TOXICOLOGICAL SCIENCES **107(2)**, 376–384 (2009)
doi:10.1093/toxsci/kfn232
Advance Access publication November 13, 2008

Does Atrazine Influence Larval Development and Sexual Differentiation in *Xenopus laevis*?

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Received June 20, 2008; accepted October 30, 2008

Debate and controversy exists concerning the potential for the herbicide atrazine to cause gonadal malformations in developing *Xenopus laevis*. Following review of the existing literature the U.S. Environmental Protection Agency required a rigorous investigation conducted under standardized procedures. *X. laevis* tadpoles were exposed to atrazine at concentrations of 0.01, 0.1, 1, 25, or 100 μg/l from day 8 postfertilization (dpf) until completion of metamorphosis or dpf 83, whichever came first. Nearly identical experiments were performed in two independent laboratories: experiment 1 at Wildlife International, Ltd. and experiment 2 at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB). Both experiments employed optimized animal husbandry procedures and environmental conditions in validated flow-through exposure systems. The two experiments demonstrated consistent survival, growth, and development of *X. laevis* tadpoles, and all measured parameters were within the expected ranges and were comparable in negative control and atrazine-treated groups. Atrazine, at concentrations up to 100 μg/l, had no effect in either experiment on the percentage of males or the incidence of mixed sex as determined by histological evaluation. In contrast, exposure of larval *X. laevis* to 0.2 μg 17β-estradiol/l as the positive control resulted in gonadal feminization. Instead of an even distribution of male and female phenotypes, percentages of males:females:mixed sex were 19:75:6 and 22:60:18 in experiments 1 and 2, respectively. These studies demonstrate that long-term exposure of larval *X. laevis* to atrazine at concentrations ranging from 0.01 to 100 μg/l does not affect growth, larval development, or sexual differentiation.

Key Words: **atrazine; amphibians; endocrine; sexual differentiation; development; *Xenopus laevis*.**

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) is a widely used triazine herbicide that inhibits photosynthesis in plants and algae. The ecotoxicity profile of atrazine has been well characterized and a recent comprehensive, probabilistic risk assessment concluded that direct effects on aquatic animals were very unlikely (Giddings *et al*., 2005). Although atrazine has been reported to affect sexual development and gonadal differentiation in the African clawed frog, *Xenopus laevis*, the results of different studies have been inconsistent. One report indicated that exposure of *Xenopus* larvae to concentrations of atrazine ranging from 0.1 to 200 μg/l (Hayes *et al*., 2002) caused 16–20% of the males (subsequently revised as 32–40% in Hayes *et al*., 2006) to form multiple gonads. Other gonadal findings were reported as indicative of adverse development such as decreased ovarian melanophores. In a single dose study with a nominal atrazine concentration of 21 μg/l, histological effects on gonads were reported in both male and female *X. laevis* (Tavera-Mendoza *et al*., 2002a,b). Carr *et al*. (2003) reported a significant increase in intersex animals and discontinuous gonads at 25 μg atrazine/l, however, re-evaluation of the morphological findings by histology confirmed that neither intersex, defined as ovarian and testicular tissue present as separate structures within an animal, or mixed sex, defined as ovarian and testicular tissue present within the same gonad occurred in that study (http://www.epa.gov/oscpmont/sap/meetings/2007/october/transcript2007-10-11.pdf). Further studies concerning potential effects of atrazine on *X. laevis* (Hecker *et al*., 2005a,b) have not detected any effect on gonads.

In 2003, the U.S. Environmental Protection Agency (EPA) reviewed all available pertinent scientific literature to assess potential effects of atrazine on amphibian development and concluded "there is not sufficient scientific evidence to indicate that atrazine consistently produces effects across the range of amphibian species examined" (EPA White Paper on Potential Developmental Effects of Atrazine on Amphibians, FIFRA Scientific Advisory Panel, June 17, 2003; http://www.epa.gov/scipoly/sap/2003/june/finaljune2002telconfreport.pdf); (http://www.epa.gov/scipoly/sap/2003/june/junemeetingreport.pdf).

EPA required that design, methods, and quality limitations exhibited by the existing studies be addressed and additional investigations conducted. To resolve uncertainties and discrepancies among various published reports, a dual-experiment laboratory study to assess potential effects of atrazine on larval development and sexual differentiation in *X. laevis* was

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conducted. The study was conducted under "blinded" conditions, in compliance with Good Laboratory Practice (GLP) standards and utilized validated flow-through exposure systems (Lutz *et al*., 2007). Both negative and positive control groups were included in each experiment. Endpoints evaluated histologically included sex ratio, and the prevalence of mixed sex (defined as both gender tissues within a single gonad), intersex (defined as left/right gonads of different gender), and testicular oocytes (testicular ovarian follicles).

MATERIALS AND METHODS

The two experiments that constitute the in-life, exposure portion of the study were conducted independently at the facilities of Wildlife International, Ltd. (WLI, MD) and the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB, Berlin, Germany). Experimental Pathology Laboratories, Inc. (EPL, Sterling, VA) performed the histopathological evaluations for both experiments. Sielken and Associates (Bryan, TX) conducted all statistical analyses. Water and stock samples were analyzed for E2 concentrations by WLI and for atrazine concentrations by Syngenta Crop Protection, Inc. (Greensboro, NC).

Preliminary studies were performed in both laboratories to develop and validate a test protocol for the evaluation of gonadal development in *X. laevis* exposed to test substances in a flow-through test system. The optimized protocol for the two experiments reported here has been previously described in detail (Lutz *et al*., 2007) and therefore are described only briefly here (Table 1).

Test solutions were generated by delivering streams of test substance and dilution water to flow-through mixing cups and then passed to the test tanks. Samples of the water in the test tanks were collected weekly, except during the first three weeks of the experiment when samples were taken twice weekly.

Atrazine analyses were performed using a validated direct aqueous injection (DAI) liquid chromatography-electrospray ionization mass spectrometry/mass spectrometry method. A Perkin-Elmer Series 200 LC was interfaced to an Applied Biosystems/MDS Sciex API 4000 tandem quadrupole mass spectrometer using +ion mode electrospray in the Multiple Reaction Monitoring (MRM) mode. The separation employed an Agilent Zorbax SB-AQ column (2.1 × 50 mm, 5 μm). The limit of quantification (LOQ) was 0.010 μg/l.

The estradiol (E2) analyses were performed using a validated DAI/Agilent Series 1100 LC coupled with a MDS SCIEX API 3000 tandem quadrupole mass spectrometer method and an MDS SCIEX API Heated Nebulizer ion source (in the MRM mode). The separation employed a Keystone Betasil C-18 column (2 × 50 mm, 3 μm). The LOQ was 0.100 μg/l.

