

RESPONSE OF LARVAL *XENOPUS LAEVIS* TO ATRAZINE: ASSESSMENT OF GROWTH, METAMORPHOSIS, AND GONADAL AND LARYNGEAL MORPHOLOGYJAMES A. CARR,\*†‡ ANGIE GENTLES,†‡ ERNEST E. SMITH,‡ WANDA L. GOLEMAN,† LINA J. URQUIDI,†  
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**Abstract**—Larval *Xenopus laevis* were exposed to one of four concentrations of atrazine (0, 1, 10, or 25 µg/L, 11 replicate tanks per treatment, 60–65 larvae per replicate) dissolved in an artificial pond water (frog embryo teratogenesis assay- *Xenopus* [FETAX]) medium beginning 48 h after hatching until the completion of metamorphosis. Separate groups of larvae (six replicate tanks per treatment, 60–65 larvae per replicate) were exposed to estradiol (100 µg/L), dihydrotestosterone (100 µg/L), or ethanol vehicle control dissolved in FETAX medium. None of the treatments affected posthatch mortality, larval growth, or metamorphosis. There were no treatment effects on sex ratios except for estradiol, which produced a greater percentage of female offspring. Exposure to either estradiol or 25 µg atrazine/L increased the incidence of intersex animals based on assessment of gonadal morphology. Atrazine did not reduce the size of the laryngeal dilator muscle, a sexually dimorphic muscle in this species. We conclude that environmentally relevant concentrations of atrazine do not influence metamorphosis or sex ratios and do not inhibit sexually dimorphic larynx growth in *X. laevis*. The incidence of atrazine-induced intersex animals was small (<5%) and occurred only at the greatest concentration of atrazine tested, a concentration that is rarely observed in surface waters in the United States.

**Keywords**—Amphibian Anuran Atrazine Reproduction Metamorphosis

## INTRODUCTION

Recent declines in the population size of some amphibian species have been reported [1–4]. Potential causes include changes in the amount and quality of habitat, climate changes, parasites, bacterial and fungal infections, and agricultural and industrial chemicals, all of which might influence the fitness of amphibian populations. One agricultural chemical that has received particular attention for its potential effects on amphibians is atrazine (2-chloro-4-ethylamino-6-isopropylamino-*S*-triazine), which is one of the most widely used agricultural herbicides in the United States [5]. Although atrazine has been widely detected in surface waters, concentrations in freshwater rivers and streams rarely exceed 20 to 25 µg atrazine/L [5]. These concentrations are less than the lowest-observable-adverse-effect concentration (LOAEC, 1.1 mg atrazine/L) for deformities in acute (96-h) exposure testing in *Xenopus laevis* [6] and a no-observed-effect concentration ([NOEC], 200 µg atrazine/L) for larval growth and metamorphosis in the northern leopard frog (*Rana pipiens*) [7]. While predicted NOECs for lethality in *R. pipiens* and *Bufo americanus* larvae [8] fall within the upper part of a range of atrazine concentrations that may occur in temporary ponds as a result of runoff [9–11], these values are based on 30-d exposures under laboratory conditions, and the probability of sufficient duration of exposure during intermittent runoff events is small [5]. These

observations and the fact that median lethal concentrations (LC50) in acute toxicity tests with a variety of amphibians range from 15.8 to 126 mg atrazine/L [6,8] suggest that atrazine concentrations that are likely to be encountered in permanent freshwater environments are not lethal or directly toxic to developing anurans.

There are conflicting reports regarding the potential for atrazine to influence reproductive development in amphibians at environmentally relevant concentrations. Atrazine (21 µg atrazine/L, the only concentration tested) was reported to reduce testicular volume and number of testicular nest cells in larval *X. laevis* exposed for 48 h [12]. In a different study [13], atrazine concentrations as small as 0.1 µg atrazine/L were reported to cause gonadal abnormalities in *X. laevis* larvae and 1 µg atrazine/L was reported to reduce the size of the laryngeal dilator muscles in developing males. In contrast, a field study of cricket frogs (*Acris crepitans*) in Illinois found no correlation between incidence of intersex and atrazine usage [14]. Similarly, Beasley et al. [15] found no relationship between historical incidence of intersex and atrazine use. The incidence of intersex during the 1990s was virtually the same (3%) as it was during the period of 1852 through 1929 (2%) and was considerably less than the incidence of intersex (16.5%) in the years (1946–1959) immediately prior to the registration of atrazine for use in the United States (1959).

It has been suggested that atrazine affects reproductive development in frogs [13] at concentrations that are several hundredfold less than those reported to cause acute toxicity [6].

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Aside from the frog embryo teratogenesis assay-*Xenopus* (FETAX) procedure, which is limited to a 96-h exposure window immediately after fertilization, there are no standardized approaches to assessing developmental exposure effects on development in *X. laevis*, and differences in the strain of animals, the type of rearing medium used, and replicate sample size can add to the normal variability that is associated with differences in testing methodologies. In light of these uncertainties, we undertook a study to determine the response of larval *X. laevis* to environmentally relevant concentrations of atrazine during a full developmental period exposure in FETAX medium, a standardized rearing medium that has been extensively used to assess toxicity and teratogenicity in *X. laevis*. Survival, growth, and proportion of frogs undergoing metamorphosis were determined. In addition, laryngeal dilator muscle size and gonadal developmental morphology were assessed as indicators of potential reproductive effects. Because it has been proposed that atrazine may influence reproductive development by altering sex steroid hormone production [13], we also investigated the response of developing *X. laevis* to dihydrotestosterone (DHT) and estradiol. Estradiol exposure during gonadal differentiation leads to a greater percentage of phenotypic female offspring in *X. laevis* [16]. Although larval exposure to androgens such as DHT does not influence gonadal differentiation in *X. laevis*, DHT has well-documented stimulatory effects on growth of the sexually dimorphic laryngeal dilator muscle in this species [17].

## METHODS

### Test materials

Atrazine (CAS No. 1912-24-9, purity 98.6%) was obtained from Syngenta (Greensboro, NC, USA). Estradiol (CAS No. 50-282, purity >99%) and DHT (CAS No. 521-18-6, purity >99%) were purchased from Sigma Chemical (St. Louis, MO, USA). Absolute ethanol (CAS No. 64-17-5, purity 100%, USP grade) was purchased from AAPER alcohol (Shelbyville, KY, USA).

### Animals

Sexually mature male and female *X. laevis* imported from South Africa were purchased from Xenopus Express (Homosassa, FL, USA). Adults were maintained in dechlorinated water on a 12:12 h light:dark regime (lights on 0700) at 19°C at a density of three animals per 18 L in 40-L glass aquaria. Frogs were fed frog brittle (Nasco, Ft. Atkinson, WI, USA) three times weekly immediately following each water change.

Breeding pairs were allowed to acclimate in 40-L glass aquaria containing 18 L FETAX medium [18] for 7 d prior to breeding. Deionized water was passed through a 1.2-ft<sup>3</sup> carbon filter immediately before it was used to prepare FETAX medium.

Naturally fertilized eggs were obtained from five pairs of adults as previously described [19] and transferred to individual 250-ml glass beakers containing 100 ml FETAX medium and maintained at 22 ± 1°C on a 12:12 h light:dark regimen. Starting on posthatch day 5, larvae were fed 0.4 g of powdered frog brittle (Nasco) mixed in 2 ml of FETAX solution per beaker/tank every 72 h immediately following a 50% water change. All procedures involving *X. laevis* were approved by the Texas Tech Animal Care and Use Committee (Texas Tech University, Lubbock, TX, USA).

