RESPONSE OF THE AMPHIBIAN TADPOLE (XENOPUS LAEVIS) TO ATRAZINE DURING SEXUAL DIFFERENTIATION OF THE TESTIS

LUZ TAVERA-MENDOZA,† SYLVIA RUBY,*‡ PAULINE BROUSSEAU,‡ MICHEL FOURNIER,‡ DANIEL CYR,‡ and DAVID MARCOGLIESE§
†Department of Biology, Concordia University, Montreal, Quebec H3G-1M8, Canada
‡INRS-Institut Armand-Frappier, Point-Claire, Quebec H9R-1G6, Canada
§Environment Canada, Montreal, Quebec H2Y-2E7

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Abstract—Xenopus laevis tadpoles were exposed for 48 h during sexual differentiation to atrazine at 21 μg/L under static laboratory conditions at 21 ± 0.5°C. After this exposure period, tadpoles were fixed and the kidney–gonad complex was microdissected. Quantitative histological analysis of the gonad revealed a 57% reduction in testicular volume among atrazine-exposed tadpoles. In addition, primary spermatogonial cell nests that represent germ cells for the life of the organism were reduced by 70%. Nursing cells, which provide nutritive support for the developing germ cells, had declined by 74%. Testicular resorption was observed among 70% and aplasia or failure of full development of the testis was recorded in 10% of the atrazine-exposed tadpoles. Because cell nests represent the pool of primordial germ cells for the reproductive life of the organism, the combined reduction in spermatogonial cell nests and nursing cells suggest that a pulse exposure to 21 μg/L of atrazine during sexual differentiation could significantly reduce reproduction during the reproductive life of these animals.

Keywords—Atrazine Amphibian Tadpole Sexual differentiation Testis

INTRODUCTION

Amphibian population declines exceeding normal population fluctuations have been perceived globally in recent years and extinction has occurred in some populations [1,2]. Although no single cause has been identified, current studies raise concern regarding the potential role that pesticides may play in these declines [3].

Atrazine (2-chloro-4-ethylamino-6-isopropyl-aminos-triazine) is a heavily utilized herbicide in North America [4]. Atrazine primarily is used for weed control in corn and sorghum production [5]. Approximately 36,000 tons of atrazine were applied to crops in the United States in 1993. At the same time, more than 584 tons were applied to corn in the province of Ontario in Canada [6]. Atrazine inhibits photosynthesis by blocking electron transport within the Hill reaction of photosystem II [7]. Reports suggest that atrazine is more toxic to plants than to animals [5]. Atrazine is relatively persistent in naturally occurring freshwater. The half-life of atrazine is variable and ranges from 8 to 350 d, depending upon the ecological factors existing in the ecosystem [5]. The no-observed-ecological-effect concentration for atrazine in surface waters has been estimated at 21 μg L⁻¹ [6]. Concentrations of atrazine as high as 21 μg/L have been measured in the St. Lawrence River Valley region of Quebec, Canada [8]. Although concentrations in rivers and streams rarely exceed 20 μg/L, concentrations at the field edge can reach 250 μg/L and concentrations as high as 740 mg/L have been detected in runoff waters from treated cornfields after spring application [9].

Numerous laboratory and field studies have examined the effects of atrazine on amphibian larval development [5,9–13].
experimental tanks. Each of the four tanks contained 16 tadpoles. They were randomly assigned to experimental tanks. The total number of tadpoles for the experiment was 48. After histological examination, the total number of males was 24 and the distribution of males in each of the tanks was 7 and 9 in control and 8 and 8 in treated tanks. No mortalities occurred among larvae in either control or treatment tanks. Tadpoles were fed a specially prepared tadpole diet purchased from Boreal (St. Catharines, ON, Canada). The tadpoles were fed twice a week during the acclimation period. More frequent feeding resulted in too high a particulate concentration in the water. *Xenopus* tadpoles are suspension-feeding vertebrates and excess particulate matter will result in clogging of their gill filters [17]. Tadpoles were not fed during the 48-h exposure period.

