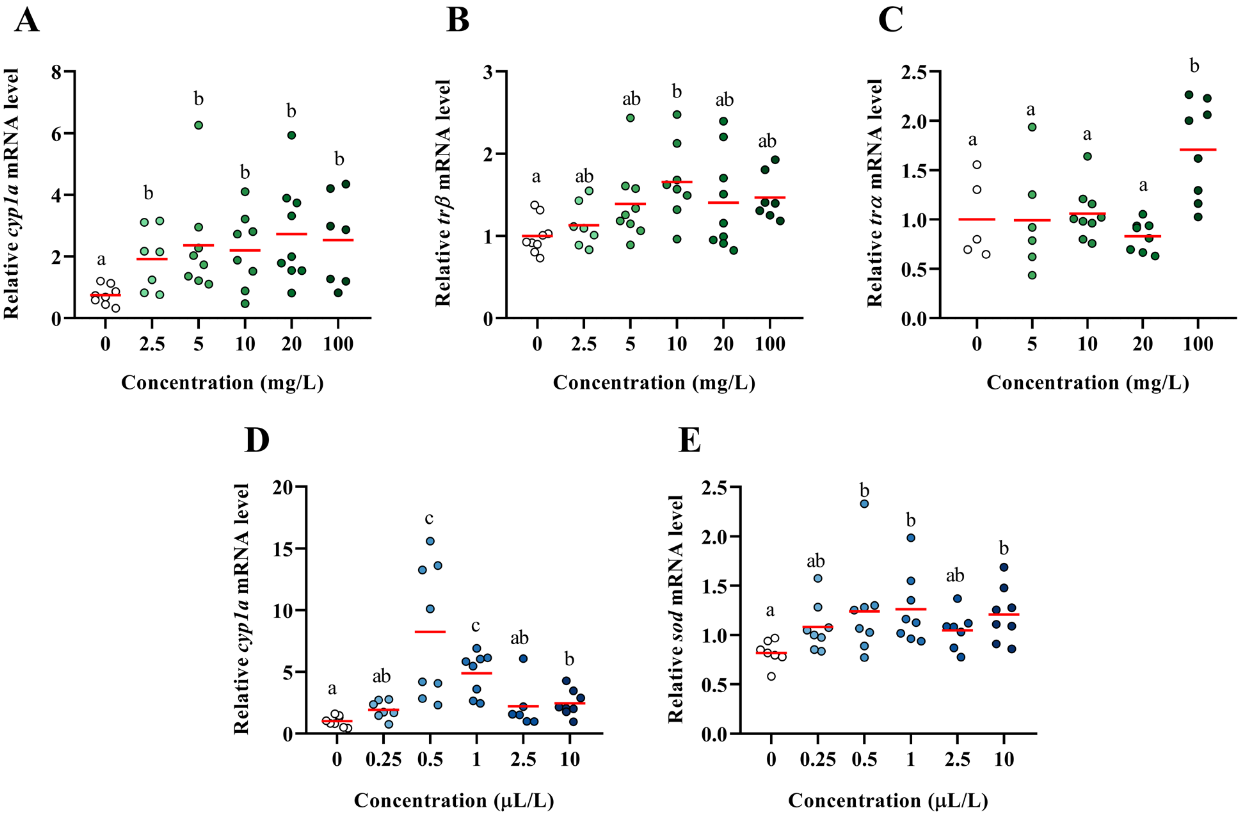

Figure 3. Gene expression analysis for *cyp1a* and *trβ* in the tail of *L. sylvaticus* exposed chronically to VectoBac® 200G (A and B), for *trα* in the liver of *A. americanus* exposed chronically to VectoBac® 200G (C), and for *cyp1a* and *sod* in the tail of *L. sylvaticus* exposed chronically to VectoBac® 1200L (D and E). The red line indicates the mean and the different letters indicate groups that are significantly different ($p < 0.05$).

Intestinal Histopathology.

We examined intestine samples under the microscope to observe if VectoBac® 200G and 1200L induced detachment of the tissue layers. We classified the intestine samples into three classes according to the level of detachment between tissue layers (Fig. 4A–F). There were no significant differences ($\chi 2$, $p > 0.05$) in the detachment level of the tissue layers between controls and the different concentrations of the tested formulations of Bti (Figure 4G–J). These results suggest that the commercial formulations of Bti at the concentrations tested herein do not affect the level of the detachment of tissue layers on *L. sylvaticus* and *A. americanus* intestines. These results are in agreement with a previous study of the potentially toxic effects of VectoBac® WG on *R. temporaria* tadpoles. In this article, the histological examination of the tadpole intestines did not show any impact of Bti on cellular, tissue, or organ integrity.[20] However, Lajmanovich et al. (2015) observed infiltration in the connective tissue and dilation of blood vessels of the intestine of the South American common frog (*L. latrans*) tadpoles exposed to a commercial liquid formulation of Bti (Introban®); which is considered as a defense mechanism against a stressor.[18] Yet, they did not mention a concentration-relationship effect in their study nor statistical analysis to support their observations.

The insecticidal properties of Bti are based on the ingestion, the dissolution in an alkaline environment and activation of insecticidal proteins by proteases.[9–12] However, in anuran herbivorous tadpoles the stomach pH is neutral and acidic in carnivorous ones.[89–91] Thus, the pesticide proteins of Bti might not be active if ingested by tadpoles. Herein, we analyzed only the intestines of the surviving individuals. Thus, exposure to Bti might have affected the intestine integrity of the dead tadpoles. Future studies should include the analysis of the intestines of individuals that died during exposure to Bti formulations. Also, some samples were damaged during preparation thus reducing the number of samples that could be included in the analysis.

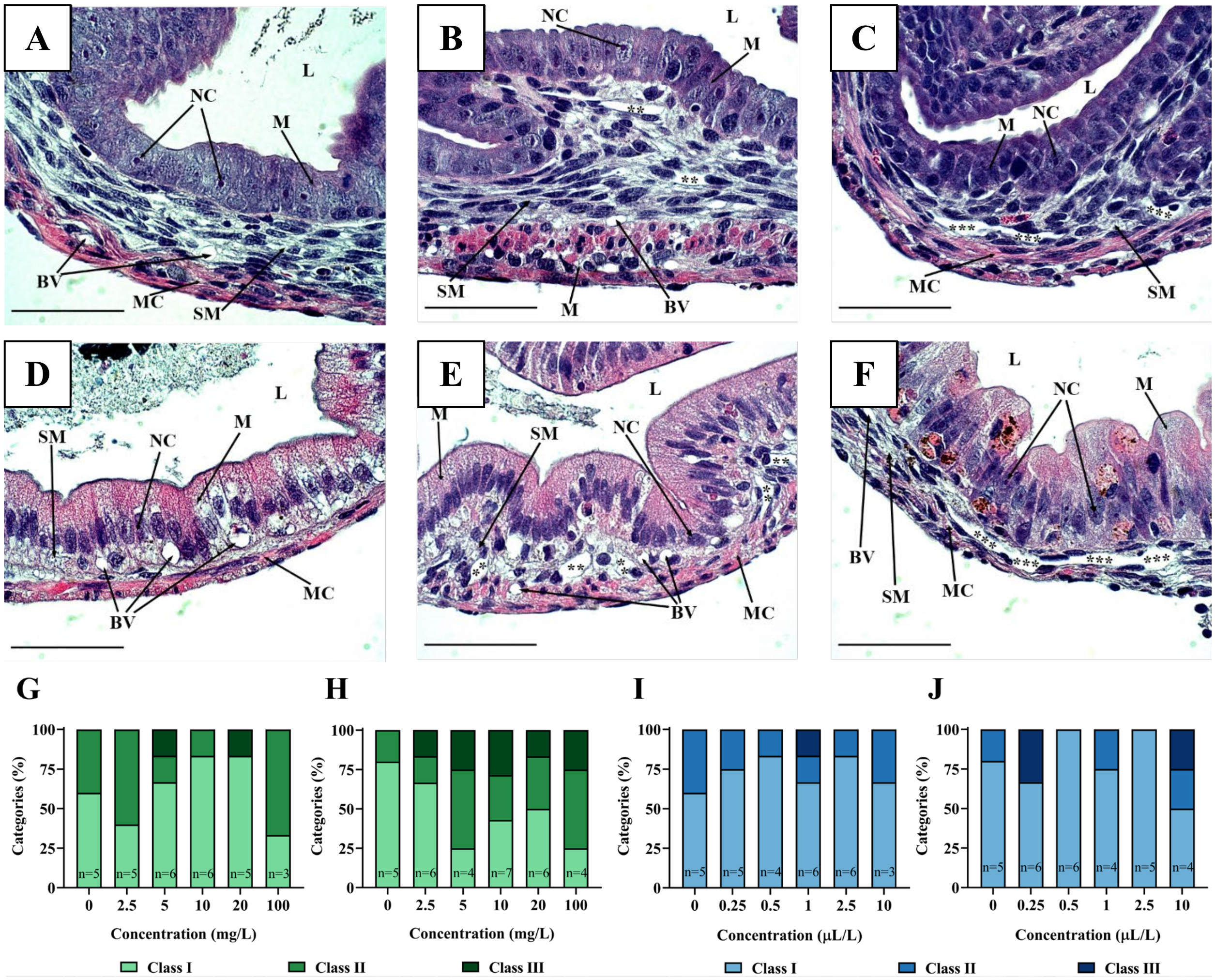

Figure 4. Intestine histology of *L. sylvaticus* (A-C) and *A. americanus* (D-F) at GS 42. An example of Class I in A and D. In class I, the mucosa, the submucosa and the muscularis layers are attached, the cells have a cylindrical shape and their structure is normal. An example of Class II in B and E. In class II, there is a slight dilation of blood vessels and slight detachment of the submucosa and muscularis (**). An example of Class III in C and F. In class III, the detachment and dilation are very pronounced (***). NC = nucleolus, M = mucosa, SM = submucosa, MC = muscular, BV = blood vessel, L = lumen. The scale bar is equal to 50 µm. Effect of the exposure to VectoBac®

200G on the proportions of the different levels of detachment between the tissue layers of the intestinal wall of *L. sylvaticus* (G) and *A. americanus* (H). Effect of the exposure to VectoBac® 1200L on the proportions of the different levels of detachment between the tissue layers of the intestinal wall of *L. sylvaticus* (I) and *A. americanus* (J).

