

Effects of Atrazine on *CYP19* Gene Expression and Aromatase Activity in Testes and on Plasma Sex Steroid Concentrations of Male African Clawed Frogs (*Xenopus laevis*)

Markus Hecker,^{*1} June-Woo Park,^{*} Margaret B. Murphy,^{*} Paul D. Jones,^{*} Keith R. Solomon,[†] Glen Van Der Kraak,[‡] James A. Carr,^{§¶} Ernest E. Smith,[¶] Louis du Preez,^{||} Ronald J. Kendall,[¶] and John P. Giesy^{*¶||}

^{*}Department of Zoology, National Food Safety & Toxicology Center, Center for Integrative Toxicology, Michigan State University, East Lansing, Michigan 48824; [†]Centre for Toxicology and Department of Environmental Biology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada; [‡]Department of Integrative Biology, University of Guelph, Ontario, N1G 2W1, Canada; [§]Department of Biological Sciences, Texas Tech University, Lubbock, Texas 79409; [¶]Department of Environmental Toxicology, and The Institute of Environmental & Human Health, Texas Tech University, Lubbock, Texas 79416; ^{||}North West University for Christian Higher Education, Potchefstroom 2520, South Africa; ^{|||}Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong, SAR, China

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Some investigators have suggested that the triazine herbicide atrazine can cause demasculinization of male amphibians via upregulation of the enzyme aromatase. Male adult African clawed frogs (*Xenopus laevis*) were exposed to three nominal concentrations of atrazine (1, 25, or 250 µg atrazine/l) for 36 days, and testicular aromatase activity and *CYP19* gene expression, as well as concentrations of the plasma sex steroids testosterone (T) and 17β-estradiol (E2), and gonad size (GSI) were measured. There were no effects on any of the parameters measured, with the exception of plasma T concentrations. Plasma T concentrations in *X. laevis* exposed to the greatest concentration of atrazine were significantly less ($p = 0.034$) than those in untreated frogs. Both *CYP19* gene expression and aromatase activities were low regardless of treatment, and neither parameter correlated with the other. We conclude that aromatase enzyme activity and gene expression were at basal levels in *X. laevis* from all treatments, and that the tested concentrations of atrazine did not interfere with steroidogenesis through an aromatase-mediated mechanism of action.

Key Words: estrogen synthesis; gene expression; amphibians; endocrine modulation; Q-RT-PCR; aromatase.

INTRODUCTION

Together with other factors such as habitat fragmentation, introduction of predatory species, wetland losses, UV-B radiation, diseases, and other environmental chemicals, pesticides have been suggested as possible causes for worldwide amphibian declines (Allran and Karasov, 2000, 2001; Corn,

2000). Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine; ATZ) is a widely used triazine herbicide that is applied in many areas around the world to control broad-leaf weed species in a number of crops (Hopenhayn-Rich *et al.*, 2002; Solomon *et al.*, 1996). It has been hypothesized that ATZ can interact with the endocrine system of amphibians and may have the potential to cause demasculinization of male frogs (Bisson and Hontela, 2002; Hayes *et al.*, 2002; Larson *et al.*, 1998). Possible interactions of ATZ with various endocrine functions have been the subject of numerous studies (Carr *et al.*, 2003; Cooper *et al.*, 2000; Crain *et al.*, 1999; Gluth and Hanke, 1985; Hayes *et al.*, 2002; Oulmi *et al.*, 1995; Stoker *et al.*, 2000) that are reviewed in a series of papers (Hecker *et al.*, 2004; Spano *et al.*, 2004). The most recent issue raised about ATZ is its potential to cause demasculinization of male frogs through an estrogenic or an anti-androgenic mechanism at environmentally relevant concentrations (Hayes *et al.*, 2002). On the basis of results from a study where male adult *X. laevis* had decreased plasma T concentrations after exposure to 25 µg/l atrazine, Hayes *et al.* (2002) suggested that this decrease in plasma T was due to an increase in testicular aromatase activity that depleted T levels via increased E2 synthesis. Although ATZ does not bind to the estrogen receptor (ER), at relatively high concentrations it has been found to upregulate *CYP19* gene expression in a human adrenocarcinoma cell line (H295R) (Sanderson *et al.*, 2000). In another study with a human choriocarcinoma cell line (JEG-3), exposure to the ATZ metabolite diaminochlorotriazine (DACT) but not ATZ resulted in a decrease of aromatase activity and an increase in EROD activity for both ATZ and DACT (Oh *et al.*, 2003). Another study using an artificial mammalian *in vitro* system found that ATZ is a competitive inhibitor of phosphodiesterase and can increase concentrations of the second messenger cAMP (Roberge *et al.*, 2004), which was

