

PLASMA SEX STEROID CONCENTRATIONS AND GONADAL AROMATASE
ACTIVITIES IN AFRICAN CLAWED FROGS (*XENOPUS LAEVIS*) FROM
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Abstract—Adult African clawed frogs (*Xenopus laevis*) were collected from a corn-growing region (CGR) and a non-corn-growing region (NCGR) with different exposure profiles for atrazine and related triazines. Physical, chemical, and biological parameters from the catchment areas were also measured. Frogs were surveyed for possible effects of exposure to triazine herbicides on plasma testosterone (T) and estradiol (E2) titers, gonadal aromatase activity, and gonad growth (GSI). Concentrations of both T and E2 varied among locations and were correlated to some accessory factors, such as pH, several ions, and metals. Greatest median plasma T concentrations (males: 19 ng/ml; females: 16 ng/ml) occurred in frogs inhabiting NCGR as compared to those from the CGR (males: 4 ng/ml; females: 1 ng/ml). Median E2 concentrations were also greater in frogs collected from the NCGR (males: 3 ng/ml; females: 28 ng/ml) than those in frogs from the CGR (males: 2 ng/ml; females: 5 ng/ml). Because some exposure to agricultural chemicals at both regions occurred, as did simultaneous exposures to multiple chemicals, a regression analysis was employed. Negative correlations were observed between plasma T concentrations and concentrations of atrazine, deisopropylatrazine, deethylatrazine, and tertbutylazine in females and between T and diaminochlorotriazine in males. Estradiol in females exhibited a significant negative correlation with atrazine and deethylatrazine. No correlations were observed between gonadal aromatase activity or GSI and any of the agricultural chemicals measured. Median aromatase activities in ovaries varied among sampling sites ranging from 7 to >3,000 times greater than those in males when measurable. Testicular aromatase activity was below the detection limit of the assay in male frogs at most of the sites. Although exposure to agricultural inputs did not affect aromatase activities, effects of atrazine or coapplied pesticides on sex steroid homeostasis cannot be excluded at this point.

Keywords—Amphibians Aromatase Agriculture Frog Triazines

INTRODUCTION

Considerable interest and controversy have recently surrounded the issue of potential endocrine-disrupting effects of agricultural chemicals on development and reproduction in amphibians, particularly in habitats adjacent to application [1]. It has been suggested that these chemicals contributed to frog population declines that occurred in several areas since the 1960s [2]. In particular, concern has been raised about the widely used herbicide atrazine (6-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) [3–7]. Since the early 1960s, atrazine has been used extensively in corn-growing regions of many areas of the world, including North America and South Africa [8]. Although less atrazine is now being used and it is being used in combination with other chemicals, it is still the most widely used herbicide in the United States, and probably the most widely used herbicide in the world for the past four decades [9]. Atrazine was not predicted to have measurable effects on the survival or growth of most fish, amphibians, or invertebrates at the concentrations at which it is found in the environment [10–14]. Atrazine does not have a high potential

to bioaccumulate or biomagnify [14,15] but is persistent in the environment, with an estimated half-life in water ranging from 40 to 240 d [16] and field dissipation half-lives ranging from 8 to 99 d [17], depending on environmental conditions. As a result of both its widespread use and its persistence, atrazine has been widely detected in surface waters and rainfall with peak concentrations ranging up to approximately 20 µg/L [11,16].

Recently, several studies have suggested that atrazine may disrupt the endocrine systems of frogs [3,4,18,19]. It has been reported that environmentally relevant concentrations of atrazine, as low as 0.1 µg/L, caused both gonadal abnormalities and demasculinization of the larynx, as indicated by reduced size of the laryngeal dilator muscle in male *Xenopus laevis* exposed from hatching through completion of metamorphosis [3]. However, other studies with *X. laevis* have found no effects of atrazine on the size of the laryngeal dilator muscle, gonadal development, or plasma hormone concentrations [20,21]. It has been hypothesized by Hayes et al. [3] that atrazine can affect the development of frogs, including *X. laevis*, by induction of aromatase activity. Aromatase is the enzyme that catalyzes the aromatization of testosterone (T) to estradiol (E2). Atrazine has been reported to up-regulate the activity of

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the CYP-19 gene in a human adrenocarcinoma (H295R) cell line [22]. This hypothesis—the decrease of androgen levels as a consequence of increased estrogen production through up-regulation of aromatase enzyme activity—was proposed to explain the observed smaller laryngeal dilator muscles in male *X. laevis* exposed to atrazine than in those of unexposed individuals [3]. Since laryngeal development is an androgen-dependent process and a decrease in plasma concentration of plasma T was observed in adult frogs, the authors proposed that up-regulation of aromatase resulted in a decrease in plasma T, which then resulted in a smaller laryngeal dilator muscle [3]. To date, no direct evidence has been found linking induction of aromatase activity in anuran species with atrazine or related pesticides exposure [3,21].

