

## Plasma concentrations of estradiol and testosterone, gonadal aromatase activity and ultrastructure of the testis in *Xenopus laevis* exposed to estradiol or atrazine

Markus Hecker<sup>a,\*</sup>, Wan Jong Kim<sup>b</sup>, June-Woo Park<sup>a</sup>, Margaret B. Murphy<sup>a</sup>, Daniel Villeneuve<sup>a</sup>, Katherine K. Coady<sup>a</sup>, Paul D. Jones<sup>a</sup>, Keith R. Solomon<sup>c</sup>, Glen Van Der Kraak<sup>d</sup>, James A. Carr<sup>e</sup>, Ernest E. Smith<sup>f</sup>, Louis du Preez<sup>g</sup>, Ronald J. Kendall<sup>f</sup>, John P. Giesy<sup>a,h</sup>

<sup>a</sup> Department of Zoology, Aquatic Toxicology Laboratory, 218C National Food Safety and Toxicology Center, Center for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, USA

<sup>b</sup> Department of Biology, Soonchunhyang University College of Natural Sciences, 336-745 Asan-si, Chungcheongnam-do, South Korea

<sup>c</sup> Centre for Toxicology and Department of Environmental Biology, University of Guelph, Guelph, Ont., Canada N1G 2W1

<sup>d</sup> Department of Zoology, University of Guelph, Ont., Canada N1G 2W1

<sup>e</sup> Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409, USA

<sup>f</sup> Department of Environmental Toxicology, Texas Tech University, Lubbock, TX 79416, USA

<sup>g</sup> Potchefstroom University for Christian Higher Education, Potchefstroom 2520, South Africa

<sup>h</sup> Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong, SAR, China

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### Abstract

The ultrastructure of testicular cells of adult male African clawed frogs (*Xenopus laevis*) exposed to either estradiol (0.1 µg/L) or 2-chloro-4-ethylamino-6-isopropyl-amino-*s*-triazine (atrazine; 10 or 100 µg/L) was examined by electron microscopy and compared to plasma concentrations of the steroid hormones, testosterone (T) and estradiol (E2), testicular aromatase activity and gonad growth expressed as the gonado-somatic index (GSI). Exposure to E2 caused significant changes both at the sub-cellular and biochemical levels. Exposure to E2 resulted in significantly fewer sperm cells, inhibition of meiotic division of germ cells, more lipid droplets that are storage compartments for the sex steroid hormone precursor cholesterol, and lesser plasma T concentrations. Although not statistically significant, frogs exposed to E2 had slightly smaller GSI values. These results may be indicative of an inhibition of gonad growth and disrupted germ cell development by E2. Concentrations of E2 in plasma were greater in frogs exposed to E2 in water. Exposure to neither concentration of atrazine caused effects on germ cell development, testicular aromatase activity or plasma hormone concentrations. These results suggest that atrazine does not affect testicular

\* Corresponding author. Tel.: +1 517 712 6258; fax: +1 517 432 2310.

E-mail address: heckerm@msu.edu (M. Hecker).

function. In contrast, exposure of male *X. laevis* to E2 led to sub-cellular events that are indicative of disruption of testicular development, and demasculinization processes (decrease of androgen hormone titers). These results indicate that atrazine does not cause responses that are similar to those caused by exposure to E2.

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## 1. Introduction

There is concern about natural and synthetic substances that have the potential to interfere with the “synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for maintenance of homeostasis, reproduction, development, and/or behavior” (Ankley et al., 1998). It has been hypothesized that such compounds may elicit a variety of adverse effects in both humans and wildlife, including promotion of hormone-dependent cancers, reproductive tract disorders, and reduction in reproductive fitness (Gray et al., 1997). Substances that can mimic estrogen through either a direct (hormone receptor-mediated) or indirect mechanism have been of particular concern (Kavlock et al., 1996). 2-Chloro-4-ethylamino-6-isopropylamino-*s*-triazine (atrazine) is a herbicide used in many areas of the world (Hopenhayn-Rich et al., 2002). Although atrazine was not found to bind to the estrogen receptor (ER), relatively great concentrations of atrazine (71 µg/L) resulted in up-regulation of the *in vitro* activity of aromatase in a human carcinoma cell system (H295R) (Sanderson et al., 2000). However, in a rat Leydig cell carcinoma cell line (R2C) atrazine did not induce aromatase activity (Heneweer et al., 2003). Since aromatase converts testosterone (T) to estradiol (E2), it has been speculated that up-regulation of this enzyme could result in increased estrogen production leading to a net estrogenic response.

Reports of amphibian population declines (Carey and Bryant, 1995; Stebbins and Cohen, 1995; Weller and Green, 1997) and an increased incidence of deformities in some frog populations (Kaiser, 1997; Burkhart et al., 1998; Gardiner and Hoppe, 1999) have been attributed to a number of possible causes including the effects of endocrine disrupting compounds in recent years (Kavlock, 1998). Some authors have attempted to link these responses with exposure to atrazine. For example, concentrations as low as 0.1 µg/L have been

reported to cause both gonadal abnormalities (e.g. occurrence of oocytes in testes) and demasculinization of the larynx, as indicated by reduced size of the laryngeal dilator muscle, in male African clawed frogs (*Xenopus laevis*) exposed from hatching through completion of metamorphosis (Hayes et al., 2002). However, other studies with juvenile *X. laevis* have not found effects on the size of the laryngeal dilator muscle, gonadal development, plasma sex steroid concentrations or gonadal aromatase activities at such low (0.1–1 µg/L) atrazine concentrations (Carr et al., 2003; Coady et al., 2004, *in press*). Based on experiments with adult male *X. laevis* that showed reduced plasma T concentrations after treatment with atrazine it was hypothesized by Hayes et al. (2002) that the above demasculinization effects were caused through the same mechanism, up-regulation of gonadal aromatase activity, as described earlier for a mammalian cell system (Sanderson et al., 2000). In contrast, under field conditions no such effects of atrazine on plasma T as well as on plasma E2 and gonadal aromatase activity could be observed (Hecker et al., 2004). This study was conducted to further elucidate the potential of atrazine to decrease plasma T and increase plasma E2 through up-regulation of aromatase activity in the male African clawed frog. In addition, the potential of atrazine to cause subtle ultrastructural changes in testicular cells was investigated. Furthermore, an objective of the study was to demonstrate whether these changes, if present, were indicative of histological changes, such as reduction in the size of the larynx and occurrence of testicular oocytes that have been reported by some authors. Since the proposed mechanism of feminization of *X. laevis* is through increased production of E2 and decreased plasma T the results of exposures to atrazine were also compared to effects on these same endpoints that were caused by aqueous exposure to E2. E2 was used as a model compound for estrogenic effects. While there have been numerous studies on the effects of E2 on testicular ultrastructure in a series of species including fish

and mammals (Islinger et al., 2003; Kizilay and Uygun, 2003), nothing is known about effects in amphibians. Therefore, in addition to the above goal – identify possible effects of atrazine on testicular ultrastructure in *X. laevis* – this study aimed to establish typical effects of exposure to E2 on testicular ultrastructure in adult *X. laevis* as a sub-cellular marker for estrogen exposure in amphibians.

