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The herbicide atrazine increases mucopolysaccharides and decreases the thickness of the epithelium of the frog *Lithobates spectabilis* (Ranidae)

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Abstract. The application of herbicides is one of the leading causes of the decline of amphibians in the world. Atrazine is widely used to control herbaceous weeds in crops. In Mexico, it is not regulated and widely applied. More information regarding the effects of atrazine in aquatic systems and on amphibians in this country is therefore needed. Amphibians have a smooth skin coated with excretions that protect them from harmful environmental influences, yet still facilitates the exchange of gases, ions, and thermoregulation, but is especially susceptible to absorbing toxic substances, including atrazine. A few studies have revealed that atrazine modifies the thickness of the epithelial layer in fish, but there are few studies to that effect in frogs. No studies have investigated whether atrazine exposure affects mucopolysaccharides or glucans, which are essential for hydrating the skin. We hypothesized and found confirmed that the herbicide atrazine reduces the thickness of the dorsal epithelium and promotes a greater presence of mucopolysaccharides in the dorsal epithelium and glands in *Lithobates spectabilis*. Our findings suggest that this frog is likely more vulnerable to dehydration, ultraviolet radiation, and will suffer impaired wound healing when exposed to atrazine.

Key words. Amphibia, aquatic, epithelial layer, glands, Mexico, pesticide, skin.

Introduction

Amphibians are one of the most endangered vertebrate groups in the world. One of the causes of its decline is pesticide contamination (Brühl et al. 2011). Atrazine (6-chloro-N-ethyl-N'-(1-methyl ethyl)-1,3,5-triazine-2,4-diamine is the most widely used pesticide in the world (Cheng et al. 2016) and is the most widely used herbicide to control herbaceous weeds in crops (Iriel et al. 2014). Atrazine contamination in aquatic and terrestrial habitats may vary around the world (Schwab et al. 2006), but can reach 50 ppb in rivers and streams, 4000 ppb in runoffs, and 2.5 ppb in rain (U.S. epa, 2003; Graziano et al. 2004). In terrestrial habitats, atrazine concentrations can vary from 0.2 to 0.01 mg/kg^{-1} in surface and subsoil layers, respectively (Vonberg et al. 2014). In different types of sandy loam and clay soils, 5 to 5000 mg/kg^{-1} have been measured (Gan et al. 1996). In Mexico, few studies have investigated atrazine concentrations in aquatic and terrestrial systems: in the surface waters of agricultural areas, atrazine concentrations are 4.62 to 15.01 µg/L, and in groundwater, 6.23 to 21.26 µg/L (Hernández-Antonio & Hansen 2011).

Several studies have revealed that amphibians are susceptible to contaminants due to their permeable skin (Quaranta et al. 2009). However, to date there is little information on the general dermal toxicity of atrazine (Brühl et al. 2011, Storrs et al. 2009), but to amphibians,

atrazine is a toxic substance that is absorbed via the skin within hours (Storrs et al. 2009). Amphibian skin is a mucosal organ with various functions, including physical protection from environment influences, and facilitates dermal respiration, osmoregulation and thermoregulation (Duellman & Trueb 1994).

Excretions from the epithelium and glands are essential for moisturizing the skin, particularly in organisms such as amphibians that lack a thick fibrous covering. It has been reported that these excretions mainly comprise proteoglycans, glycoproteins (glycoconjugates), ions, and sugars (Dapson 1970). Importantly, mucopolysaccharides (MP) are glycosaminoglycans that maintain organ turgidity, serve as lubricants, and provide viscoelasticity to tissues (Meyer 1969).

Most studies on the effects of herbicides on the epithelium of fish have shown that they affect epithelium thickness; for example, glyphosate causes it to increase in thickness (Vasanth et al. 2022). In addition, atrazine affects the mucosubstances of the gill epithelium in *Prochilodus lineatus* (Prochilodontidae; Paulino et al. 2012). Few studies have so far been published on the effects of herbicides on the epithelial thickness in frogs. In Bullfrogs, epithelial thickness decreased when exposed to Roundup Transorb R® (Rissoli et al. 2016), specimens of *Pelophylax* kl. *esculenta* collected from highly contaminated rice paddies had superficial keratinized cells in the epidermis (Fenoglio et al. 2006). Given these studies, and since the changes in epithelial thickness and the presence or distribution of MP in frogs exposed to atrazine are unknown, the present work aimed at identifying such possible effects in males of the frog *Lithobates spectabilis*.