The present study was conducted to test the hypothesis that atrazine or related triazines might affect aromatization of T to E2 under field conditions. Specifically, aromatase activities in the gonads and plasma concentrations of T and E2 were measured in adult male and female *X. laevis* from corn-growing region (CGR) in the Potchefstroom region of South Africa and compared to frogs from an area where corn is not grown (NCGR). In addition, various other measures of water quality, such as ions, metals, temperature, and pH, were made as potential confounding factors that may interfere with the animal's physiology.

MATERIALS AND METHODS

Sampling locations

A total of eight sites were studied in two adjacent regions in the vicinity of Potchefstroom, South Africa [23]. One region was in the Viljoenskroon area, which is a corn-growing region (CGR) where predominantly corn (*Zea mays*) is cultivated. The other area, in an adjacent watershed near Potchefstroom where corn is not cultivated, was designated as a non-corn-growing region (NCGR). The sites were selected to give a range of concentrations of atrazine and other pesticides used in corn production that were likely to run off into catchment ponds adjacent to the fields [23]. Compounds of major interest were atrazine and its degradation products deethylatrazine (DEA), deisopropylatrazine (DIA), diaminochlorotriazine (DACT), and terbuthylazine. In addition, concentrations of simazine and acetochlor were also measured [23]. During the sampling period, various environmental parameters were measured at each field site to describe the habitat, including a brief description of vegetation and water depth. In addition, a series of ions and metals were routinely measured as parameters for water quality.

NCGR. Site NCGR3 is a small (surface area 2,000 m²), heavily vegetated site with clear water. Maximum depth was 38.5 cm and secchi depth was 32 cm. Sites NCGR1 (surface area 20,500 m²; maximum depth 261 cm) and NCGR6 (surface area 14,860 m²; maximum depth 104 cm) are large, earth-walled farm ponds with muddy water and secchi depths of 11.5 and 6.5 cm, respectively. Vegetation was limited to the periphery of the ponds. Conductivity varied from 47 μ S/cm at site NCGR1 and NCGR3 to 38 μ S/cm at site NCGR6. The pH varied between 5.1 at site NCGR3 and 8.8 at site NCGR6 [23]. The pH was slightly alkaline, ranging from 7.3 to 8.3 at site NCGR3 and NCGR1, respectively. Site NCGR3 dries up frequently, while sites NCGR1 and NCGR6 are semipermanent ponds. All three sites have fairly large catchment areas (170–480 ha), and sites NCGR1 and NCGR3 have in-flowing springs.

CGR. Pond surface areas in the Viljoenskroon CGR were from 2,400 (sites CGR4 and CGR8), 4,700 (site CGR1), 46,000 (site CGR3), and 68,000 m² (site CGR6). All sites had vegetation around the periphery and had floating aquatic plants. Conductivity ranged from 210 (site CGR3) to 1,567 μ S/cm (site CGR4). The pH ranged from 7.2 (site CGR3) to 10.8 (site CGR8). All sites had clear water. Light penetrated to the bottom in sites CGR1, CGR3, CGR4, and CGR8. Site CGR6 had a secchi depth of 207 cm. Water and sediment grab samples were analyzed for the presence of atrazine, related triazines, other pesticides, and metals.

Frog collection

Xenopus laevis has an extended breeding season from early spring to late summer. In the study area, this ranges from October to April. Reproductive activity is stimulated by rainfall. Several thousand small sticky eggs are deposited and attached to plants and other objects. Wild *X. laevis* of both sexes were collected after the rainy season in April and May 2002. Sampling events took place once or twice and within a few days of each other. In the NCGR, 20 males and 20 female individuals were collected per site. In the CGR, in this particular year, it was more difficult to obtain a similar number of *X. laevis* as in the NCGR. This was most likely due to an introduction of predatory catfish from the adjacent river flooding CGR ponds during the rainy season earlier that spring/summer. In order to maintain sampling within the necessary time window, the number of frogs caught was reduced to 10 males and 10 females per pond (except from CGR8, where only three males and eight females could be caught). Corn in this region of South Africa is usually planted in October/November and is treated with atrazine and the related triazine terbuthylazine. Frogs were collected using 10 bucket traps per site baited with liver and marrow bones. Traps were collected 2 d later and the frogs were transferred to a container labeled with a number corresponding to the site. Frogs were then transported to the laboratory at Potchefstroom University for further processing.