¹ To whom correspondence should be addressed at 218c National Food Safety & Toxicology Center, Michigan State University, East Lansing, MI 48824. Fax: +1 (517) 381-1435. E-mail: heckerm@msu.edu.

shown to induce aromatase in H295R cells (Sanderson *et al.*, 2000, 2002). Because aromatase is the enzyme that converts testosterone (T) to estradiol (E2), it has been speculated that upregulation of this enzyme in the testis could result in increased E2 production and a concomitant decrease in T, leading to demasculinization effects. However, a series of studies conducted to investigate this mode of action have not found any significant effects of ATZ on aromatase *in vitro* in a rat cell line (Heneweer *et al.*, 2004) or *in vivo* in juvenile fish (Kazeto *et al.*, 2004), developing (Coady *et al.*, 2005) and adult (Hecker *et al.*, 2004) frogs, and juvenile reptiles (Crain *et al.*, 1999). This inconsistency is further complicated by the fact that the above research was conducted using different species which, as demonstrated by the comparison of a rat and a human cell line (Heneweer *et al.*, 2004), may respond differently to ATZ. It is known that there are substantial differences in steroid hormone regulatory mechanisms among different species but also among developmental stages and between genders of the same species, and even among different tissues in the same animal. The inconsistencies in the literature regarding the effects of atrazine on estrogen synthesis are likely to be due to such differences. Therefore, the present study was designed to specifically analyze the mechanism of ATZ action for amphibians proposed by Hayes *et al.* (2002), induction of aromatase in testis of male adult *X. laevis*.

In a series of studies conducted to assess potential effects of ATZ on male amphibians *in vivo* (Coady *et al.*, 2004; Hecker *et al.*, 2004, 2005), there were no changes in gonadal aromatase activity in ATZ-treated animals when compared to controls (CON). The reason for the negative results may be due to one of two factors: (1) ATZ does not affect aromatase in the investigated amphibian species, or (2) because of the very low enzyme activities in testicular tissues, it was not technically feasible to determine subtle changes in aromatase with the applied test system. Therefore, in the present study we used a multiple line of evidence approach that included highly sensitive direct and indirect measures of effects on estrogen synthesis.

MATERIALS AND METHODS

Test materials and animals. Atrazine (CAS number 1912-24-9; purity 97.1%) was obtained from Syngenta Crop Protection Inc. (Greensboro, NC). UV-treated and carbon-filtered laboratory freshwater was used for acclimatization of frogs in the laboratory and for all subsequent exposures. Adult sexually mature male *X. laevis*, 30–50 g, were purchased from Xenopus Express (Plant City, FL). Prior to exposure, all animals were immersed for 3–4 h in 0.06% NaCl solution to reduce the risk of possible infections, as recommended by the supplier (Xenopus Express). Frogs were acclimatized for several weeks at Michigan State University's Aquatic Toxicology Laboratory before the experiment was initiated. During acclimatization, animals were held in 600-l fiberglass tanks under flow-through conditions. Average water temperature was $19.6^{\circ} \pm 1.3^{\circ}\text{C}$ in all treatments over the entire course of the experiment. The photoperiod was 12 h light:12 h dark. Animals were fed Nasco frog brittle (Nasco, Fort Atkinson, WI) three times per week *ad libitum*.

Experimental design. The exposures were conducted under static renewal conditions for 36 days, with 50% test solution renewal every 3 days. Feeding regimen, temperature, and photoperiod during the exposures were consistent with acclimatization conditions. In this system, African clawed frogs (*X. laevis*) were exposed to concentrations of 0, 1, 25, and 250 μg ATZ/l (nominal). Atrazine stock solutions (12 mg/l) were made up in UV-treated laboratory freshwater before each water renewal, and diluted appropriately in 5 gallon carboys before being added to the different treatment aquaria. Frogs were exposed individually in 40-l aquariums with 15 replicates per ATZ treatment, and 20 replicates for the control (CON) group. Volume of the exposure solution was 10 l per aquarium. There were no differences in water quality (NO_2 , NH_3 , dissolved oxygen, pH, hardness, and temperature) among tanks or treatments.