### Analytical procedures

Ammonia, pH, and specific conductance of the tank water were monitored every 7 d. Dissolved oxygen was monitored every 2 d and water temperature monitored once daily. A YSI® model 85 meter (Yellow Springs, OH, USA) was used to monitor water temperature, percent saturation with dissolved oxygen, specific conductivity, and salinity for each tank. Free ammonium ion concentration and pH of the water in each tank were determined with a Hach® spectrophotometer model DR/2000 (Loveland, CO, USA) and an Oakton® pH meter (Gresham, OR, USA), respectively. Water temperature and pH in the test tanks averaged 21.2°C (range of 19.0–22.8°C) and 7.8 (range of 6.9–8.3), respectively, while specific conductivity and dissolved oxygen averaged 1,794 µS/cm (range of 1,114–2,347 µS/cm) and 6.7 mg/L (range of 3.9–9.3 mg/L), respectively. Average values for conductivity and dissolved oxygen are within the ranges previously reported for aerated FETAX medium [18].

Samples of concentrated and diluted stock solutions as well as aliquots of reference and test tank media collected throughout the experiment were analyzed for atrazine and estradiol content. The atrazine content of (100×) stock solutions was verified in diluted samples prior to the onset of dosing by enzyme-linked immunosorbent assay (ELISA) using a RaPID® atrazine test kit (Strategic Diagnostics, Newark, DE, USA). The method detection limit for this assay was 0.046 µg atrazine/L and the limit of quantitation was 0.1 µg atrazine/L. Atrazine analysis of diluted stock solutions (prepared every 72 h during the exposure period) was performed using the Beacon™ atrazine plate kit (Beacon Analytical Systems, Portland, ME, USA) as described previously [20]. Samples were analyzed in duplicate at 1- and 10-fold dilutions. The kit has a measurement range of 0.05 to 5.00 µg atrazine/L [20]. Actual atrazine in the 1-, 10-, and 25-µg/L stock solutions averaged (± standard error [SE], *n* = 25) 1.02 ± 0.03 µg atrazine/L, 9.96 ± 0.29 µg atrazine/L, and 21.3 ± 0.67 µg atrazine/L, respectively. Atrazine in tank samples (11 per treatment) from the 1-, 10-, and 25-µg/L treatments collected once per week for eight weeks during the exposure period averaged (± SE, *n* = 88) 1.07 ± 0.02 µg atrazine/L, 10.31 ± 0.15 µg atrazine/L, and 19.53 ± 0.21 µg atrazine/L. Atrazine was not detected in tank samples (*n* = 88) from the FETAX medium control treatment or tank samples (*n* = 48) from the ethanol control or steroid treatments. Estradiol was measured using a radioimmunoassay kit (Diagnostic Products, Los Angeles, CA, USA). The detection limit was 20 pg/ml and the median effective dose (ED50) was 157 pg/ml. All samples were analyzed in the same assay to avoid interassay variation. Intraassay variation averaged 9%. Estradiol was detected only in diluted estradiol stock solutions and estradiol test tank medium samples. Concentrations of DHT are reported as nominal.

### Experimental design

At 48 h posthatch, 60 to 65 *X. laevis* larvae per replicate beaker were exposed to a single concentration of atrazine (nominal concentrations of 0, 1, 10, and 25 µg atrazine/L diluted in FETAX medium), DHT (100 µg/L, nominal), estradiol (100 µg/L, nominal), or ethanol alone diluted in FETAX medium. Steroids were dissolved in ethanol and diluted in FETAX medium. Ethanol was added to the ethanol control tanks and tanks containing steroid treatments at a concentration of 0.0025% (v/v).

On posthatch day 5, larvae were transferred from the 250-

ml glass beakers to 2-L glass beakers containing 1 L of treatment-matched solutions for 14 d. On day 19, larvae were transferred to treatment-matched 10-L glass tanks containing 4 L FETAX medium and appropriate test or reference solutions. Eleven replicates of each atrazine and untreated FETAX control treatment were performed. Six replicates each were used for the ethanol vehicle control, DHT, and estradiol treatments. Exposures continued for 80 d posthatch. Contents of the test chambers were aerated continuously throughout the experiment. A 50% change of test and control solutions was performed every 72 h. All stock solutions, mixing containers, tanks, nets, and tubing were color coded and personnel performing tank changes did not know the identity of the reference and test tanks.

Larvae were monitored daily for changes in general health. Dead or moribund animals were removed and stored in 10% neutral buffered formalin. Tanks were observed for water quality, and the number of dead larvae as well as the number exhibiting bent tails (bent axial skeleton), asymmetrical tails, edema, or abnormal swimming (defined as tadpole lying on bottom, swimming upside down, swimming on its side, or swimming in circles) were recorded. Forelimb emergence ([FLE], both forelegs visible) and complete tail resorption were recorded daily. Tanks were checked on a daily basis for depletion of food, and additional food was provided to tanks as needed.

At completion of metamorphosis (Nieuwkoop–Faber [NF] stage 66 [21]) animals were removed from tanks, euthanized by immersion in 3-aminobenzoic acid ethyl ester, rinsed in distilled water, and immediately weighed. Each animal was given a unique identification number that included the study number, treatment color, replicate tank letter, and animal number. A midline incision was made in the abdomen to allow fixative penetration. Animals were then placed in plastic tissue cassettes and immersion fixed in Bouin's fixative for 48 h followed by storage in 70% ethanol. Snout–vent lengths (SVLs) and sex (based on gonadal morphology) [22] were recorded on preserved NF-stage 66 specimens. The experiment was terminated on posthatch day 80. Any remaining tadpoles were processed as described for NF stage 66 animals above. Only data from NF stage 66 animals were used for the present study.

#### *Assessment of larynx size*

Animals were selected randomly for larynx analysis by means of a spreadsheet developed specifically for this purpose (Sielken and Associates, Bryan, TX, USA). A prespecified number of NF stage 66 frogs of each sex in a tank were randomly selected sequentially as the animals completed metamorphosis and were sexed. In essence, it was the position (or order) in the processing list that was randomly chosen. After determining gonadal phenotype, each frog was quickly identified for larynx analysis if it was listed in one of the selected positions. The Excel® (Microsoft, Redmond, WA, USA) spreadsheet randomly selected the positions and facilitated bookkeeping of this process.

In the initial analysis, 40 to 45 males and 11 females were analyzed per treatment from the FETAX medium control and 25 µg atrazine/L groups. Heads were removed from preserved NF stage 66 frogs, processed for routine paraffin embedding, coronally sectioned at 8 µm, and stained with hematoxylin and eosin. Every 20th section through the laryngeal dilator muscle was digitally photographed using an Olympus P11 dig-

ital camera attached to an Olympus BH-2 microscope (Olympus, Tokyo, Japan). Cross-sectional areas were computed from digital images using SimplePCI software (Compix Imaging Systems, Cranberry Township, PA, USA). Total muscle volume [23,24] was first calculated and compared with the largest cross-sectional area of muscle, determined by ranking all area measurements for a single animal and selecting the largest area. We found that the largest cross-sectional area was a more robust indicator of sex differences in muscle size. Thus, cross-sectional area was used as the primary measure of androgen-induced muscle differentiation. Left and right muscle cross-sectional areas were measured independently for all animals. There were no significant differences between right and left muscle cross-sectional areas, so the values were combined to provide a single area measurement (total muscle cross-sectional area) for each animal.

