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Spectrochemical Analysis of Tissues of Frog *Dryophytes plicatus* Tadpoles (Amphibia: Hylidae) Developing under Lead and Iron Pollution

ABSTRACT

This study conducted a bioassay on frog tadpoles of the Mexican endemic species Dryophytes plicatus exposed to lead and iron. This species was used because some of its populations live near urban areas that may exposed them to pollutants, such as heavy metals due to industrial processes or mining industry. Specimens in a post-embryonic stage of Dryophytes plicatus were collected in water bodies near El Chico National Park. For the bioassay, the collected samples were grouped into three namely; tadpoles in contact with steel cloves (99% Fe, 1% C); the second group in contact with lead plates; and the last was the control group (without heavy metals). A spectrochemical analysis was held to identify the concentrations of these elements in the liver, intestines, and gills. This study shows that Dryophytes plicatus can bioaccumulate these heavy metals in their tissues, particularly in the liver and the intestine. The concentration of lead and iron was similar in both the control and experimental groups, due to the use of tap water of a mining place, but the concentration in the tadpoles tissues indicates a bioaccumulation process.

David Ramiro Aguillón-Gutiérrez[1]
Aurelio Ramírez-Bautista[2]
Claudia Romo-Gómez[3*]

[1] Facultad de Ciencias Biológicas. Universidad Juárez del Estado de Durango. Av. Universidad s/n. Fracc. Filadelfia. C.P. 35010. Gómez Palacio, Durango, México
[2] Laboratorio de Ecología de Poblaciones, Centro de Investigaciones Biológicas, Instituto de Ciencias Básicas e Ingeniería, Universidad Autónoma del Estado de Hidalgo. Ciudad del Conocimiento, Carretera Pachuca-Tulancingo km. 4.5, Colonia Carboneras. C.P. 42184, Mineral de la Reforma, Hidalgo, México
[3] Centro de Investigaciones Qímicas, Instituto de Ciencias Básicas e Ingeniería, Universidad Autónoma del Estado de Hidalgo

*Corresponding author:
davidrag@gmail.com

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INTRODUCTION

A significant decline in amphibian populations has been reported around the world in the last decade. This phenomenon is attributed to a confluence of factors, such as habitat loss, introduced species, diseases, global climate change, and pollution (*Young et al. 2001*).

Amphibians are good experimental models in ecotoxicology because of their life cycles and the permeability of their skin (*Demichelis et al. 2001; Newman and Unger 2003; Selvi, Gul and Yilmaz 2003; Mitchell et al. 2005; Marques et al. 2008, 2009; Burlibasa and Gavrila 2011*). One example of this is the African Clawed Frog *Xenopus laevis*, which is used for the "Frog Embryo Teratogenesis Assay Xenopus" (FETAX) and the "Xenopus Metamorphosis Assay" (XEMA) (*Opitz et al. 2005; Mouche, Malesic and Guillardeaux 2011*). Amphibians also give useful information about environmental conditions; therefore, they are considered as bioindicators, especially during its development.

As in other organisms, amphibians are more sensitive to the environmental pollution at early developmental stages; for example, anomalies in spine, gut, skin, pigmentation, and limbs have been identified in *Rana temporaria, Rana arvalis, Bufo bufo,* and *Dryophytes plicatus* (*Severtsova et al. 2012a; Aguillón-Gutiérrez and Ramírez-Bautista 2015*). While some amphibian species may adapt to a new environmental scenario of anthropogenic pollution, others might become extinct. Consequently, a good approach to this problem is the study of early stages of development of this amphibian (*Severtsova et al. 2012b*). While some amphibian

species may adapt to a new environmental scenario of anthropogenic pollution, others might become extinct. Consequently, a good approach to this problem is the study of early stages of development of this amphibian (*Severtsova et al. 2012b*).

About 119,000 Mg of Pb and about 1,925,000 Mg of Fe (*Moiseyenko 2009*) are dumped into the environment each year. These heavy metals can enter into trophic chains and are accumulated in tissues and organs of different vertebrate organisms. High concentrations of heavy metals can be lethal to amphibians (*Blaustein et al. 1997; Blaustein and Kiesecker 2002; Blaustein and Johnson 2003*).