Effects on the frog intestine microbiota.

We analyzed the diversity and composition of intestine bacterial communities of *L. sylvaticus* and *A. americanus* to understand the potential effects of the chronic exposure to VectoBac® 200G and 1200L. Firstly, we used the betadisper function of the vegan package to know if there were significant differences between the intestine microbiota diversity of non-treated *L. sylvaticus* and *A. americanus* tadpoles. The betadisper function tests for homogeneity of variances and it can be interpreted as a measure of beta-diversity or how diversity varies among samples.[92] The analysis of the microbiota diversity using genomic DNA in control individuals shows no significant difference between *L. sylvaticus* and *A. americanus* (Beta-disper; F = 0.701; $p = 0.705$; Fig. S10).

The gDNA and cDNA microbiota data showed that the dominant phyla in the intestine of *L. sylvaticus* and *A. americanus* tadpoles reared in the laboratory were *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidota* (Fig. 5 A, Fig S11 A), and *Alphaproteobacteria*, *Gammaproteobacteria* and *Firmicutes* (Fig. 5 B, Fig. S11 B), respectively. Similarly, in a previous report, the *Proteobacteria* followed by *Firmicutes* were the dominant phyla in the gut microbiome of *Lithobates pipiens* (Leopard frog) stage 36 ± 2 tadpoles.[93] *Proteobacteria* are Gram-negative bacteria that include heterotrophic and phototrophic bacteria[94] and this phylum has been suggested as a microbial signature of the modification of the gut microbiome or dysbiosis in mammals.[95] *Bacteroidota* are anaerobic-Gram-negative bacteria that do not form endospores widely spread in the biosphere and *Firmicutes* are Gram-positive spore-forming bacteria, including Bti, with a low DNA mol% G + C, both commonly found as part of the gut microbiome of vertebrates.[96,97]

While there was no significant difference between the microbiota diversity in control *L. sylvaticus* and *A. americanus*, the gDNA and cDNA data showed that the chronic exposure to VectoBac® 200G and 1200L differentially changed the intestinal microbial composition in the two amphibian species (PERMANOVA; gDNA: $p = 0.005$; cDNA: $p = 0.005$). This indicates that the intestinal microbial communities of *L. sylvaticus* and *A. americanus* respond differently to the chronic exposure to the Bti formulations. In *L. sylvaticus*, there was no significant change in the gDNA and cDNA profile of intestinal bacterial communities following the chronic exposure to VectoBac® 200G and 1200L (PERMANOVA; gDNA: $p = 0.094$, Fig. 5 C; cDNA: $p = 0.138$, Fig. S11 C). However, in *A. americanus*, the gDNA and cDNA profile of the intestinal bacterial communities significantly changed (PERMANOVA; gDNA: $p = 0.028$, Fig. 5 D; cDNA: $p = 0.022$, Fig. S11 D). In the exposed *A. americanus* tadpoles, the relative abundance of *Verrucomicrobia*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* increased (Fig. 5 B and Fig. S11 B). This is important since the modification of the gut microbiome could affect the susceptibility to parasitic infections in amphibians,[98] and dysbiosis has been linked to the development of chronic diseases in other amphibians.[27] Further studies should investigate if the changes in the microbiota have implications in the host physiology, fitness and potential alterations of life-history traits, as well as its potential impact on the anuran populations and eventually the functioning of the ecosystem. For example, there is evidence of changes in the gut microbiota during metamorphosis,[93] and a failed gut microbiota restructuration could cause death.[99] Therefore, we would suggest investigating if the exposure to Bti formulations impacts the adaptation to a carnivorous diet in *A. americanus*. Also, the intestine microbiota of *A. americanus* tadpoles responded to the exposure to VectoBac® 200G and 1200L, however, the effects of the formulations are not the same (Fig. 5D) suggesting that the additives may have a potential role in the effects on the microbiota changes and this should be further explored. Of note, diet influences the gut microbiome,[100] and thus, likely the diet and laboratory conditions affected the natural microbiome of *L. sylvaticus* and *A. americanus* tadpoles. We suggest for future analysis to compare the intestine microbiota of wild-caught tadpoles living in non-treated versus Bti-treated sites. Moreover, it should be considered to track the intestine microbiota changes over the exposure to Bti formulations to observe a potential acclimation. Overall, this study shows that the use of Bti has a potential impact on the two North American anuran larvae. We then recommend that proposed Bti-spraying activities in amphibian-rich ecosystems should be kept minimal until there is more research to assess if the changes in the time to metamorphosis and microbiota can lead to negative outcomes in amphibian populations, and eventually, the functioning of ecosystems. Furthermore, new methodologies should be developed for Bti quantification, as of now, there is no standardized method to measure Bti in the environment upon spraying activities, and this would be an important step to move towards environmental Bti monitoring.

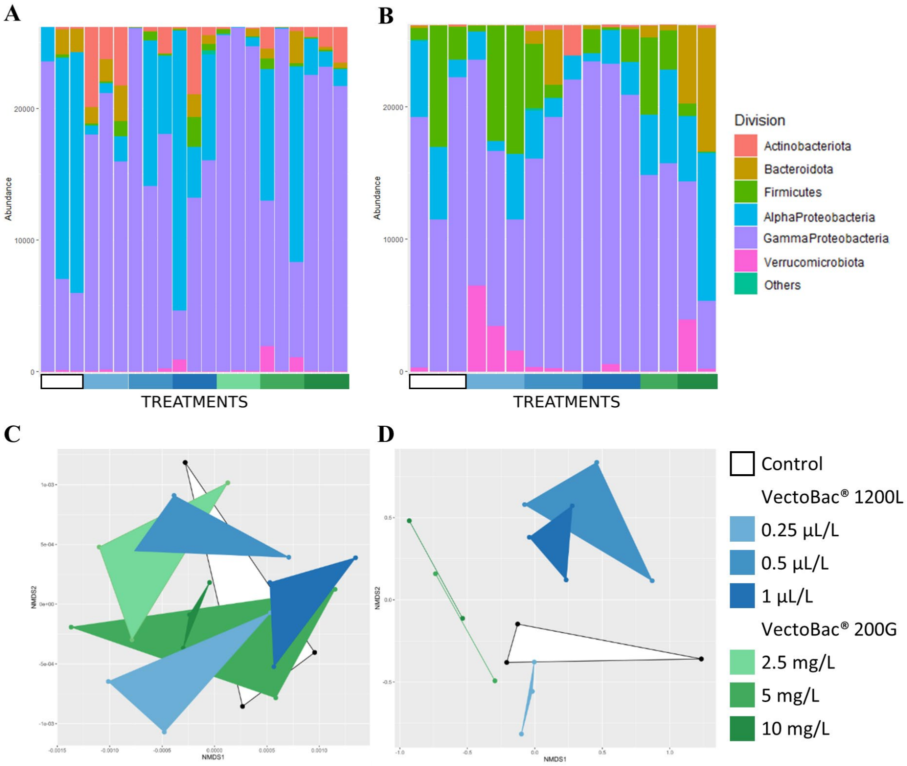

Figure 5. Taxonomic stacked bar charts showing the relative abundance of bacteria in the intestine of *L. sylvaticus* (A) and *A. americanus* (B) exposed chronically to VectoBac® 200G and 1200L. Non-metric multidimensional scaling visualization of the changes in bacterial community composition in the intestine of *L. sylvaticus* (C) and *A. americanus* (D) exposed chronically to VectoBac® 200G and 1200L. The results presented are based on the analysis of the gDNA.

ASSOCIATED CONTENT

Supporting Information. A document is provided with methodology details, the control room conditions during the exposure, description of primers, insecticide potency verification, acute mortality, size development, qPCR data, and metabarcoding data (PDF).

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Author Contributions

JMGV: data curation, formal analysis, investigation, visualization, writing, review and editing. GP: conceptualization, methodology, investigation. TAT: investigation, writing and review. MLR: investigation, writing and review. LRLJ: investigation and review. JC: investigation, formal analysis, writing and review. BK: review and conceptualization. VSL: conceptualization, methodology, resources, review, editing, funding acquisition. All authors have approved the final version of the manuscript.

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Conflict of Interest Disclosure

The authors declare no competing interests.

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ABBREVIATIONS

Bacillus thuringiensis var. *israelensis* (Bti); Crystal toxin (Cry), Cytolytic toxin (Cyt); International Toxic Units (ITU); Colony-forming units per milliliter (CFU/mL); Hepatosomatic index (HSI); Gonadosomatic index (GSI); Genomic DNA (gDNA); Complementary DNA (cDNA); messenger RNA (mRNA); Amplicon sequence variants (ASV); Non-metric Multidimensional Scaling (nMDS); Permutational multivariate analysis of variance (PERMANOVA); Median (Mdn); Interquartile range (IQR).

