Archives of Environmental Sciences and Environmental Toxicology

OPEN ACCESS

Hernández-Pérez K, et al. Arch Environ Sci Environ Toxicol 8: 150.

www.doi.org/10.29011/2688-948X.100150

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Short Communication

Effects of Atrazine in The Seminiferous Tubules and Spermatozoa in The Leopard Frog (*Lithobates Spectabilis*)

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Citation: Hernández-Pérez K, Medina-Bello KI, Morales-Cruz C, Ortiz-Ortiz E, Méndez-Tepepa M (2025) Effects of Atrazine in The Seminiferous Tubules and Spermatozoa in The Leopard Frog (*Lithobates Spectabilis*). Arch Environ Sci Environ Toxicol 8: 150. DOI: 10.29011/2688-948X.100150

Received Date: 12 December 2024; **Accepted Date**: 11 February 2025; **Published Date**: 14 February 2025

Abstract

Some analyses have determined that amphibian populations are at risk due to exposure to chemical contaminants, such as herbicides, present in their natural environments. Atrazine (6-chloro- N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine) is one of the most widely used herbicides worldwide. In recent decades, it has been documented that environmental concentrations of atrazine significantly affect the function and performance of reproductive structures in African frogs and toads. This study evaluated the effect of atrazine at environmental concentrations reported for Mexico (15 µg/L) on the area of seminiferous tubules and the area occupied by spermatozoa in the leopard frog (*Lithobates spectabilis*). For this purpose, a control group without herbicide exposure was established, while an experimental group was exposed to atrazine for 90 days. After exposure, individuals were euthanized to analyze the area of the seminiferous tubules, the area occupied by spermatozoa, and the histology of spermatogenic cells. We found that atrazine increases both the area of the seminiferous tubules and the area occupied by spermatozoa. The results of this study suggest that modifications in testicular histomorphology could alter other reproductive physiological mechanisms. Therefore, we recommend conducting additional molecular and endocrine studies across the different spermatogenic phases.

Keywords: Amphibians; Aquatic system; Herbicide; Mexico; Spermatogenesis; Spermatozoa; Reproduction; Testicle

Introduction

Exposure to environmental contaminants, such as herbicides, poses a significant threat to biodiversity [1]. One of the most widely used herbicides globally is atrazine (6-chloro- N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine) [2]. Atrazine is employed for weed management in agricultural crops, domestic gardens, and urban parks [3]. Consequently, recent years have seen an increase in reports detailing the adverse effects of atrazine on wildlife, particularly concerning the reproductive systems of aquatic vertebrates, such as amphibians [4,5].

In this context, experimental analyses conducted under laboratory conditions have documented that even minimal concentrations of atrazine adversely affect the function and processes of reproductive traits in toads and frogs [6]. For instance, at the testicular level, it has been reported that male African frogs, such as the northern leopard frog (*Rana pipiens*) and the African clawed frog (*Xenopus laevis*), when exposed to environmental concentrations of atrazine (10 and 21 µg/L, respectively), develop gonadal malformations and experience a reduction in spermatogenesis [2,7]. These changes may compromise the reproductive capacity of males, negatively impacting population density [5,8]. However, this issue has been overlooked in current studies. Therefore, it is essential to focus on how environmental contaminants adversely influence the reproductive processes of amphibians to address critical aspects of species survival and conservation [4,5,8].

Given the significance of reproductive traits in mating systems, any alterations in the structure and function of the testicles can affect an individual's reproductive capacity, potentially impacting reproductive success and long-term population growth [9]. In this study, the aim was to describe the spermatic cells and evaluate the effect of atrazine on both the area of the seminiferous tubules and the area occupied by spermatozoa in the testes of male leopard frogs (*Lithobates spectabilis*) inhabiting central Mexico. To achieve this, we exposed a group of individuals to a concentration previously recorded in aquatic systems in Mexico [10], while a control group remained unexposed. This experimental design provided a reference point for directly comparing the effects of the herbicide on the testicular structures of the individuals.