In particular, lead can be accumulated in the tissues of amphibians (*Berzins and Bundy 2002*) affecting nervous, circulatory system and behavior (*Perez-Coll and Herkovits 1990;Herkovits and Perez-Coll 1991; Rowe et al. 1996; Herkovits et al. 1997; Nixdorf et al. 1997; Lefcort et al. 1998; Sparling et al. 2006.* Lead can affect the liver and the blood vessels at a histopathological level in the toad *Bufo maculatus* due to the toxic effects of lead on the hepatocytes (*Ezemonye and Enuneku 2012*). Its bioaccumulation is significant in the liver of *Xenopus laevis* larvae even from the lowest concentration of exposure (*Mouchet et al. 2007*).

On the other hand, iron concentrations can affect several species in numerous ways at different stages. For example, in *Bufo boreas* tadpoles, iron concentrations in water from 20 to 30 $mg \cdot L^{-1}$ causes a mortality of 100% (*Porter and Hakanson 1976*). In *Xenopus laevis*, iron can lead to a slow development (*Dawson et al.1985*).

Lead and iron can also delay the development process in three species of anurans (*Rana temporaria, Rana arvalis* and *Bufo bufo*) (*Severtsova and Aguillón-Gutiérrez 2013*), and it can be bioaccumulated in the liver and intestines of amphibian species (*Severtsova, et al. 2013*). The influence of Pb and Fe on the postembryonic development of the tree frog *Dryophytes plicatus* in experimental conditions was analyzed in this work.

MATERIALS AND METHODS

The tree frog *Dryophytes plicatus* Brocchi 1877 (family Hylidae) is endemic to Mexico; its distribution occurs in the central part of the country (Sierra Madre Oriental, Mexican Valley, Hidalgo, Tlaxcala, and Veracruz) (*Duellman 2001*) (**Figure 1A**). It is a small frog, mean snout-vent length is 38.8 ± 4.2 mm; its reproduction occurs in spring and summer (*Ramírez-Bautista et al. 2009*) (**Figure 1B**).

Sample sizes

In this study 300 specimens of the tree frog *Dryophytes plicatus* in the postembryonic stage of development from the municipality Mineral del Chico, State of Hidalgo, México (20°09'54.7"N 98°41'25.2"W) were collected in 2013 (**Figure 2**).

The habitat where the organisms were collected was in El Chico National Park, which is part of the Trans-Mexican Volcanic Belt. Its altitude is from 2350 to 3086 MASL. The average annual temperature ranges from 10 to 14°C, with maximum temperature at 36°C, and the minimum at -6°C. Average annual rainfall is 1567.9 mm. The rainy season goes from May to October. The dominant type of vegetation is oyamel-oak (*Abies, Quercus*) (**Figure 3**) (*Gallina et al., 1974, Villavicencio*

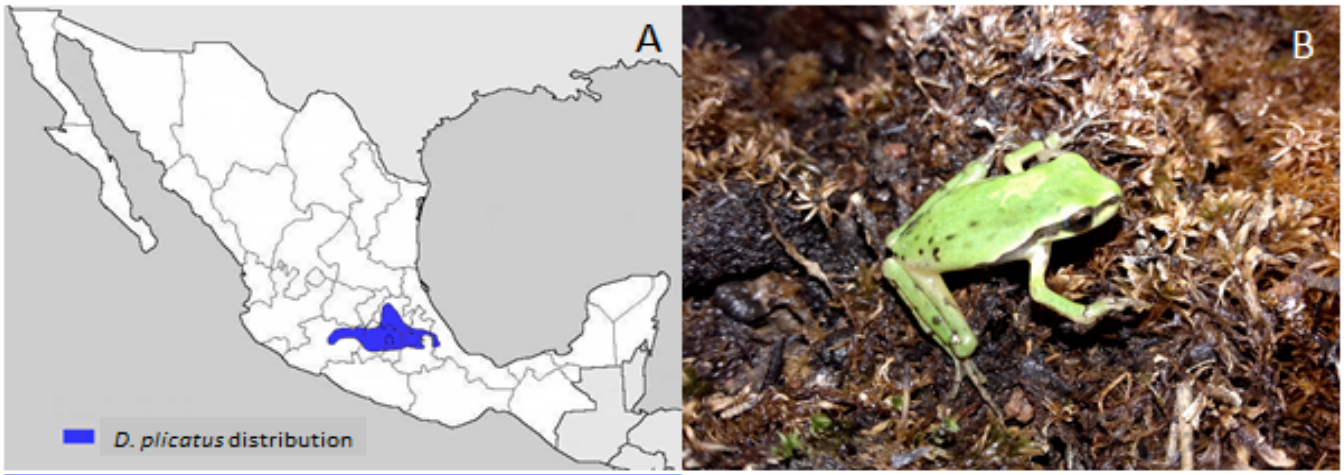

Figure 1. A) *Dryophytes plicatus* distribution area; B) *Dryophytes plicatus*.