#### *Assessment of gonadal morphology*

Assessment of gonadal morphology was performed on all animals that reached NF-stage 66 during the 78-d exposure. Sex ratios were determined by direct visual inspection of 276 to 334 animals per treatment for the atrazine and FETAX medium controls (1,215 animals total) and 135 to 160 animals per group from the ethanol vehicle controls and steroid treatments (446 animals total) as described previously [22]. Previously fixed specimens were rinsed in deionized water and pinned to a dissecting dish of hardened paraffin wax placed under a binocular dissecting microscope with a high intensity illuminator as a light source. The abdominal cavity was opened and the intestines removed. The kidneys were located in the retroperitoneal region. Gonads appeared as thin whitish strips of tissue on the medial side of each kidney. Ovaries were long and lobular, with small areas of dark pigmentation visible. Testes were shorter and lacked both lobes and pigmentation. Gonadal abnormalities were scored using the following criteria: intersex gonads were categorized as left/right intersex (a testis on one side and an ovary on the opposite side), rostral/caudal intersex (testicular characteristics rostrally and ovarian characteristics caudally or vice versa), or mixed sex (mixed testicular/ovarian tissue). For purposes of analysis, all three subcategories were combined and referred to as intersex. Discontinuous gonads exhibited abnormal segmentation of the gonad along the rostral–caudal axis.

After assessment of gonadal morphology, the dorsal wall of the abdominal cavity with kidneys and gonads attached was processed for routine paraffin embedding. Gonads were sectioned at 10 µm, stained with hematoxylin and eosin, and gonadal morphology examined using a light microscope.

#### *Statistical methods*

Our study design (tanks nested within treatments, frogs nested within tanks) required that we carefully assess the contribution of tank-to-tank variation in all statistical analyses. For quantal data, tank-to-tank differences within treatments were tested by a chi-square test of homogeneity. The *p* values for all *m* treatments were summed as  $-2 \ln(p)$  and compared to a chi-squared distribution with  $2m$  *df*. If there were no tank effects for a quantal characteristic, the incidences in all tanks within each treatment were pooled. For quantal characteristics with evidence of clear tank effects, the tank percentages were computed and the data analyzed as continuous. The quantal characteristics analyzed as incidences (i.e., with tanks pooled within treatments) were bent tails, edema, abnormal swim-

Table 1. Hatching, mortality, metamorphosis, and gross developmental abnormalities in Nieuwkoop–Faber stage 66 *Xenopus laevis* exposed to atrazine or sex steroids<sup>a</sup>

Treatment	Hatching (%)	Mortality <sup>b</sup> (%)	Forelimb emergence (%)	Tail resorption (%)	Bent tails (%)	Edema (%)	Abnormal swimming (%)
FETAX medium control <sup>c</sup>	94.9 ± 1.02	10.4 ± 1.07	60.8 ± 3.00	51.5 ± 2.91	0.94 ± 0.43	0.15 ± 0.15	1.37 ± 0.44
1 µg atrazine/L	93.6 ± 1.12	11.4 ± 1.20	59.0 ± 3.31	48.4 ± 3.00	0.49 ± 0.36	0.30 ± 0.20	2.15 ± 0.53
10 µg atrazine/L	94.5 ± 0.93	14.1 ± 0.99	52.2 ± 3.85	42.3 ± 3.42	2.13 ± 0.46	0.44 ± 0.23	3.12 ± 0.68
25 µg atrazine/L	96.0 ± 0.82	12.4 ± 1.58	52.5 ± 2.39	44.4 ± 2.14	1.22 ± 0.33	1.04 ± 0.33	3.75 ± 0.64*
Ethanol control	93.7 ± 1.47	11.7 ± 1.62	53.8 ± 4.93	46.1 ± 3.84	0.84 ± 0.56	0.57 ± 0.36	1.99 ± 0.73
Dihydrotestosterone	94.6 ± 2.32	12.2 ± 2.84	48.8 ± 5.36	37.2 ± 4.86	0.80 ± 0.36	1.38 ± 0.49	2.98 ± 0.94
Estradiol	92.9 ± 2.21	15.2 ± 4.16	51.2 ± 1.91	42.8 ± 2.28	0.82 ± 0.37	0.57 ± 0.36	1.69 ± 0.60

<sup>a</sup> Values are the mean ± SE of 11 (aqueous vehicle) or 6 (ethanol vehicle) replicates. Sample size per replicate ranged from 20 to 30 animals.

<sup>b</sup> 80-d posthatch mortality.

<sup>c</sup> Frog embryo teratogenesis assay-*Xenopus*.

\* Significantly different from FETAX medium control,  $p < 0.05$ .

ming, sex ratios, and discontinuous gonads. The quantal characteristics analyzed in analysis of variance (ANOVA) as tank percentages were percent hatching, percent mortality, FLE, complete tail resorption, and intersex fraction. Incidence data were transformed (arcsine of the square root) before ANOVA [25] and proportions of 0/ $n$  were replaced with 1/4 $n$  to improve the arcsine transformation [26]. For general ANOVA, data were tested for homogeneity of variance using Bartlett's test or with an  $F$  test for equal standard deviations. If the assumptions of parametric tests were not met, then the Mann-Whitney nonparametric  $t$  test or the Kruskal–Wallis (KW) ANOVA by ranks followed by Dunn's multiple comparisons test were used.

For continuous data (body weight, SVL, and dilator muscle cross-sectional area), tank-to-tank variation was handled as described for quantal data or by using a nested (mixed-model) one-way ANOVA that incorporated a tank effect term. Atrazine dose-order trends were tested either by the Cochran–Armitage test (for incidence data, when there were little or no difference among tanks within treatments) or by a simple linear contrast  $F$  test within ANOVA (for data analyzed as continuous). All statistical analyses were performed using InStat® software (GraphPad Software, San Diego, CA, USA) or SAS® (Ver 8, SAS Institute, Cary, NC, USA).

## RESULTS

Atrazine did not affect posthatch mortality or growth of larvae (Table 1). There were no significant differences in hatching success among any of the pretreatment groups (ANOVA,  $F_{3,40} = 1.0$ ,  $p = 0.4$  for FETAX medium controls and atrazine treatments;  $F_{2,15} = 0.2$ ,  $p = 0.8$  for ethanol controls and steroid

treatments) and no effects of any treatment on posthatch mortality (ANOVA,  $F_{3,40} = 1.6$ ,  $p = 0.2$  for FETAX medium controls and atrazine treatments;  $F_{2,15} = 0.4$ ,  $p = 0.7$  for ethanol controls and steroid treatments; Table 1). Percent hatching was greater than 90% in all pretreatment groups (Table 1). Eighty-day posthatch mortality ranged from 10 to 14% in all groups except for the estradiol group, which exhibited a mortality of 15% (Table 1). There were no differences in body weight or SVL among FETAX control medium and atrazine-exposed animals (Table 2). Time to complete metamorphosis (NF stage 66) varied inversely with both body weight and SVL ( $r^2 = 0.36$  for body wt,  $n = 1,211$ , and SVL,  $n = 1,215$ ). Although body weight accounted only for 36% of the variation in time to metamorphosis, those animals reaching NF stage 66 first in each tank were significantly larger than those animals that were the last to reach NF stage 66 in every treatment (paired  $t$  test,  $p < 0.05$ ; Fig. 1). Body weight and SVL also were inversely correlated with time to complete metamorphosis in ethanol-vehicle and steroid-treated animals ( $r^2 = 0.31$  for body wt and SVL,  $n = 446$ ). Although mean body weight did not differ significantly between ethanol- and steroid-treated *X. laevis*, estradiol-treated females but not males were significantly longer than ethanol-treated females based on differences in SVL (mixed model ANOVA,  $F_{2,15,6} = 4.9$ ,  $p = 0.02$ ).