Exposure verification. Concentrations of ATZ in the aquaria were confirmed by taking water samples randomly from aquaria every 5–7 days from all treatments during the exposure period. Atrazine concentrations in the aqueous samples were quantified by GC/MS. The average procedural recovery rates were $92 \pm 7\%$. The limit of quantification for the applied method was 0.025 μg ATZ/l.

Tissue collection. Frogs were sampled on exposure day 36 during a time window of 4 h between 0700 and 1100 hours to minimize diurnal effects on plasma hormone titers. *X. laevis* were anesthetized by immersion in 250 mg/l MS-222 (tricaine methanesulfonate). Immediately thereafter, blood was collected by cardiac puncture with heparinized syringes, and plasma was separated by centrifugation at $10,000 \times g$ for 10 min. Plasma was then stored at -80°C until further analysis. Wet weight and snout-vent length were measured. Gonads were removed, measured, weighed, and morphological abnormalities were recorded if present. Each gonad was split into two equal portions, and the portions from the left and right gonad were mixed to be able to capture possible effects on both gonads with each sample. After dissection, the gonads were immediately stored in liquid nitrogen for subsequent measures of aromatase activity (arom) and *CYP19* gene expression (*CYP19*gen). The gonadosomatic index (GSI) and the condition index (CI) were calculated (equations 1 and 2).

$$\text{GSI}(\%) = (\text{gonad weight}/\text{body weight}) \times 100 \quad (1)$$

$$\text{CI}(\%) = (\text{body weight}/\text{snout vent length}) \times 100 \quad (2)$$

Aromatase activity. Aromatase activity was measured by use of the tritiated water release assay according to the protocol of Lephart and Simpson (1991), with modifications. Between 0.3 g and 0.5 g of gonadal tissue (one testis) was homogenized in 600 μl of ice-cold gonad buffer (50 mM KPO_4 , 1 mM EDTA, 10 mM glucose-6-phosphate, pH 7.4). The homogenate was incubated with 300 nM ^3H -androst-4-ene-3,17-dione (25.9 Ci/nmol; Lot No. 3467-067; Cat. No. NET-926; New England Nuclear, Newton, MA), 0.5 IU/ml glucose-6-phosphate dehydrogenase (Sigma Cat. # G6378), and 10 mM NADP (Sigma Cat. # N-0505) at 37°C and 5% CO_2 for 90 min. Tritiated water released from each sample was extracted, and its activity was determined by liquid scintillation. Aromatase activity was expressed as femtomoles of androstenedione converted per hour per milligram of protein. The specificity of the reaction for the substrate was determined by use of a competitive test with non-labeled androstenedione, and the use of the specific aromatase inhibitor fadrozole (provided as a gift by Novartis Pharma AG, Basel, Switzerland). Addition of large amounts of androstenedione reduced tritiated water formation to the concentrations found in the tissue blanks. Furthermore, addition of fadrozole during the tritium-release assay reduced aromatase enzyme activity in a dose-dependent manner at concentrations of 5 μM and greater, resulting in complete inhibition of enzyme activity to the levels measured in the blanks. This demonstrated that the activity being measured was specific for aromatase. Protein concentrations were determined using the Bradford assay (Bradford, 1976) with bovine serum albumin as the protein standard (Sigma, St. Louis, MO).

TABLE 1
Primer Sequences (forward and reverse) Used for the Amplification of the CYP19 and GAPDH Genes

	CYP19	GAPDH ^a
Forward primer	5' CCGTTCCATATCGTTACTTCC3'	5' GCTCCTCTCGCAAAGGTCAT3'
Reverse primer	5' GCATCTTCCTCTCAATGTCTG3'	5' GGGCCATCCACTGTCTTCTG 3'
Amplicon length (bp)	140	101

^aPrimers were designed in accordance with the procedure of Wiechmann and Smith (2001).