Figure 2. Collection site. Municipality of Mineral el Chico, State of Hidalgo, Mexico (http://www.inafed.gob.mx).

et al. 1992).

The specimens were captured directly from the lake, and were transported to the Laboratory of Population Ecology in the Center of Biological Sciences Research at the Universidad Autónoma del Estado de Hidalgo. The tadpoles were captured at the 32-46 stages of development based on *Gosner* (*1960*). Specimens were collected under the scientific permit issued by SEMARNAT with the number SGPA/DGVS/02419/13.

Bioassay

Three groups were formed (Control, Experimental group "Fe", and Experimental group "Pb"), each one with 100 specimens. Before the experimental phase, all animals were subjected to a period of one week of acclimation. Two plastic aquariums with a capacity of 50 dm^3 per group were used, without aeration pump. In each aquarium, 50 tadpoles (100 per group) were placed. Tap water, sat for three days for dechlorination was used.

In each aquarium of the group "Fe", 20 non rusty steel cloves (99% Fe, 1% C), 10 cm long and a weight of 13 g each (260 g altogether) were added. In the aquariums of the group "Pb", 10 lead plates (98% purity) of 3.5 x 3.5 cm per each side, and a weight of 26 g each (260 g altogether) were added. The metallic objects were used in order to imitate the process of pollution in ponds, where usually car batteries, pipelines, lead bullets used by hunters, steel cloves, and other similar items are thrown.

In the aquariums of the "Control" group, nothing was added. Tadpoles were in exposed with these heavy metals for four weeks. The metal objects were placed in the bottom of the aquariums, where tadpoles frequently look for food. As a result, tadpoles would have contact with metals by oral, respiratory or cutaneous means since some metals could be dissolved in the water.

Figure 3. Habitat of *D. plicatus*. Municipality of Mineral el Chico.

Tadpoles were fed three times a week with algae taken from the lakes they inhabit. Once a week, half of the water was changed in each aquarium.

At the end of the experiment, the tadpoles were euthanized and fixed in formaldehyde (10%). After that, tadpoles were dissected in order to obtain the liver, the intestine (without the content) and the gills. These tissues were placed in a furnace at 60°C for three days. Once the tissues were dried, they were grounded and stored

in microcentrifuge tubes. The spectrochemical analysis was made by group and by tissue, making three lectures per group. In addition, water was analyzed. These tissues and water were digested following the Standard Method 3051A (*USEPA 2007a*), and Method 3015A (*USEPA 2007b*) using 69% high-purity HNO and 37% HCl. Metals (lead and iron) were quantified by atomic absorption spectrometry with a graphite furnace (Agilent Technologies model 200 Series AA, Malaysia). For quality control, the reference standard SRM 1640 for water certified by the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) was used, with recoveries of 95 %–98 % (*NMX 2013*).

Statistical analysis were made by PASW STATISTICS 18.0. Differences among groups were examined with t-student test (*Zar 2009*).

RESULTS

Spectrochemical analysis showed that in the "Control" group, the concentrations of lead were lower than in the group "Pb" (t=3.1, 8 d.f., p=0.006); in contrast, iron concentrations between "Control" and "Fe" groups were similar (t=1.03, 8 d.f., p>0.05) (**Table 1**). However, the average of iron concentration was considerably higher in the case of the liver and the intestines in the group "Fe" than in the "Control" group (about 10 times more), but very similar in the case of the gills.

The concentration of lead in the liver was higher in the group "Pb" than in the "Control" (t=6.01, 2 d.f., p=0.01). However, in the case of intestines and gills, there was no statistical difference (t=1.49, 2 d.f., p>0.05), although there are also remarkable differences in the lead concentrations between these groups, being higher in the group "Pb" (**Figure 4A**).

The concentration of iron was higher in the intestines of the group "Fe" than in the "Control" (t=7.1, 2 d.f., p=0.009), but there were no statistical differences between the iron concentrations in the liver and the gills in the experimental and the "Control" groups (t=0.94, 2 d.f., p>0.05) (**Figure 4B**).

In the group "Pb", the tissue with the biggest concentration of lead was the intestine, while in the group "Fe", it was the liver. In the "Control" group, the levels of lead were similar in the different tissues, but the levels of iron were higher in the liver.