Atrazine (99% purity; Sigma Chemical, St. Louis, MO, USA) was dissolved in distilled water by ultrasonication. The solution for 6 h in an ice bath. A stock solution was freshly prepared on the evening before the 48-h pulse exposure. Atrazine (0.015 g) was weighed and diluted into 1 L of ultradistilled water. Twenty-one milliliters of stock solution was added to the treatment tanks and Montreal city water was added up to 15 L to produce a final concentration of 21 μg/L. The pH of the water was 7.6. Water samples were collected from control and exposure tanks at the onset of the experiment and after the 48-h exposure period. They were transported to Bodycote Technitrol (Pointe Claire, PQ, Canada) on the day of sampling and were subsequently analyzed by mass spectrometry–high-performance liquid chromatography for determination of atrazine. Concentrations of atrazine in control tanks remained <0.05 μg/L throughout the experiment. At the onset of the experiment and after a 48-h exposure period, atrazine concentrations of 18 μg/L were recorded in water collected from exposure tanks.

After a 48-h exposure period, tadpoles were anesthetized with tricaine methane sulfonate (MS-222, 0.6 g/L) and fixed in Bouin’s solution (Sigma). The gonad–kidney complex was microdissected, dehydrated, embedded and sectioned (9 μm), stained with hematoxylin, and counterstained with eosin by routine procedures. The brain along with the attached pituitary gland was removed by microdissection and subsequently sectioned at 9 μm and stained with azocarmine [18] to determine if cells of the pituitary were actively secreting hormones.

Three indicators of reproductive impairment in males were evaluated to assess the effect of atrazine on gonadal differentiation. These included an index for total testicular volume and an index that estimated numbers of spermatogonial cell nests. These were measured because spermatogonial cell nests represent the primary source of germ cells for the life of the organism. A third indicator measured nurse cell integrity. Nurse cells in the amphibian testis are analogous to the Sertoli cell in mammals and perform both a nutritive and endocrine function for the developing spermatogonial cells. For calculation of the volume of each cone-shaped testis, the volume formula of the right circular cone was applied: \( V = \frac{1}{3} \pi r^2 h \), where \( V \) is volume of the testis, \( h \) is testis length of the largest section, and \( r \) is distance between the first section and the largest section. Measurements were recorded in control and atrazine-exposed testes and the total volume of each testis was expressed in mm³. For calculation of spermatogonial cell nests, the total number was recorded in the largest section located in the middle of each testis. Calculations from two additional sections at the anterior and posterior end of the testis were also included. This was done to ensure homogeneity of the tissue throughout the developing gonad. The mean number is reported for control and atrazine-exposed testes. Nurse cell scores were assigned in the following manner. A grid was designed (0.1 × 0.01 mm) that produced 100 squares. A number was assigned to each square. Ten random numbers were selected from each of three histological sections. Sections were selected from the anterior, middle, and posterior parts of the testis. Cell nests in each of the 10 random squares were examined. If the nurse cells were absent, or showed signs of swelling, vacuolation, karyolysis (decreased staining of the dissolving nucleus), karyorrhexis (nuclear fragmentation), or pyknosis (nuclear shrinking to produce densely staining masses) [19], a score of 0 was assigned. If the nurse cell appeared normal, a score of 1 was assigned. Ten random numbers were selected to provide a variance less than 5% in each section. All tadpole testes were scored by this method.

For the three testicular parameters, the data were ranked to satisfy the assumptions of a normal distribution. For statistical analysis, an SPSS10 program (SPSS, Chicago, IL, USA) was utilized and a one-way analysis of variance was performed followed by Tukey’s test.