Test organisms. *X. laevis* larvae were obtained from a commercial supplier, Xenopus I (Dexter, MI). Larvae originated from 10 pairs of adult breeders for each experiment. At the supplier's facility, eggs were reared until day 3 postfertilization (dpf) and shipped to each laboratory. The experimental design and husbandry conditions are summarized in Table 1. The larvae were acclimated in 50-l glass tanks and fed with Sera micron (Sera GmbH, Heinsberg, Germany). On dpf 8, tadpoles were randomly assigned to test tanks each containing finally 25 individuals. Tadpoles were fed three times daily with 300 mg Sera micron per tank and adjusted according to their development up to 900 mg per feeding. Exposures were initiated at dpf 8 and continued until metamorphosis was completed or until tadpoles had been exposed for dpf 83. Each experiment consisted of a total of seven groups, a positive control group [0.2 μg E2/l dissolved according to Lutz *et al*. (2007) without solvent], a negative control group (dilution water), and five groups exposed to technical grade atrazine (0.01, 0.1, 1.0, 25, and 100 μg atrazine/l). Each group consisted of eight tanks per group, except the negative control group, which included 16 tanks. The tanks in each treatment group were arranged in two clusters of four tanks, whereas negative control exhibited four clusters of four tanks. In experiment 1 (WLI), trace levels of atrazine were found in one cluster of four negative control tanks. A microbial bloom occurred in an additional cluster of negative control tanks and consequently data derived from both affected clusters were excluded from all statistical analyses. Despite the elimination of these two clusters, the built-in redundancy in the experimental design ensured that there were still eight negative control tanks and eight tanks per treatment group, and therefore the statistical analysis and interpretation were not adversely affected.

The clusters of tanks in the water baths (IGB) or environmental chamber (WLI) were randomly assigned to treatments. 25 larvae were randomly assigned to each test tank ($n = 200$ larvae per E2 or atrazine group and $n = 400$ larvae in the negative control group). Thus, a total of 1600 larvae were used in each laboratory. The biological loading rate was less than 1 g/l/day.

Observations of health, survival, metamorphosis, and growth. Tadpoles were monitored daily for changes in general health, feeding, swimming behavior, appearance, and survival. Each day, the numbers of animals completing metamorphosis and the numbers of dead or moribund in each tank were recorded.

Each frog was removed from its test tank when it completed metamorphosis or at dpf 83 if metamorphosis was incomplete. After removal, frogs were euthanized by immersion in Finquel (MS-222, Argent Chemical Company, Redmond, WA) and given a unique identifier. Each frog was then examined for external abnormalities and the stage of each tadpole that did not complete metamorphosis by dpf 83 was determined. Tadpoles found dead prior to reaching Nieuwkoop and Faber stage 58 of metamorphosis were not examined for sexual differentiation.

The length of each frog (snout-to-vent) was measured to the nearest millimeter using a graduated grid. Each frog was blotted dry and then weighed. Weights were recorded to the nearest mg.

Gonadal assessments. The ventral body wall and viscera of each frog were excised to expose the gonads, which appear as thin pale tan strips on the ventromedian side of each kidney (Lutz *et al*., 2007). The gonads of each frog were observed using a dissecting stereomicroscope, gonadal, and extragonadal anomalies were reported, and gonads from each individual were photographed *in situ* using a digital camera. Each photograph included a millimeter measurement scale placed adjacent to the gonads so that measurements of gonadal image area could be made from the image. The area of each gonad was calculated using Image-Pro Plus, Media Cybernetics, Silver Spring, MD.

After gross assessment of the gonads, each frog was fixed for 18–36 h in Bouin's solution, rinsed with 70% ethanol and transferred to 10% neutral-buffered formalin. Following tissue processing, the trunk segment of each frog containing the gonads was embedded in paraffin. Step sections of 4- to 5-μm thickness were microtomed from each block at 12-μm intervals until gonads were completely sectioned. All sections that contained gonadal tissue were mounted on glass slides and stained with hematoxylin and eosin. Prior to evaluation by the pathologist, slides were randomized and masked with coded numbers so that the pathologist was unaware ("blinded") of the treatment group for each frog. The pathologist evaluated for potential abnormalities that included, but were not limited to, mixed sex, intersex, and testicular oocytes.

Terminology. Differences in descriptive terminology have contributed to the difficulties in interpreting the results of studies of gondal abnormalities in *X. laevis* and therefore, a consistent set of terms was developed to describe both gross and histological gonadal features (Lutz *et al*., 2007). For example, the term mixed sex was used to identify the co-occurrence of both ovarian and testicular tissue in a single gonad, whereas the term intersex was reserved to describe the contralateral occurrence of one testis and one ovary in one individual.

Sex determination and histopathology. The phenotypic sex of each frog was identified as male, female, or mixed sex (intersex frogs were not observed in this study). Sex determinations were made based on both gross and histological basis, but only the histologically determined gender was used for as the basis for grouping frogs for analysis. A comparison between the gross and histopathological findings demonstrated that histopathology was far more accurate and sensitive for detecting gonadal abnormalities in *X. laevis*; therefore only the histopathological results are reported here.