Blood and tissue collection

After capture, frogs were kept individually in 2-L plastic containers containing laboratory water for 48 h to minimize possible variances resulting from immediate capture stress before blood and gonad tissue were collected. Blood was collected by cardiac puncture with syringes coated with 7.5% ethylenediaminetetraacetic acid to prevent clotting. Blood samples were centrifuged at 2,000 g for 3 min at room temperature to separate out the plasma fraction. Plasma was stored at -80°C until further analyses. Gonads were removed, measured, and weighed. One gonad was fixed in 10% neutral-buffered formalin or Bouin's for 48 h and then transferred to ethanol before preparation for sectioning and staining for histological examination. The second gonad of each specimen was snap frozen in liquid nitrogen for subsequent biochemical analyses. The gonadosomatic index was calculated.

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

Biochemical analyses

Steroid hormones. Plasma samples were extracted twice with diethyl ether, and concentrations of E2 and T were measured by competitive enzyme-linked immunosorbent assay (ELISA) as described by Cuisset et al. [24] with modifications

[25]. In this competitive ELISA, plasma steroid competes with acetylcholinesterase-labeled steroid for the binding site on the polyclonal rabbit antisteroid antibody. Antiserum to T was obtained from D.E. Kime (University of Sheffield, Sheffield, UK). Cross reactivities of the T antiserum are described in Nash et al. [26]. The antiserum to E2 (Cayman Chemical, Ann Arbor, MI, USA) has been reported to cross-react with estradiol-3-glucuronide (17%), estrone (4%), estriol (0.57%), T (0.1%), and 5 α -dihydrotestosterone (0.1%). For all other steroids, cross-reactivities were reported as less than 0.1%. The steroid ELISAs were performed using Costar high binding plates (Costar, Bucks, UK). The working ranges of these assays were determined to be as follows:

T: 0.78–800 pg/well
17 β -estradiol: 0.78–800 pg/well

Both E2 and T ELISAs were validated for use with plasma from *X. laevis* by use of standard additions methods. Plasma of male and female frogs from both regions (NCGR and CGR) was spiked with T and E2 at various dilutions before extraction and processed in exactly the same manner as unspiked samples. In addition, a series of samples was split and one subsample was analyzed for T by the endocrinology laboratory of the Veterinary Medical Department at Michigan State University (East Lansing, MI, USA) by a commercially available radioimmunoassay (Diagnostic Products, Los Angeles, CA, USA), while the second subsample was analyzed in our laboratory using the previously described ELISA. A total of three male and three female plasma samples from both regions (CGR and NCGR) were subjected to this interlaboratory verification of the T ELISA. Each sample was split, and each subsample was extracted separately with the second extract being spiked with a T solution as an internal control.

CYP19 aromatase activity. Aromatase activity was measured following the protocol of Lephart and Simpson [27] with minor modifications. Less than 0.5 g of gonadal tissue was homogenized in 600 μ l of ice-cold gonad buffer (50 mM KPO₄, 1 mM ethylenediaminetetraacetic acid, 10 mM glucose-6-phosphate, pH 7.4). The homogenate was incubated with 21.33 nM ³H-androst-4-ene-3,17-dione (25.9 Ci/nmol; lot 3467-067; NET-926; New England Nuclear, Newton, MA, USA), 0.5 IU/ml glucose-6-phosphate (Sigma, St. Louis, MO, USA, G6378), and 1 mM NADP (Sigma N-0505) at 37°C and 5% CO₂ for 120 min. Tritiated water released from each sample was extracted and activity determined by liquid scintillation. Aromatase activity was expressed as pmoles of androstenedione converted per hour per milligram protein. The specificity of the reaction for the substrate was determined by use of a competitive test with nonlabeled androstenedione. Addition of large amounts of androstenedione reduced tritiated water formation to the levels found in the tissue blanks, which demonstrated that the activity being measured was specific for aromatase. The within-day and between-day coefficients of variations for the assay were 0.3 and 2.8%, respectively.