The Nieuwkoop staging system for *Xenopus* development was used throughout this study [16]. This system includes 66 stages from fertilization to completion of metamorphosis. Sexual differentiation in *Xenopus* occurs at stage 56. During gonadal differentiation in the male, the testis enlarges and forms an exterior cortex and an interior medulla. The primordial germ cells, which represent the stock of germ cells for the reproductive life of the organism, migrate into the interior medulla. This is contrary to females, where the interior medulla is transformed to a simple epithelium, a cavity is formed, and the primary germ cells are confined to the gonadal cortex [20].

RESULTS

Figure 1 illustrates a normally developing testis from a control male at stage 56. Total testicular volume decreased from 0.026 ± 0.003 mm³ in controls to 0.01 ± 0.001 mm³ in atrazine-exposed tadpoles (Fig.2). This represented a 57% decrease in testicular volume among the atrazine-exposed tadpoles after a 48-h exposure period. The decrease in testicular volume was significant \( F = 5.62, df = 3, 21, p = 0.004 \). Similarly, number of spermatogonial cell nests decreased in atrazine-exposed tadpoles compared to controls, from a mean
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Fig. 2. Mean testicular volume (mm$^3$) ± standard error (SE) in *Xenopus laevis* tadpoles exposed to atrazine at 21 μg/L for 48 h during sexual differentiation; n = 24 for each group (p = 0.004).

Fig. 4. Frequency of nursing cells ± standard error (SE) in stage 56 *Xenopus laevis* tadpole testes exposed to atrazine at 21 μg/L for 48 h during sexual differentiation; n = 24 for each group (p = 0.000).

Fig. 3. Mean number of primary spermatogonial cell nests ± standard error (SE) in stage 56 *Xenopus laevis* tadpoles exposed to atrazine at 21 μg/L for 48 h during sexual differentiation; n = 24 for each group (p = 0.000).

Fig. 5. Outline of a testis (T) from a stage 56 *Xenopus laevis* tadpole undergoing testicular resorption (tr) after exposure to atrazine at 21 μg/L for 48 h during sexual differentiation. Note that the testis is fully formed and was originally the same size as that of the control (Fig. 1). Loss of testicular form is evident along with breakdown of the tissue composing the testis (×250).

of 242.4 ± 35.7 in control to 72.9 ± 21.8 in atrazine-exposed tadpoles. This represented a 70% reduction in spermatogonial cell nests (Fig. 3). The decrease was significant (F = 9.38, df = 3, 21, p = 0.000). Number of nursing cells declined in atrazine-exposed testes relative to controls from a mean value of 9.62 ± 0.17 in control tadpoles to 2.35 ± 0.36 in treated tadpoles (Fig. 4). This represents a 74% decline. The decline was significant (F = 40.95, df = 3, 21, p = 0.000).

Testicular resorption and aplasia both occurred in developing larvae exposed to atrazine. Testicular resorption was observed in 70% of male tadpoles exposed to atrazine (Fig. 5) relative to control tadpoles (Fig. 1). Testicular resorption is marked by the loss of normal testicular form and breakdown of the tissue composing the testis, as outlined in Figure 5. Testicular resorption involves resorption of a fully formed testis. Failure of full development of the testis or aplasia was recorded in 10% of the testes examined. The testis forms as a small club-shaped structure and then ceases further development, as depicted in Figure 6. Histological sections of the pituitary revealed only undifferentiated chromophobes present in pituitary glands from both control and treated tadpoles. No evidence was found of chromophores, indicating that the pituitary was actively secreting hormones.

DISCUSSION

Exposure of *Xenopus* tadpoles to atrazine at 21 μg/L for 48 h during gonadal differentiation resulted in decreased testicular volume among males. Declines in number of spermatogonial cell nests combined with lower numbers of nursing cells indicate that at this concentration reproductive and somatic cells both were affected. Testicular aplasia and resorption were also recorded among atrazine-exposed tadpoles.