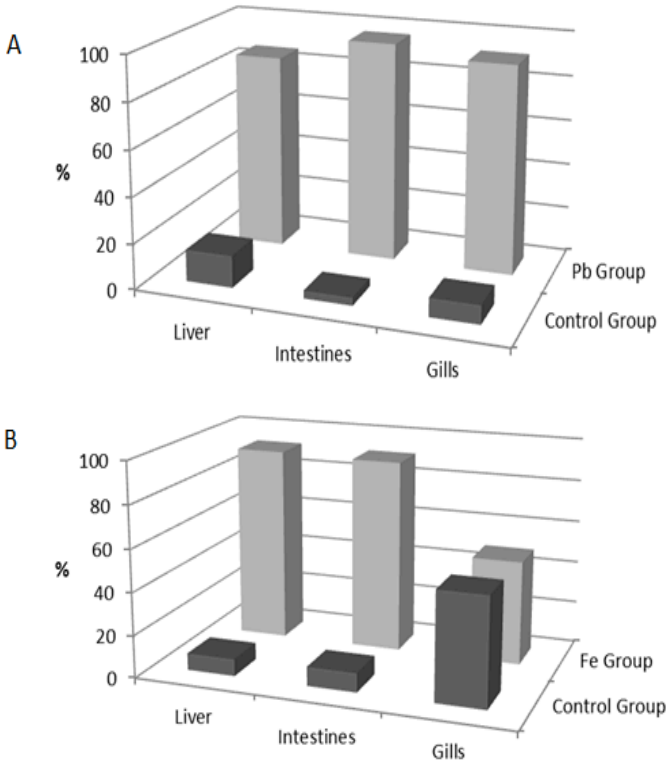

Figure 4. Comparison in percentage of lead (A) and iron (B) concentration between the Control and the experimental groups of the bioassay.

Table 1. Spectrochemical analysis of tissues of *Dryophytes plicatus* tadpoles. (L- liver, I- intestine, G- gills) and spectrochemical analysis of water of the experimental groups (Pb, Fe), and the control group in the bioassay after four weeks.

Lecture in tissues	Group Pb Pb (mg g^{-1})			Group Fe Fe (mg g^{-1})			Control					
							Pb (mg g^{-1})			Fe (mg g^{-1})		
	L	I	G	L	I	G	L	I	G	L	I	G
Lecture1	0.336	1.585	0.261	2.058	4.428	0.568	0.037	0.041	0.055	0.484	0.436	1.693
Lecture2	0.220	0.283	0.114	0.720	6.580	0.645	0.025	0.040	0.051	8.849	0.799	0.495
Lecture3	0.449	1.214	0.938	194.825	3.969	1.196	0.093	0.030	0.012	7.742	0.161	0.295
Average	0.335	1.027	0.437	65.867	4.992	0.803	0.051	0.037	0.039	5.691	0.465	0.827
Lecture in water	Pb (mg L^{-1})			Fe (mg L^{-1})			Pb (mg L^{-1})			Fe (mg L^{-1})		
Water	0.04			0.16			0.04			0.15		

The comparison among tissues (regardless of the groups) in their capacity to concentrate these elements showed a difference between the intestines and the gills, being a higher concentration in the intestines (t=1.8, 11 d.f., p=0.04), even though the higher concentration of iron was detected in the liver of the experimental group "Fe" (194.825 mg g^{-1}) (**Figure 5**). Concentrations of lead and iron were quite similar in the experimental groups as in the "Control" (**Table 1**).

DISCUSSION

Our results showed that in the "Control" group, the concentrations of lead were lower than in the group "Pb" ($p<0.05$). This pattern is similar to the results by *Ezemonye and Enuneku* (*2012*), who reported that in the toad *Bufo maculatus* hepatic bioaccumulation occurred after an exposure period to lead for 28 days, causing liver and blood vessels damage. *Sparling et al.* (*2006*) concluded that lead produce skeletal developmental abnormalities, *Nixdorf et al.* (*1997*) reported that lead exposure in bullfrog (*Lithobates catesbeianus*) tadpoles affects the monoamine neurotransmitters that are implicated in the learning process and *Berzins and Bundy* (*2002*) showed that lead inhibited the normal development of *Xenopus laevis*.