The percentage of *X. laevis* reaching FLE and complete tail resorption varied between 49 and 60% and 37 and 52%, respectively, during the 78-d day exposure period. Despite weak trends toward fewer atrazine-exposed animals reaching FLE (ANOVA trend test,  $F_{1,40} = 5.0$ ,  $p = 0.03$ ) and completing tail resorption (ANOVA trend test,  $F_{1,40} = 4.5$ ,  $p = 0.04$ ), the general ANOVA  $F$  test revealed no significant effect of at-

Table 2. Body weights and snout–vent lengths in Nieuwkoop–Faber stage 66 *Xenopus laevis* exposed to atrazine or sex steroids<sup>a</sup>

Treatment	Body weight (g)		Snout–vent length (mm)	
	Male	Female	Male	Female
FETAX medium control <sup>b</sup>	0.328 ± 0.004	0.320 ± 0.008	13.9 ± 0.10	13.9 ± 0.17
1 µg atrazine/L	0.311 ± 0.006	0.325 ± 0.010	13.7 ± 0.11	13.8 ± 0.15
10 µg atrazine/L	0.347 ± 0.019	0.356 ± 0.007	14.1 ± 0.24	14.2 ± 0.13
25 µg atrazine/L	0.323 ± 0.010	0.327 ± 0.010	13.8 ± 0.17	14.1 ± 0.15
Ethanol control	0.309 ± 0.016	0.320 ± 0.015	13.6 ± 0.19	13.6 ± 0.22
Dihydrotestosterone	0.322 ± 0.012	0.331 ± 0.013	13.9 ± 0.20	14.1 ± 0.18
Estradiol	0.291 ± 0.015	0.345 ± 0.019	13.7 ± 0.25	14.5 ± 0.24*

<sup>a</sup> Values are the mean ± SE of 11 (aqueous vehicle) or 6 (ethanol vehicle) replicates. Samples size per replicate ranged from 20 to 30 animals.

<sup>b</sup> Frog embryo teratogenesis assay-*Xenopus*.

\* Significantly different from ethanol control,  $p < 0.05$ .

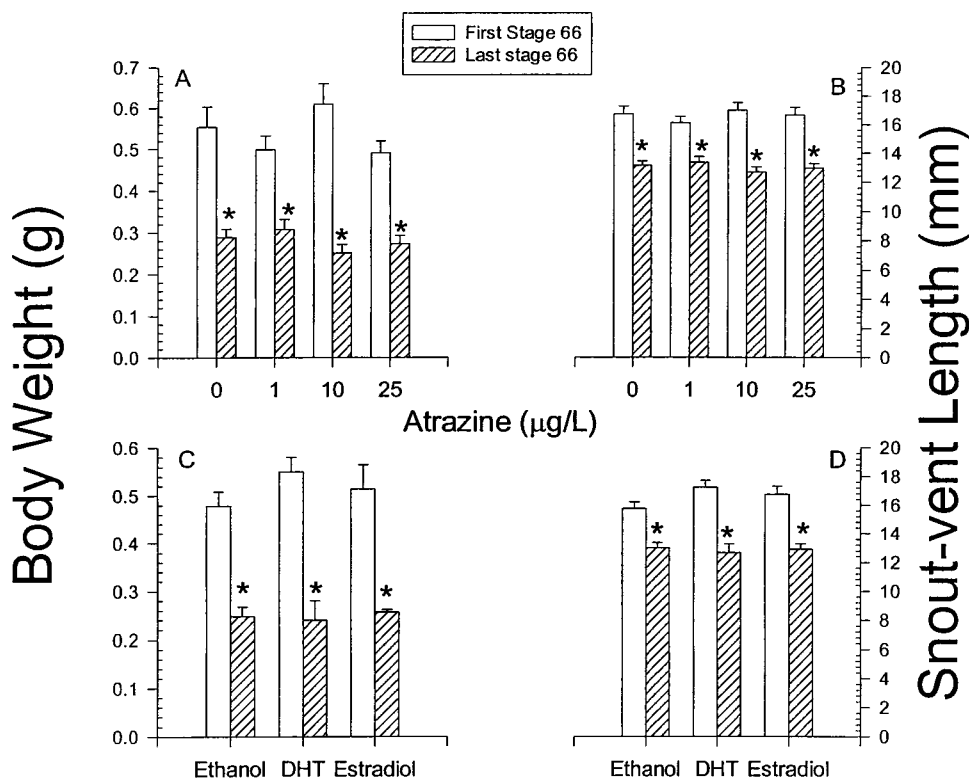


Fig. 1. Relationship between days to Nieuwkoop-Faber stage 66 and body weight (A, C) and snout-vent length (B, D) in *Xenopus laevis* exposed to atrazine (A, B) or steroids (C, D). The bars represent the mean + standard error of 6–11 animals per group. Asterisks indicate significant differences within treatment as determined by paired *t* tests.

razine on either parameter compared with FETAX medium controls ( $F_{3,40} = 1.95$ ,  $p = 0.14$  for FLE;  $F_{3,40} = 2.0$ ,  $p = 0.13$  for tail resorption).

The incidence of gross developmental abnormalities was small (<5%) in all treatments. Although incidence of edema was weakly correlated with atrazine concentration (Cochran-Armitage trend test,  $Z = -2.3$ ,  $p = 0.02$ ), a chi-square homogeneity test revealed that none of the atrazine concentrations significantly increased the incidence of edema compared with FETAX medium controls (chi-square = 6.18, 3 *df*,  $p = 0.1$ ). Incidence of abnormal swimming also was correlated with atrazine concentration (Cochran-Armitage trend test,  $Z = -2.90$ ,  $p = 0.004$ ; Table 1), but only larvae exposed to 25 µg atrazine/L exhibited a significantly greater incidence of abnormal swimming (chi-square homogeneity test, chi-square = 8.5, 3 *df*,  $p = 0.04$ ).

There were no detectable effects of atrazine treatment on sex ratios (Fig. 2). There was a slight reduction in percentage males in the 25-µg atrazine/L group (45% males from the 25-µg atrazine/L group vs. 48–50% males for other treatments). However, exposure to 25 µg atrazine/L did not increase the percentage of females, and the sex ratio in this group did not differ statistically from a 50:50 ratio of males:females. Neither ethanol nor DHT significantly affected sex ratios. Estradiol significantly reduced the percentage of males to 26% and increased the percentage of females to 67%. Approximately 7% of the animals exposed to estradiol were classified as intersex based on gross morphology of the gonads (Fig. 2).