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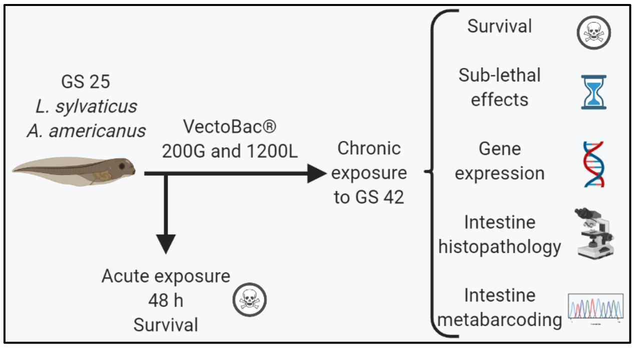
TOC


GS 25
L. sylvaticus
A. americanus
VectoBac®
200G and 1200L
Chronic exposure to GS 42
Acute exposure
48 h
Survival
Survival
Sub-lethal effects
Gene expression
Intestine histopathology
Intestine metabarcoding

Supplementary

Frogs Respond to Commercial Formulations of the Biopesticide Bti, especially their Intestine Microbiota

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MATERIAL AND METHODS

Chemicals

For exposures, to ensure the water purity a reconstituted solution was made with distilled water, NaCl (7.2 g/L), CaCl (0.17 g/L), and KCl (0.37 g/L). The pH of the solution was adjusted with $NaHCO_3$ to pH 7.8.[1] The purity of the salts for the preparation of reconstituted water solution was higher than 99% (Fisher Scientific and Sigma-Aldrich). Both formulations (VectoBac® 200G and Vectobac® 1200L) were prepared as concentrated stock solutions in reconstituted water, then used to prepare the exposure solutions.

Viable Cells Count

Samples of 0.5 mL were taken and diluted in 4.5 mL of a sterile saline solution (0.85% NaCl) followed by 10-fold serial dilutions and 0.1 mL of each dilution was spread in nutrient agar plates in triplicates. The composition of the nutrient agar was as follows: yeast extract 2 g/L, beef extract 1 g/L, peptone 5 g/L, NaCl 5 g/L, and agar powder 17 g/L. The dilutions and agar plate inoculation were performed under a microbiological safety cabinet previously cleaned with alcohol and UV light. The plates were incubated under aerobic conditions at 30 °C for 24 h. The morphology of Bti colonies in Petri dish is a circular beige colony. Colonies with other morphologies were not considered in the colony counting.

Frog Egg Collection

The egg masses of *L. sylvaticus* were collected on May 9, 2019 from a pond located at the Base plein-air de Sainte-Foy, Quebec City, Quebec, Canada. The water of this pond had a conductivity of 58 µS/cm and a temperature of 11.6 °C. The egg masses of *A. americanus* were collected on May 18, 2019 from a pond located at the Domaine de

Maizerets, Quebec City, Quebec, Canada. The water of this pond had a conductivity of 216 µS/cm and a temperature of 14.9 °C.

Exposures and Sample Collection

Tadpoles were fed twice a day with lyophilized spirulina and krill (SERA MICRON). The amount of food was estimated calculated based on the tadpoles' mass in the aquariums.

To start the exposures, on May 22 and June 1, 2019, 1,200 tadpoles of *L. sylvaticus* and *A. americanus* GS 25 were randomly placed in different aerated glass tanks, respectively. Every three days, water conductivity, ammonia level, pH, and temperature were assessed to ensure water quality.

During the collection time, the length, the total weight, the liver weight and the gonads weight were recorded. The hepatosomatic index (HSI) and the gonadosomatic index (GSI) were calculated using Equations S1 and S2. The individuals were dissected and intestines, liver, and tail were collected in RNase/DNase-free tubes. A subsample of intestines was randomly selected, placed in histological cassettes and conserved in a buffered 3.7% formalin (Fischer Scientific) for histological analysis. The rest of the intestines, liver, and tail samples were placed in dry ice and later stored in a -80 °C freezer for transcriptomic and microbiome analysis.

RNA Extraction and cDNA Synthesis

Before cDNA synthesis, the concentration and the RNA integrity of all samples were assessed using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific) and an agarose gel. No reverse transcriptase (NRT) and no template controls (NTC) were included during the cDNA synthesis. For *L. sylvaticus* tail tissue exposed to VectoBac® 200G and VectoBac® 1200L, 3,000 and 4,000 ng of total RNA was used for the cDNA synthesis, respectively. For *A. americanus* tail tissue exposed to VectoBac® 200G and VectoBac® 1200L, 1,000 and 2,500 ng of total RNA was used for the cDNA synthesis, respectively. *For A. americanus* liver tissue exposed to VectoBac® 200G and VectoBac® 1200L, 3,500 ng of total RNA was used for the cDNA synthesis in both cases.

Degenerate Primers and PCR

The degenerate primers were synthesized by Sigma Aldrich. The PCR SuperMix (Invitrogen) was used to amplify the regions of interest. The cycling conditions were 2 min at 94 °C; 35 cycles of 15 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C. The PCR products of each sample were resolved on 1% agarose gel stained with Sybr Safe (Invitrogen). The product was purified using the QIAquick PCR & Gel Cleanup Kit (Qiagen). If the amount of DNA was low, the purified product was submitted to a second PCR reaction using the same cycling conditions described above. The PCR products were sent to the Centre hospitalier Laval University and sequenced using 3730 DNA Analyzer (Applied Biosystems). The obtained sequences were analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine nucleotide identities.

Real-Time Quantitative Polymerase Chain Reaction

To confirm the specificity of the primers the PCR products were observed in a 2% agarose gel and one single band was observed in the gels. To confirm the amplification of the regions of interest, the PCR products were purified using the NucleoSpin® Gel and PCR Clean-Up kit (Takara), sent to the Centre Hospitalier de l’Université Laval and sequenced using 3730 DNA Analyzer (Applied Biosystems).

The qPCR thermal cycling parameters were as suggested by the manufacturer; an activation step at 95 °C for 10 min, followed by 40 cycles of 95 °C denaturation step for

15 s and one primer annealing/extension temperature depending on the primer set for 60 s (Table S3). After 40 cycles, a melt curve was performed over a range of 60–95 °C with increments of 1 °C to ensure a single amplified product. The concentration of each primer in all of the RT-qPCR reactions was 0.3 µM. The final volume in all of the reactions was 20 µL. Progene® thin-wall PCR strip tubes and PCR 8-Strip flat optically clear caps for qPCR were used for the reactions.

Histopathology

The fixed tissues were dehydrated in a solution ethanol-formalin (70 and 3.7%), then in a series of ethanol (100%) washes and three washes of toluene, then embedded in paraffin blocks, serially sectioned at 3 µm and stained with hematoxylin and eosin. Seven intestines per treatment were prepared and three to seven samples were analyzed depending on the quality of the sections. The sections were analyzed under a microscope Zeiss Axio Plan 2 connected to a camera Axio cam 305 colour using a 100× magnification. Pictures were taken using the Zen 3.0 software with a 40× magnification.

The tadpoles intestine tissues were observed for the presence of infiltration on the connective tissue and dilation of blood vessels under the intestine epithelium. The intestinal tube wall is formed by three distinct layers: the mucosa, the submucosa, and the muscularis. The mucosa is made of epithelial cells and goblet cells that intercalate and form a prismatic or pseudostratified cell layer. The submucosa contains blood vessels and connects the epithelium and the muscularis. The muscularis is made of circular and longitudinal muscle cells.

Metabarcoding and Bioinformatics

The intestine samples were pre-homogenized in a Mixer mill MM400 RETSCH® for 30 s at 30 Hz using a 5 mm stainless steel bead and then during 30 s at 30 Hz in a ZR BashingBead Lysis Tube (0.1 and 0.5 mm). The DNA and RNA purification was performed in parallel in two different columns and the RNA column was treated with a DNase I treatment. The samples were later eluted with 50 μL of RNAse/DNAse free water. The concentration and the purity of the samples were assessed with a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific). The amplification of the 16S V3-V4 hypervariable regions was performed using a two-step double-indexing PCR approach specifically designed for Illumina instruments. In the first step, the specific sequence of the gene is fused to the Illumina TruSeq sequencing primers. The PCR was carried out in a total volume of 25 μL that contained 1×Q5 buffer (NEB), 0.25 μM of each primer, 200 μM of each dNTP, 1 U of high fidelity Q5 DNA polymerase (NEB) and 1 μL of the template. The thermal cycling parameters were an initial denaturation at 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 55 ° C for 10 s, extension at 72 °C for 30 s and a final extension at 72 °C for 2 min. The PCR reaction was purified using the Axygen® PCR cleaning kit (Axygen). The quality of the purified PCR product was assessed on a 1% agarose gel. A 50-100 fold dilution of this purified product was used as a template for a second PCR to add barcodes (double index) and the missing sequence required for Illumina sequencing. The cycles of the second PCR were identical to those of the first PCR, but with 12 cycles. The PCR reactions were purified as previously described, their quality was assessed on a DNA7500 bioanalyzer chip (Agilent), then quantified by spectrophotometry with a Nanodrop 1000 (Thermo Fisher Scientific).

To remove low-quality base pairs, the forward and reverse sequences were cut at 260 and 190 bp, respectively. In the end, 1,757,499 sequences were retained for the combined DNA and RNA fractions. A total of 1,525 amplicon sequence variants (ASV) were observed after the different quality filters.

Table S1. Environmental conditions used to set the room conditions for the exposure of the frog *Lithobates sylvaticus* and the toad *Anaxyrus americanus* tadpoles to Bti formulations.

Week	Date range	Temperature (°C)	Humidity (%)	Photoperiod		
				Sunrise	Sunset	Light: dark cycle
1	May 6 - 12	15	54	05:40	19:40	14:10
2	May 13 -19	16	54	05:26	20:22	14:10
3	May 20 - 26	17	54	05:15	20:20	15:09
4	May 27 - June 2	18	54	05:10	20:25	15:09
5	June 3 - 9	19	54	04:50	20:30	15:09
6	June 10 - 16	21	54	04:48	20:38	16:08
7	June 17 - 21	21	54	04:48	20:38	16:08

Table S2. Sets of degenerate primers for the frog *Lithobates sylvaticus* and the toad *Anaxyrus americanus*.