While standard light microscopy provides some information on histology at the tissue level, electron microscopy has the advantage of elucidating subtle changes in function related structures in cell compartments that can be more directly related to physiological processes. Thus, ultrastructural examination was used to address these issues.

## 2. Materials and methods

### 2.1. Test materials and animals

Atrazine (CAS number 1912-24-9; 97.1% pure) was provided by Syngenta Crop Protection Inc. (Greensboro, NC, USA). 17 $\beta$ -estradiol (E2) (CAS number 50-28-2; 98% pure) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethanol (EtOH) (CAS number 64-17-5, 100% USP grade) was purchased from AAPER Alcohol (Shelbyville, KY, USA). Atrazine stock solutions (12 mg/L) were made up in UV-treated laboratory water before each water renewal, diluted appropriately in 5 gallon carboys and then added to the different treatment tanks. EtOH was used as a carrier solvent to deliver the E2 treatment. E2 stock solutions were diluted in laboratory freshwater at the appropriate concentration directly in the exposure tanks. The final EtOH concentration in the E2 treatment groups was 0.005%. Due to logistical constraints, an EtOH solvent control was not included in the study design. Adult male *X. laevis*, 30–50 g, were purchased from Xenopus Express (Plant City, FL, USA). Prior to exposure, all animals were acclimated for several weeks at Michigan State University's Aquatic Toxicology Laboratory. During acclimation, animals were held in 600 L fiberglass tanks under flow-through conditions. Water temperature was 18.5–23.5 °C. Photoperiod was 12-h light/12-h dark. Animals were fed Nasco frog brittle (Nasco, Fort Atkinson, WI, USA) three times per week ad libitum.

### 2.2. Experimental design

Animals were exposed in 600 L fiberglass tanks filled with 120 L of the appropriate test solution. Exposure was conducted under static renewal conditions, with 50% test solution renewal every 3 d. Feeding regimen, temperature, and photoperiod during the exposures were consistent with acclimation conditions. Average frog wet wt ( $\pm 1$  standard deviation (S.D.)) was  $42 \pm 6.6$  g. Initial loading was 14 frogs per tank with three replicate tanks per treatment (approximately 4.9 g frog/L test solution). Mortalities were one individual in one E2 treatment tank over the entire course of the experiment. Except for one control treatment tank, from which eight animals were collected, seven frogs were sampled on exposure day 49 from all other tanks, and anesthetized by immersion in 250 mg/L MS-222 (tricaine methanesulfonate). The remaining seven frogs were used in a different experiment the results of which will be reported elsewhere. Immediately thereafter, blood was collected from all animals by cardiac puncture into heparinized syringes, and plasma was separated by centrifugation at  $10,000 \times g$  for 10 min. In some cases not enough blood could be drawn which resulted in slightly lower overall sample sizes for the hormone data (CTR:  $n = 19$ ; ATZ-10:  $n = 19$ ; ATZ-100:  $n = 18$ ; E2:  $n = 19$ ). Wet weight and snout-vent length were measured, and the gonado-somatic index (GSI) was calculated (Eq. (1)).

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

From a subset of these animals (two per treatment), testes were saved for ultrastructural analyses. Water samples (15 mL) were collected from each of the three replicate tanks for each treatment both immediately before and shortly after each solution renewal (every third day). This made it possible to characterize variability in atrazine concentrations among replicate tanks. Phenotypic sex was determined by size and by external sexually dimorphic characteristics, such as the absence of cloacal folds. Sex was definitively confirmed by inspection of the gonads at the termination of each exposure. All animals used for the study were adult males.

The treatments tested in this study included two nominal concentrations of atrazine (ATZ-10, ATZ-100; 10 and 100  $\mu\text{g/L}$  in laboratory freshwater), control

(CTR; laboratory freshwater), and E2 (E2; 0.1 µg/L in laboratory freshwater) with three replicate tanks per treatment.

### 2.3. Exposure verification

Atrazine concentrations in the aqueous samples were quantified using Envirogard® Triazine 96-well Plate Kits (Strategic Diagnostics, Newark, DE, USA). The method detection limit (MDL) and limit of quantification (LOQ) for the plate kits were determined based on guidance presented elsewhere (Eaton et al., 1995). The manufacturer's estimated lower limit of detection (LLD) was 0.02 µg/L (Strategic Diagnostics kit #72110). The standard deviation of replicate measures ( $n=16$ ) of water spiked with 0.1 µg/L atrazine (approximately,  $5\times$  LLD) was determined. The resulting S.D. was multiplied by a Student's  $t$ -value for  $p=0.01$ , 15 degrees of freedom to estimate the MDL (0.074 µg/L). The LOQ was defined as  $3\times$  the MDL which resulted in a reportable concentration of 0.22 µg/L.

### 2.4. Tissue preparation for ultra structural analyses

Testes from specimens for electron microscopy analysis were removed immediately after anesthetization, and fixed in Karnovsky's fixative (2% glutaraldehyde and 2.5% para-formaldehyde in 0.1 M phosphate buffer, pH 7.2) for 4 h and followed by post-fixation in 1% osmium tetroxide for 1 h at 4 °C. Samples were then dehydrated in ethanol, rinsed in propylene oxide and embedded in Araldite mixture. Blocks were sectioned with diamond knives on an ultra microtome (Reichert Supernova, Leica Co.). Ultra-thin sections (70 nm in thickness) were mounted on copper grids and double stained with uranyl acetate and lead citrate (Stampal and Ward, 1964). Sections from each group were randomly selected, viewed and photographed in a JEM transmission electron microscope for ultra structural analysis. Multiple sections were randomly picked per sample for observation (approximately 5–10). Developing germ and Leydig cells were assessed through random selection on the fluorescent screen within an area of  $5\ \mu\text{m} \times 7\ \mu\text{m}$ . Observations were made at a magnification of 10,000 $\times$ .