Previous studies suggested that atrazine does not pose a
significant risk to the aquatic environment. Although some inhibitory effects on algae, phytoplankton, or macrophyte production have been reported, and may occur in small streams vulnerable to agricultural runoff, it is generally accepted that these effects would be transient and ecological systems could be expected to recover quickly [6]. Our findings suggest that the developing germ cells in tadpole testes during testicular differentiation are highly sensitive to atrazine at 21 μg/L. Because these are the primary source of germ cells for the life of the organism [14], the effects would not be transient and recovery could not occur.

The specific mechanisms by which atrazine alters sexual differentiation in Xenopus are unknown. Mechanisms of sexual differentiation in vertebrates in general are poorly understood [21], but in lower vertebrates, sex steroids are believed to play a pivotal role [22,23]. Increasing evidence from studies of both mammalian and lower vertebrates indicates that atrazine may disrupt sexual differentiation by altering testosterone metabolism through the enzyme aromatase. Aromatase catalyzes the conversion of androgens to estrogens. Estrogen biosynthesis in the ovary, adipose tissue, and brain is catalyzed through the expression of the enzyme aromatase P450. This enzyme is the product of the CYP19 gene. This gene is a member of a superfamily of genes containing more than 300 members in some 36 gene families [24]. Aromatase converts androgens to estrogen by binding the C17, androgen substrate and catalyzing several reactions, thus leading to a phenolic ring characteristic of estrogens [25]. Increased levels of aromatase were recorded in vitro when gonad–adrenal–mesonephros complexes were excised from neonatal alligators (Alligator mississippiensis) after treatment of eggs with a topical solution containing 14 ppm atrazine [26]. Preliminary results from our laboratory also support this mechanism. Female Xenopus treated identically to males during gonadal differentiation demonstrated no evidence of gonadal resorption or reduced ovarian volume. In addition, female ovaries revealed enhanced levels of oogonial development during sexual differentiation and increased levels of atresia (L. Tavera-Mendoza et al., unpublished data).

Atrazine could also disrupt normal endocrine function of the differentiating testis by directly blocking testosterone and dihydroxytestosterone at the receptor level. In rats, atrazine has been shown in both in vivo and in vitro studies to have antitestosterone and antidihydroxytestosterone effects at the receptor level [27,28]. Similarly, in rats, atrazine has been shown to strongly inhibit 5α-reductase, the enzyme that converts testosterone to dihydroxytestosterone [29].

The potential for extrapolation of the results of our experiments with X. laevis to other vertebrates is high. Development and its regulation by hormones is the single most heavily conserved feature throughout the vertebrates [30]. Testosterone and estrogen receptor–ligand binding sites, as well as the mRNA nucleotide sequence for both 5α-reductase and aromatase are well conserved. They have demonstrated a very high alignment in the amino acid sequences among vertebrates sequenced to date [http://www.ncbi.nlm.nih.gov/BLAST/]. Receptors and enzymes with high similarity in composition, function, and structure throughout the vertebrates could be expected to respond similarly to atrazine with respect to their mechanisms of action.

The high level of sensitivity of the primary germ cells observed in Xenopus tadpoles during sexual differentiation in the present study suggests that the use of Xenopus in the frog embryo teratogenesis assay continues to provide an excellent model for extrapolating the effects of xenobiotics on other vertebrates during development.

In conclusion, the present findings suggest significant effects of atrazine at 21 μg/L on early testicular development in Xenopus tadpoles after a 48-h exposure period during gonadal differentiation. Primordial germ cells are set aside during the early stages of development and subsequently give rise to the definitive germ cells [14]. Because of the high level of reabsorption of the testes, recovery would be unlikely. Atrazine may act as an endocrine disrupter at this sensitive stage in the developmental process and may subsequently significantly reduce the reproductive capacity of the organism for life. Given that development and its regulation by hormones is the single most heavily conserved feature throughout the vertebrates, our findings would suggest that the present no-effect concentration suggested for atrazine in surface waters should be reconsidered.

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REFERENCES

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