Gene name	Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
Cytochrome P450 Family 1 Subfamily A	*cyp1a*	GAGCACTACAAAACATTTGAC	ACTTGCCACTGGTTGATC	408
Superoxide Dismutase	*sod*	TATGGAGATAACACYAAYGGCTG	CCTTCTCATGGACCACCGC	169
Glutathione Peroxidase	*gpx*	CCMGTAAACTACACTCAGCTYG	CTTTAGGYTGSKCCTTCATC	229
Glutathione-Disulfide Reductase	*gsr*	GTTGAYWGCTTACTGTGGGC	GAATCCTCCTGACCTTCAAAC	236
Thyroid Hormone Receptor Alpha	*trα*	GGTGAGATGGCAGTGAAGC	GTTCAAAGGCGAGGAGGTAAG	220
Thyroid Hormone Receptor Beta	*trβ*	GGCAACAGATTTGGTTTTGGAC	CCAGTGACTTCCCTGTGC	178
Ribosomal Protein L8	*rpl8*	CAGAGTYAAGCTGCCRTCTGG	TGGAGCATCTCTCCTGATAGT	253
Ornithine Decarboxylase	*odc*	GGMTCWTTCAACTGCATCTTGTTT	TCSCTGAAATCCATTGAATG	236

Table S3. Primer sets validated and used for gene expression analysis of the frog *Lithobates sylvaticus* and the toad *Anaxyrus americanus*.

Species	Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Temperature (°C)
Lithobates sylvaticus	*cyp1a*	TCAGAGGGTTCCTCCTGGTA	ATGGGGATTCTTGCTTAGGG	91	59
	sod	TCGAGCAGGAAGAAGATGGA	TGCCTTGGGGGTTGAAATG	144	60
	gpx	GGTTTACACATCCTCGGGTTTC	TGCACCATCTCCATTGACC	141	63
	gsr	ACCTGGACTACCTGGGTCT	TCTGTGAGCCAATCTTCTTCC	161	61
	trα	AGATGGCAGTGAAGCGAGAAC	GGTCTGAGGACATGAGCAGGA	150	59
	trβ	AAGGAACCAGTGCCAAGAATGT	AACGCTTGCTGTCATCCAAA	86	61
	rpl8	GTGTAGAAGAGAAGCCAGGTGAT	GGATTGTGGGAGATGACGGTAG	79	59
	odc	TGCCCATGTCAAACCCATAC	TCAAACAGCATCCAGTCCCC	153	63
Anaxyrus americanus	*cyp1a*	GCATTGACTCCTGCTCATTC	CAAGTACCTCTGTGGCAGATA	104	61
	sod	CAGATGGCGTTGCAGATTTGAG	GCGACCAATGATGCCGTGT	73	61
	gpx	GTTTACGCATCCTCGGGTTTC	CAACTTTGTAGGACGCAGCAA	96	63
	gsr	GGAGAACTGGGTCTTGAGTTAGA	CTGCCACACACATCACCTAC	104	61
	trα	CCTAACACTGAGTGGTGAGATGG	GTCCCAAGTCAAATATGGCATCAG	92	61
	trβ	CGAGCTGCCATGTGAAGA	GTTTCACTCTCTGGGTCATATCTC	99	63
	rpl8	CCGTGGTGTGGCTATGAA	GGAGCATCTCTCCTGATAGTTG	94	61
	odc	GCCATTATACAGTAGCAGCTTATGGG	CAGGAGCCAGTCACCAACTT	102	65

Table S4. Effect of the chronic exposure of VectoBac® 200G and 1200L on the mortality, total length, total weight, hepatosomatic index (HSI) and gonadosomatic index (GSI) of *L. sylvaticus* and *A. americanus* at the end of the chronic exposure. Significant differences to the control are marked in black and an asterisk (*)

Formulation	Species	Concentration	Mortality (%)	Total length Mean ±SD (mm)		Total weight Mean ±SD (mg)		HSI Mean ±SD		GSI Mean ±SD	
VectoBac® 200G	*Lithobates sylvaticus*	0 mg/L	3	37.2	±3.9	407.5	±87.7	2.76	±0.60	0.74	±0.31
		2.5 mg/L	3	38.0	±4.8	390.9	±55.1	2.27	±0.49	0.82	±0.17
		5 mg/L	3	38.7	±2.5	410.4	±58.5	2.38	±0.44	0.69	±0.23
		10 mg/L	5	**42.1**	**±2.2***	417.4	±67.8	2.33	±0.33	0.91	±0.30
		20 mg/L	3	41.3	±3.8	404.8	±60.6	2.35	±0.67	0.79	±0.16
		100 mg/L	8	40.0	±5.4	447.1	±78.1	2.47	±0.49	0.84	±0.18
	Anaxyrus americanus	0 mg/L	6	24.0	±2.6	165.6	±36.4	3.47	±1.17	0.92	±0.31
		2.5 mg/L	3	24.4	±2.0	147.1	±27.7	4.24	±0.74	1.12	±0.74
		5 mg/L	13	25.4	±2.4	**138.7**	**±23.3***	3.81	±1.20	2.26	±1.72
		10 mg/L	11	24.8	±2.4	145.6	±22.5	3.62	±1.14	0.92	±0.30
		20 mg/L	6	21.6	±3.8	**137.6**	**±33.3***	4.11	±0.93	1.71	±0.89
		100 mg/L	**20***	24.2	±3.1	144.0	±27.3	3.97	±1.01	1.22	±0.76
VectoBac® 1200L	*Lithobates sylvaticus*	0 µL/L	3	37.2	±3.9	407.5	±87.7	2.76	±0.60	0.74	±0.31
		0.25 µL/L	3	37.9	±4.1	390.8	±53.8	**2.17**	**±0.31***	0.69	±0.23

		0.5 μL/L	13	38.9	±3.9	392.5	±78.3	**2.09**	**±0.47***	0.66	±0.43
		1 μL/L	13	37.8	±4.0	384.1	±65.3	**2.18**	**±0.45***	0.60	±0.30
		2.5 μL/L	0	39.8	±4.4	399.9	±74.5	2.64	±0.51	0.83	±0.22
		10 μL/L	3	39.3	±3.0	440.1	±74.8	2.50	±0.65	0.55	±0.20
	Anaxyrus americanus	0 μL/L	10	24.0	±2.6	165.6	±36.4	3.47	±1.17	0.92	±0.31
		0.25 μL/L	19	23.5	±3.5	166.9	±26.6	3.86	±0.91	1.13	±0.60
		0.5 μL/L	5	24.5	±2.2	**131.6**	**±33.0***	4.00	±1.11	1.21	±0.47
		1 μL/L	**29 ***	25.0	±1.8	**134.7**	**±22.5***	2.39	±0.81	1.47	±0.62
		2.5 μL/L	**35 ***	23.2	±2.0	146.2	±32.0	3.71	±1.22	1.19	±0.52
		10 μL/L	3	23.4	±1.4	**133.8**	**±22.8***	3.49	±0.72	1.26	±0.49

SD: standard deviation.

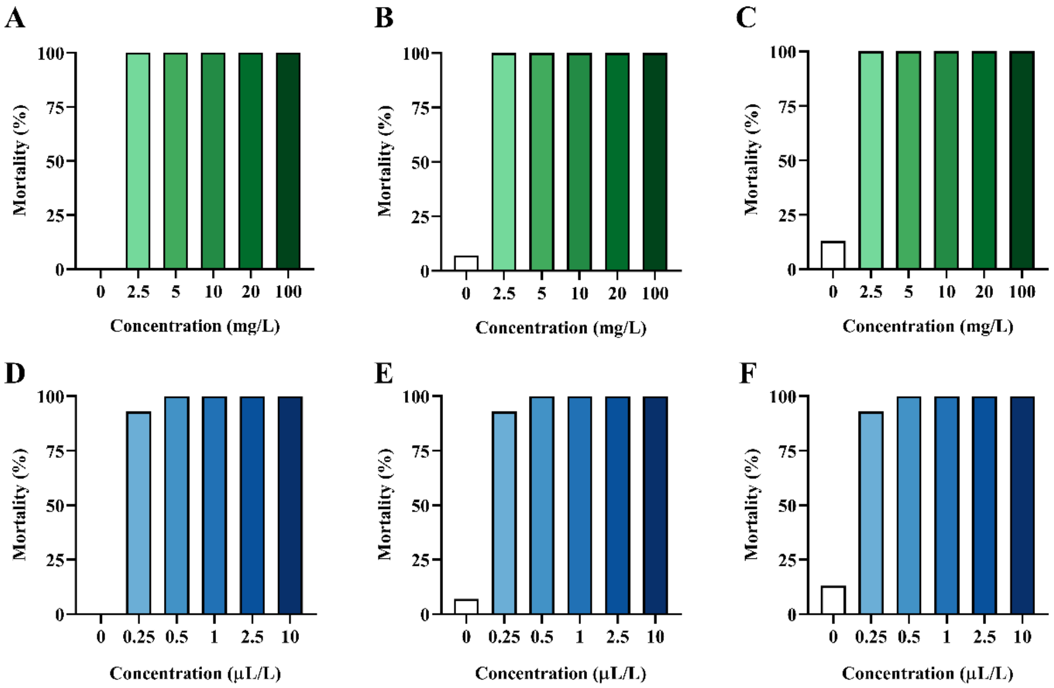

Figure S1. Mortality of *Ochlerotatus sp.* mosquito larvae induced by the exposure to VectoBac® 200G after 24 h (A), 48 h (B) and 72 h (C). Mortality of *Ochlerotatus sp.* mosquito larvae induced by exposure to VectoBac® 1200L after 24 h (D), 48 h (E) and 72 h (F).