Materials and methods

Capture site and study model

We used *Lithobates spectabilis* (Hillis & Frost, 1985) as our study model.This species is listed as being of 'Least Concern' due to its wide distribution and presumably large population size (IUCN 2020); it is native to, and widely distributed throughout, central Mexico (Hillis & Frost 1985). Twelve adult males of *L. spectabilis* were captured using an entomological net in a water body in Tlaxcala, Mexico (19°23'60" N, 98°21'53.99" W; 2,919 m a.s.l.). The climate at this site is cold-temperate and sub-humid. The predominant vegetation is made up mainly of oak forests (*Quercus* spp.), the average annual temperature is between 17 and 20°C, and the average annual precipitation is between 1,800 and 2,000 mm (INEGI 2014–2016). The frogs were captured near the beginning of the species' daily activity period, between 18:00 and 20:00 h, during the rainy season of 2021 (August and September). All individuals were captured under a scientific collection permit issued by the Secretaría de Medio Ambiente y Recursos Naturales de México (SEMARNAT; SGPA/DGVS/03662). The species identity was validated using the guide for Mexico provided by Hillis & Frost (1985).

Laboratory conditions

The captured individuals (n = 12) were placed in glass terraria offering climatic conditions that were similar to those in nature. They were converted from 40-L aquaria (51 × 29.5 × 26 cm), lined with uncontaminated grass that was kept moist with potable water, and sported a glass container filled with water (25.5 × 25.5 × 8 cm). Physicochemical analysis verified that the potable water provided did not contain any toxic substances (NOM 127-SSA1-2001, CE-CA, 1989; Supplementary Information). Relative humidity was maintained at between 75 and 90%, and room temperature was controlled to range between 22.3 and 24.9°C. Temperature and humidity were constantly monitored and recorded using Arduino-cloud software. In each tank, a photoperiod of 12/12 h light/dark was maintained using a lamp controlled by a digital timer (lights on at 6:00 am) (Rimayi et al. 2018).

Acclimatization and exposure to atrazine

All 12 frogs were acclimatized in a tank filled with potable water for one hour before being placed in the terrarium, then spent ~21 days in the terraria prior to our experiment (Oliveira et al. 2018). During the latter period, the individuals were exposed only to potable water, which was exchanged daily to prevent the accumulation of frog feces and urine. Once the individuals had acclimatized to the laboratory conditions, we randomly divided them into two groups, control and atrazine-exposed, in two separate terraria. The control group (n = 6) was exposed to potable water only. In the atrazine-exposure treatment (n = 6), individuals were exposed to a commercial formulation of Gesaprim® (90% purity) at a concentration of 15 µg/L dissolved in potable water that was added to the glass containers in the terrariums for 90 days (Rimayi et al. 2018). The concentration of atrazine applied in our experiment matched what has already been reported from an aquatic system in Mexico (Hernández-Antonio & Hansen 2011). The atrazine solution (exposure group) and potable water (control group) were exchanged once every day throughout the duration of our experiment. In addition, during this period, the grass lining the terrarium hosting the exposure group was also sprayed daily with the solution (15 µg/L) to ensure that the individuals were in contact with the herbicide. All individuals were fed ad libitum with live crickets (Petmmal S.A de C.V) during the experimental period; this is a commonly used diet for frogs in captivity.

Anesthesia and removal of the dorsal epithelium

The frogs were anesthetized by immersion in 200 mg/L MS-222 (tricaine methane sulfonate, Sigma-Aldrich) for 15 min. Subsequently, we removed a portion of the dorsal epithelium for histological processing, and euthanized the individuals with an overdose of MS-222.