TABLE 1
Experimental Design Specifications

Parameter	Characteristics
Water quality (ASTM standard)	
Supply water: filtered, contaminant free	Filtered (0.45 μm)/UV sterilized; tested for chemical contaminants
Dissolved oxygen	≥ 60% of saturation
pH	7.9–8.3
Ammonia	0.0–0.35 mg/l
Nitrate	0.04–2.29 mg/l
Hardness	90–130 mg $CaCO_3$/l
Alkalinity	133–172 mg $CaCO_3$/l
Specific conductance	726–817 μS/cm
Supplemental aeration	Yes
Animal husbandry and exposure conditions	
Animal supply	XENOPUS I, Dexter, MI
Shipped from supplier	dpf 3
Acclimation period	dpf 4–7
Treatment period	dpf 8–83
Feed: sera micron	Sera micron (contaminants and estrogenic potential evaluated). Contents: 50% protein, 8% fat; 4% fiber; 11% ash
Feeding rate—acclimation period	dpf 4–5, per 300 larvae: 200 mg; dpf 6–8, per 300 larvae: 200 mg twice daily
Feeding rate—exposure period	dpf 8–83, per 25 larvae: three times daily, 300 mg/tank at 8 dpf, increasing to 900 mg per tank at 33 dpf, then adjusted downward proportionately as frogs were removed from tanks
Tank volume	7 l
Tank flow rate	50 l/day
Number of larvae per group	200 larvae per treatment group; negative control group: 400 larvae
Larval loading density	< 1 g/l/day; 25 larvae per tank
Exposure tank cleaning	Twice daily
Exposure tank replacement	As needed
Temperature	22 ± 1°C
Light intensity	100–500 lux
Light:dark cycle	12 h:12 h; 30 min transition between light:dark:light
Experimental design and quality control	
Negative control	Water (16 replicates (tanks), 4 clusters)
Positive control (17β-estradiol [E2])	0.2 μg/l (eight replicates, two clusters)
Atrazine concentrations	0.01, 0.1, 1, 25, 100 μg/l (eight replicates, two clusters for each group)
HPLC/MS MS verification for E2 and atrazine	dpf 8 and 14 all tanks; dpf 21–83 alternating tanks
Study compliance with GLP standards	Compliant
Color coded (blind) observations	Yes
Randomization of tadpoles	Yes
Randomization of tank clusters	Random placement
Statistical procedures	
Unit of analysis	Tank (cluster effects were not significant); tadpole (E2 males only, tank effects were not significant)
Data transformed?	Only for continuous endpoints when there was evidence of substantial non-normality (Shapiro-Wilk test, $p < 0.01$) or variance heterogeneity (Levine test, $p < 0.01$), for tadpole (incidence data for E2 males by Fisher's Exact Test)
Protective screening tests for atrazine (E2 group excluded)	Analysis of variance *F*-test (continuous endpoints); Kruskal-Wallis test (incidence endpoints)
Comparison to negative control	Pair-wise ANOVA contrasts (continuous, atrazine); *t*-tests (continuous E''); Wilcoxon-Mann-Whitney (incidence, atrazine, nonmale E2); Fisher's Exact test (incidence, male E2)
Trend tests (negative control and atrazine groups)	Step-down Jonckheere-Terpstra tests for monotonic trend

Statistical analysis. The tank was considered to be the primary experimental unit and statistical analyses used tank means (continuous endpoints) or tank percentages (incidence-based endpoints). To control the experiment-wise error rate for each endpoint at $\alpha = 0.05$, statistical analysis of atrazine effects was performed in a stepwise fashion. First, a protective screening test comparing all groups except the positive control was performed. If this protective test detected significant heterogeneity among groups, comparisons of each atrazine group with the negative control were performed. For endpoints requiring two-sided control-treatment comparisons, the protective test used a 5% significance level threshold. When one-sided comparisons with control were appropriate, the protective test

TABLE 2
Mean Atrazine and 17β-Estradiol Concentration (Percent of Nominal) during the Closing of the Sensitive Window for Gonadal Sex Reversal (NF Stage 52–56) Covering dpf 21–42

	Experiment 1 (WLI)		Experiment 2 (IGB)	
Treatment (μg/l)	Study mean %	Critical window mean %	Study mean %	Critical window mean %
Estradiol (0.2 μg/l)	84	62	81	66
Neg. con.	< LOQ	< LOQ	< LOQ	< LOQ
0.01 Atz	101	90	64	53
0.1 Atz	92	91	56	52
1.0 Atz	95	99	72	74
25 Atz	94	97	88	81
100 Atz	87	93	88	79

Note. Sample values < LOQ are assumed to be 50% of the LOQ (0.01 μg/l) for computational purposes.

used a 10% significance threshold. Continuous variables (such as body weight) were analyzed using the one-way ANOVA as the preliminary test. When the ANOVA *F*-test was significant at the 5% significance level, each atrazine treatment was then compared with the negative control using pair-wise ANOVA contrasts at the 5% significance level. Incidence-based endpoints were analyzed using nonparametric statistical methods. The protective test for such endpoints was the Kruskal-Wallis test. If significant, this was followed by Wilcoxon-Mann-Whitney treatment-control comparisons at the 5% significance level.

Comparisons of the single E2-positive control with the negative control did not require protective tests. For female and combined-sex endpoints, the E2-control comparisons were analyzed using either a *t*-test (continuous) or a Wilcoxon-Mann-Whitney test (incidence) at the 5% level of significance. Analysis of incidence data among males in the E2-positive control group was an exception to these above procedures. Because feminization of the males in this group reduced the number of males per tank, analysis of incidence data for these frogs was performed using Fisher's Exact Test with individual frogs treated as the experimental unit.

All endpoints were also analyzed using a step-down procedure based on the Jonckheere-Terpstra nonparametric trend test. This procedure was used to determine if there was evidence of a monotonic dose related trend in responses for atrazine, and also to determine the concentration at which any existing trend test was no longer significant.

RESULTS

Environmental Conditions and Test Compound Concentrations

Environmental parameters remained within acceptable ranges throughout both experiments. The mean concentrations of atrazine in the three highest treatments (1.0, 25, and 100 μg/l) in both experiments ranged from 74 to 99% of nominal concentrations during the sensitive window for sex reversal (Table 2) that is considered to close gradually between Nieuwkoop and Faber stages 52 and 56 (Villalpando and Merchant-Larios, 1990; Witschi, 1971). In treatment groups with nominal atrazine concentrations below 1 μg/l, measured concentrations were generally more variable, ranging from means of 52–91% of nominal concentrations during the critical window.

During a period of approximately dpf 21–42, 17β-estradiol (E2) levels in the positive control tanks declined to 43% (WLI) and 25% (IGB) of the nominal concentration of 0.20 μg E2/l as tadpoles grew and feeding increased. As the frogs completed metamorphosis and were removed, E2 concentrations rebounded and approximated nominal concentrations. A similar pattern was observed in previous studies with E2 in both laboratories, as reported by Lutz *et al.* (2007). Records of E2 stock concentrations and operation of the diluter system from the current experiments suggest that delivery of E2 to the test tanks remained relatively constant during the experiments. The decline in the E2 concentration in the positive control tanks was related to the biomass in the tanks, and probably can be attributed mainly to biological uptake and metabolism by tadpoles and to a lesser extent to microbial degradation.

Survival, Growth, Development, and Gonad Area

Atrazine had no effect on survival. Survival in the negative control group of experiment 1 was 93.5% and was 98.5, 95.0, 95.4, 94.0, and 96.5% in the 0.01, 0.1, 1.0, 25, and 100 μg atrazine/l treatments, respectively. Survival in experiment 2 was 97.5% in the negative control and 96.0, 98.0, 97.5, 97.5, and 97%, in the respective atrazine treatments. Survival in the E2 positive control groups was 97.5 and 97.0% in experiments 1 and 2, respectively.