Exposure to either 25 µg atrazine/L or estradiol significantly increased the percentage of individuals with intersex gonads in each tank replicate as determined by gross morphology of the gonads (Fig. 3). Although incidence of intersex

increased with increasing atrazine concentration (Cochran-Armitage,  $Z = 3.6$ ,  $p = 0.0003$ ), only 25 µg atrazine/L significantly increased the average incidence of intersex animals per tank compared with FETAX medium controls ( $n = 11$  per treatment, KW = 12.4,  $p = 0.0061$ ; Fig. 3). Exposure to 25 µg atrazine/L resulted in a total number of 14 of 296 animals (4.7%) with intersex gonads versus a total of 2 intersex animals out of 334 (0.6%) in the FETAX medium controls, a total of 3 intersex animals out of 309 (0.97%) in the 1-µg atrazine/L group, and a total of 1 intersex animal out of 276 (0.36%) in the 10-µg atrazine/L treatment group. There was a significant correlation between incidence of discontinuous gonads and atrazine concentration (Cochran-Armitage,  $Z = 2.9$ ,  $p = 0.0042$ ), although 25 µg/L was the only atrazine concentration to increase the incidence of discontinuous gonads compared with the FETAX medium controls. The percentage of intersex gonads per tank also was significantly greater (KW = 8.1,  $p = 0.01$ ; Fig. 3) in the estradiol-treated group (10 intersex out of 135 total, or 7.4%) compared with the ethanol-treated (0 intersex out of 160 total) group. Examples of gonadal morphology in intersex animals from the estradiol and 25-µg atrazine/L treatment groups are shown in Figure 4.

Intersex animals were processed for routine histological analysis to compare histological features of gonadal structure with the gross morphological differences that were observed in these animals. Animals classified as intersex in the 25-µg atrazine/L treatment group tended to have an obvious testicular or ovarian morphology when examined by light microscopy, although the testes at times appeared flat and somewhat smaller than those from animals classified as males based on gross morphology (Fig. 5). In contrast, histological analysis of es-

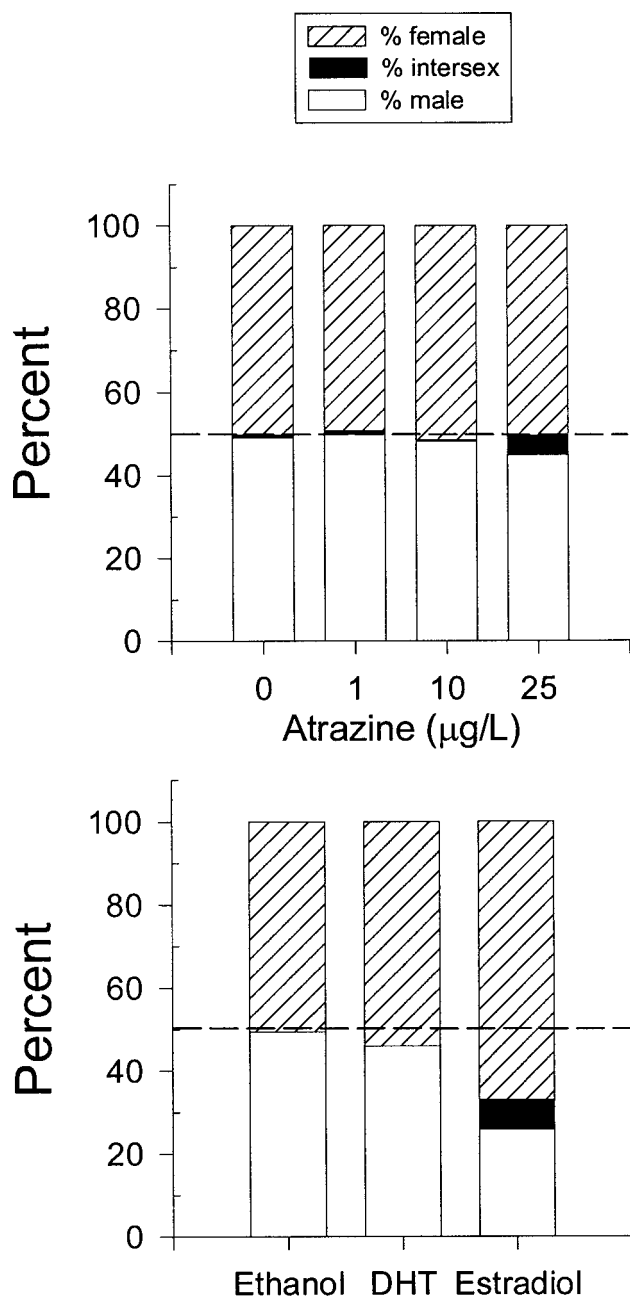


Fig. 2. Sex ratios based on assessment of gonadal morphology for atrazine and steroid-exposed Nieuwkoop-Faber stage 66 *Xenopus laevis*. Ratios were constructed from 276 to 334 animals per treatment for the atrazine experiment and 135 to 160 animals per treatment for the steroid experiment. The broken line represents the 50% mark.

tradiol-treated animals revealed ambiguous gonadal tissues in some cases (Fig. 5).

Atrazine did not decrease laryngeal dilator muscle size in developing *X. laevis* (Fig. 6, Table 3). There were no significant between-tank differences in male dilator muscle size within any of the FETAX medium control or atrazine treatments, so data from individual animals were pooled within each treatment and analyzed by one-way ANOVA ( $F_{3,168} = 2.4, p = 0.07$ ). Total muscle cross-sectional area was 30 to 40% larger in males than females in all atrazine treatment groups (Fig. 6). There were no significant effects of atrazine on total muscle cross-sectional area in females ( $p = 0.5$ ). The utility of our method for assessing androgenic effects on dilator

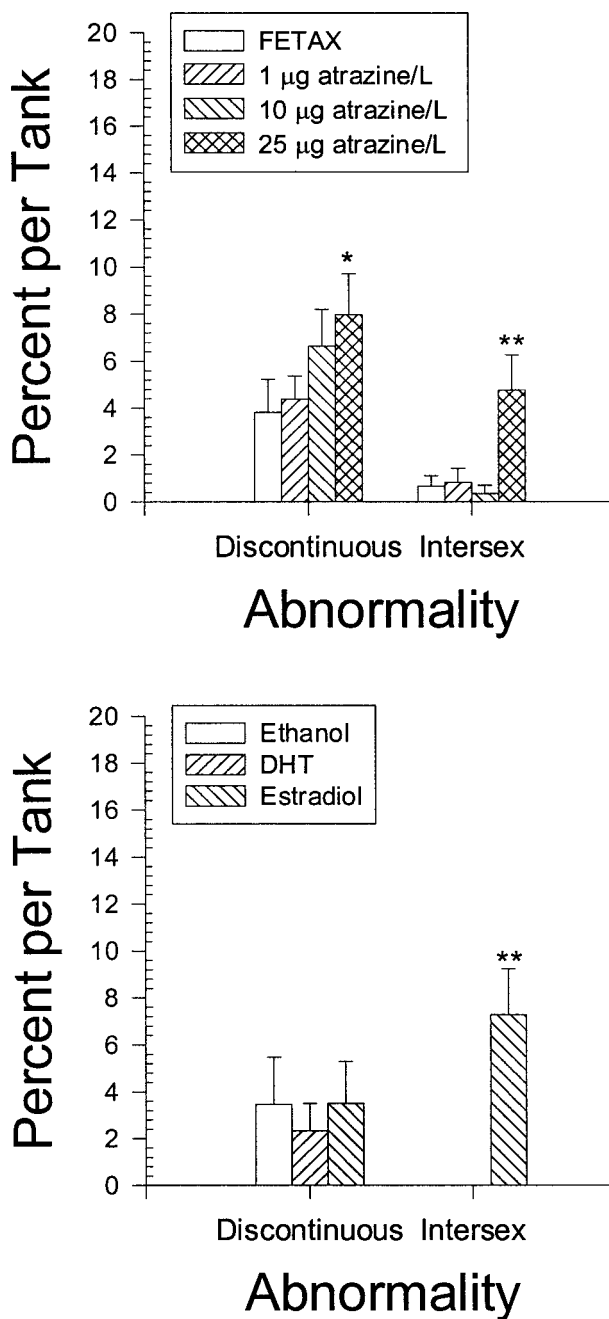


Fig. 3. Percentage of gonadal abnormalities per treatment tank in Nieuwkoop-Faber stage 66 *Xenopus laevis* exposed to atrazine (top panel) or steroids (bottom panel). The bars represent the mean + standard error of 6 (steroids and control) to 11 (atrazine and control) replicates per group. Each replicate consisted of 20 to 30 animals. One asterisk (\*) indicates significantly different from control by chi-square analysis when incidence per tank was pooled within treatment. Two asterisks (\*\*) indicate significantly different from control when percentage per tank was analyzed by Kruskal-Wallis test followed by Dunn's multiple comparisons test. FETAX = frog embryo teratogenesis assay-*Xenopus*; DHT = dihydrotestosterone.