### 2.5. Steroid hormone analyses

Plasma samples were extracted twice with diethyl ether, and concentrations of E2 and T were measured by competitive enzyme-linked immunosorbent assay (ELISA) as described by Cuisset et al. (1994) with modifications (Hecker et al., 2002). In this competitive ELISA, plasma steroid competes 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), and cross-reacted with 5-dihydrotestosterone (46%), 5-dihydrotestosterone (19%), 5-androstane-3,17-diol (3.7%), 11-hydroxytestosterone (3.3%), 5-androstane-3,17-diol (2.7%), 5-androstane-3,17-diol (2.5%), 11-ketotestosterone (0.85%), estradiol (0.54%), 4-androstenedione (0.47%), 4-androstenetrione (0.31%), and 17,20P (0.18%) at the 50% displacement level (Nash et al., 2000). The antiserum to E2 (Cayman Chemical, Ann Arbor, MI, USA) was reported to cross-react with estradiol-3-glucuronide (17%), estrone (4%), estriol (0.57%), T (0.1%) and 5 $\alpha$ -dihydrotestosterone (0.1%). For all other steroids cross-reactivities were reported as less than 0.1%. The steroid ELISAs were performed using COSTAR high binding plates (COSTAR, Bucks, UK). The working ranges of these assays were determined as follows: testosterone (0.78–800 pg/well); 17 $\beta$ -estradiol (0.78–800 pg/well). Validation of this test method for use with plasma from *X. laevis* was reported elsewhere (Hecker et al., 2004).

### 2.6. Aromatase activity

Aromatase activity was measured following the protocol of Lephart and Simpson (1991) with minor modifications. Less than 0.5 g of gonadal tissue was homogenized in 600 µL of ice-cold gonad buffer (50 mM KPO<sub>4</sub>, 1 mM EDTA, 10 mM glucose-6-phosphate, pH 7.4). The homogenate was incubated with 21.33 nM <sup>3</sup>H-androst-4-ene-3,17-dione (25.9 Ci/nmol; lot no. 3467-067; cat. no. NET-926; New England Nuclear, Newton, MA, USA), 0.5 IU/mL glucose-6-phosphate (Sigma cat. no. G6378), and 1 mM NADP (Sigma, cat. no. N-0505) at 37 °C and 5% CO<sub>2</sub> for 120 min. Tritiated water released from each sample was extracted and activity determined by liquid scintillation counting. Aromatase activity was expressed as fmol of androstenedione converted per hour per milligram

protein. The specificity of the reaction for the substrate was determined by use of a competitive test with non-labeled androstenedione. Addition of  $7.5 \mu\text{l}$  of  $5.6 \times 10^2 \mu\text{M}$  4-androsten-4-ol-3,17-dione reduced tritiated water formation to the levels found in the tissue blanks, which demonstrated that the activity being measured was specific for aromatase. The within- and among-day CVs for the assay were determined to be 3.8 and 11.3%, respectively (Hecker et al., 2004).

### 2.7. Statistical analyses

Statistical analyses were performed on log-transformed atrazine, E2 and T data. GSI data was not transformed. Kolmogorov–Smirnov’s one sample test was used to assess whether data sets were normally distributed. A combination of Cochran’s C-test, Bartlett’s test, and Levene’s test were used to test for homogeneity of variance across data sets being compared. When data (or log-transformed data) were normally distributed and variance was homogenous, analysis of variance (ANOVA) and the *t*-test were used to detect significant differences among tank means or geometric means. When data violated parametric assumptions, the non-parametric Kruskal–Wallis test was used to detect differences among treatment groups. If a significant difference was indicated, the Mann–Whitney *U*-test was used to determine differences between treatments. The power (based onto a non-central *t*-distribution) to detect significant differences between the different exposure groups was determined for the biochemical parameters and GSI considering possible tank effects, which were estimated using the intra-class correlation (ICC) derived from a nested analysis of variance. To test for significant differences between before-renewal and after-renewal atrazine concentrations, the non-parametric Wilcoxin signed-rank test was used to test the hypothesis that the median difference between log-transformed before-renewal and after-renewal atrazine concentrations was zero. The criterion for significance in all statistical tests was  $p < 0.05$ .

## 3. Results

### 3.1. Exposure verification

Average atrazine concentration in the ATZ-10 and ATZ-100 treatment tanks were approximately

Table 1

Mean atrazine concentrations in water sampled from exposure tanks

Treatment	Mean ( $\mu\text{g/L}$ )	S.D.
CTR	<0.074	0.057
E2	<0.074	0.020
ATZ-10 $\mu\text{g/L}$	11.6	1.68
ATZ-100 $\mu\text{g/L}$	107	20.7

All samples were analyzed in duplicate. Samples were collected from each of the three replicate tanks for each treatment, both immediately before and shortly after solution renewal, every 3 d over the 49-d exposure. Means represent the average concentration among all three replicate tanks. There were no significant differences in atrazine concentrations among replicate tanks within a treatment ( $p = 0.485\text{--}0.948$ ).

100–120% of nominal (Table 1). Atrazine was not detectable in the CTR or E2 tanks. There were no significant differences among replicate tanks for any of the exposure treatment groups (CTR:  $p = 0.730$ ; E2:  $p = 0.333$ ; ATZ-10:  $p = 0.790$ ; ATZ-100:  $p = 0.866$ ). The median difference between before- and after-renewal atrazine concentrations was not significantly different from 0 for any of the treatments (CTR:  $p = 0.127$ ; E2:  $p = 0.061$ ; ATZ-10:  $p = 0.384$ ; ATZ-100:  $p = 0.853$ ). Thus, it can be concluded that concentrations of atrazine were not depleted during the exposures.

### 3.2. Ultra structural analyses

#### 3.2.1. Control group (CTR)

Active spermatogenesis was observed in seminiferous tubules. Sertoli cells were associated with each other or adjacent germ cells by intercellular junctions, and various stages of developing germ cells were orderly distributed in tubules (Fig. 1a and b). Leydig cells were observed in the interstitium near the blood vessels (Fig. 4a). Spermatogonia were in small, synchronously developing groups. Each spermatogonium contained a single large nucleus with some indentations, well developed endoplasmic reticulum and numerous polysomes. Primary spermatocytes showed chromosome condensation patterns and were identified by the presence of synaptonemal complex indicating crossing over at early prophase of the first meiosis (Fig. 1a). Different stages of spermatids were observed as indicated by different shapes ranging from round (earlier stage) to elongated (later stage). The average number of spermatids was  $27.2 \pm 1.7$  (mean  $\pm$  S.E.M.)