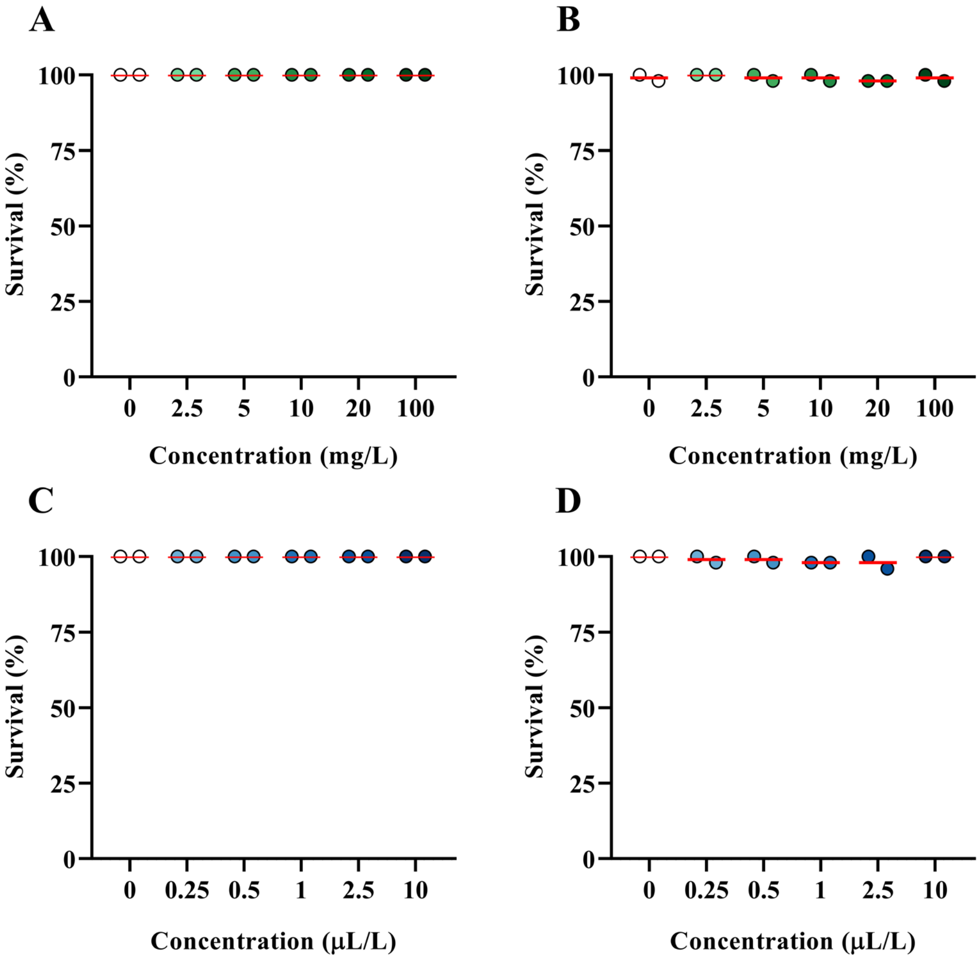

Figure S2. Effect of VectoBac® 200G in the survival of *L. sylvaticus* (A) and *A. americanus* (B) after 48 h exposure. Effect of VectoBac® 1200L in the survival of *L. sylvaticus* (C) and *A. americanus* (D) after 48 h exposure. Red line indicates the median.

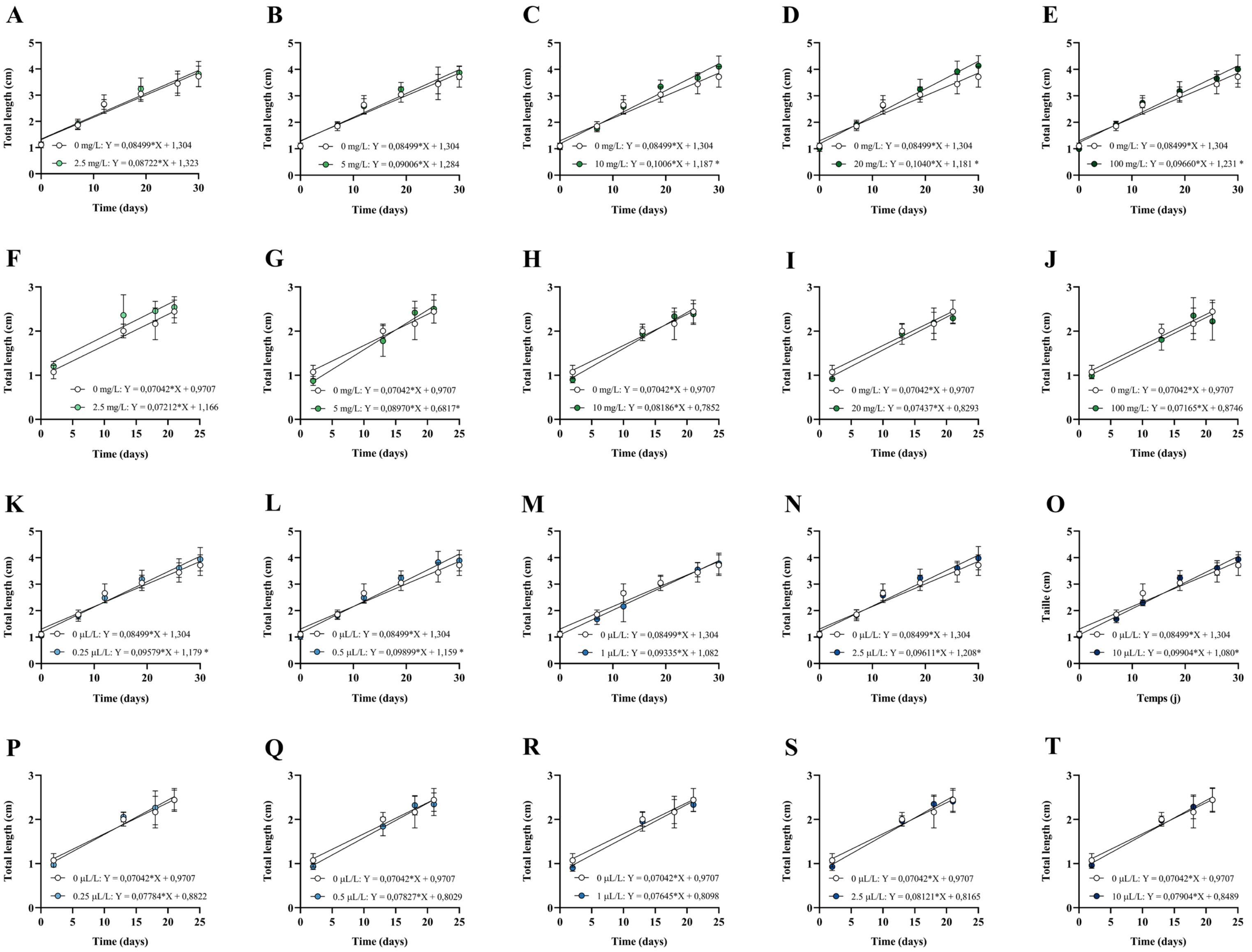
A
Total length (cm)
0 1 2 3 4 5
0 mg/L: Y = 0,08499*X + 1,304
2.5 mg/L: Y = 0,08722*X + 1,323
0 10 20 30
Time (days)
B
Total length (cm)
0 1 2 3 4 5
0 mg/L: Y = 0,08499*X + 1,304
5 mg/L: Y = 0,09006*X + 1,284
0 10 20 30
Time (days)
C
Total length (cm)
0 1 2 3 4 5
0 mg/L: Y = 0,08499*X + 1,304
10 mg/L: Y = 0,1006*X + 1,187 *
0 10 20 30
Time (days)
D
Total length (cm)
0 1 2 3 4 5
0 mg/L: Y = 0,08499*X + 1,304
20 mg/L: Y = 0,1040*X + 1,181 *
0 10 20 30
Time (days)
E
Total length (cm)
0 1 2 3 4 5
0 mg/L: Y = 0,08499*X + 1,304
100 mg/L: Y = 0,09660*X + 1,231 *
0 10 20 30
Time (days)
F
Total length (cm)
0 1 2 3
0 mg/L: Y = 0,07042*X + 0,9707
2.5 mg/L: Y = 0,07212*X + 1,166
0 5 10 15 20 25
Time (days)
G
Total length (cm)
0 1 2 3
0 mg/L: Y = 0,07042*X + 0,9707
5 mg/L: Y = 0,08970*X + 0,6817*
0 5 10 15 20 25
Time (days)
H
Total length (cm)
0 1 2 3
0 mg/L: Y = 0,07042*X + 0,9707
10 mg/L: Y = 0,08186*X + 0,7852
0 5 10 15 20 25
Time (days)
I
Total length (cm)
0 1 2 3
0 mg/L: Y = 0,07042*X + 0,9707
20 mg/L: Y = 0,07437*X + 0,8293
0 5 10 15 20 25
Time (days)
J
Total length (cm)
0 1 2 3
0 mg/L: Y = 0,07042*X + 0,9707
100 mg/L: Y = 0,07165*X + 0,8746
0 5 10 15 20 25
Time (days)
K
Total length (cm)
0 1 2 3 4 5
0 µL/L: Y = 0,08499*X + 1,304
0.25 µL/L: Y = 0,09579*X + 1,179 *
0 10 20 30
Time (days)
L
Total length (cm)
0 1 2 3 4 5
0 µL/L: Y = 0,08499*X + 1,304
0.5 µL/L: Y = 0,09899*X + 1,159 *
0 10 20 30
Time (days)
M
Total length (cm)
0 1 2 3 4 5
0 µL/L: Y = 0,08499*X + 1,304
1 µL/L: Y = 0,09335*X + 1,082
0 10 20 30
Time (days)
N
Total length (cm)
0 1 2 3 4 5
0 µL/L: Y = 0,08499*X + 1,304
2.5 µL/L: Y = 0,09611*X + 1,208*
0 10 20 30
Time (days)
O
Taille (cm)
0 1 2 3 4 5
0 µL/L: Y = 0,08499*X + 1,304
10 µL/L: Y = 0,09904*X + 1,080*
0 10 20 30
Temps (j)
P
Total length (cm)
0 1 2 3
0 µL/L: Y = 0,07042*X + 0,9707
0.25 µL/L: Y = 0,07784*X + 0,8822
0 5 10 15 20 25
Time (days)
Q
Total length (cm)
0 1 2 3
0 µL/L: Y = 0,07042*X + 0,9707
0.5 µL/L: Y = 0,07827*X + 0,8029
0 5 10 15 20 25
Time (days)
R
Total length (cm)
0 1 2 3
0 µL/L: Y = 0,07042*X + 0,9707
1 µL/L: Y = 0,07645*X + 0,8098
0 5 10 15 20 25
Time (days)
S
Total length (cm)
0 1 2 3
0 µL/L: Y = 0,07042*X + 0,9707
2.5 µL/L: Y = 0,08121*X + 0,8165
0 5 10 15 20 25
Time (days)
T
Total length (cm)
0 1 2 3
0 µL/L: Y = 0,07042*X + 0,9707
10 µL/L: Y = 0,07904*X + 0,8489
0 5 10 15 20 25
Time (days)

Figure S3. Effect of the chronic exposure of VectoBac® 200G at 2.5, 5, 10, 20 and 100 mg/L on the mean size of tadpoles of *L. sylvaticus* (A–E) and *A. americanus* (F–J). Effect of the chronic exposure of VectoBac® 1200L at 0.25, 0.5, 1, 2.5 and 10 μL/L on the mean size of tadpoles of *L. sylvaticus* (K–O) and *A. americanus* (P–T). An asterisk in the equation (*) indicates that the slopes of the linear regressions are significantly different ($p < 0.05$).