muscle size were confirmed in the DHT-treatment group. Exposure to DHT increased total muscle cross-sectional area approximately twofold in both male ( $F_{2,15} = 67, p < 0.0001$ ) and female ( $F_{2,15} = 41, p < 0.0001$ ) frogs (Fig. 6B). Total muscle cross-sectional area was larger in males than females in the ethanol control group but not in the estradiol and DHT-treatment groups (Fig. 6B).

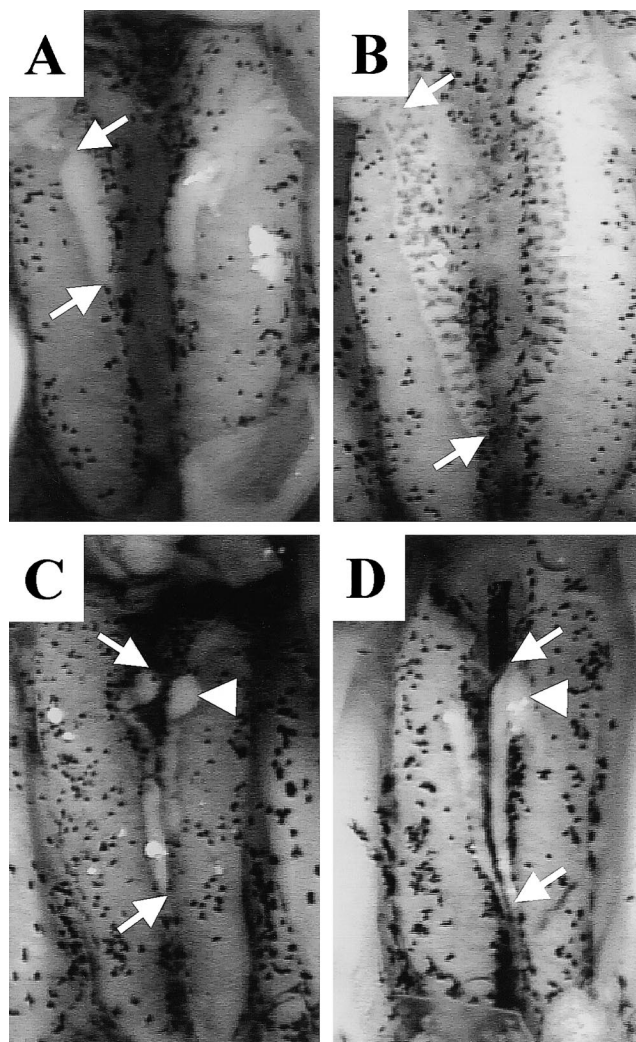


Fig. 4. Photomicrographs of gonads from Nieuwkoop-Faber stage 66 *Xenopus laevis* exposed to estradiol or 25 µg atrazine/L. Rostral is located at the top of the photomicrographs. Arrows mark the rostral-caudal extent of each gonad. (A) The appearance of the testes in a normal male. The testes are short, unpigmented, unlobed, and are bilaterally symmetrical in length and shape. (B) The appearance of the ovaries in a normal female. The ovaries are transparent, lobed, pigmented, and are much longer than the testes. (C) Gonads from an estradiol-exposed frog scored as mixed sex based on gonadal morphology. Note the swollen mass of tissue (arrowhead) at the rostral tip of the gonad. (D) Gonads from a 25-µg atrazine/L-exposed frog scored as mixed sex based on gonadal morphology. Note the swollen mass of tissue (arrowhead) at the rostral tip of the gonad.

#### DISCUSSION

Our findings are in general agreement with FETAX data as well as data collected from full developmental period exposure of *R. pipiens* [7] to atrazine, suggesting that environmentally relevant concentrations of atrazine are not lethal and not directly toxic to developing frogs. Atrazine at concentrations as great as 25 µg/L did not affect posthatch mortality, larval growth, or incidence of bent tails in developing *X. laevis* exposed from 48 h posthatching until NF stage 66. The fact that hatching success was >90% for all groups suggests that healthy embryos were used for this study. Although the FETAX assay is an efficient means of estimating acute toxicity and teratogenicity, this assay may not accurately predict chronic exposure scenarios during larval development due to its short exposure duration (96 h) and the fact that the assay is

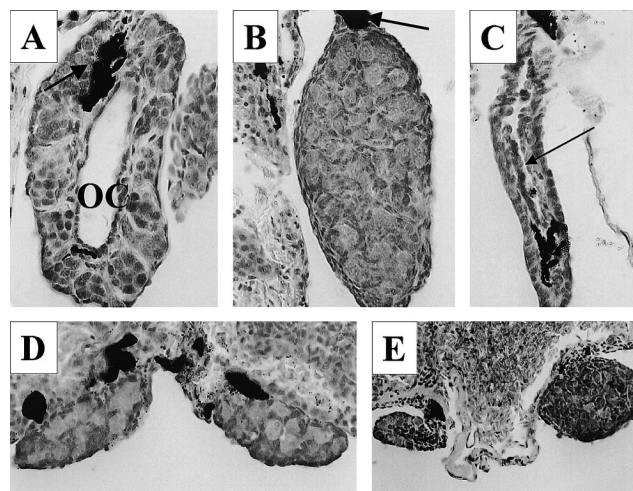


Fig. 5. Photomicrographs of gonadal tissue from Nieuwkoop-Faber stage 66 *Xenopus laevis* exposed to 25 µg atrazine/L or estradiol. (A) Ovarian tissue from a normal female based on gonadal morphology. Note the prominent ovarian cavity (OC) and the melanophore (arrow). Original magnification, ×200. (B) Testes from a normal male based on gonadal morphology. The testes appear normal, with no obvious cortical tissue present. Melanophores (arrow) are present adjacent to the testes. Original magnification, ×200. Ovary (C) and testes (D) from animals exposed to 25 µg atrazine/L and scored as mixed sex based on gonadal morphology. The ovary (C; original magnification, ×200) is underdeveloped, with no clear oogonia present (compare with Fig. 5A). The testes (D; original magnification ×200) appear flat and are considerably smaller than the testis of the normal male (compare with Fig. 5B, photographed at the same magnification). (E) Gonadal tissue from an animal exposed to estradiol and scored as mixed sex based on gonadal morphology. What appears to be a testis is located on the right side but gonadal tissue with a clear cortex, resembling ovarian tissue, is located on the left side of the photomicrograph. Original magnification, ×100.

terminated during early embryonic development. Although data exist on the response of *X. laevis* to atrazine in the FETAX assay [6], there have been few studies examining the response of *X. laevis* or any other frog to atrazine during the entire larval period. Our data indicate that exposure to 25 µg atrazine/L resulted in a slight but statistically significant increase in the incidence of abnormal swimming. Normally, undisturbed larval *X. laevis* exhibit schooling behavior in which siblings orient themselves parallel to one another in the water column [27]. In contrast, tadpoles exhibiting abnormal swimming behavior can be found motionless on the aquarium bottom or swimming in circles. Whether the slight increase in abnormal swimming would affect survival or fitness of *X. laevis* larvae under natural conditions is not known, as the adaptive significance of schooling behavior in *X. laevis* is not entirely clear [27]. However, it is unlikely that the small increase in abnormal swimming in the 25-µg atrazine/L group adversely affected larvae in the present study because there were no treatment-related effects on posthatch mortality and posthatch survivorship in this treatment group was roughly 88%, compared with 90% in FETAX medium controls.