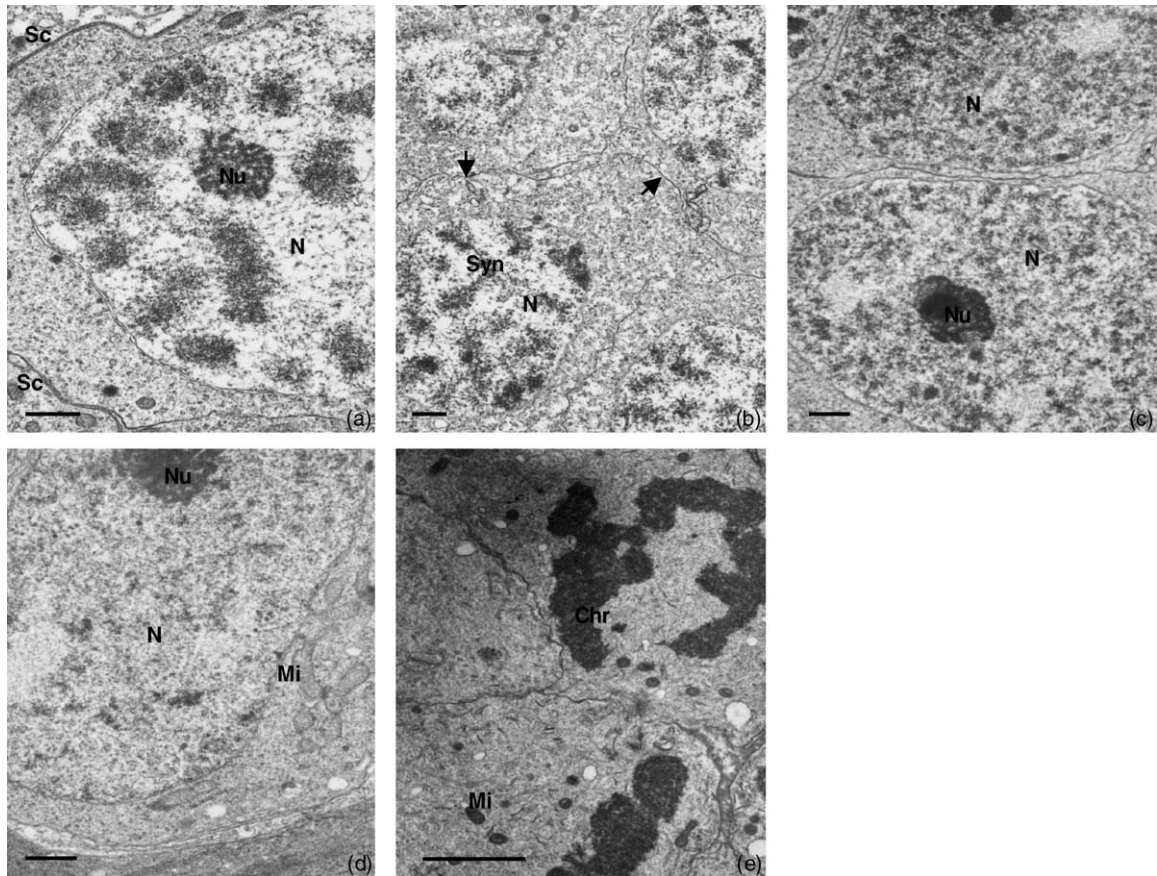


Fig. 1. Electron photomicrographs of *X. laevis* spermatocytes. Each scale bar on the figures represents 1  $\mu\text{m}$ . N: nucleus; Nu: nucleolus; Mi: mitochondria. (a) CTR: primary spermatocyte within Sertoli cell (SC) shown in early prophase in terms of condensing chromatin. Gap junctions can be seen between the two cells. (b) CTR: intercellular junctions (arrows) among primary spermatocytes and the synaptonemal complexes (Syn) of chromosomes. (c) E2: primary spermatocytes with evenly distributed chromatin and the prominent nucleolus (Nu) within the nucleus (N). (d) ATZ: spermatogonium with large nucleus (N) and prominent nucleolus (Nu) and organelles such as mitochondria (Mi) and ribosomes are present. (e) ATZ: dividing primary spermatocytes at prophase of the first meiosis. Nuclear envelope is already disintegrated with highly condensed chromosomes (Chr) clearly present.

cells within an area of  $5 \mu\text{m} \times 7 \mu\text{m}$  (observed at a magnification of 10,000-fold) (Fig. 2). The heads of late spermatids were very electron dense. The nuclear material of sperm was condensed to its greatest extent, and the axoneme of the tail showed the typical 9+2 arrangement of microtubules (Fig. 3a). There were evenly distributed ribosomes, and Golgi apparatus near the rounded nuclei, which were electron dense in a homogeneous manner. Mitochondria were elongated. The organelles of Leydig cells, which are involved in T production stood out in the interstitial tissue. Leydig cells contained oval nuclei with moderate heterochromatin

formation, tubular mitochondria and well-developed smooth endoplasmic reticulum (Fig. 4a).

### 3.2.2. Estradiol treatment group (E2)

A variety of ultrastructural changes were observed in the testes of E2-treated *X. laevis* relative to those of the controls. Prominent nucleoli and no chromosome condensation (chromatin evenly distributed throughout nucleus) could be observed in primary spermatocytes (Fig. 1c). Significantly fewer dividing germ cells, particularly spermatids and sperm, were observed (Fig. 2). In the E2-treated frogs the average number ( $22.3 \pm 1.5$ )

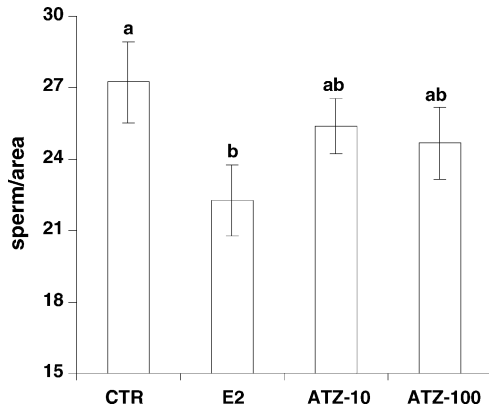


Fig. 2. Average number of sperm per area of  $5 \mu\text{m} \times 7 \mu\text{m}$  (observed at a magnification of 10,000-fold) in the control (CTR), estradiol (E2), and atrazine (ATZ-10, ATZ-100) treatment groups in *X. laevis*. Number of observations = 12–17 per treatment group. Error bars = standard error. Letters (a, b) signify tests for statistically significant differences at  $p < 0.05$  between treatments.