Materials and Methods

Frog care and housing

The frogs were captured in a water body in Tlaxcala, Mexico (19°23'60" N, 98°21'53.99" W; 2919 m.a.s.l.) using a beating net at dusk during the breeding season (August-September 2021). Six male individuals of *L. spectabilis* were selected after confirming that all were in the reproductive stage. Following capture, we recorded their body mass (g) and snout-vent length (mm). Sexual maturity was verified based on body size and specific male characteristics, such as vocalizations and nuptial pads [11]. The species identity was validated using the amphibian guide for species distributed in Mexico by Hillis and Frost [12]. The selected individuals were immediately placed in containers with aeration and water from the capture site and transported to the laboratory. The frogs were physically inspected to rule out diseases and malformations. The capture and handling of the animals were conducted under a permit from Secretaría de Medio Ambiente y Recursos Naturales de México (SEMARNAT; SGPA/DGVS/03662).

In the laboratory, we randomly divided the captured frogs into two groups and placed them in individual glass terrariums (51 cm x 29.5 cm x 26 cm) containing contaminant-free grass. We maintained the temperature, humidity, and photoperiod conditions to closely resemble their natural environment (i.e., 22.3–24.9 °C and 75–90% relative humidity, with a 12/12-hour light-dark cycle) to facilitate a 21-day habituation period to the laboratory conditions. During this period of time, the frogs were allowed to acclimate and reduce handling stress before the experimental trials, ensuring that any changes in their behavior or physiology could be attributed to the experimental treatment. All individuals were fed ad libitum with live crickets and provided with water daily throughout this period.

Exposure to the Herbicide Atrazine

Once habituated, we classified each terrarium into a control group (n=3) and an experimental group (exposed to atrazine) (n=3). The control group was maintained using potable water, free of any toxic compounds (Juaréz-Santacruz, unpublished data). In contrast, the experimental group was exposed to a commercial formulation of Gesaprim® (90% purity) at a concentration of 15 µg/L dissolved in potable water for a duration of 90 days.

Testicular Histology

After 90 days, the frogs were euthanized by immersion in an overdose of tricaine methanesulfonate for 15 minutes (200 mg/L, Sigma-Aldrich [2]). To ensure the well-being of the animals during the experiments, we adhered to appropriate measures and methods for handling, anesthesia, and surgical and diagnostic procedures in amphibians [13,14]. We then removed the testicles of each individual. This organ was fixed in Bouin-Duboscq solution for 24 hours, dehydrated with alcohol in increasing concentrations (60% to 100%), and embedded in paraffin (Paraplast, SIGMA-ALDRICH). Subsequently, 7 µm histological sections were prepared using a microtome (Leica model RM 2125) and stained with Periodic Acid-Schiff (PAS). Finally, the tissue samples were dehydrated again and mounted with Cytoseal TM60 before being covered with coverslips.

Histology of spermatogenic cells, area of the seminiferous tubules and area occupied by spermatozoa

We selected a sample consisting of intact slides of testicular tissue that were neither torn nor folded for histological analysis. To

determine the area of the seminiferous tubules (100 seminiferous tubules per individual), photomicrographs of the testis were captured at 40x magnification using an optical microscope. Five random fields of the testis were analyzed, resulting in a total of forty photomicrographs at 40x magnification. Additionally, to assess the area occupied by spermatozoa within the 100 seminiferous tubules, 40 photomicrographs were taken at 40x for each individual. For the identification and histological description of spermatogenic cells [15,16], photomicrographs were captured at100x magnification. We utilized the Axio Vision Rel 4.8 software to measure the area of the seminiferous tubules and the area occupied by spermatozoa. All photomicrographs were captured using a Leica DM750 optical microscope connected to a LEICA ICC50 E camera, with the LAS EZ 3.3.0 software.

Statistical Analysis

All statistical analyses were conducted using RStudio software (version 1.4.1717). To evaluate the differences in means for the area of seminiferous tubules (μm^2) and the area occupied by spermatozoa (μm^2) between individuals in the control group and those exposed to atrazine concentrations, a student's t-test (function "t.test"). Prior to this analysis, the assumptions of normality of the data were assessed using the Shapiro-Wilk test (function "shapiro.test") for small samples, and the homogeneity of variances was verified using Levene's test (function "levene.test") before conducting the analyses. Significant differences were considered when the p-value was less than 0.05. Data are presented as means ± SEM unless otherwise stated.