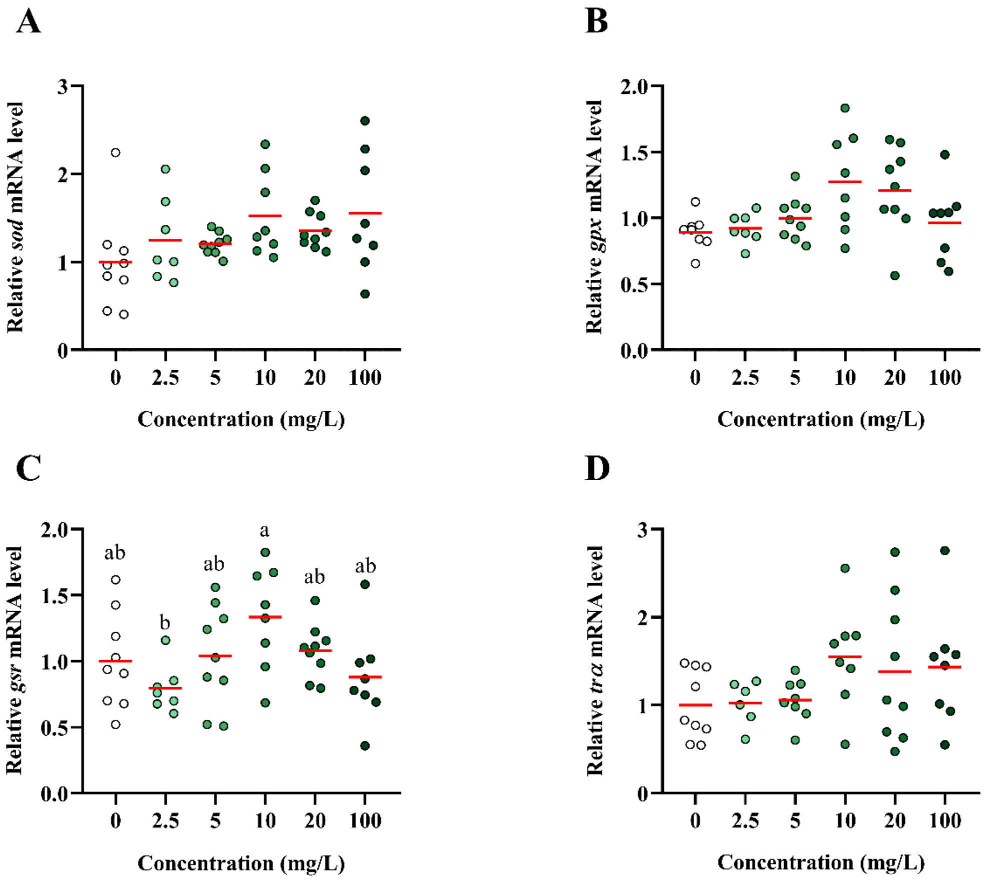

Figure S4. RT-qPCR analysis for *sod*, *gpx*, *gsr* and *trα* in tail of *L. sylvaticus* exposed chronically to VectoBac® 200G (A - D). The red lines indicate the mean and different letters indicate groups that are significantly different ($p < 0.05$).

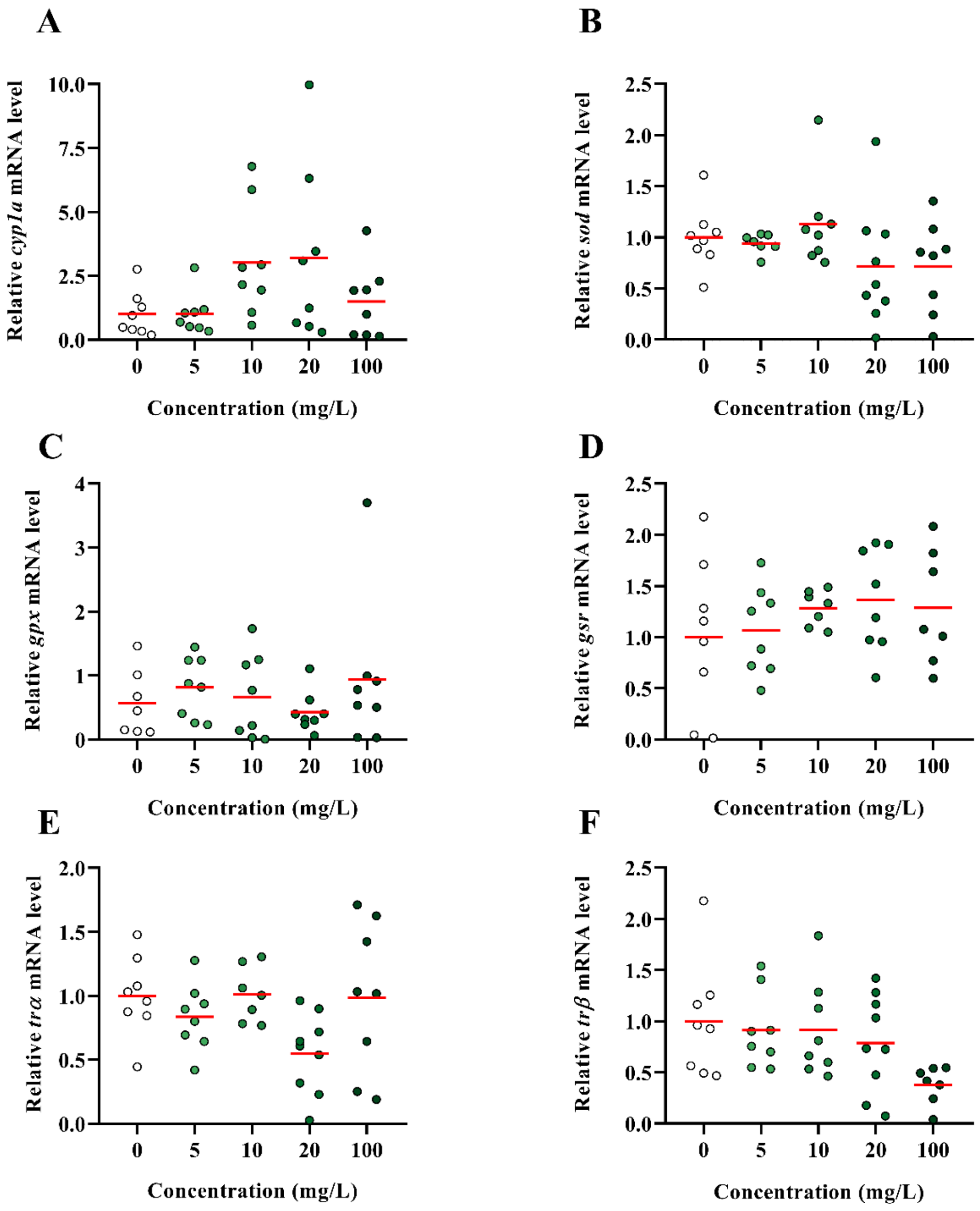

Figure S5. RT-qPCR analysis for *cyp1a*, *sod*, *gpx*, *gsr*, *trα* and *trβ* in tail of *A. americanus* exposed chronically to VectoBac® 200G (A - F). The red lines indicate the mean and different letters indicate groups that are significantly different ($p < 0.05$).