Reverse transcription real-time PCR to measure CYP19 gene expression. CYP19gen in testes of male African clawed frogs was measured by means of a quantitative reverse transcription real-time polymerase chain reaction (Q-RT-PCR) technique. Briefly, total RNA was isolated from the gonad tissue through lysis in guanidine thiocyanate and β-mercaptoethanol (Cat No. Z3100, Promega, Madison, WI). The concentration of total RNA was determined in a TD700 laboratory fluorometer (Turner Design, Sunnyvale, CA) with RiboGreen RNA quantitation reagent (Molecular Probes, Inc., Eugene, OR). A total amount of 500 ng RNA was used to synthesize single-strand cDNA by reverse transcription (Cat. No. 11904-018, Invitrogen, Carlsbad, CA). Prior to reverse transcription, total RNAs to be reverse-transcribed were treated with DNase I to remove potential chromosomal DNA.

To determine accumulation of the PCR product, SYBR Green I dye was used as a real-time reporter that measured double-stranded DNA. In the Q-RT-PCR assay, the level of expression of the CYP19 gene was normalized to an internal control "housekeeping" gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Because GAPDH is a relatively consistently expressed gene, it is suitable for use as an internal standard to correct for internal variation caused by a range of parameters in real-time RT-PCR assays (Raaijmakers *et al.* 2002). GAPDH was selected as the internal control because it has been reported to be less expressed than other housekeeping genes such as β-actin and 18S rRNA (Wiechmann and Smith, 2001), a condition that is preferred when making comparisons to genes such as CYP19 that are expressed at a low level. Primer sequences are presented in Table 1.

Reverse-transcribed samples were subjected to real-time PCR at dilutions of 1:4 and 1:20 for CYP 19 and GAPDH, respectively. All PCR reactions were performed using a Smart Cycler II (Cepheid, Sunnyvale, CA). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 59°C for CYP19, and 64°C for GAPDH for 50 s and 72°C for 30 s.

Data were analyzed using the standard curve method. A standard curve for each transcript was generated from a dilution series of synthesized plasmid DNA standards and a linear regression model was applied to quantify the data (equation 3):

$$Y = aX + b, \quad (3)$$

where Y is the C_T value, a is the slope of the standard curve, X is the logarithm of the total copy numbers, and b represents the y-intercept.

Real-time PCR data were expressed as a C_T value, the cycle number obtained when a reaction reaches the threshold (level of detection of increasing fluorescence) (Girault *et al.*, 2002). Determination of transcript abundance (mean of C_T value) of the CYP19 and the GAPDH gene was conducted in triplicate. Expression ratio (ER) of mRNA copy numbers between CYP19 and GAPDH in the same sample was calculated (equation 4).

$$ER = 10^{(X_{CYP19})/10} / 10^{(X_{GAPDH})} \quad (4)$$

Where X_{CYP19} and X_{GAPDH} are the logarithms of the copy numbers in the same sample for CYP19 and GAPDH, respectively. The fold change in CYP19gen due to exposure to ATZ was then calculated by dividing the mean ER in an ATZ treatment group by that of the CON group (equation 5).

$$\text{Fold change ATZ} = ER_{ATZ} / ER_{CON} \quad (5)$$

Plasma sex steroids. Plasma samples (50 μl) were extracted twice with 2.5 ml diethyl ether. Ether fractions were nitrogen evaporated to dryness, and the hormone extract was re-constituted in 250 μl phosgel buffer for use in the enzyme-linked immunosorbent assay (ELISA). A ³H-labeled steroid was added to each plasma sample before extraction as an internal recovery standard. After the extraction procedure, a fraction of the final extract was quantified in a liquid scintillation counter to test for recoveries. Concentrations of T and E2 in plasma extracts were measured by competitive ELISA as described by Cuisset *et al.* (1994) with modifications (Hecker, 2001). In the competitive ELISA, steroids of the sample extract compete with acetylcholinesterase-labeled steroid for the binding site on the polyclonal rabbit anti-steroid antibody. Antiserum to T was obtained from Dr. D. E. Kime (Sheffield, UK). Cross reactivities of the T antiserum are described in Nash *et al.* (2000). The antiserum to E2 (Cayman Chemical, Ann Arbor, MI) cross-reacted with estradiol-3-glucuronide (17%), estrone (4%), estriol (0.57%), T (0.1%), and 5α-dihydrotestosterone (0.1%). For all other steroids, cross-reactivities were less than 0.1%. The steroid ELISAs were performed using 96-well COSTAR high binding plates (Corning Inc, product # 3590).