There were no apparent general effects of either atrazine or E2 on larval growth in either experiment, as indicated by measures of body weight and snout-to-vent length at completion of metamorphosis (Figs. 1a and 1b). However, statistically significant reductions of body weight and snout-to-vent length were observed in the 0.01, 1, and 100 μg/l atrazine-treated females in experiment 2, but not in experiment 1. In addition, these slight differences were not concentration dependent and thus they were attributed to random variation.

In both experiments, the mean age of males and females at metamorphosis (Fig. 1c), and the cumulative percentages of frogs reaching metamorphosis on each study day (Figs. 2a–d), were similar in the atrazine-treated and negative control groups. In contrast, the onset of metamorphosis in E2-treated males and females consistently occurred later than in the negative control groups (Figs. 2a–d). The mean age at completion of metamorphosis was significantly increased for males and females in both studies (Fig. 1c). Only three of the frogs that survived until 83 dpf failed to complete metamorphosis (one frog each in the 0.1 and 100 μg/l atrazine treatments of experiment 1 and one frog from the 25 μg/l atrazine treatment of experiment 2), which suggests that husbandry conditions were ideal.

In either male or female frogs, there were no significant differences between the gonad areas of atrazine-treated frogs and negative controls, as measured in digitized photographic images. Conversely, in both experiments the gonad areas of

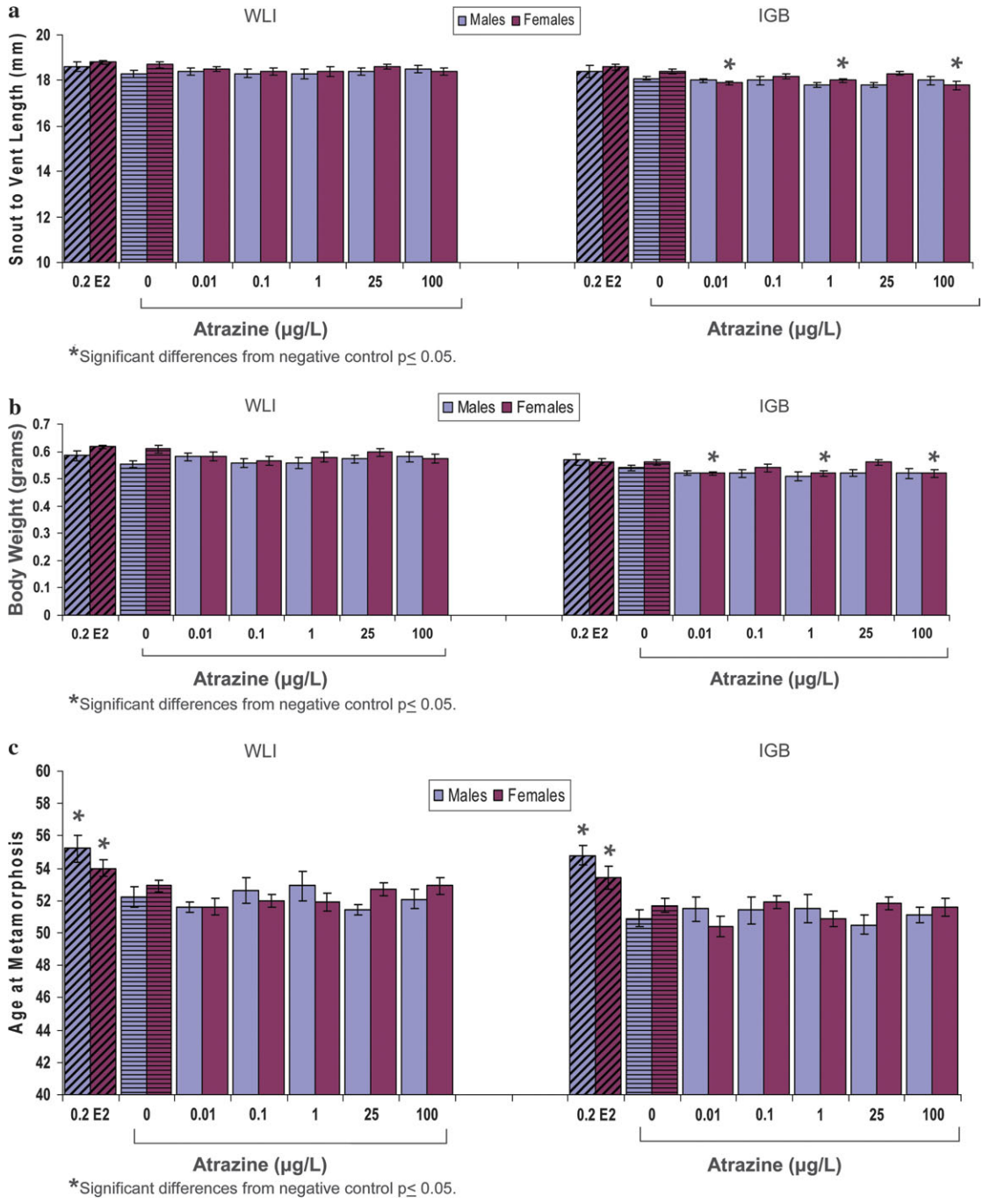

FIG. 1. (a) Snout-to-vent length (mean ± SE) in experiment 1 (WLI) (left panels) and 2 (IGB) (right panels). Statistical significance is marked by asterisk ($p < 0.05$). (b) Weight (mean ± SE) in experiment 1 (WLI) (left panels) and 2 (IGB) (right panels). Statistical significance is marked by asterisk ($p < 0.05$). (c) Time to metamorphosis (mean ± SE) in experiment 1 (WLI) (left panels) and 2 (IGB) (right panels). Statistical significance is marked by asterisk ($p < 0.05$).

E2-treated positive controls were significantly greater than those of negative controls.

Sexual Differentiation

Atrazine treatment (0.01–100 µg/l) had no effect on the relative percentages of males, females, or animals displaying mixed sex. Approximately 50% of the frogs were male and 50% were female (Fig. 3). One animal (25 µg/l group, experiment 2, IGB) out of approximately 2000 atrazine-treated frogs had mixed sex gonads. In contrast, E2 treatment in the positive control groups resulted in feminization near the expected EC50 value for E2 as reported by Lutz *et al.* (2007). The percentages of male:female:mixed sex were 19:75:6 and 22:60:18 in experiments 1 and 2, respectively.