of sperm heads within an area of  $5 \mu\text{m} \times 7 \mu\text{m}$  (observed at a magnification of 10,000-fold) was 18% less than that in the control group ( $27.3 \pm 1.7$ ) ( $t$ -test:  $p = 0.033$ ). Condensation of sperm nuclei of this group was incomplete, and vacuoles and inclusion bodies in spermatid and sperm head were increased (Fig. 3b and c). Furthermore, Leydig cells were less differentiated when compared to CTR frogs. There were swellings and irregularities of the nuclear envelope, and less developed smooth endoplasmic reticulum (Fig. 4b). Also, an increased number of vacuoles and lipid droplets could be observed in the cytoplasm of Leydig cells (Fig. 4b and c).

### 3.2.3. Atrazine treatment groups (ATZ-10 and ATZ-100)

There were no ultrastructural differences between control and atrazine-treated frogs. The distribution of developing germ cells exhibited a pattern similar to that of the control group. Primary spermatocytes containing condensed chromosomes with a well-developed synaptonemal complex were present (Fig. 1d and e). As in the controls, sperm had a spiral slender nucleus with a high electron density (Fig. 3d).

Other cell organelles and constituents of developing germ cells and sperm were also similar to those of the CTR group. Leydig cells of the ATZ-10 and ATZ-100 treatment groups showed oval nuclei, well de-

veloped smooth endoplasmic reticulum and secretory vesicles (Fig. 4d). Numbers of sperm heads (ATZ-10:  $25.4 \pm 1.1$ ; ATZ-100:  $24.7 \pm 1.4$ ) were neither significantly different from the controls ( $t$ -test:  $p = 0.38$  and  $0.28$ , respectively) nor from the E2 treatment group ( $t$ -test:  $p = 0.1$  and  $0.26$ , respectively) (Fig. 2).

### 3.3. Plasma T and E2 concentrations

Exposure to either concentration of atrazine did not significantly affect plasma T or E2 concentrations compared to the controls. Exposure to E2 resulted in significantly lesser concentrations of plasma T (Mann–Whitney  $U$ -test:  $p < 0.001$ ), and significantly greater concentrations of plasma E2 (Mann–Whitney  $U$ -test:  $p < 0.001$ ) when compared to the controls (Fig. 5). The variation in the plasma E2 and T concentrations detected among individuals, along with moderate tank effects, limited statistical power for the available sample sizes and increased the magnitude of difference between treatments that could be resolved statistically. Intra-class correlations associated with the tank effects detected for T and E2 were estimated to be approximately 0.3. Assuming tank effects and an ICC of 0.3, a power analysis was conducted to determine the minimum ratio of geometric means that could be detected with 80% power given the sample sizes used and variation observed in this study (Table 2).

### 3.4. Aromatase activity

Waterborne exposure to neither atrazine nor E2 affected testicular aromatase activity. Samples with detectable aromatase activity were defined as those that yielded activities (in disintegrations per minute; dpm) that were at least 2-fold greater than the non-specific activity (in dpm) detected for bovine serum albumin blanks. Thus, the method detection limit (MDL) for the tritiated water release assay was calculated to be  $0.22 \pm 0.1$  fmol/h/mg protein (mean  $\pm$  S.D.). No testicular homogenates from CTR frogs yielded activities that were greater than twice that detected in the blanks. This suggested that there was no background level of detectable aromatase activity in the *X. laevis* testicular tissue. Exposure to neither 10 nor 100  $\mu\text{g}$  atrazine/L (nominal) resulted in detectable activities of aromatase in adult *X. laevis* testicular tissue. The performance of the tritiated water release assay was validated by ana-