Statistical analyses. Data were tested for normality using a Kolmogorov-Smirnov one-sample test. If necessary, data were transformed to the logarithms (\log_{10}) to approximate a normal distribution, as was the case for plasma T, aromatase activity, and CYP19gen. When data (or log transformed data) were normally distributed and variance was homogeneous, analysis of variance (ANOVA) and Fisher's least-significant-difference (LSD) test were applied to test for significant differences among treatments. When data violated parametric assumptions, as was the case for plasma E2, the non-parametric Kruskal Wallis test followed by the Mann-Whitney U -test were used to determine differences among treatments. Possible relationships between the different parameters investigated were analyzed with the correlation model described by Spearman. Statistical significance was accepted when $p < 0.05$.

RESULTS

Exposure Verification

Over the entire course of the exposure, 12 water samples were taken from the CON group, and five samples were drawn from each of the ATZ treatments. Average ATZ concentrations measured in the different treatments were close to nominal concentrations at 0.8 ± 0.11 , 24.6 ± 2.1 , and 258.6 ± 29.1 μg ATZ/l (mean ± SEM). All water samples taken from the CON tanks had non-detectable ATZ concentration (<0.025 μg/l). Atrazine concentrations were stable in all treatments over the entire duration of the experiment.

Biological Responses

A total of $n = 61$ frogs (CON: 19; 1 μg/l ATZ: 15; 25 μg/l ATZ: 14; 250 μg/l ATZ: 13) were analyzed. The mortality rate

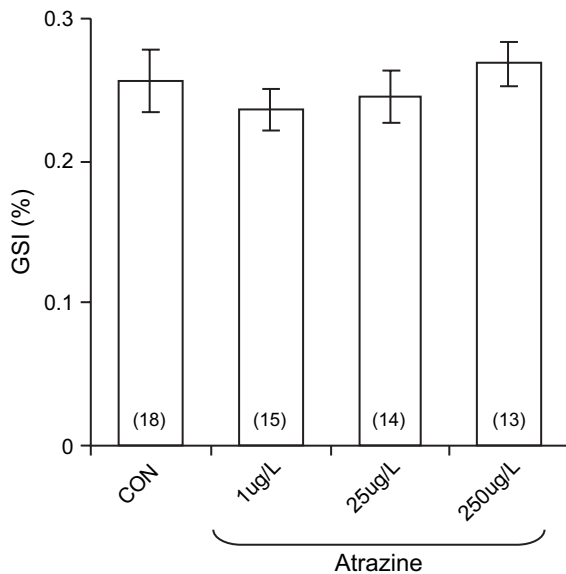


FIG. 1. Mean gonadal somatic index (GSI) for adult male *Xenopus laevis* exposed to control water (CON), and atrazine. Error bars = standard error. There were no statistically significant differences in GSI between treatments (ANOVA). Numbers in parentheses indicate number of individuals per treatment.

was low over the entire course of the experiment: one frog from the CON group died, and one and two frogs, respectively, from the 25 and 250 $\mu\text{g/l}$ ATZ treatments died. The condition of frogs was similar throughout all treatments, with average CIs between $0.68 \pm 0.03\%$ (ATZ-250) and $0.73 \pm 0.02\%$ (ATZ-25). No morphological abnormalities of the testes such as discontinuous gonads or occurrence of ovarian tissue were observed for any of the analyzed frogs.