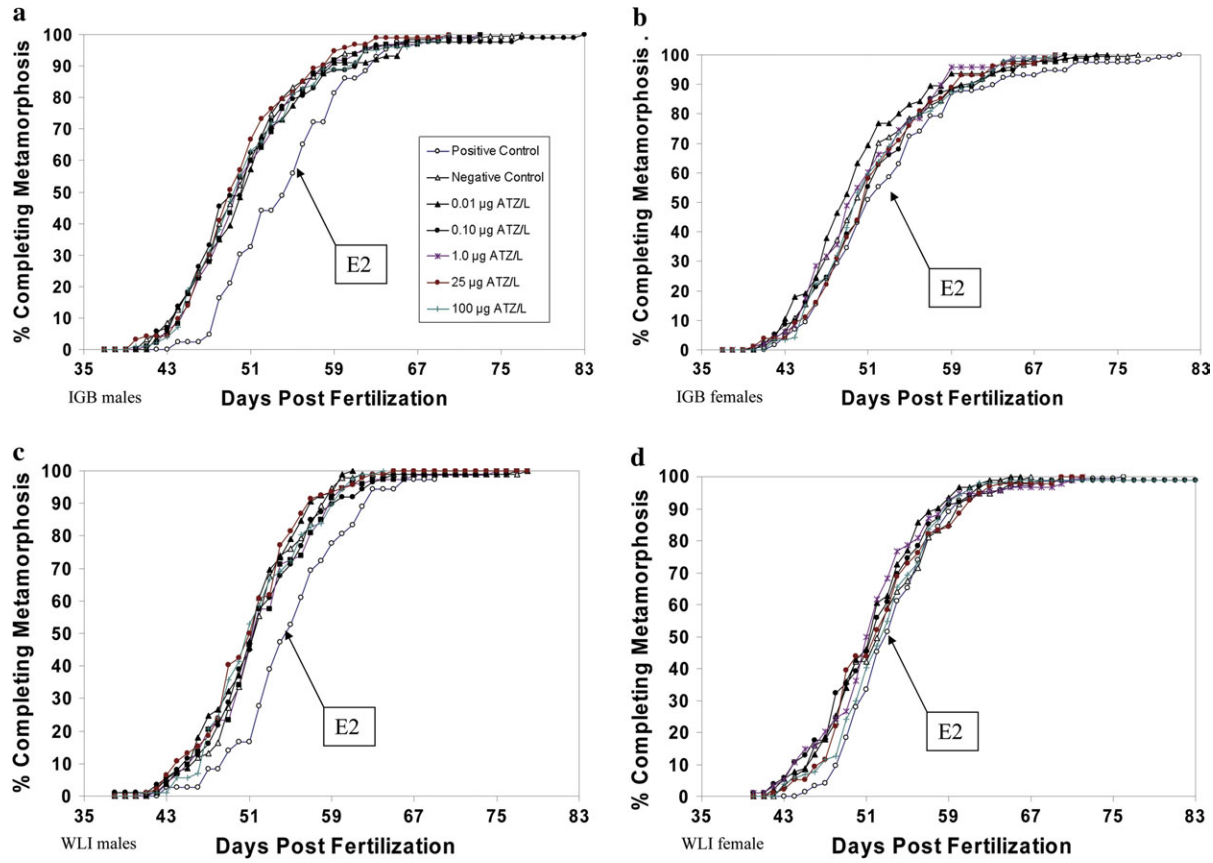

FIG. 2. (a) Cumulative percentage of males completing metamorphosis on each study day in both experiments at IGB. (b) Cumulative percentage of females completing metamorphosis on each study day in both experiments at IGB. (c) Cumulative percentage of males completing metamorphosis on each study day in both experiments at WLI. (d) Cumulative percentage of females completing metamorphosis on each study day in both experiments at WLI.

Figure 4 demonstrates the gross appearance of mixed sex in an E2-exposed positive control frog as compared with normal female and male gonads from negative control frogs. No frogs displaying intersex or testicular oocytes were observed.

Gonadal Abnormalities

Histopathological comparisons of the testes of negative control to E2 treated frogs revealed statistically significant differences in the prevalences of dilated tubules, dividing

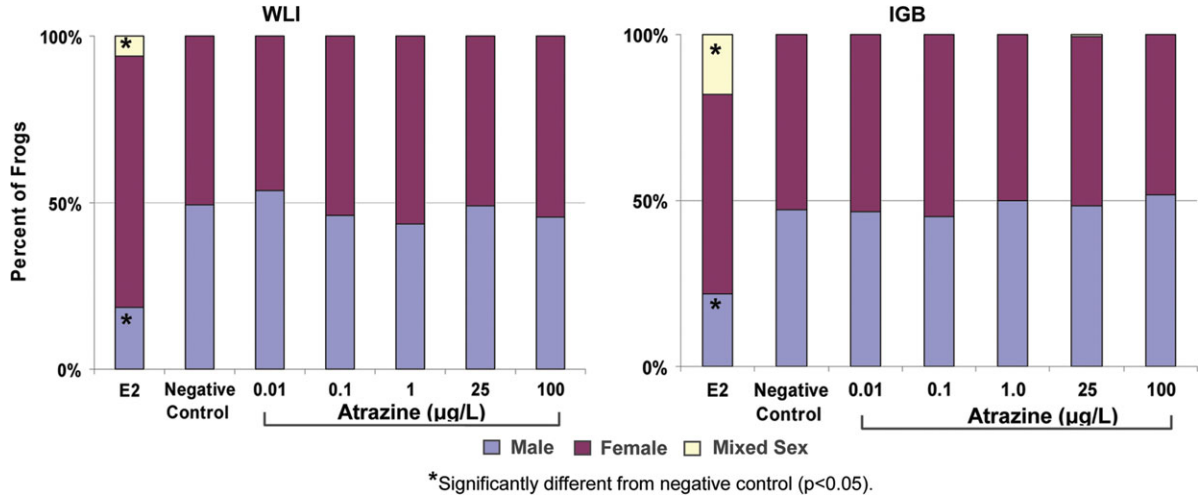

FIG. 3. Proportion of frogs (%) that were histologically determined to be male, female, or mixed sex. Statistical difference are marked by asterisk $*p < 0.05$.