Statistical analyses

Because of the nature of the parameters measured, several statistical models were used for data analyses. Data for males and females were analyzed separately. The study was designed to be analyzed by both fixed-effects models and regression types of statistics. This type of analysis was necessitated because the specific locations could not be classified as exposed or unexposed. A range of concentrations of the primary res-

idues of concern existed, with greater concentrations in the CGR. However, residues were also detected in the NCGR. Because most of the investigated parameters were not normally distributed (one-sample Kolmogorov–Smirnov test) and to make the analyses insensitive to outliers, statistical comparisons were made between the median values of plasma T and E2 concentrations as well as the E2/T ratio, the GSI, and gonadal aromatase activity. Differences between the different exposure groups were tested by comparing medians of the CGR with medians of the NCGR using both a two-sided, two-sample *t* test assuming equal variances and the nonparametric Mann–Whitney *U* test. The power to detect significant differences between the two groups (CGR and NCGR) was determined for each biological parameter at the given sample size and number of sites investigated. Furthermore, necessary sample sizes to be able to detect the observed differences between the geometric means were calculated based on the given within- and among-site variances. The among-site and a within-site variance components were estimated from the log-transformed data using a simple linear mixed-effect model [28]. The site-group (NCGR or CGR) was included as a fixed effect to ensure that any site-group difference was not included in the background variance. The mixed procedure [29] in Version 8.2 of SAS® (SAS Institute, Cary, NC, USA) was used to fit these mixed models to the data. Because a primary objective of this study was to investigate the relationships between exposure to atrazine and/or its degradation products and other triazine herbicides, correlations between atrazine or concentrations of degradation products and each parameter were also investigated. When necessary, data were log-transformed to approximate a normal distribution before calculating linear regressions and Pearson correlation coefficients. Physiological parameters such as plasma T and E2 or aromatase activity react quickly to environmental changes in as little time as a few hours, days, or weeks. Therefore, the four-week means (4WM) of the period before collecting frogs at the individual sites for pesticide concentrations and environmental cofactors were used for the calculations of significances and correlations/regressions in this study. Values were considered to be statistically significant when $p < 0.05$.

RESULTS

Sex steroid ELISA verification

Accuracy and precision of the assays for E2 and T were determined. Recoveries were $96 \pm 13\%$ ($n = 4$) and $87 \pm 13\%$ ($n = 7$) for E2 in males and females, respectively, and $90 \pm 28\%$ ($n = 10$) and $113 \pm 19\%$ ($n = 19$) for T in males and females, respectively. Except for one single outlayer in the RIA experiment (206% recovery), the average recoveries of T spiked into *X. laevis* blood plasma of male and female *X. laevis* for the interlaboratory verification were similar between the ELISA and RIA methods (RIA = $123.6 \pm 26.7\%$; ELISA = $125.6 \pm 9.6\%$). The absolute concentrations in the spiked samples in both tests were also similar (average deviation = 23%). No difference was observed in the CV for the RIA and ELISA methods for either males (26%) or females (25%). No trend was observed of negative or positive bias for either the RIA or the ELISA. These results suggest that the concentrations reported are both accurate and precise.

Pesticide concentrations

Concentrations of pesticide residues varied among locations and among seasons. With the exception of site CGR8, the

Table 1. Concentrations of triazine herbicides and degradation products ($\mu\text{g/L}$) in non-corn-growing regions (NCGR) and corn-growing regions (CGR) in South Africa measured by GC/MS[23]. Values represent two individual samplings that were conducted four weeks before collecting *Xenopus laevis* within a time period in 2002^a

	Date (all 2002)	Simazine ($\mu\text{g/L}$)	DACT ($\mu\text{g/L}$)	DIA ($\mu\text{g/L}$)	DEA ($\mu\text{g/L}$)	ATZ ($\mu\text{g/L}$)	Tert ($\mu\text{g/L}$)
NCGR1	April 2	<0.5	3.91	0.23	0.18	0.28	<0.1
	April 15	<0.5	<0.1	0.27	<0.1	0.26	<0.1
	Mean ^b	0.25	1.98	0.25	0.12	0.27	0.05
NCGR3	March 18	<0.5	<0.1	0.30	0.15	0.36	<0.1
	April 2	<0.5	<0.1	0.61	<0.1	0.57	<0.1
	Mean ^b	0.25	0.05	0.46	0.1	0.47	0.05
NCGR6	May 1	<0.5	<0.1	0.13	0.12	<0.1	0.51
	May 13	<0.5	<0.1	0.15	0.13	0.2	0.59
	Mean ^b	0.25	0.05	0.14	0.13	0.13	0.55
CGR1	April 15	<0.5	<0.1	0.7	1.29	4.14	3.66
	May 1	<0.5	<0.1	0.93	1.16	3.5	2.95
	Mean ^b	0.25	0.05	0.82	1.23	3.82	3.31
CGR3	April 2	<0.5	0.38	0.47	0.43	1.05	0.97
	April 15	<0.5	<0.1	0.28	0.38	1.01	1.07
	Mean ^b	0.25	0.22	0.38	0.41	1.03	1.02
CGR4	April 2	<0.5	<0.1	0.18	0.11	0.32	0.19
	April 15	<0.5	<0.1	0.64	<0.1	0.29	0.24
	Mean ^b	0.25	0.05	0.41	0.08	0.31	0.22
CGR6	April 15	<0.5	5.45	0.49	1.09	3.78	2.46
	May 1	<0.5	<0.1	0.32	0.90	3.90	2.40
	Mean ^b	0.25	2.75	0.41	0.10	3.84	2.43
CGR8	April 15	1.70	1.24	0.70	1.04	3.53	0.86
	May 1	1.20	<0.1	0.88	0.91	3.12	0.72
	Mean ^b	1.45	0.65	0.79	0.975	3.33	0.79