Possible effects of ATZ treatment on gonad size were determined using the gonadosomatic index (GSI). The GSI measured between $2.4 \pm 0.01\%$ (mean \pm SEM; ATZ-1) and $2.7 \pm 0.02\%$ (ATZ-250) (Fig. 1). None of the tested ATZ concentrations had a statistically significant effect on the GSI

($p = 0.633$, ANOVA). Neither gonadal aromatase activity nor gonadal *CYP19*gen were significantly different among any of the treatments (Fig. 2). Aromatase activities were detectable at average activities between 6.3 (ATZ-25) and 8.1 fmol/h/mg protein (ATZ-1), and, in many cases, were only slightly greater than the detection limit of the applied assay (approx. 1.8 fmol/h/mg protein; Fig. 2a). The average relative expression of *CYP19* mRNA was not significantly different among ATZ treatments (Fig. 2b). No correlations between aromatase activity and *CYP19*gen were observed, except in the greatest exposure group (250 $\mu\text{g/l}$ ATZ), where a significant negative correlation was observed between *CYP19* mRNA and aromatase activity ($R^2 = 0.675$, $p = 0.001$). Except for GSI and *CYP19* mRNA copy number in the 1 μg ATZ/l treatment, none of the other parameters were correlated with either aromatase activity or *CYP19*gen.

With the exception of plasma T concentrations of frogs from the 250 $\mu\text{g/l}$ treatment group, none of the ATZ treatments significantly affected plasma T or E2 concentrations in *X. laevis* (Fig. 3a and b). There was a concentration-dependent negative relationship between average plasma T concentrations and ATZ exposure concentration. The mean plasma T concentration for frogs exposed to 25 μg ATZ/l was slightly less (10.8 ng T/ml plasma) than that of the control frogs (15.3 ng T/ml plasma), but the effect was not statistically significant. The mean plasma concentration of T (7.1 ng T/ml plasma) of frogs exposed to the 250 μg ATZ/l treatment was significantly lower than that of the controls (Fisher's LSD test: $p = 0.036$) (Fig. 3a).

No statistically significant effects were observed for plasma E2 concentrations in male *X. laevis* among treatments. E2 concentrations in male *X. laevis* were relatively constant throughout all treatments, and ranged from 3.1 ng/ml (CON) to 3.6 ng/ml (25 $\mu\text{g/l}$ ATZ) (Fig. 3b). Neither plasma T nor E2 was correlated with GSI, aromatase activity, *CYP19*gen, or with each other.

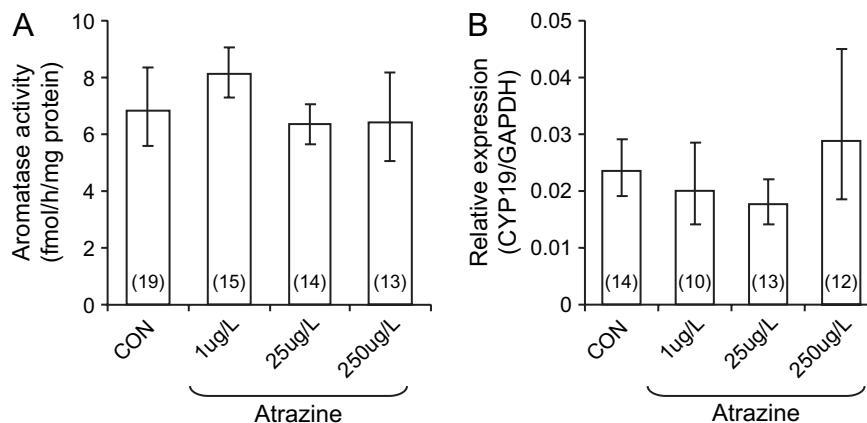


FIG. 2. Geometric mean aromatase activities (A) and *CYP19* mRNA gene expression (B) detected in adult male *X. laevis* exposed to control water (CON), and atrazine. Error bars = standard error. There were no statistically significant differences in aromatase activity or gene expression between treatments (ANOVA). Numbers in parentheses indicate number of individuals per treatment.