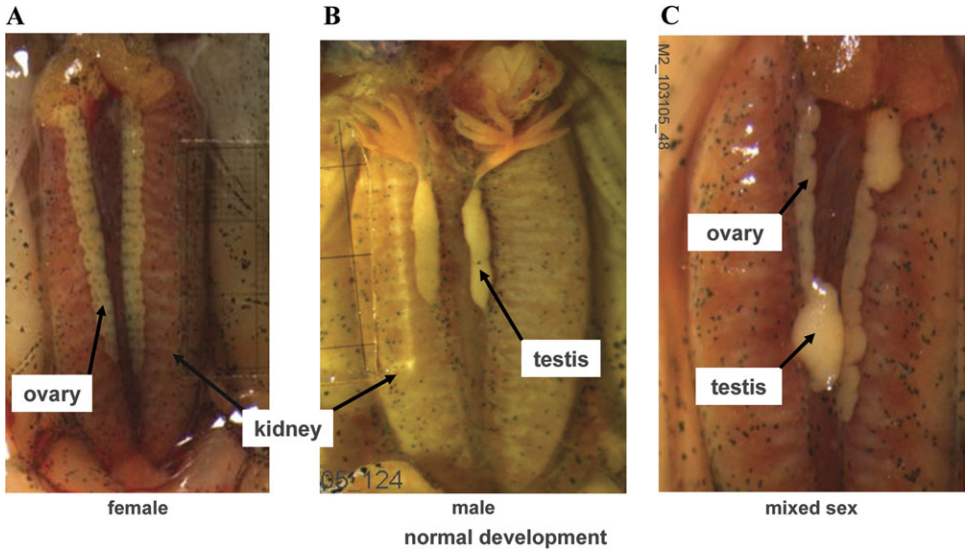

FIG. 4. Gonads of *Xenopus laevis* after completion of metamorphosis demonstrating clearly differentiated male and female phenotypes by testes (a) and ovaries (b) of negative control animals. The mixed sex gonad (c) exhibiting testicular as well as ovarian tissue was obtained from the positive control of 0.2 µg E2/l treatment.

gonocytes, and internal melanophores (Table 3). For phenotypic females, in experiment 1 both increased and decreased ovarian cavity sizes were related to E2 exposure, whereas in experiment 2 only increased ovarian cavity size was E2 related. Other inconsistent effects of E2 included segmental hypoplasia of the testes and ovaries which was only observed in experiment 2. It is important to note that no statistically significant effects of atrazine were observed among the endpoints that demonstrated significant responses to E2 treatment.

DISCUSSION

X. laevis has long been regarded as a sensitive model for the study of the effects of steroid hormones on sexual development, including feminization of males by estrogenic compounds (Chang and Witschi, 1955a,b; Gallien, 1953; Kloas and Lutz, 2006; Kloas *et al.*, 1999; Witschi, 1971). Therefore, *X. laevis* is a suitable model for testing the potential effects of atrazine. Consistent with this contention is our observation that E2 readily causes feminization of frogs under experimental conditions similar to those used in the current experiments (Lutz *et al.*, 2007). The clear feminization of *X. laevis* tadpoles exposed to 0.2 µg E2/l in a flow-through exposure system, demonstrated that these animals were very sensitive to moderate estrogenic influences.

Long-term exposure (75 days) of *X. laevis* to atrazine at concentrations ranging from 0.01 to 100 µg/l had no effects upon the developmental endpoints (general condition, body weight, snout-to-vent length, time to completion of metamorphosis) or upon endpoints related to sexual differentiation (percentage of males and females, occurrence of mixed sex, intersex, or testicular oocytes). The absence of effects in atrazine exposed *X. laevis* indicates that atrazine neither feminizes nor demasculinizes frogs, as has been previously reported (Hayes *et al.*, 2002). The results from the present study are also contrary to those of Hayes *et al.* (2006) who reported that 32–40% of frogs exposed to atrazine at concentrations ranging from 0.1 to 200 µg/l had multiple gonads. There is no obvious explanation for the differences between Hayes findings and the findings of the current study, however, the latter clearly indicates that atrazine does not cause gonadal feminization of *X. laevis*. More recently, Oka *et al.* (2008) demonstrated also clearly that atrazine had no estrogenic effects including a lack of potential to induce aromatase expression. Despite the clear absence of any estrogenic mode of action at two concentrations of atrazine, 10 and 100 µg/l, the authors noted a higher percentage of female phenotypes after larval exposure compared with negative

TABLE 3
Secondary Gonadal Histopathological Observations of 17β-Estradiol and Atrazine Treatments compared with Controls

		WLI		IGB	
Histopathologic finding		E2	ATZ	E2	ATZ
Frequency of males		*	NS	*	NS
Mixed sex		*	NS	**	NS
Testis	Dilated tubules	**	NS	**	NS
	Dividing gonocytes	**	NS	**	NS
	Internal melanophores	*	NS	*	NS
Ovary	Increased ovarian cavity size	*	NS	*	NS

Note. *$p \leq 0.05$, **$p < 0.001$, NS = not significant.

controls having a higher percentage of male phenotypes and no mixed sex has been determined in any atrazine treatment. It has also been proposed that atrazine causes feminizing effects in males via upregulation of aromatase in testicular tissue, resulting in reduced testosterone and increased estrogen levels (Hayes, 2005; Hayes *et al*., 2002, 2006). Induction of an increased aromatase expression or activity has been reported *in vitro* at near saturated atrazine concentrations in an adrenal chromatin and a placental cell line (Sanderson *et al*., 2000, 2001) but not in other cell lines such as the MCF7 breast cancer cell (Sanderson *et al*., 2001), the R2C Leydig cell (Heneweer *et al*., 2004), the ovarian granulosa cell (Ohno *et al*., 2004), an immortalized sea turtle cell line (Keller and McClellan-Green, 2004) or in chicken egg (Matsushita *et al*., 2006). Furthermore, no relationship between atrazine dose and aromatase activity or gene expression has been found in any *in vivo* study conducted with amphibians (Hecker *et al*., 2004, 2005a,b; Murphy *et al*., 2006) or rodents (Rayner *et al*., 2004). In three studies concerning impacts of atrazine on aromatase induction conducted in fish two studies were negative (Kazeto *et al*., 2004; Nadzialek *et al*. 2008) and one was reported to be positive (Suzawa and Ingraham, 2008). The weight of the evidence indicates that atrazine does not induce aromatase *in vivo*. This is consistent with the fact that atrazine has failed to show estrogenic potential in either *in vitro* or *in vivo* models (Eldridge *et al*., 2008).

Strengths of the present study, some of which are unique to this particular area of research, include: the optimization of environmental testing conditions, the conduct of parallel experiments in two laboratories using the same protocol, the use of a large number of replicates in order to increase the statistical power of each experiment, the use of procedures that blinded investigators with respect to treatment groups, the inclusion of both positive and negative control groups, the standardization of descriptive and quantitative gross and histopathologic terminology and criteria, the assessment of the histopathology slides of both experiments by an independent board-certified veterinary pathologist, full GLP compliance, and the utilization of comprehensive statistical analysis procedures that were defined *a priori*. These provisions should have allowed detection of any effects of atrazine on the sexual development of *X. laevis*, had such effects existed.