Forelimb emergence and tail resorption are thyroid-hormone dependent features of metamorphosis in *X. laevis* and many other anuran species [28]. None of the atrazine concentrations tested in the present study significantly influenced the proportion of animals reaching FLE or completing tail resorption, suggesting that atrazine does not influence thyroid status in developing *X. laevis*. Our results are consistent with previous reports in which atrazine concentrations as great as

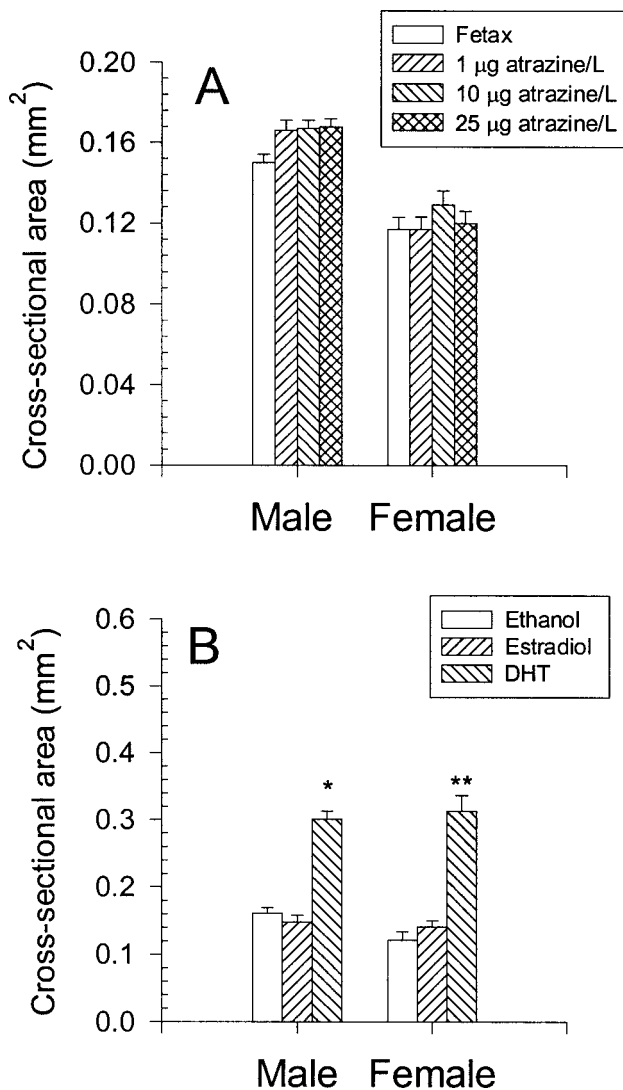


Fig. 6. Largest cross-sectional area through the combined left and right laryngeal dilator muscles in Nieuwkoop–Faber stage 66 *Xenopus laevis* exposed to atrazine (A) or sex steroids (B). (A) Bars are the mean  $\pm$  SE of measurements from 10 to 45 animals per treatment. Total muscle cross-sectional area was significantly larger in males than females in every treatment. (B) Bars are the mean  $\pm$  SE of 5 to 6 animals per treatment. Asterisks indicate significantly different from ethanol-vehicle control based on one-way analysis of variance: \* $F_{2,15} = 67$ ,  $p < 0.0001$ ; \*\* $F_{2,15} = 40$ ,  $p < 0.0001$ . Total muscle cross-sectional area was larger in males than females in the ethanol-vehicle controls but not in the estradiol or dihydrotestosterone (DHT) treatment groups.

200  $\mu\text{g/L}$  did not affect metamorphosis in *X. laevis* [13] or *R. pipiens* [7]. In contrast, atrazine has been reported to delay metamorphosis in larval tiger salamanders (*Ambystoma tigrinum*) exposed to 75  $\mu\text{g}$  atrazine/L [29]. Despite slower metamorphosis in salamander larvae exposed to 75  $\mu\text{g}$  atrazine/L, the authors reported elevated plasma thyroxine in larvae exposed to 75 and 250  $\mu\text{g}$  atrazine/L [29]. The authors also reported that atrazine altered plasma corticosterone concentrations and hypothesized that this may have counteracted any potential affect of elevated thyroxine on metamorphosis, although the mechanism whereby this might occur is not currently known.

A greater incidence of intersex and discontinuous gonads in *X. laevis* larvae exposed to 25  $\mu\text{g}$  atrazine/L was observed

Table 3. Mean ( $\pm$  SE) of total dilator muscle cross-sectional area (mm<sup>2</sup>) in Nieuwkoop–Faber stage 66 *Xenopus laevis* exposed to atrazine or sex steroids<sup>a</sup>

Treatment	Males	Females
FETAX medium control <sup>b</sup>	0.154 $\pm$ 0.004 ( <i>n</i> = 45)	0.117 $\pm$ 0.006 ( <i>n</i> = 11)
1 $\mu\text{g}$ atrazine/L	0.167 $\pm$ 0.005 ( <i>n</i> = 41)	0.118 $\pm$ 0.006 ( <i>n</i> = 10)
10 $\mu\text{g}$ atrazine/L	0.168 $\pm$ 0.004 ( <i>n</i> = 44)	0.129 $\pm$ 0.007 ( <i>n</i> = 11)
25 $\mu\text{g}$ atrazine/L	0.168 $\pm$ 0.004 ( <i>n</i> = 42)	0.122 $\pm$ 0.006 ( <i>n</i> = 12)
Ethanol control	0.161 $\pm$ 0.008 ( <i>n</i> = 6)	0.121 $\pm$ 0.013 ( <i>n</i> = 6)
Dihydrotestosterone	0.301 $\pm$ 0.012* ( <i>n</i> = 6)	0.313 $\pm$ 0.024* ( <i>n</i> = 6)
Estradiol	0.148 $\pm$ 0.010 ( <i>n</i> = 6)	0.141 $\pm$ 0.009 ( <i>n</i> = 6)

<sup>a</sup> Combined largest cross-sectional area through the right and left laryngeal dilator muscles.

<sup>b</sup> Frog embryo teratogenesis assay-*Xenopus*.