Results

Our results indicated that the morphology of spermatogenic cells in the seminiferous tubules of *L. spectabilis* was consistent with findings from previous studies conducted on other frog species [15]. We identified spermatogonia (Figure 1b), spermatocytes (Figure 1c), meiosis I spermatocytes (Figure 1d), spermatids (Figure 1e), and spermatozoon loops (Figure 1f). In comparison, the control group exhibited smaller seminiferous tubules (Figure 2a) and a reduced number of spermatozoa (Figure 2c) compared to the atrazine-exposed group (Figures 2b and 2d illustrate the seminiferous tubules and spermatozoa, respectively). In this study, we observed significant differences in the seminiferous tubule area ($t = -5.03$, $p < 0.05$; Figure 3a) and the area occupied by spermatozoa ($t = -5.62$, $p < 0.05$; Figure 3b) between individuals in the control group and those exposed to atrazine. Specifically, individuals exposed to atrazine exhibited a larger seminiferous tubule area ($732.3 \pm 49.8\ \mu m^2$) and a greater area occupied by spermatozoa ($191.1 \pm 9.2\ \mu m^2$) compared to control group individuals, who had a seminiferous tubule area of $288.5 \pm 8.8\ \mu m^2$ and an area occupied by spermatozoa of $159.8 \pm 3.3\ \mu m^2$.

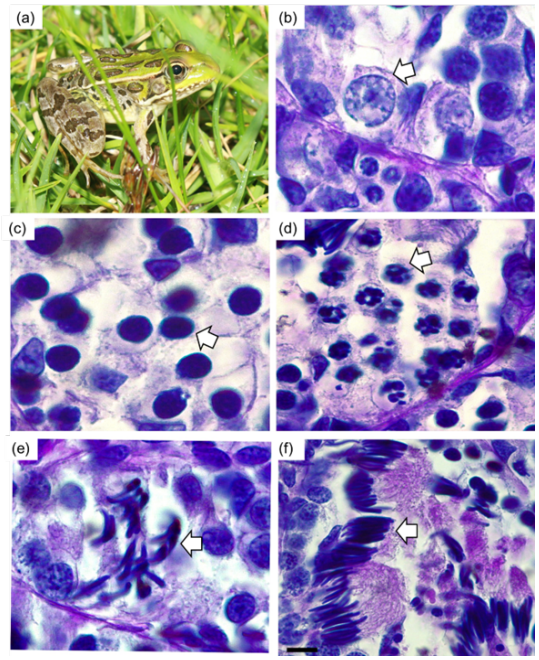

Figure 1: Morphology of the spermatogenic cells of *L. spectabilis* (a). Spermatogonia (b), spermatocytes (c), spermatocyte nodules in Meiosis I (d), spermatids (e), and spermatozoon loops (f). The arrows indicate the type of spermatogenic cell, respectively. PAS stain. Scale bars = 10 µm.

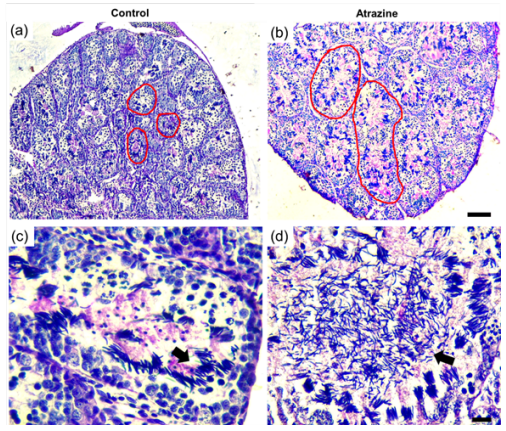

Figure 2: Effect of the herbicide atrazine on the seminiferous tubules and spermatozoa of *L. spectabilis*. Smaller seminiferous tubule area in the control group (a; outlined in red), compared to seminiferous tubule area in the atrazine group (b; outlined in red), seminiferous tubule with fewer spermatozoa in control group (c, arrow), compared to seminiferous tubule full of spermatozoa in individuals exposed to the atrazine group (d, arrow). PAS staining. Scale bars = 10 µm (a-b) and 20 µm (c-d).