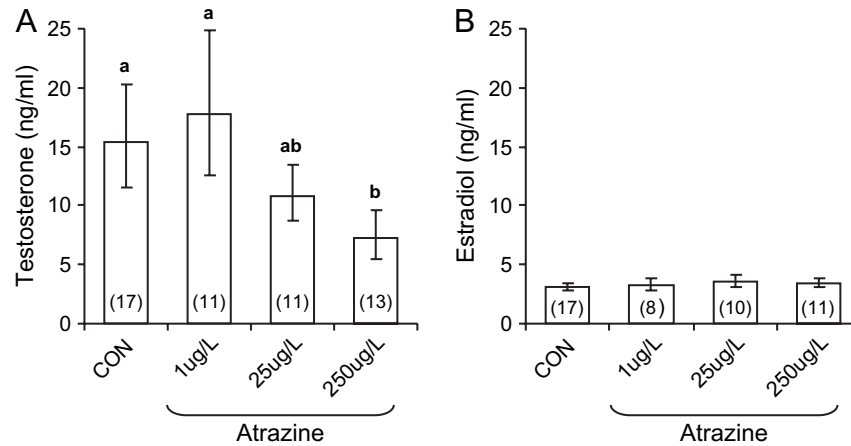


FIG. 3. Geometric mean plasma testosterone (A) and estradiol (B) concentrations detected in adult male *X. laevis* exposed to control water (CON) and atrazine. Error bars = standard error. Letters (a–b) signify tests for statistically significant differences at $p < 0.05$ between treatments. Numbers in parentheses indicate number of individuals per treatment.

DISCUSSION

The objective of this study was to identify possible effects of ATZ concentrations (ranging from ecologically relevant to very great) on steroidogenic functions in male *X. laevis*. Except for plasma T concentrations in frogs from the greatest (250 µg ATZ/l) treatment group, none of the tested ATZ concentrations resulted in a significant effect on any of the biological parameters measured relative to unexposed frogs.

The mean gonadosomatic index (GSI) of unexposed frogs (0.26%) was similar to that reported from a different laboratory study with male *X. laevis* under different housing conditions (0.28%; Hecker *et al.*, 2005), which also did not find any significant effects of ATZ on gonad size at similar exposure concentrations (10 µg ATZ/l and 100 µg ATZ/l). This shows that ATZ, at the concentrations and exposure time (36 days) tested in the present study, does not affect gonad size of adult male *X. laevis*.

The statistically significant reduction of plasma T concentration observed in frogs exposed to the greatest concentration of ATZ (250 µg/l) was similar to decreases in plasma T concentrations in male adult frogs, as well as other vertebrate species, that have been reported previously (Hayes *et al.*, 2002; Spano *et al.*, 2004). While Hayes *et al.* (2002) reported a significant decrease of plasma T concentration in male adult *X. laevis* at an ATZ concentration of 25 µg/l, we observed a significant decrease in plasma T only at the highest ATZ concentration tested (250 µg/l). This result is similar to that reported for a study with goldfish (*Carassius auratus*) in which a significant decrease of plasma T titers of male fish was observed when they were exposed to 1000 µg ATZ/l but not at 100 µg ATZ/l (Spano *et al.*, 2004). Although Spano *et al.* (2004) found a concomitant increase of plasma E2 in goldfish exposed to the greatest concentration of ATZ, no effect on plasma estrogen was observed in our study. These authors

hypothesized that the reduction in T was due to an induction of aromatase activity resulting in a depletion of T and an increased formation of endogenous E2. Hayes *et al.* (2002) suggested a similar mechanism of action as being possible for frogs, but they did not report E2 concentrations. Thus, this conclusion was based only on the observation of reduced plasma T in males. That all concentrations of ATZ tested in their study did not affect aromatase activity or *CYP19*gen suggests that the theory proposed by Hayes *et al.* (2002) is not correct. This is also similar to a different study with a cyprinid fish species, the zebrafish (*Danio rerio*), that did not find any effects of atrazine up to 100 µg/l on any of the two *CYP19* isoforms in this species (Kazeto *et al.*, 2004). However, differences in the regulation and sensitivity to disturbance of steroidogenic gene expression among groups of animals such as teleost fish and frogs must be considered, and therefore, great care must be taken when comparing these results. Finally, the lack of statistically significant effects of ATZ on either aromatase activity or expression of the *CYP19* gene suggests that any effects of atrazine on plasma hormone concentrations occurring only at concentrations that are tenfold greater than the effect level of 25 µg/l reported for male adult *X. laevis* by Hayes *et al.* (2002) are unlikely to be mediated through an upregulation of aromatase (Hecker *et al.*, 2004, 2005). Interestingly, a significant and negative correlation between *CYP19*gen and testicular aromatase activity was observed in frogs exposed to 250 µg/l atrazine. This could be due either to catalytic inhibition of aromatase or to an increase in gene expression that was not followed by the catalytic activation of the enzyme. However, there was neither a trend toward greater *CYP19*gen nor toward lesser catalytic activities in the 250 µg/l group when compared to the controls, a finding that could explain the negative correlation between enzyme activity and gene expression. The exact cause for this relationship remains unclear, and the possibility that exposure to 250 µg/l or greater