\* Significantly greater than ethanol control ( $p < 0.0001$ ).

in the present study. There are two other recent reports of atrazine affecting gonadal differentiation in *X. laevis*. Tavera-Mendoza et al. [12] found that 21  $\mu\text{g}$  atrazine/L reduced testicular volume and the number of spermatogonial cells in the developing testes after a 48-h exposure of tadpoles at NF stage 55. The authors report that the observed effects of atrazine on testicular histology are due to “resorption” of testicular tissue [12]. However, pretreatment controls were not analyzed in this study [12], and it is unclear whether the control and exposed larvae began at the same stage of testicular development. In a more recent study, Hayes et al. [13] found that atrazine concentrations as small as 0.1  $\mu\text{g}$  atrazine/L caused gonadal abnormalities in developing *X. laevis*. Similar abnormalities were observed in the present study but only at a 250-fold greater concentration. Furthermore, the percentage of intersex gonads (<5%) that we observed in *X. laevis* treated with 25  $\mu\text{g}$  atrazine/L was less than the 16 to 20% intersex reported in the previous study [13]. Differences between our results and those reported by Hayes et al. [13] may be related to differences in the source of animals, the rearing medium, and the delivery vehicle used. Preliminary data from others [30] suggest that the ionic composition of the rearing medium can dramatically affect the sensitivity of *X. laevis* embryos to atrazine. Furthermore, Hayes et al. [13] used ethanol as a vehicle for atrazine delivery, even though atrazine is soluble in water at concentrations as great as 30 mg atrazine/L [5]. It is unlikely that the ethanol alone caused a greater percentage of intersex, as we observed no intersex in our ethanol control animals. Ethanol at greater concentrations than those used in the present study is teratogenic in *X. laevis* [31], but the interaction between atrazine and low ethanol concentrations is presently unknown. Finally, the response of larval *X. laevis* to atrazine can vary depending on the source of the animals [32]. Hayes et al. [32] reported that the incidence of gonadal abnormalities in response to atrazine exposure varied from 3 to 5% in offspring from one strain of *X. laevis* to 30% in offspring from a different strain of *X. laevis*.

The greater incidence of gonadal abnormalities observed in the estradiol-treated *X. laevis* larvae was likely a result of the feminizing effect of estradiol in this species. Exposure to estradiol during the larval period results in a sex ratio skewed



toward more females in *X. laevis* [16]. Estradiol has been observed to cause gonadal abnormalities [16] that are identical to those observed in the present study (Fig. 4). Whether endogenous steroids play a role in amphibian sex differentiation remains unknown. A recent study [33] suggests that endogenous estradiol is required for ovary development in *X. laevis*. Treatment of gonadal explants in vitro with the aromatase inhibitor CGS 16949A leads to a male phenotype in developing gonads [33]. However, there have been no reports in which estradiol content has been successfully determined in developing *X. laevis* tadpoles. Unlike other species such as *Hyporolius argus*, which respond to androgen treatment with a sex ratio skewed toward males [34], androgens have no effect on sex ratio in developing *X. laevis*, a finding confirmed by our data.

The fact that there was a greater incidence of mixed-sex animals exposed to either 25 µg atrazine/L or estradiol is noteworthy given evidence that atrazine can affect the activity of aromatase, the enzyme responsible for converting testosterone to estradiol. Atrazine and other triazines (simazine and propazine) and metabolites (atrazine-desethyl and atrazine-desisopropyl) induced aromatase activity in H295R (human adrenocortical carcinoma) cells [35,36] and JEG-3 (placental choriocarcinoma) cells [36]. Although it is sometimes difficult to extrapolate directly from in vitro studies to whole-animal exposures, there are data, although somewhat equivocal, suggesting that atrazine may influence gonadal aromatase in developing animals. Atrazine exposure induced gonadal/adrenal aromatase activity in hatchling male alligators [37], although a subsequent study investigating in ovo exposure of alligators to atrazine reported no effect of the herbicide on hepatic aromatase or gonadal differentiation [38]. Although data from Miyata and Kubo [33] indicate that aromatase is active in the undifferentiated larval gonad of *X. laevis* and that altering the activity of aromatase can affect gonad differentiation in this species, there is insufficient evidence to indicate whether induction of aromatase is a plausible mechanism underlying the atrazine-induced gonadal abnormalities observed in the present study.

Larynx size and shape are androgen-dependent sexually dimorphic features in a number of anuran species. The small size of the NF stage 66 frogs (<1 g) used for the present study made it impossible to reliably remove the entire laryngeal complex for analysis of larynx weight, a sensitive means of gauging sexual dimorphism in larger frogs [39]. Instead, we histologically reconstructed larynxes and analyzed the cross-sectional area of the largest section through the dilator muscle. This proved to be a sensitive indicator of the sexual dimorphism in larynx size, as the dilator muscle was 30 to 40% larger in NF stage 66 males compared with females in all of the atrazine-treatment groups. Our finding that the laryngeal dilator muscle is sexually dimorphic at completion of metamorphosis in *X. laevis* is consistent with data from Hayes et al. [13] and suggests that the dilator muscle in *X. laevis* responds to androgens during larval life earlier than previously thought [39]. Previous work by Tobias et al. [39] suggested that sexually dimorphic features of the *X. laevis* laryngeal complex do not appear until after metamorphosis. Data from Kang et al. [40] indicating that *X. laevis* tadpoles can synthesize androgens as early as NF stage 47 support our findings. The laryngeal dilator muscle is capable of responding to androgens prior to NF stage 66 because exposure to DHT (Fig. 6B) increased total muscle cross-sectional area approximately

twofold in both male and female frogs, a finding that is consistent with a previous report that both male and female post-metamorphic frogs respond to testosterone with an increase in larynx size [41].

Although our analytical technique was sufficiently sensitive to detect sexually dimorphic differences in the dilator muscle at NF-stage 66, we found no reduction of dilator muscle cross-sectional area in atrazine-exposed animals. This finding contradicts the results of a recent study [13] reporting that exposure to as little as 1 µg atrazine/L reduces dilator muscle size in male *X. laevis*. The differences between our findings and those of Hayes et al. [13] may be explained by differences in sampling, both at the level at which animals were selected for analysis as well as how laryngeal size was determined. First, our data indicate that body weight is inversely correlated with time to reach metamorphosis in *X. laevis*, and we selected frogs over a range of different body weights for the larynx analysis. Second, we measured the largest area through the dilator muscle by serially sectioning through the entire muscle of each animal and measuring cross-sectional area every 160 µm. In contrast, Hayes et al. [13] selected tissue sections for analysis based on muscle shape, after determining in preliminary studies that the largest section through the muscle generally occurs one third of the way through the larynx, although the authors do not specify whether this was determined from the rostral or caudal pole of the muscle. Finally, differences in the source of animals used also must be taken into consideration. Indeed, Hayes et al. [13] point out large differences in larynx size in *X. laevis* obtained from different sources and even suggest that sensitivity of the larynx to atrazine may differ depending on the source of the animals used [32].

In the present study, atrazine exposure appeared to increase the frequency of gonadal abnormalities, although the frequency was only statistically different from FETAX medium controls at 25 µg atrazine/L. These effects did not significantly alter the sex ratio. The effects of 25 µg atrazine/L on gonadal differentiation seem to be restricted to males because the percentage increase in intersex offspring in this treatment group was offset by a proportional decrease in the percentage of male offspring. Atrazine concentrations in freshwater rivers and streams seldom exceed 25 µg atrazine/L [5], but the likelihood of natural frog populations in farm ponds being exposed to atrazine concentrations as great as 25 µg/L is uncertain. Many frog species breed in temporary ponds that may receive runoff from agricultural sites, so exposure will vary in periodicity and duration with rainfall events. The results of the present study, together with data on the reproductive impact of these gonadal abnormalities and data from frogs exposed to atrazine under field conditions, will form an important basis for future assessment of the risk faced by natural frog populations exposed to atrazine.

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