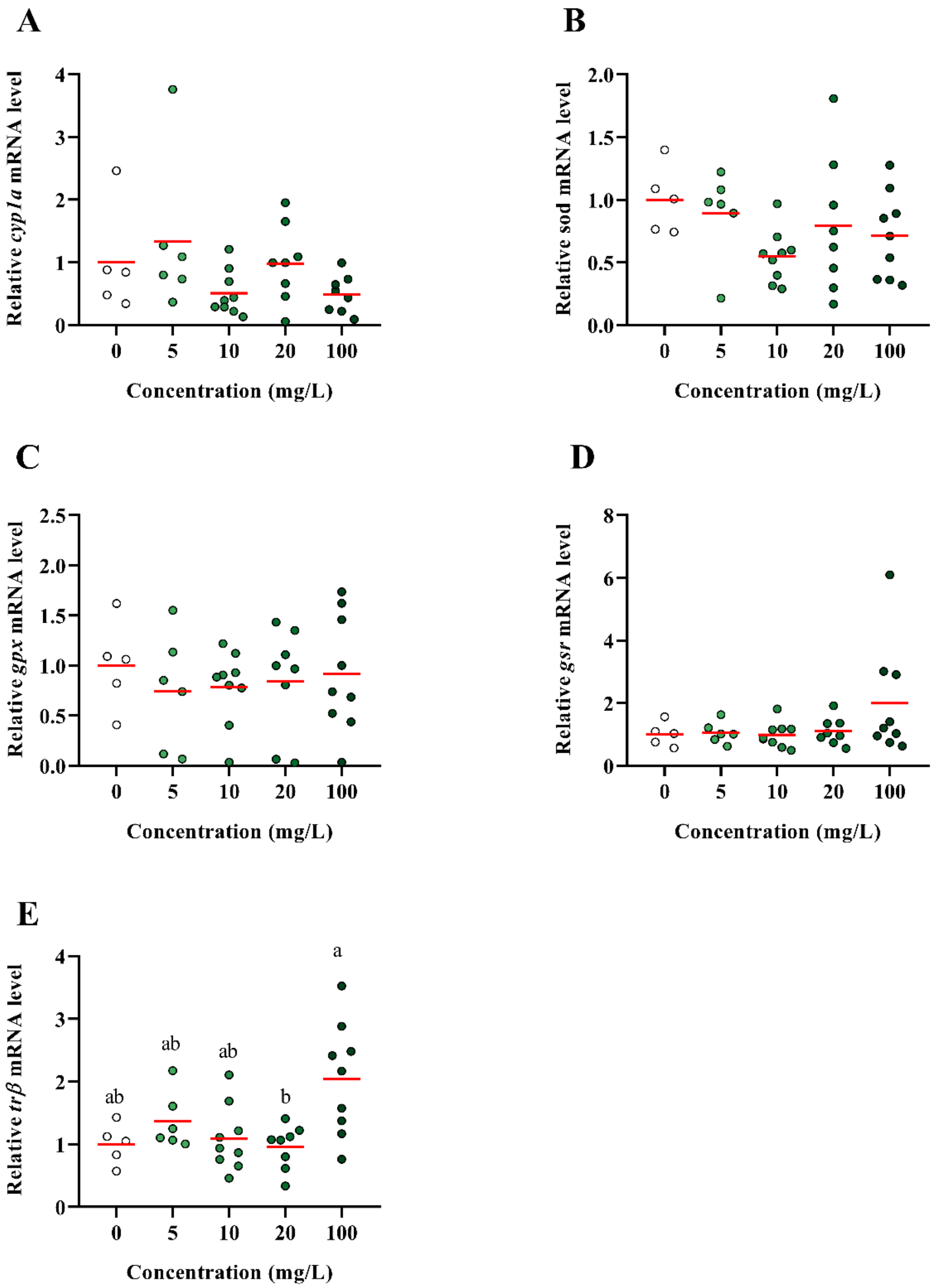

Figure S6. RT-qPCR analysis for *cyp1a*, *sod*, *gpx*, *gsr* and *trβ* in liver of *A. americanus* exposed chronically to VectoBac® 200G (A - E). The red lines indicate the mean and different letters indicate groups that are significantly different ($p < 0.05$).

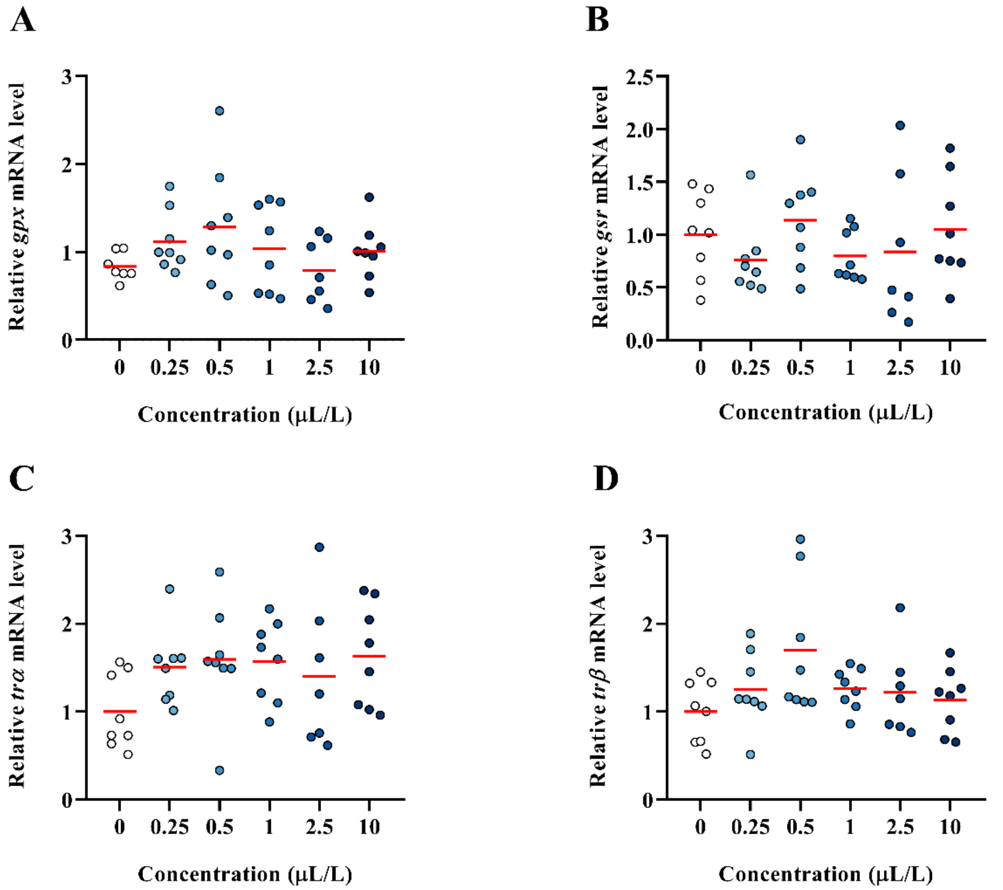

Figure S7. RT-qPCR analysis for *gpx*, *gsr, trα* and *trβ* in tail of *L. sylvaticus* exposed chronically to VectoBac® 1200L (A - D). The red lines indicate the mean and different letters indicate groups that are significantly different ($p < 0.05$).

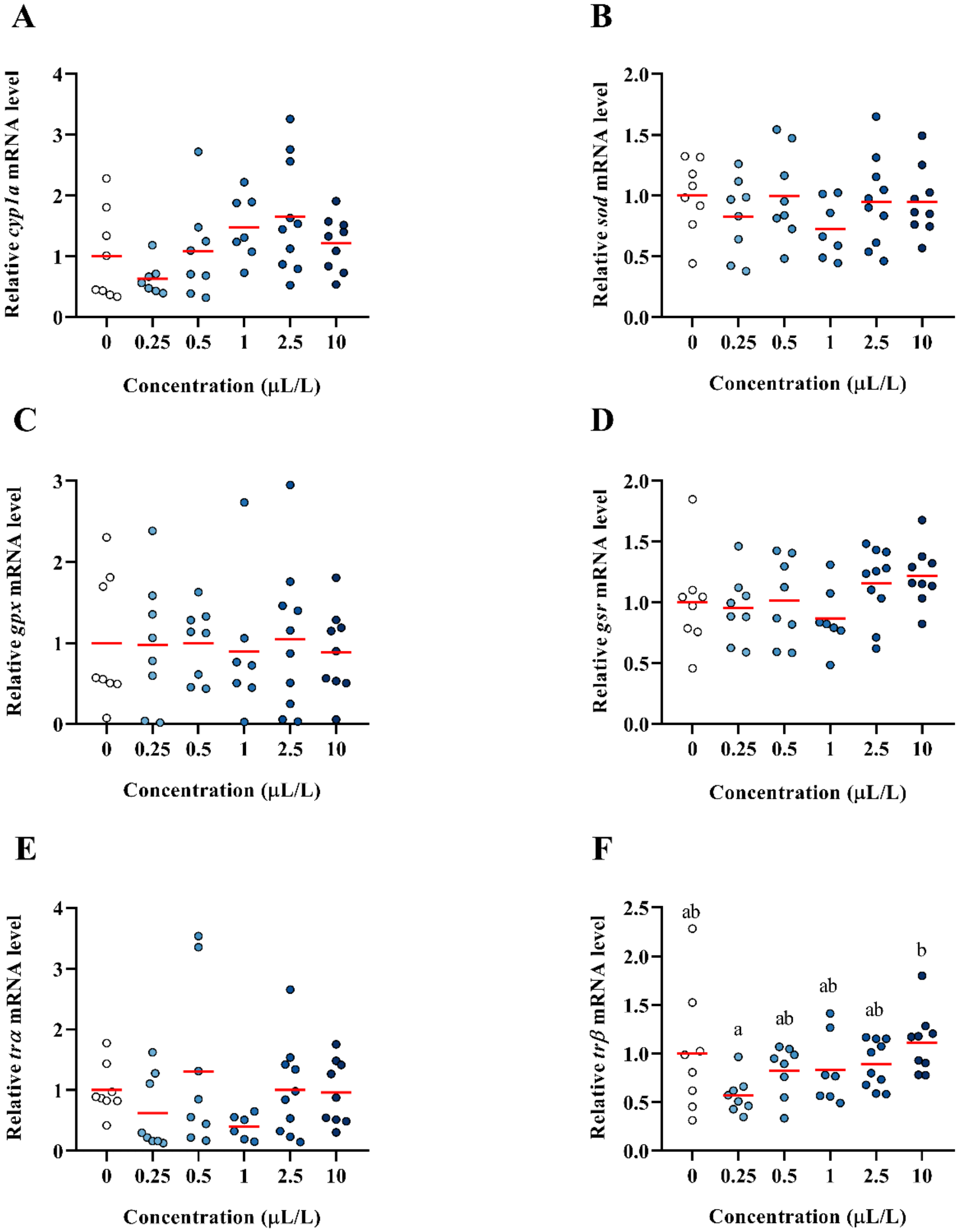

Figure S8. RT-qPCR analysis for *cyp1a*, *sod*, *gpx*, *gsr*, *trα* and *trβ* in tail of *A. americanus* exposed chronically to VectoBac® 1200L (A - F). The red lines indicate the mean and different letters indicate groups that are significantly different ($p < 0.05$).