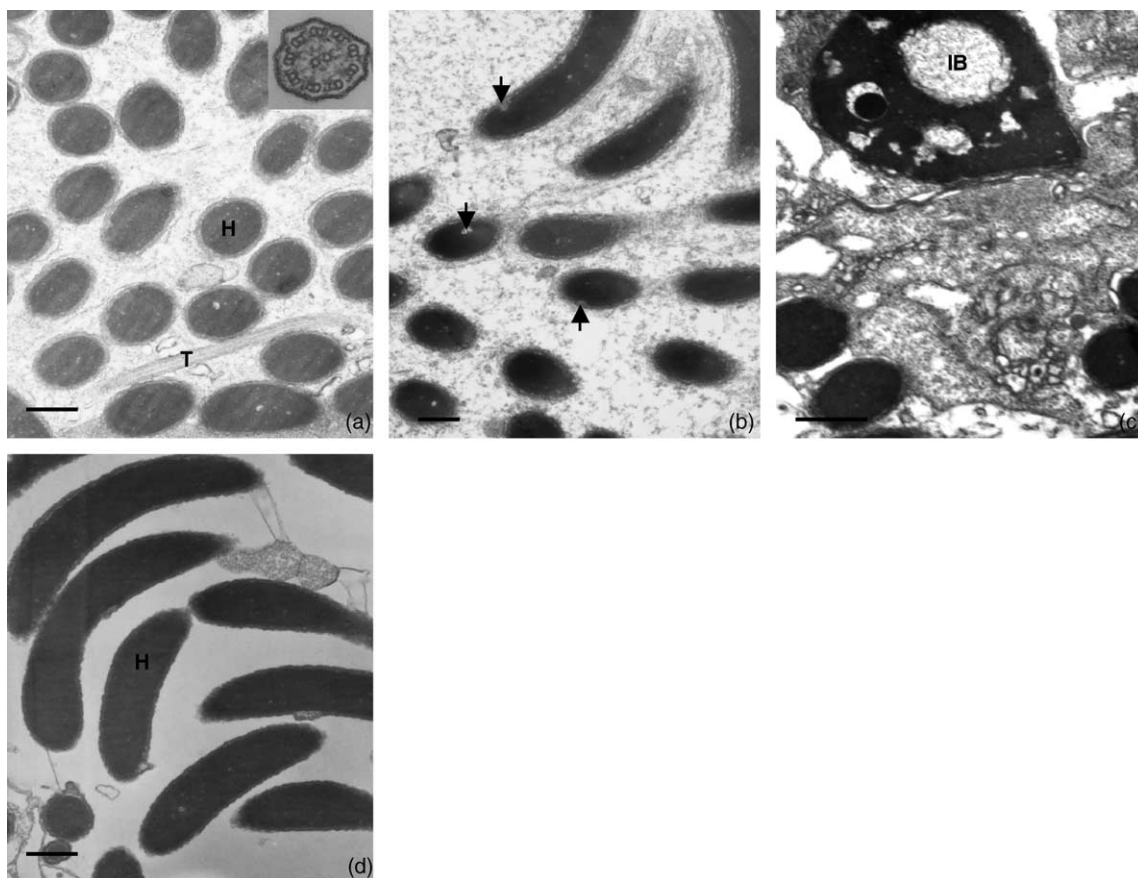


Fig. 3. Electron photo-micrographs of *X. laevis* sperm heads. Scale bars = 1  $\mu\text{m}$ . (a) CTR: Cross sectioned sperm heads (H) and a longitudinally sectioned tail (T). Cross sectioned axoneme of sperm tail shows typical 9 + 2 arrangement of microtubules (insert). (b) E2: vacuoles (arrows) in sperm heads. (c) E2: severely vacuolized sperm nuclei with inclusion body (IB) in the nucleus. (d) ATZ: tangentially sectioned sperm heads (H) that are normally elongated and curved.

lyzing *X. laevis* ovary homogenates, as a positive control, along with each set of testicular homogenates. Detectable aromatase activity was consistently observed for positive control *X. laevis* ovarian tissue with ac-

tivities ranging from  $3.5$  to  $2.6 \times 10^1$  fmol/h/mg protein. Positive control activities were at least 120-fold greater than the MDL determined for the males. Addition of  $7.5 \mu\text{l}$  of  $5.6 \times 10^2 \mu\text{M}$  4-androsten-4-ol-3,17-

Table 2

Minimum ratio of true geometric mean (GM) estradiol (E2) and testosterone (T) concentrations, or E2/T ratios that could be detected with 80% power given the samples sizes used in each experiment and the variation observed

Endpoint	Minimum detectable GM ratio	Observed GM ratio <sup>a</sup>		
		CTR and ATZ-10	CTR and ATZ-100	CTR and E2
Testosterone	6.6	1.4	1.3	17
Estradiol	1.6	1.3	1.2	2.6
GSI	1.7	1.14	1.11	1.13

GM ratio between control (CTR) and atrazine (10  $\mu\text{g/L}$ :ATZ-10; 100  $\mu\text{g/L}$ :ATZ-100) or E2 (0.1  $\mu\text{g/L}$ : E2) treatment groups also shown. Power analysis assumed tank effects with an intraclass correlation of 0.3 (T and E2) and 3.7 (GSI).

<sup>a</sup> Italic font indicates CTR > treatment; standard font indicates CTR < treatment.



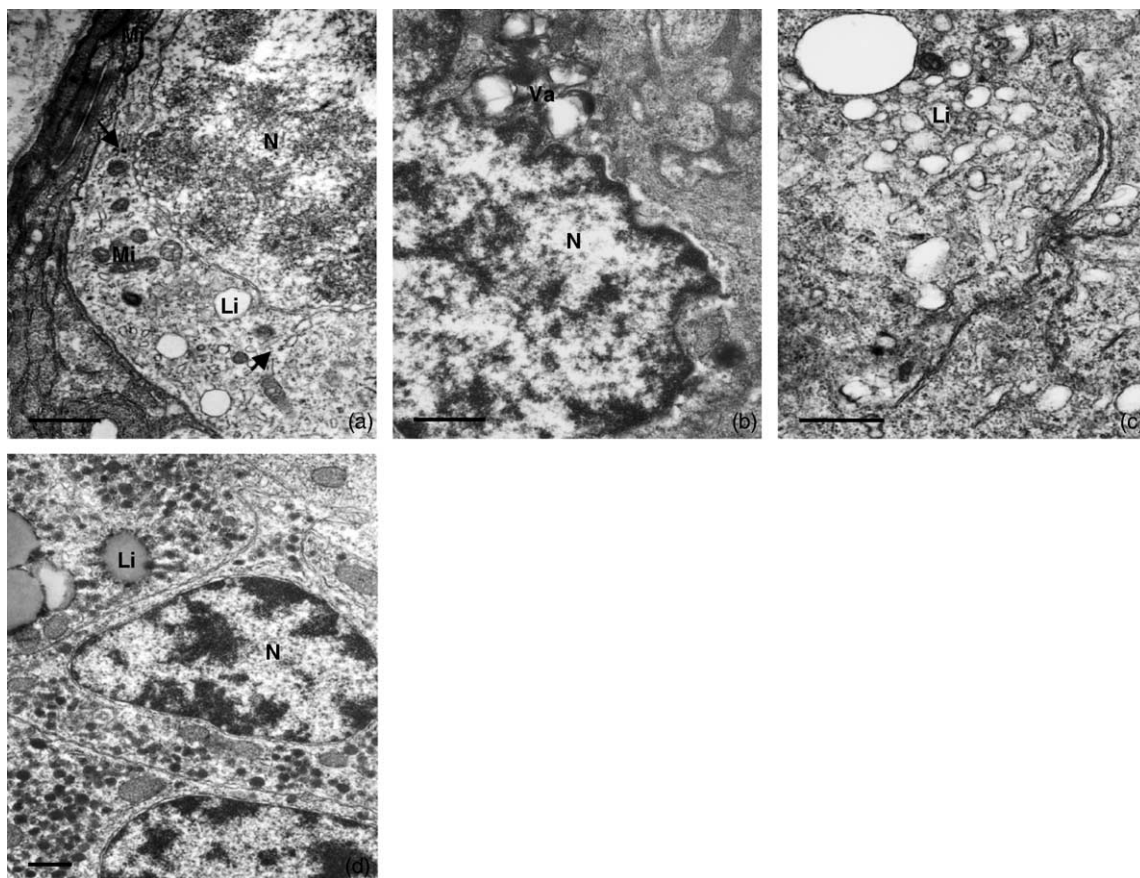


Fig. 4. Electron photo-micrographs of leydig cells of *X. laevis*. Each scale bar on the figures represents 1  $\mu\text{m}$ . Li: lipid droplets; Mi: mitochondria; N: nucleus (a) CTR: normal Leydig cell. Leydig cell containing the nucleus (N) with moderate heterochromatin, mitochondria with tubular cristae (Mi), lipid droplets (Li) and smooth endoplasmic reticulum (arrows) in the cytoplasm. (b) E2: part of a Leydig cell. Nuclear (N) outline is irregular and swollen. Free ribosomes are evenly distributed with few vacuoles (Va) in the cytoplasm. (c) E2: part of a Leydig cell that shows accumulation of lipid droplets (Li). (d) ATZ: Leydig cells with oval nuclei containing moderate amounts of heterochromatin in the cytoplasm.

dione, a specific, competitive inhibitor of aromatase, reduced the activity to 3.9–7.7% of that observed for homogenates without inhibitor added. Thus, the assay was determined to be functional and specific for aromatase activity.