The concentration of lead in the liver was higher in the group "Pb" than in the "Control group ($p<0.05$), indicating that the tadpole got most of the lead orally, heading towards the intestine, and once there, the lead is hurled but some of it is sent to the liver. That result is opposite to the findings by *Sparling et al.* (*2006*) who found that the liver in *Rana sphenocephala* tadpoles did not appear to be a particular depot for lead; but it coincides with the results of *Mouchet et al.* (*2007*) who report that in the tadpoles of *Xenopus laevis*, the liver is a relevant lead bioaccumulator. The liver plays a very important role in the physiology of organisms because it is the detoxification center of the body, as a result, all kind of toxic molecules could come to the liver (*Repetto-Jiménez 2009*). However, inside the group "Pb" the tissue with a higher concentration of lead was the intestine, which was expected because a big part of the lead is discarded through the intestinal tract.

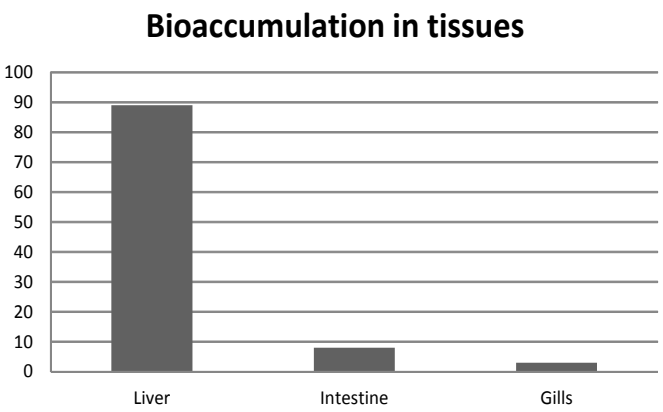

Figure 5. Comparison in percentage of the bioaccumulation potential among the liver, the intestine and the gills of the *Dryophytes plicatus* tadpoles, considering the control and experimental groups.

Average iron concentration was considerably higher in the case of the liver and the intestines in the group "Fe" than in the "Control" group (about 10 times more), but very similar in the case of the gills. This indicates that the principal means of obtaining iron is the mouth when feeding, coinciding with the results of *Severtsova et al.* (*2013*). Molecules of heavy metals tend to go to the bottom of the waterbodies, so tadpoles can acquire these molecules when they seek for food.

The concentration of iron was higher in the intestines of the group "Fe" than in the "Control" ($p<0.05$), here it occurs the same that with the lead. In ecotoxicology iron is not as studied as lead because unlike lead, it has a biological role, but in higher concentrations, it can produce slow development and alterations in the body size (*Dawson et al. 1985*). Steel objects are commonly one of the most thrown out items in the environment, so these elements can enter in living organisms.

The higher concentration of iron was detected in the liver of the group "Fe" (194.825 mg g^{-1}), it is a very high concentration data, showing the high bioaccumulation capacity in this species. Iron is not mortal in humans, but it is in some amphibians (*Porter and Hakanson 1976; Pauli et al. 2000*), hence, the importance of including it in this experiment.

The fact that the gills are the tissue with lower accumulation of these heavy metals in this study is consistent with the results by *Severtsova et al.* (*2013*), who also reported the gills as the tissue with the smallest concentration of lead and iron compared with other tissues (**Figure 5**). The tadpoles filter the water through the gills, and thus can keep polluting particles, but not as significantly as through the mouth.

The results in the water spectrochemistry among the control and experimental groups were similar; that indicated that those metallic objects do not always dissolve in the environment, in this case, in a waterbody. But due to feeding behavior in tadpoles, in which they eat

usually at the bottom of the waterbody, they are in constant contact with those objects and acquire the metal orally, this is also the conclusion by *Severtsova et al.* (*2013*) in their bioassay research with *Rana temporaria* and *Bufo bufo*. The amount of iron and lead in the control group can be due to the fact that tap water was used, the pipes are made of steel, and the old pipes of lead. Other than that, Mineral del Chico, Hidalgo is a mining historical place, but as we showed, despite the similar heavy metals concentration in the water among the groups, the situation is quite different in the tissues, that indicates the bioaccumulation potential of these organisms, and the possibility of using them as a bioindicators of the environmental health.

CONCLUSION AND RECOMMENDATIONS

Bioassays in ecotoxicological studies can help to clarify the phenomena of population decline and extinction of some amphibian species, in this case, the tree frog *Dryophytes plicatus*. Heavy metals are among the most common pollutants in urban and industrial areas, especially in mining zones. Lead and iron are well documented about their effects on other amphibian species, especially in the African clawed frog *X. laevis*. These elements can accumulate in different tissues like the liver, intestine, and gills. The study recommends considering those amphibian species in nature with population declines to be used as bioindicators through ecotoxicological studies by biomonitoring and bioassays.

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