Histological analysis of dorsal epithelium

We fixed the removed samples of dorsal epithelium in a Bouin-Duboscq solution for 24 h. The skin was dehydrated with ascending alcohol concentrations (60–100%), fixed in xylol, and embedded in paraffin (Paraplast. SIGMA-ALDRICH). Then, longitudinal sections 7 µm thick were cliced off with a microtome (Leica 2115). Serial tissue sections were stained with periodic acid-Schiff (PAS) to identify neutral MP (Lillywhite et al. 1997, Yamabayashi, 1987). After staining, the epithelial tissue was further dehydrated with alcohol in ascending concentration (60–100%). Masson's trichrome stain was used for describing the histology of the skin of *L. spectabilis* (Lefkowitch 2006) after fixing the samples with cytoseal TM60 and coverslipping them.

Thickness of the dorsal epithelium and presence of MP

First, we selected a whole dorsal epithelial tissue sample that was not torn or folded. Fifty randomly selected fields were considered to measure the thicknesses of the dorsal epithelia in the control and atrazine groups. Microphotographs at 40× magnification of both the dorsal epithelia and glands were taken under a light microscope (Leica; DM750) with a camera (Leica ICC50 E) using the LAS EZ 3.3.0 software. The thickness of the dorsal epithelium was determined using the AxionVision Rel.4.8 program. The presence of MP was evaluated qualitatively, as described previously (Rodríguez-Castelán et al. 2018, Méndez-Tepepa et al. 2020). We considered a relative scale of the abundance of MP in the dorsal epithelium and glandular epithelium of *L. spectabilis*. For that, arbitrary categories were established: indicating lower presence (+) and higher presence (++) of MP.

Data analysis

All statistical analyses were conducted in Rstudio software (v. 1.4.1717). We estimated the extent of epithelial damage caused by the exposure to atrazine. First, we used a two-way analysis of variance (ANOVA) to evaluate the differences in 1) the presence of MP in the dorsal epithelium, and 2) the presence of MP in the epithelial glands (function "aov"; library "car" and "multcomp"). We selected this type of analysis because our experimental design was primarily aimed at assessing differences in the control and atrazine-exposed groups. Furthermore, in our models, we included a second categorical explanatory variable ("lower" and "higher" presence) based on the abundance of MP. The assumptions of the ANOVA were determined utilizing a test of normality (Shapiro Wilk) and of homoscedasticity of variances (Barlett's test). Subsequently, we applied a post hoc test (Bonferroni correlation) to quantify the differences in the levels of our explanatory variables (function "pairwise.t.test" library "WRS"). On the other hand, we identified the differences in the thickness of the dorsal epithelium between the groups using a Student's t-test. We considered differences to be significant when the p-value was lower than 0.05. Data are presented as means ± SEM unless otherwise stated.

Results

Histology of the dorsal epithelium

The dorsal epithelium of the frog *L. spectabilis* comprises the epidermis (a stratified epithelium), the spongy layer, the compact layer, and the serous and mucous glands (Fig. 1). Morphologically, the frogs exposed to atrazine possessed a thinner dorsal epithelium and had an increased presence of MP in it (Fig. 2B) and in the glands (Fig. 2D) compared to the control group (Figs 2A and 2C, respectively).

Dorsal epithelium thickness

We found that the thickness of the dorsal epithelium was different between the study groups ($t = 7.6$, $gl = 481.06$, $p < 0.001$; Fig. 3); with the individuals of the atrazine group presenting a decrease in the thickness of the dorsal epithelium (47.6 ± 4.1 µm), compared to the individuals in the control group (63.4 ± 5.3 µm).

MP in the dorsal epithelium and glands

The presence of MP in the dorsal epithelium was different between the study groups, too (ANOVA two-way: $F = 38.1$,

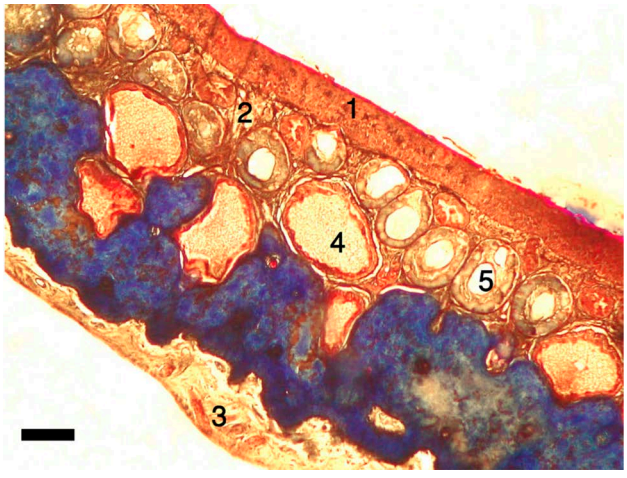

Figure 1. (a) Morphology of the skin of the frog *L. spectabilis*. The skin is composed of epidermis (1), spongy layer (2), compact layer (3), serous glands (4), and mucous glands (5). Masson's trichrome staining. Scale: 20 µm.