### 3.5. GSI

There were some treatment related differences in GSI among tanks. Frogs from both ATZ treatment groups had GSIs that were significantly greater than that observed for E2-treated frogs ( $t$ -test: ATZ-10:  $p=0.003$ ; ATZ-100:  $p=0.007$ ; Fig. 6). Median GSIs of E2-treated frogs were slightly less (0.25%) than those

in the controls (0.28%) although this difference was not statistically significant ( $t$ -test:  $p=0.145$ ; Fig. 6). No statistically significant differences between the GSIs of ATZ-100 treated (0.29%) and CTR (0.27%) *X. laevis* were observed ( $t$ -test:  $p=0.195$ ; Fig. 6). However, the mean GSI of *X. laevis* treated with 10  $\mu\text{g/L}$  atrazine were significantly greater than the mean GSI of controls ( $t$ -test:  $p=0.046$ ; Fig. 6). Here, the difference between upper (90th) and lower (10th) centile ranged from 3.1-fold (E2) to 1.8-fold (CTR, ATZ-10). Intra-class correlations associated with the tank effects detected for GSI were estimated to be approximately 0.37 indicating that moderate tank effects have occurred.

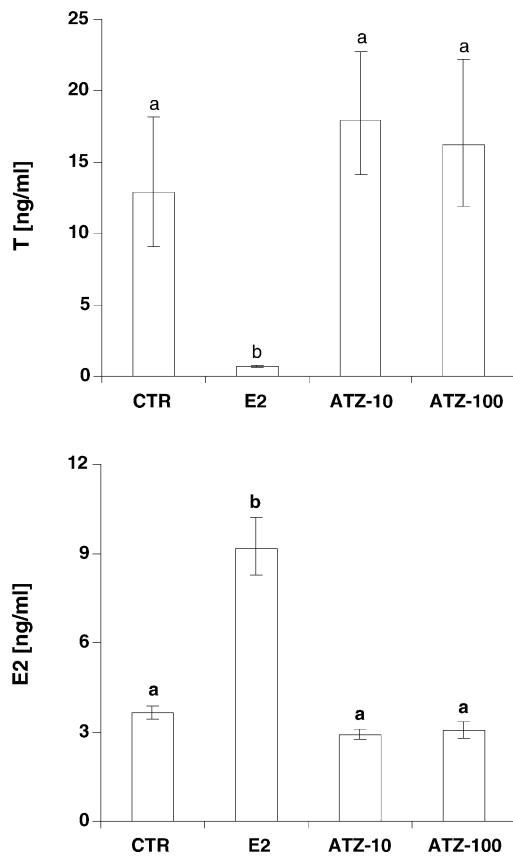


Fig. 5. Geometric mean plasma testosterone (T) (top) and estradiol (E2) (bottom) concentrations detected in adult male *X. laevis* exposed to control water (CTR), 10 or 100  $\mu\text{g/L}$  atrazine (ATZ-10, ATZ-100), or 0.1  $\mu\text{g/L}$  estradiol (E2).  $n=6-8$  frogs per tank, 18–19 frogs per treatment. Error bars = standard error. Letters (a, b) signify tests for statistically significant differences at  $p < 0.05$  between treatments.

The ratio of geometric means that would be consistently detectable with 80% power given the sample sizes used and variation observed in this study was 1.7 (Table 2).

#### 4. Discussion

In the current study environmentally relevant concentrations of atrazine did not alter the ultra structure of testicular cells, plasma concentrations of T or E2, or testicular aromatase activity in adult male *X. laevis*. There was a slight increase in gonad growth as expressed by the GSI, in atrazine-exposed frogs. In con-

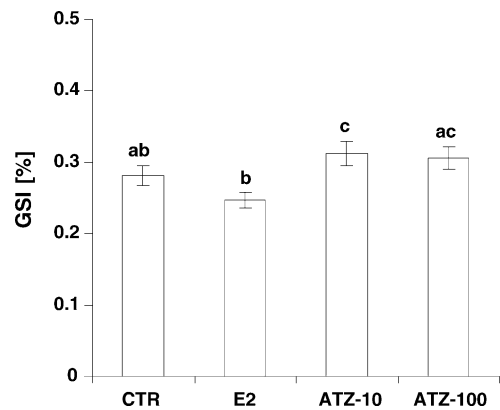


Fig. 6. Mean gonadal somatic index (GSI) for adult male *X. laevis* exposed to 10 or 100  $\mu\text{g/L}$  atrazine (ATZ-10, ATZ-100), control water (CTR), or 0.1  $\mu\text{g/L}$  estradiol (E2).  $n=20-21$ . Error bars = standard error. Letters (a–c) signify tests for statistically significant differences at  $p < 0.05$  between treatments.

trast, exposure to E2, used as a positive control, resulted in significant changes at the sub-cellular, biochemical, and tissue level in *X. laevis*.

Ultra structural characteristics of control group testes were similar to those reported for amphibians (summarized in Manochantr et al., 2003) and other lower vertebrates such as spotted ray (Prisco et al., 2002). The presence of various stages of male germ cells and the association of Sertoli cells with each other or adjacent germ cells indicate that the animals were undergoing active spermatogenesis (Manochantr et al., 2003). Interstitial Leydig cells exhibited the typical organization of steroid-secreting cells containing euchromatin in the nucleus, tubular mitochondria and a well-developed smooth endoplasmic reticulum indicating active T production (Prisco et al., 2002).