Other studies in adult male *X. laevis* have examined potential effects of atrazine exposure on hormone levels, aromatase activity and testicular ultrastructure. After 36 days of exposure to 1, 25, and 250 μg atrazine/l, significantly lower plasma testosterone levels were observed at the highest concentration only. No effects on testicular aromatase activity, aromatase gene (CYP19) expression or E2 were reported at any dose level (Hecker *et al*., 2005a). In a similar study with a 46-day exposure to 10 or 100 μg atrazine/l, there were no significant differences between atrazine-treated frogs and negative controls in endpoints such as plasma testosterone, plasma E2, testicular aromatase activity or testicular ultrastructure at either dose level (Hecker *et al*., 2005b). Coady *et al*. (2005) failed to find any effect on the incidence of mixed sex, intersex, discontinuous gonads, plasma testosterone levels, or brain aromatase activity (brain, gonads) in *X. laevis* exposed to atrazine from 72-h posthatch through metamorphosis at concentrations of 0.01–25 μg/l.

Field studies in South Africa with native African clawed frogs did not demonstrate significant differences in sex ratio, snout-to-vent length, body-mass, or age profiles between frogs exposed to atrazine in high-use areas versus nonuse control areas. The results of a mark-recapture study indicated that all control and exposed sites had robust populations, which suggested that there was no reproductive impairment (Du Preez *et al*., 2005). There were no differences in testicular cell types or laryngeal mass in frogs from either control or exposed sites (Smith *et al*., 2005). Exposure of larval *X. laevis* to atrazine (1, 10, or 25 μg/l) throughout metamorphosis in outdoor microcosms with natural photoperiod and temperature in South Africa did not have any significant effects on gonadal differentiation (Jooste *et al*., 2005).

Thus, numerous studies in the recent scientific literature suggest that atrazine does not effect growth, development, or sexual differentiation of *X. laevis*. The current study demonstrates that in *X. laevis*, sexual differentiation is sensitive to factors that might trigger the endocrine system to induce feminizing or demasculinizing effects. However, none of the effects associated with a moderate E2 exposure in the positive control frogs were seen in atrazine-treated frogs. In conclusion, the results of this large, multilaboratory, highly replicated study demonstrate that long-term exposure to atrazine at concentrations ranging from 0.01 to 100 μg/l, over a period of time that included the critical window of sensitivity for sexual differentiation of *X. laevis*, has no effect on the growth, larval development, or sexual differentiation.

FUNDING

Syngenta Crop Protection, Inc.

ACKNOWLEDGMENTS

This research was conducted under the guidance and inspection of the U.S. Environmental Protection Agency. A.H. is employed by Syngenta which is the primary registrant of atrazine. No other author has a financial interest in Syngenta.

REFERENCES

Carr, J. A., Gentles, A., Smith, E. E., Goleman, L. W., Urquidi, L. J., Thuett, K., Kendall, R. J., Giesy, J. P., Gross, T. S., Solomon, K. R., *et al*. (2003). Response of larval *Xenopus laevis* to atrazine: Assessment of growth, metamorphosis, gonadal and laryngeal morphology. *Environ. Toxicol. Chem.* **22,** 396–405.

Chang, C. Y., and Witschi, E. (1955a). Breeding of sex-reversed males of *Xenopus laevis*. *Proc. Soc. Exp. Biol. Med.* **93,** 150–151.

Chang, C. Y., and Witschi, E. (1955b). Genetic and hormonal reversal of sex differentiation in *Xenopus*. *Proc. Soc. Exp. Biol. Med.* **93,** 140–144.

Coady, K. K., Murphy, M. B., Villeneuve, D. L., Hecker, M., Carr, J. A., Solomon, K. R., Van Der Kraak, G. J., Smith, E. E., Kendall, R. J., and Giesy, J. P. (2005). Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, aromatase activity, and plasma sex steroid concentrations in *Xenopus laevis*. *Ecotox. Environ. Safe* **62,** 160–173.

Du Preez, L. H., Solomon, K. R., Carr, J. A., Giesy, J. P., Gross, T. S., Kendall, R. J., Smith, E. E., Van Der Kraak, G. J., and Weldon, C. (2005). Population structure of the African Clawed Frog (*Xenopus laevis*) in maize-growing areas with atrazine application versus non-maize growing areas in South Africa. *Afr. J. Herpetol.* **54,** 61–68.

Eldridge, J. C., Stevens, J. T., and Breckenridge, C. B. (2008). Atrazine interaction with estrogen expression systems. *Rev. Environ. Contam. Toxicol.* **196,** 147–160.

Gallien, L. (1953). Inversion totale du sexe chez Xenopus laevis Daud. A la suite du'n traitment gynoépar le benzoate of oestradiol administré pendant la vie larvaire. *C. R. Acad. Sci.* **237,** 1565–1566.

Giddings, J. M., Anderson, T. A., Hall, L. W., Hosmer, A. J., Kendall, R. J., Richards, R. P., Solomon, K. R., and Williams, W. M. (2005). Atrazine in North American surface waters: A propabilistic aquatic ecological risk assessment. Society of Environmental Toxicology and Chemistry (SETAC), Pensacola, FL.

Hayes, T. B. (2005). Welcome to the revolution: Integrative biology and assessing the impact of endocrine disruptors on environmental and public health. 2005. *Integr. Comp. Biol.* **45,** 321–329.

Hayes, T. B., Collins, A., Mendoza, M., Noriega, N., Stuart, A. A., and Vonk, A. (2002). Hermaphroditic, demasculinized frogs exposure to the herbicide atrazine at low ecologically relevant doses. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 5476–5480.

Hayes, T. B., Stuart, A. A., Mendoza, M., Collins, A., Noriega, N., Vonk, A., Johnston, G., Liu, R., and Kpodzo, D. (2006). Characterization of atrazine-induced gonadal malformations in African clawed frogs (*Xenopus laevis*) and comparisons with effects of an androgen antagonist (cyproterone acetate) and exogenous estrogen (17β-estradiol): Support for the demasculinization/feminization hypothesis. *Environ. Health Perspect.* **114**(Suppl. 1), 134–141.

Hecker, M., Giesy, J. P., Jones, P. D., Jooste, A. M., Carr, J. A., Solomon, K. R., Smith, E. E., Van Der Kraak, G. J., Kendall, R. J., and Du Preez, L. H. (2004). Plasma sex steroid concentrations and gonadal aromatase activities in African clawed frogs (*Xenopus laevis*) from the corngrowing region of South Africa. *Environ. Toxicol. Chem.* **23,** 1996–2007.