d.f. = 22, p = 0.01). The individuals of the atrazine group presented an increase in MP in the dorsal epithelium in the categories "lower presence" and "higher presence" (68 ± 5.2 and 85 ± 6.9) (post hoc: p = 0.01; Fig. 4A) unlike the control group (6.1 ± 1.7 and 11 ± 2). The presence of MP in the epithelial glands was also different between the study groups (ANOVA two-way: F = 82.9, d.f. = 22, p = 0.01). There was an increase in MP in the categories of "lower presence" and "higher presence" in individuals in the atrazine group (63 ± 5.6 and 78 ± 8.3) (post hoc: p = 0.02; Fig. 4B) unlike the control group (2.1 ± 1.1 and 6.6 ± 1.3).

Discussion

Variation in the thickness of the epidermis is due to differences in the number of cell layers (Brown et al. 1981). For example, in the Common Carp, *Cyprinus carpio L.*, atrazine causes hyperplasia and the thickening of some epithelial cells (Neskovic et al. 1993). In Common Molly, *Poecilia sphenops*, atrazine causes an increase in the thickness of the epidermis and ruptures of the gill epithelium (Vasanth et al. 2022). Bullfrogs exposed to Roundup original suffered increased epithelial thickness and hyperplasia (Rissoli et al. 2016). In contrast to these study results, we found a lowered epithelial thickness in *L. spectabilis* exposed to atrazine. Our results correspond with those relating to fishes exposed to other herbicides. For example, in *Prochilodus lineatus*, exposure to linear alkylbenzene sulfonate caused epithelial thinning and mucous cells increased, affecting mucus production (Alves et al. 2016). We propose that atrazine could cause the degeneration of epithelial cells and consequently reduce epithelial thickness and alter mucous excretion. Moreover, our previous work showed that atrazine causes hepatic cell degeneration in *L. spectabilis* (Méndez-Tepepa et al. 2023).

Decreased thickness of the epithelium could also be closely related to hypoxia. In *L. catesbeianus* tadpoles, chronic hypoxia reduces skin thickness (Burggren & Mwalukoma 1983), and we found in a previous study that atrazine induces apoptosis and the necrosis of erythrocytes in *L. spectabilis* (Méndez-Tepepa et al. 2023). In this sense,