The lesser development of spermatids and sperm cells in the E2-exposed group indicates that E2 delays or inhibits maturation of male germ cells in *X. laevis*. It has been reported for different species of laboratory rodents that E2 inhibited the function of Leydig cell (Jones et al., 1978) and spermatogenesis (Toyama et al., 2001), or induced the formation of Leydig cell tumors (Yasuda et al., 1986; Maeda et al., 2002). Since the primary function of Leydig cells is the production of T to regulate spermatogenesis, the less condensed nuclei of sperm, less differentiated Leydig cells, and more lipid droplets that were observed in the E2 group suggest abnormalities in spermatogenesis that may be

due to inhibition of T production (Blanco et al., 2001; Islinger et al., 2003). Leydig cells generally contain lipid droplets as a storage site of cholesterol, which is the precursor of T. These droplets normally disappear when production of T increases during the reproductive cycle (Prisco et al., 2002). Increases in the number and size of lipid droplets, therefore, indicate either an increase in cholesterol production or, more likely, a decrease in steroidogenic activity resulting in a decrease of the end products such as T. This was confirmed by the significantly lesser plasma T concentrations observed in the same frogs when they were exposed to exogenous E2.

One possible explanation for the reciprocal relationship between plasma T and E2 titers would be an induction of aromatase activity, the enzyme that transforms T to E2. It has been reported previously that exposure to E2 can induce gene expression of testicular aromatase activity in fish in a dose-response dependent manner (Halm et al., 2002). However, measures of aromatase activity conducted in our study did not reveal any significant alterations in the enzyme activity in the testis, indicating that there was no increased endogenous formation of E2. Although it cannot be excluded that changes in testicular aromatase activity less than the method detection limit have occurred, it is very unlikely that such minute activities, which were more than 120-fold lower than those reported for females, would have any biological relevance or result in the increase of endogenous E2 observed in this study. Thus, we conclude that the increase of plasma E2 concentrations observed in male *X. laevis* exposed to E2 treatment is not a function of increased endogenous E2 production but is likely due to other factors such as accumulation of E2 from the test solution (Kramer et al., 1998).

Synaptonemal complexes of primary spermatocytes were less frequently observed in testes of *X. laevis* exposed to E2 than in the control. The synaptonemal complex is the formation of a proteinaceous ribbon between homologous chromosomes during the first meiotic division that is essential for successful cell division (Von Wettstein, 1984). Therefore, the observation of fewer synaptonemal complexes in the E2-exposed group is indicative of poor division of primary spermatocytes, which subsequently resulted in the significant decrease of the number of sperm cells reported in this study. Also, the occurrence of nuclear vac-

uoles in sperm heads is considered an indicator of the failure of the concentration of chromatin. Vacuolated sperm heads and incomplete chromosome condensation were previously associated with apoptotic processes in mammals (Gandini et al., 2000), offering an additional explanation for decreased sperm head numbers in the E2-treated animals. A different study on zebrafish (*Danio rerio*) that were exposed to the synthetic estrogen 17 $\alpha$ -ethinylestradiol (25 ng/L) found increased phagocytosis of sperm cells by Sertoli cells in the testes as a result of this exposure (Islinger et al., 2003). Although no direct observations of phagocytotic activities of Sertoli cells were made in our study, sub-cellular indicators of autolytic activities such as vacuolated sperm heads and incomplete chromosome condensation were observed. These effects on germ cell development were also reflected by the slightly lower GSI in the E2-treated frogs compared to all other treatments, although this was not statistically significant for the controls. This lack of significance might be due to the exposure time of the experiment that was designed to capture effects on the sub-cellular structural and biochemical level, and was likely to be too short to capture effects on growth and/or development. An additional reason for the lack of significance may be the relatively low power to determine a true ratio of geometric means that would be close to that observed in our study.

For logistical reasons, no ethanol control group was tested in this experiment, and therefore, potential effects of the solvent cannot be totally excluded for E2-exposed animals. However, the physiological and cellular responses of the E2-treated *X. laevis* are similar to those reported in studies with other vertebrates (e.g. Crain et al., 1997; Kramer et al., 1998; Blanco et al., 2001; Islinger et al., 2003), indicating that E2 represents a valid positive control for the hypothesized estrogenic effects of atrazine that were tested in this study.

No changes in ultra structure relative to the controls were observed in frogs exposed to atrazine. Various stages of developing germ cells were present indicating active spermatogenesis. Primary spermatocytes exhibited a chromosome condensation pattern characteristic for early meiotic stages (Reed and Stanley, 1972) and could be identified by the presence of the synaptonemal complex that was present at a frequency similar to that in the controls. This complex is indicative of the linkage and crossing over during first meiosis, and is typical for

normal developing germ cells (Von Wettstein, 1984). The primary function of Leydig cells is the production of androgens such as T to regulate spermatogenesis. As in the controls, a smaller size and/or lower numbers of lipid droplets were observed as compared to the E2-treated frogs in both atrazine-treatment groups during the process of spermatogenesis, indicating active production of T. This was confirmed by similar plasma T concentrations in both ATZ treatments and the CTR group. Based on the relatively low power to determine significant differences between plasma hormone concentrations in atrazine treated and control frogs it cannot be ruled out entirely that there have been some minor influences of atrazine treatment on plasma E2 or T. However, given the similarities in terms of average hormone concentrations and within group variabilities it is unlikely that these – if present – would be of any biological relevance. Also, there was no negative effect on gonad growth in frogs exposed to either 10 or 100 µg atrazine/L. This indicates normal development of the testis when compared to the E2-treated frogs. Exposure to 10 µg/L atrazine resulted in a statistically significant greater GSI compared to CTR frogs. Similarly, Hecker et al. (2004) observed greater GSI values in *X. laevis* caught from an area with elevated aqueous atrazine concentrations. The authors hypothesized that the increase in relative gonad mass might have been due to better food availability in nutrient-rich farm ponds. However, in the study on which we report here there was no difference in the feeding regime of the frogs from the different treatments, but we cannot exclude possible effects of low ATZ concentrations on food availability.

## 5. Conclusions

The results of this study showed that exposure to E2, but not atrazine, affected the ultra structure of the testes as well as plasma hormone concentrations in *X. laevis*. It was hypothesized in a previous study (Hayes et al., 2002) that atrazine at a concentration (25 µg/mL, only single dose tested) similar to those tested in this study affect the endocrine system of *X. laevis* (exposed over 46 days) via an estrogen-like mechanism of action resulting in a depletion of plasma T, and an increase in plasma E2 due to up-regulation of aromatase. The results of our study do not support this hypothesis. If

there were any effects of atrazine on the testis they were weak and not related to changes in plasma sex steroid titers or aromatase activity. The greatest concentration tested (100 µg atrazine/L) in this study was greater than most concentrations of atrazine that are observed in the environment, even directly after its application when atrazine levels are the greatest (Giddings et al., 2000). Therefore, we conclude that it is unlikely that atrazine exposure leads to alterations of the endocrine system in adult male *X. laevis* that are comparable to those evoked by exposure to estrogen.

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