may have an influence on these processes cannot be rejected at this time. Nevertheless, given the great concentrations of ATZ that are rather atypical for agricultural scenarios where ATZ concentration even during application times seldom exceed 20 µg/l (Solomon *et al.*, 1996), and given the very low absolute aromatase enzyme activities or gene expression levels, it is unlikely that such an effect is of any environmental or biological relevance.

Various studies using *in vitro* and *in vivo* systems have found that ATZ has the potential to affect phase I metabolism by up-regulating cytochrome P450 enzyme activities (Chang *et al.*, 2005; Hanioka *et al.*, 1998; Oh *et al.*, 2003). Phase I enzymes such as P4501A1 and P4503A4 have been reported to be involved in steroid metabolism including androgens such as T (Liddle *et al.*, 1998; Waxman, 1988; Waxman *et al.*, 1991). Thus, induction of these enzymes by atrazine could explain the reduction of plasma T observed in this and other studies (Hayes *et al.*, 2002; Spano *et al.*, 2004). However, this study was not designed to test for a metabolic reduction of sex steroids by atrazine, and therefore, the specific mode of atrazine action in *X. laevis* remains unclear. What is clear is that atrazine did not induce aromatase activity in adult male *X. laevis* over a wide range of ecologically relevant concentrations of ATZ.

The extent to which the suppression of T observed in frogs exposed to 250 µg ATZ/l may affect reproductive functions is unclear. A different study found an increase in alterations of testis organization, signs of testicular tissue damage, and elevated levels of ovarian atresia in goldfish exposed for 21 days to the relatively great atrazine concentration of 1000 µg/l (Spano *et al.*, 2004). The authors of that study concluded that the observed effects could be due to a general toxic action of atrazine involving the induction of, *e.g.*, programmed cell death. It cannot be excluded that comparable effects may occur in frogs exposed to similarly high concentrations of ATZ. Therefore, it cannot be ruled out at this point that ATZ may affect reproductive functions under similar exposure conditions. This would also support the hypothesis that atrazine is likely to act through a more general mechanism such as increase in metabolic activity rather than specifically targeting estrogen synthesis. A different study reported that ATZ inhibited phosphodiesterase activity *in vitro* and as a result led to an increase of the second messenger cAMP (Roberge *et al.*, 2004). cAMP acts as a second messenger for the enzyme aromatase among others, and an increase may therefore explain the increase in aromatase activity observed in some *in vitro* studies (Sanderson *et al.*, 2000, 2002). cAMP, however, is also one of the most general regulating factors involved in G protein-mediated signal cascades, making it difficult to extrapolate from effects on the regulation of this second messenger to a specific type of action such as upregulation of aromatase activity. To date, the only well-established members of the P450 gene super family that are regulated by cAMP are the steroid hydroxylases; however, evidence is increasing

that cAMP also enhances other mono-oxygenases, including CYP1A1 (Nemoto and Sakurai, 1992; Zhang *et al.*, 1997). However, the cAMP-regulated activation of certain protein kinases is both a species- and tissue-specific process, and, to our knowledge, no detailed data on these pathways in gonadal tissues of amphibians are available.

Based on the results from this study, we conclude that atrazine does not affect testicular aromatase in male adult *X. laevis*, either at the gene expression level or at the enzyme activity level. It does, however, appear to affect plasma T homeostasis at the relatively high concentrations of 250 µg ATZ/l and above, and thus future studies focusing on a more general mode of toxic action such as effects on phase I metabolism are warranted. When assessing the risk that ATZ may pose to wild populations of frogs, it should be noted that the observed changes in steroid homeostasis occurred at relatively great concentrations of ATZ that were approximately ten to twenty times greater than peak concentrations observed in the environment which usually do not exceed 20 µg/l in surface waters (Solomon *et al.*, 1996).

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