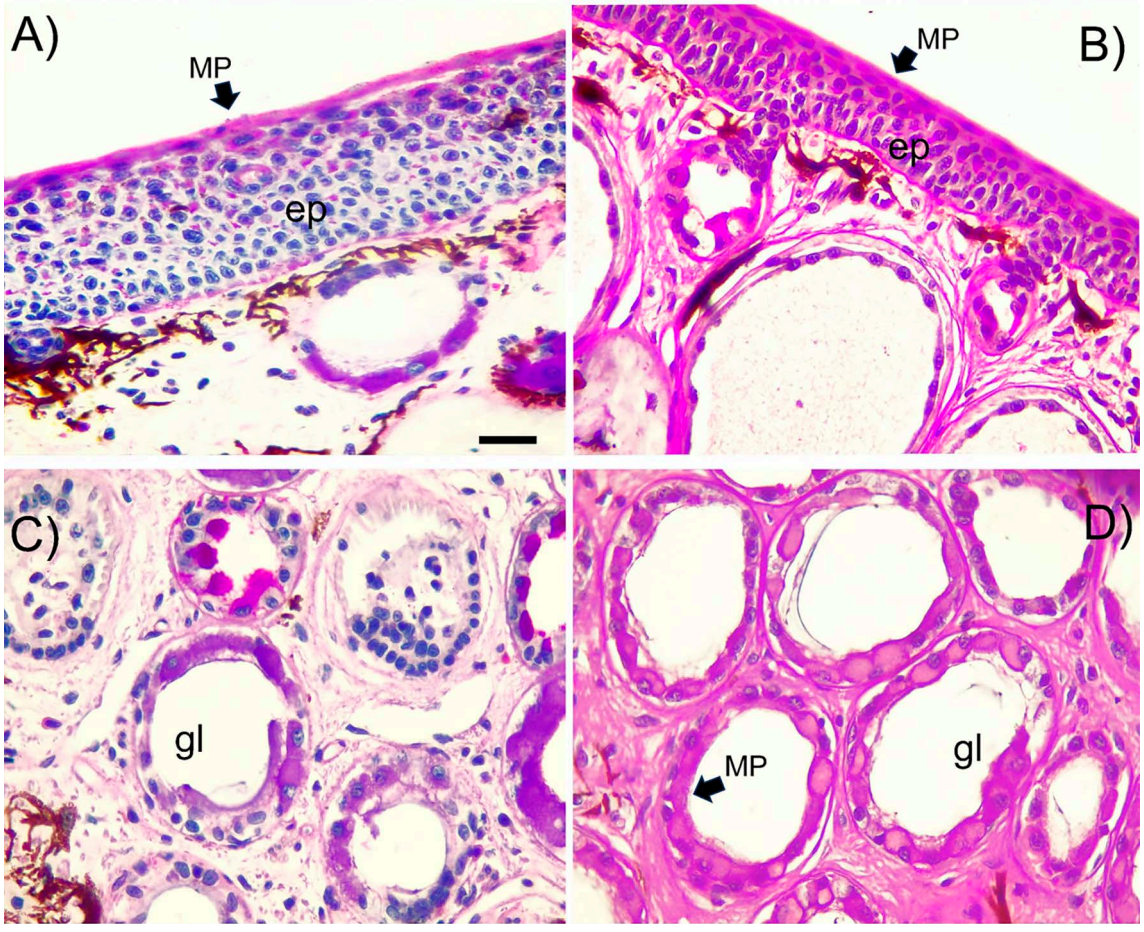

Figure 2. Effect of the herbicide atrazine on the dorsal epithelium and glands of males of the frog *L. spectabilis*. Lowered thickness of the dorsal epithelium and a higher presence of MP in the atrazine group (B) as opposed to the control group (A). In the glands of the control group, we found a lower presence of MP (C) and a higher one in the atrazine group (D). Abbreviations: MP – mucopolysaccharides, gl – glands, ep – epithelium; the black arrow indicates the presence of MP. PAS staining. Scale: 20 μm.

the lesser thickness of the epithelium could affect the individuals' ability to meet the oxygen demand (Rissoli et al. 2016), which could have long-term consequences on their survival (Burggren & Mwalukoma 1983).

On the other hand, decreased epithelial thickness could also be due to changes in the cell metabolism. For example, atrazine has been documented to reduce the height of epithelial cells due to a change in the cellular lipid metabolism in *Pelophylax nigromaculatus* (Huang et al. 2021). In this study, we found an increased presence of MP in the epithelium and glands of *L. spectabilis*. Therefore, we hypothesize that the change in the mucopolysaccharide content could be indicative of an alteration in the carbohydrate metabolism associated with a decrease in epithelial thickness.

Finally, amphibians perceive environmental changes via their skin. Increased production of skin mucus is a response to contamination in their aquatic environment (Bols et al. 2001). In this manner, the resultant skin damage, including epithelial thinning and increased presence of MP, could affect survival and tolerance to environmental changes in individuals exposed to atrazinein that they may experience dehydration and face complications such as hypoxia and decreased epithelial excretion. In addition, the possible reduction of excretion of MP could decrease the protection against pathogens, since it has been observed that this excretion protects the skin from environmental impacts and pathogens (Barbosa et al. 2022). Frogs exposed to atrazine are therefore expected to be more vulnerable to dehydration, the effects of ultraviolet radiation, and suffer from impaired wound healing. However, more analyses on the functions of glycoconjugate sugars should be undertaken in amphibians (Faszewski et al. 2008), since only a few works exist on the histology and MP in the skin of frogs exposed to environmental contaminants.