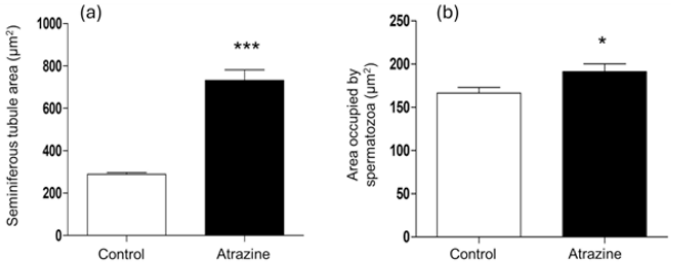

Figure 3: The effect of the herbicide atrazine on the seminiferous tubules, the area occupied by spermatozoa, and testicular area in *L. spectabilis.* Atrazine was found to increase both the area of the seminiferous tubules (a) and the area occupied by spermatozoa (b).

Discussion

In this study, we histologically examined the spermatogenic cells of *L. spectabilis* and found that the seminiferous tubules and sperm abundance in the testes are affected by concentrations of atrazine. Although we acknowledge that our results are based on a small sample size, the responses observed in both the control group and the atrazine-exposed individuals were consistent. This consistency is evident in the measures of dispersion relative to the mean (see results), which indicate controlled variability and suggest that, despite the limited sample size, the observed effects are relevant.

In our study, we found that atrazine increases the size of the seminiferous tubules and the area occupied by spermatozoa. Our results contrast with other studies conducted on the African common toad (*Sclerophrys regularis*) and the clawed frog (*Xenopus laevis*), where it has been reported that atrazine concentrations decrease the size of the seminiferous tubules and reduce the presence of mature spermatozoa [4,5]. However, we speculate that our findings may be attributed to the upregulation of estradiol, which enhances spermatogonia proliferation and increases sperm production [17]. Previous studies in amphibians suggest that atrazine-induced gonadal malformations result from androgen depletion and the induction of estrogen and aromatase production [8].

On the other hand, Cohesin Subunit SA-1 (STAG1) and Cell Division Protein kinase 2 (CDK2) proteins modulated by estrogen and atrazine biosynthesis, are associated with the cell cycle [18]. Thus, the overexpression of the CDK2 protein, which plays a role in regulating the cell cycle during the G1 phase, could accelerate spermatogenesis [19] and increase the number of spermatozoa in this species. However, the higher abundance of spermatozoa observed in our study species as a result of exposure to environmental concentrations of atrazine does not necessarily indicate greater fertility; in fact, the opposite may be true. For instance, in Japanese rice fish (*Oryzias latipes*) exposed to atrazine, there is a higher percentage of abnormal mitotic events and abnormal sperm [20]. It is crucial to recognize that spermatozoa quality is equally important for successful reproduction. Consequently, the increase in the area of the seminiferous tubules and the area occupied by spermatozoa may lead to long-term alterations in fertilization outcomes for individuals exposed to atrazine in their natural environments. Furthermore, having a higher number of spermatozoa does not guarantee that individuals are sexually active or capable of successful mating [16]. Atrazine is an endocrine disruptor that negatively impacts frog reproduction [5] and affects sperm quality and motility in fish [21]. Therefore, it is essential to consider not only the quantity of spermatozoa but also their viability, motility, and morphology.

Conclusion

Atrazine is a contaminant that contributes to an increase in the area of seminiferous tubules and the abundance of spermatozoa, potentially due to its effects on specific stages of spermatogenesis. We present novel histological findings regarding changes in testicular morphology associated with increased spermatozoa in the species *Lithobates spectabilis*. However, we believe it is essential to conduct reproductive and endocrine studies to assess the impact of atrazine on amphibians distributed throughout Mexico, focusing on all stages of spermatogenesis and the quality of spermatozoa.

Acknowledgements

All the authors express their gratitude to Xochitl Rubi Cruz Cortes for their logistical support. We also acknowledge the Laboratorio de Toxicología y Química Ambiental and Facultad de Ciencias de la salud; Universidad Autonóma de Tlaxcala.

Authors Contributions

Conceptualization, M.M.T; methodology, K.H.P and M.M.T.; data curation, K.H.P and M.M.T.; formal analysis, K.I.M.B and M.M.T; writing original draft preparation, M.M.T and K.I.M.B.; writing review and editing, M.M.T., C.M.C., E.O.O., K.I.M.B. and K.H.P. All authors have read and agreed to the published version of the manuscript.

Competing Interests

The authors declare that they have no competing interests.

Data Availability

Supplementary material is available in the following link (https://docs.google.com/spreadsheets/d/1dafonVxs-j6--hIJ2HylHm0QQZ1mExIM/edit)

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