Hecker, M., Kim, W. J., Park, J.-W., Murphy, M. B., Villeneuve, D., Coady, K. K., Jones, P. D., Solomon, K. R., Van Der Kraak, G. J., Carr, J. A., *et al.* (2005a). Plasma concentrations of estradiol and testosterone, gonadal aromatase activity, and ultrastructure of the testis in *Xenopus laevis* exposed to estradiol and atrazine. *Aquat. Toxicol.* **72,** 383–396.

Hecker, M., Park, J.-W., Murphy, M. B., Jones, P. D., Solomon, K. R., Van Der Kraak, G. J., Carr, J. A., Smith, E. E., Du Preez, L. H., Kendall, R. J., *et al.* (2005b). Effects of atrazine on CYP19 gene expression and aromatase activity in testes and on sex steroid concentrations in plasma of male African clawed frogs (*Xenopus laevis*). *Toxicol. Sci.* **86,** 273–280.

Heneweer, M., van den Berg, M., and Sanderson, J. T. (2004). A comparison of human H295R and rat R2C cell lines as in vitro screening tools for effects on aromatase. *Toxicol. Lett.* **146,** 183–94.

Jooste, A. M., Du Preez, L. H., Carr, J. A., Giesy, J. P., Gross, T. S., Kendall, R. J., Smith, E. E., Van Der Kraak, G. J., and Solomon, K. R. (2005). Gonadal development of larval male *Xenopus laevis* exposed to atrazine in outdoor microcosms. *Environ. Sci. Technol.* **39,** 5255–5261.

Kazeto, Y., Place, A., and Trant, J. (2004). Effects of endocrine disrupting chemicals on the expression of CYP19 genes in zebrafish (*Danio rerio*) juveniles. *Aquat. Toxicol.* **69,** 25–34.

Keller, J. M., and McClellan-Green, P. (2004). Effects of organochlorine compounds on cytochrome P450 aromatase activity in an immortal sea turtle cell line. *Mar. Environ. Res.* **58,** 347–351.

Kloas, W., and Lutz, I. (2006). Amphibians as model to study endocrine disrupters. *J. Chromatogr. A* **1130,** 16–27.

Kloas, W., Lutz, I., and Einspanier, R. (1999). Amphibians as model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals in vitro and in vivo. *Sci. Total Environ.* **225,** 59–68.

Lutz, I., Kloas, W., Springer, T., Holden, L., Wolf, J., Kruger, H., and Hosmer, A. (2007). Development, standardization and refinement of procedures for evaluating effects of endocrine active compounds on development and sexual differentiation of *Xenopus laevis*. *Anal. Bioanal. Chem.* **390,** 2031–2048.

Matsushita, S., Yamashita, J., Iwasawa, T., Tomita, T., and Ikeda, M. (2006). Effects of in ovo exposure to imazalil and atrazine on sexual differentiation in chick gonads. *Poult. Sci.* **85,** 1641–1647.

Murphy, M. B., Hecker, M., Coady, K. K., Tompsett, A. R., Higley, E. B., Jones, P. D., Du Preez, L. H., Solomon, K. R., Carr, J. A., Smith, E. E., *et al.* (2006). Plasma steroid hormone concentrations, aromatase activities and GSI in Ranid frogs collected from agricultural and non-agricultural sites in Michigan (USA). *Aquat. Toxicol.* **77,** 153–166.

Nadzialek, S., Spano, L., Mandiki, S. N. M., and Kestemont, P. (2008). High doses of atrazine do not disrupt activity and expression of aromatase in female gonads of juvenile goldfish (*Carassius auratus* L.). *Ecotoxicology* doi: 10.1007/s10646-008-0198-9.

Ohno, K., Araki, N., Yanase, T., Nawata, H., and Iida, H. (2004). A novel nonradioactive method for measuring aromatase activity using a human ovarian granulosa-like tumor cell line and an estrone ELISA. *Toxicol. Sci.* **82,** 443–450.

Oka, T., Tooi, O., Mitsui, N., Miyahara, M., Ohnishi, Y., Takase, M., Kashiwagi, A., Shinkai, T., Santo, N., and Iguch, T. (2008). Effect of atrazine on metamorphosis and sexual differentiation in *Xenopus laevis*. *Aquat. Toxicol* **87,** 215–226.

Rayner, J. L., Wood, C., and Fenton, S. E. (2004). Exposure parameters necessary for delayed puberty and mammary gland development in Long-Evans rats exposed in utero to atrazine. *Toxicol. Appl. Pharmacol.* **195,** 23–34.

Sanderson, J. T., Letcher, R. J., Heneweer, M., Giesy, J. P., and van den Berg, M. (2001). Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. *Environ. Health Perspect.* **109,** 1027–1031.

Sanderson, J. T., Seinen, W., Giesy, J. P., and van den Berg, M. (2000). 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: A novel mechanism for estrogenicity? *Toxicol. Sci.* **54,** 121–127.

Smith, E. E., Du Preez, L. H., Gentles, A., Solomon, K. R., Tandler, B., Carr, J. A., Van Der Kraak, G. J., Kendall, R. J., Giesy, J. P., and Gross, T. S. (2005). Assessment of laryngeal muscle and testicular cell types in *Xenopus laevis* (Anura Pipidae) inhabiting maize and non-maize growing areas of South Africa. *Afr. J. Herpetol.* **54,** 69–76.

Suzawa, M., and Ingraham, H. A. (2008). The herbicide atrazine activates endocrine gene networks via non steroidal NR5A nuclear receptors in fish and mammalian cells. *PloS One* **3,** 1–11.

Tavera-Mendoza, L., Ruby, S., Brousseau, P., Fourier, M., Cyr, D., and Marcogliese, D. (2002a). Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. *Environ. Toxicol. Chem.* **21,** 527–531.

Tavera-Mendoza, L., Ruby, S., Brousseau, P., Fourier, M., Cyr, D., and Marcogliese, D. (2002b). Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the ovary. *Environ. Toxicol. Chem.* **21,** 1264–1267.

Villalpando, I., and Merchant-Larios, H. (1990). Determination of the sensitive stages for gonadal sex-reversal in *Xenopus laevis* tadpoles. *Int. J. Dev. Biol.* **34,** 281–285.

Witschi, E. (1971). Mechanisms of sexual differentiation: Experiments with *Xenopus laevis*. In *Hormones and Development*. (M. Hamburg and E. Barrington, Eds.), pp. 601–618. Meredith, New York.