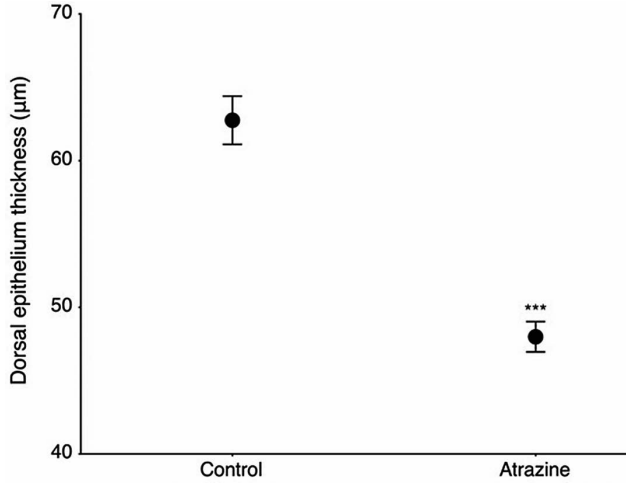

Figure 3. Effect of atrazine on the thickness of the dorsal epithelium in males of the frog *L. spectabilis*. Atrazine decreases the thickness of the dorsal epithelium compared to the control group. Standard error bars are shown. The asterisk indicates significant differences.

Conclusions

In this study, we found a thinning of the dorsal epithelium and a greater presence of MP in the skin and glands of *L. spectabilis* exposed to the herbicide atrazine. The lowered thickness of the epithelium could be due to an alteration in the metabolism of polysaccharides or cell degeneration. The skin is an important site of mucus excretion in amphibians, so that increasing MP in epithelial tissue and glands could affect skin protection and hydration. We suggest conducting more studies to identify the types of glucans in the epithelial tissue of frog species exposed to atrazine in addition to analyses of the proteins involved with degeneration and the phases of the cell cycle in frog epithelial cells.

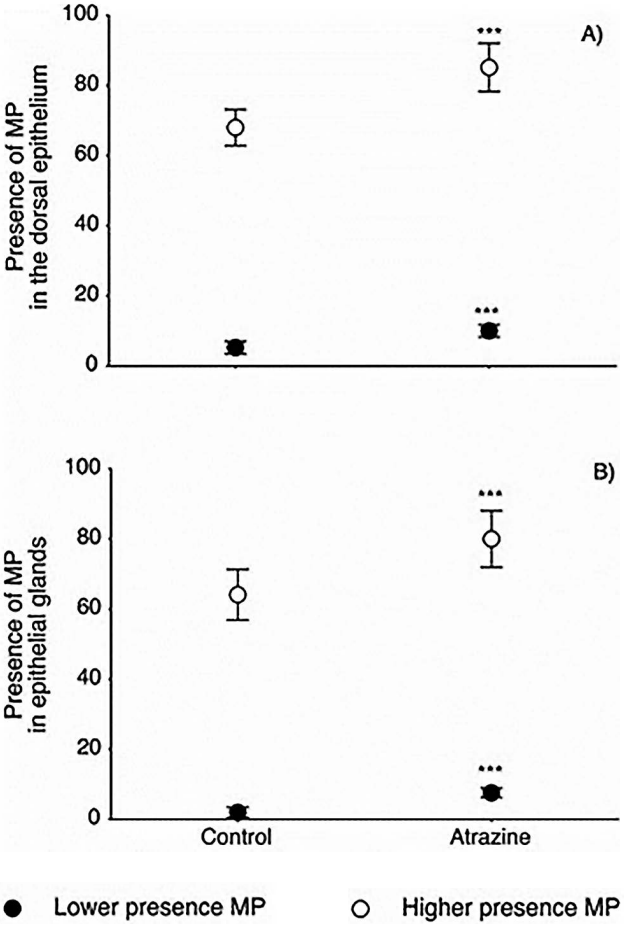

Figure 4. Effect of the herbicide atrazine on the presence of MP in the dorsal epithelium (panel A) and glands (panel B) of *L. spectabilis*. Differences between the study groups (control and atrazine) and in the categories "lower presence" (white dots) and "higher presence" (black dots) of MP in the dorsal epithelium (panel A) and the epithelial glands (panel B). The dots represent the mean, and the whiskers the standard error. Asterisks show significant differences between groups and categories.

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