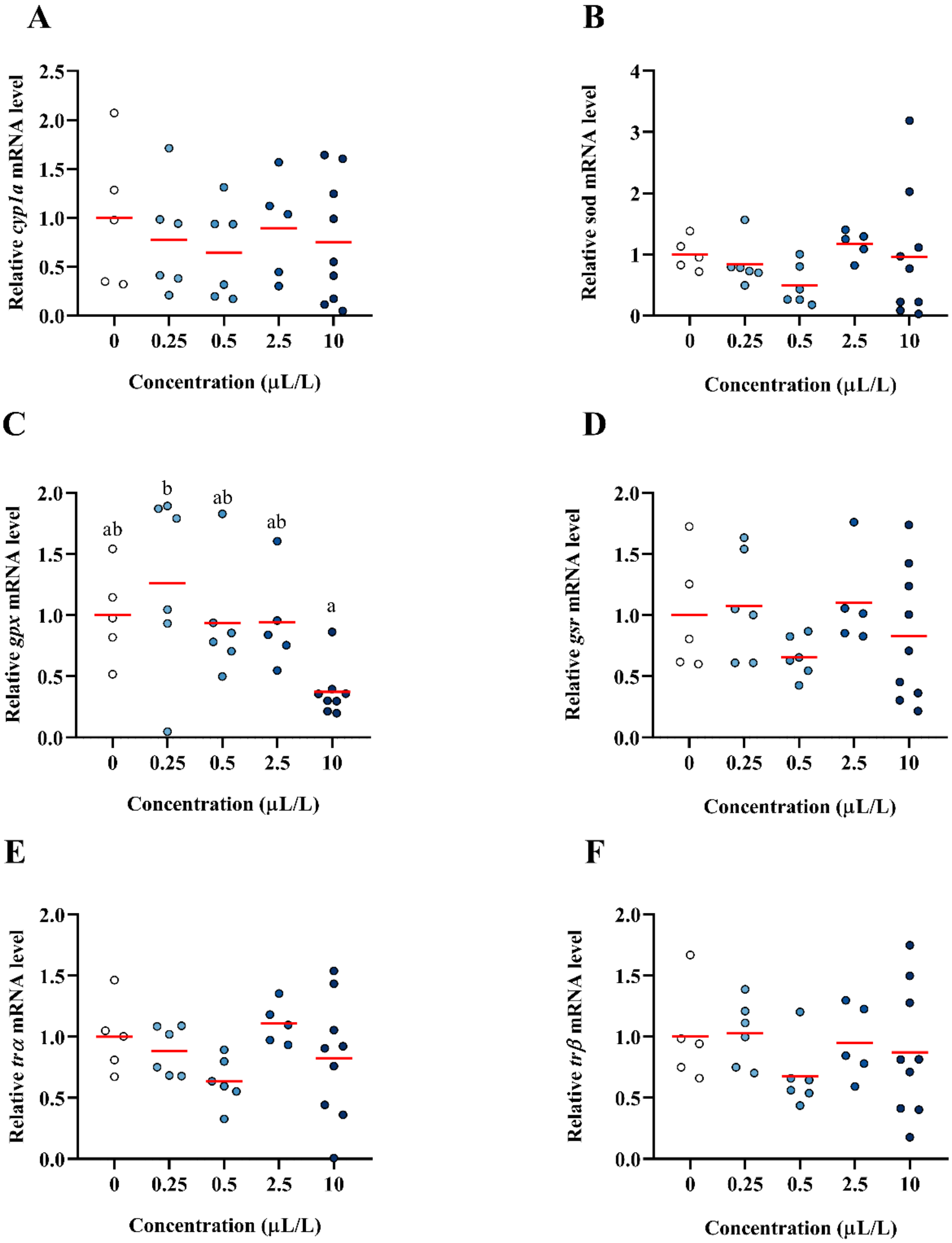

Figure S9. RT-qPCR analysis for *cyp1a*, *sod*, *gpx*, *gsr*, *trα* and *trβ* in liver of *A. americanus* exposed chronically to VectoBac® 1200L (A - F). The red lines indicate the mean and different letters indicate groups that are significantly different ($p < 0.05$).

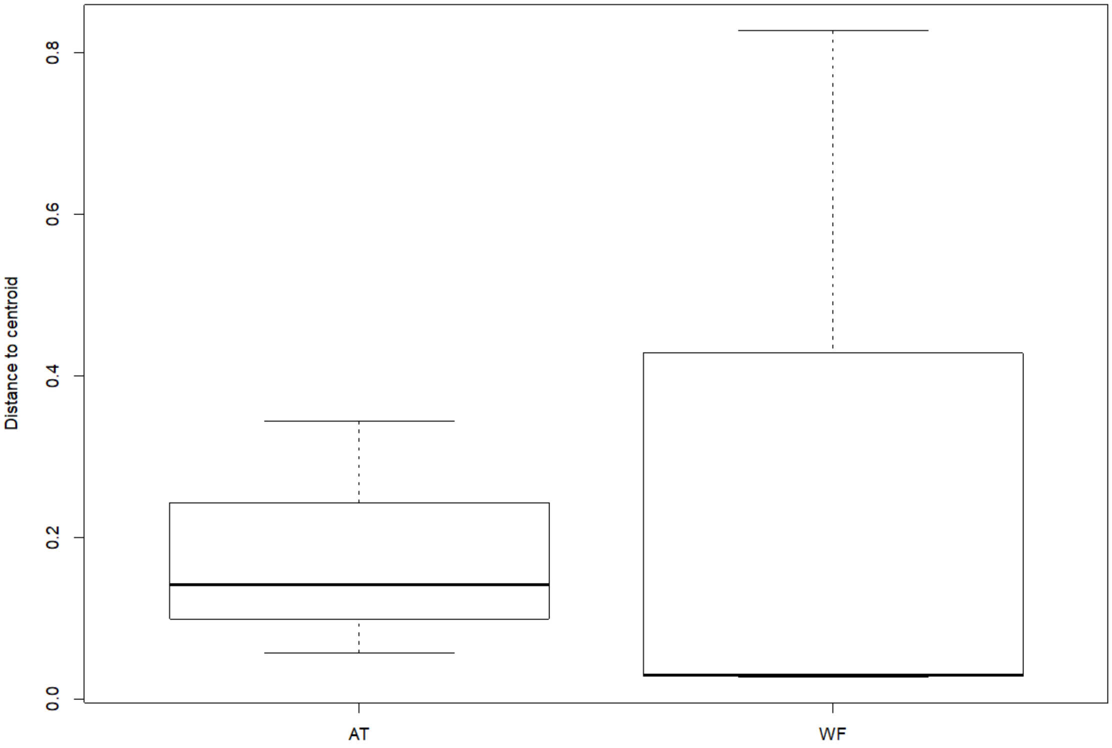

Figure S10. Box plot of the comparison of the microbiome diversity in the intestine of non-exposed *L. sylvaticus* (Wood frog; WF) and *A. americanus* (American toad; AT). Black line refers to median estimate.

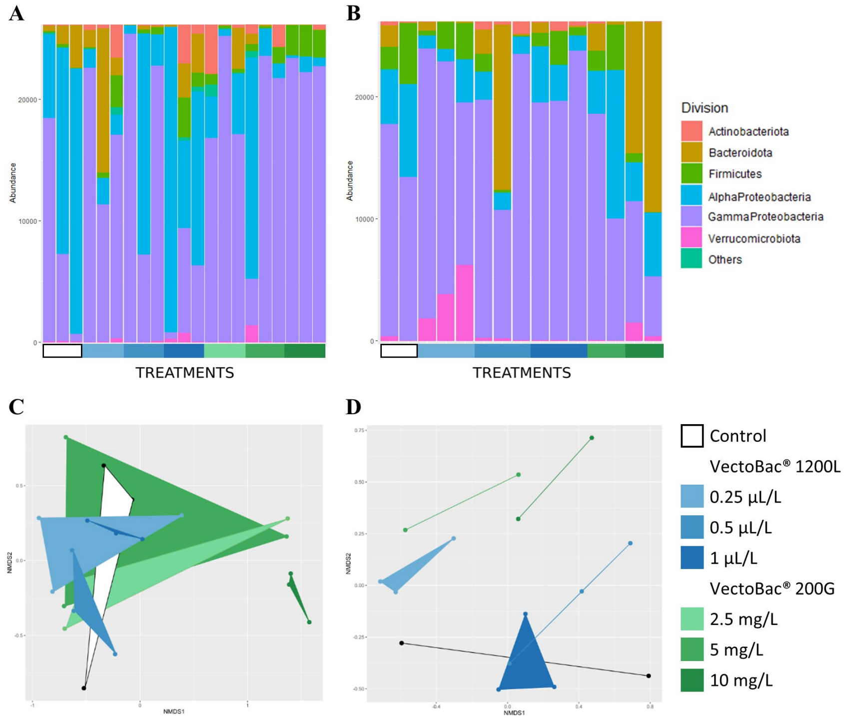

Figure S11. Taxonomic stacked bar charts showing the relative abundance of bacteria in the intestine of *L. sylvaticus* (A) and *A. americanus* (B) exposed chronically to VectoBac® 200G and 1200L. Non-metric multidimensional scaling visualization of the changes in bacterial community composition in the intestine of *L. sylvaticus* (C) and *A. americanus* (D) exposed chronically to VectoBac® 200G and 1200L. The results presented are based on the analysis of the cDNA.

Equations

Equation S1. The formula for the calculation of the hepatosomatic index (HIS).

$$HSI = \left(\frac{Liver\ weight}{Total\ weight}\right) * 100$$

Equation S2. The formula for the calculation of the gonadosomatic index (GSI).

$$GSI = \left(\frac{Gonad\ weight}{Total\ weight}\right) * 100$$

References

(1) Ringer’s Solution (PH 7.3-7.4). *Cold Spring Harb. Protoc.* **2008**, *2008* (1), pdb.rec11273.