^a DACT = diaminochlorotriazine; DIA = deisopropylatrazine; DEA = deethylatrazine; ATZ = atrazine; Tert = tertbutylazine.

^b When concentration is below the detection limit, one-half of the detection limit was used for the calculations.

greatest concentrations of atrazine in pond water were observed at the end of the rainy season (March through May). The greatest mean atrazine concentrations (4WM) were measured at sites CGR1 (4.1 $\mu\text{g/L}$), CGR6 (3.9 $\mu\text{g/L}$), and CGR8 (3.5 $\mu\text{g/L}$) (Table 1). Concentrations of atrazine at locations NCGR1, NCGR3, NCGR6, and CGR4 were low, but, in most cases, detectable. At CGR4, 4WM concentrations of pesticides were as small as in water from the reference sites. Detection limits for the different compounds measured were 0.5 $\mu\text{g/L}$ for simazine and 0.1 $\mu\text{g/L}$ for all other triazines. The log of atrazine concentration (4WM) was significantly correlated with the log concentrations of its degradation product DIA ($r = 0.829$; $p = 0.011$) and DEA ($r = 0.905$; $p = 0.002$). Tertbutylazine concentrations between 2.4 and 3.7 $\mu\text{g/L}$ were measured at CGR1 and CGR6.

The log of concentrations of the triazine metabolites DIA ($r = 0.770$; $p = 0.025$), DEA ($r = 0.746$; $p = 0.034$), and atrazine ($r = 0.802$; $p = 0.017$) were positively correlated with the pH of the water at the sampling sites. The log DIA concentration was positively correlated with log temperature ($r = 0.801$; $p = 0.017$) and the log visual depth ($r = 0.814$; $p = 0.014$). Atrazine, some of its metabolites, and tertbutylazine were also significantly correlated with a series of ions measured in water and sediments of the different ponds during this study (Table 2).

Physiological responses

GSI. Although not significant, the GSI values for both female (t test: $p = 0.082$; Mann-Whitney U test: $p = 0.053$) and male (t test: $p = 0.073$; Mann-Whitney U test: $p = 0.180$) *X. laevis* from the CGR sites appeared greater than the female and male means from the NCGR (Fig. 1). Of the sites in the CGR, CGR6 had the least median GSI in males (0.10%) and females (1.34%) that was similar to those in the NCGR (males:

0.10–0.12%; females: 1.10–1.90%). The GSI of female *X. laevis* from CGR1 and CGR8 were more variable than that at other locations. In general, less variation was observed among individuals of both sexes in the NCGR, where the median GSI values were less than that for almost all other locations (Fig. 1). The GSI of males ($r = 0.732$; $p = 0.039$) as well as the log GSI of females ($r = -0.847$; $p = 0.008$) were significantly and positively correlated with the log conductivity. A significant negative correlation was observed for the GSI in males ($r = 0.817$; $p = 0.013$) with the log visual depth. Furthermore, the GSI was positively correlated to a variety of ions, while a negative relationship occurred with molybdenum concentration in sediments only ($r = 0.697$; $p = 0.026$) (Table 2).

Testosterone. The median plasma T concentrations of females were significantly greater at the NCGR sites than at the CGR sites when using the t test ($p = 0.018$) and close to significance when compared with the nonparametric Mann-Whitney U test ($p = 0.053$). The median plasma T concentrations of males were not significantly different between the CGR and the NCGR. However, the power to detect significant differences between the two areas for males was poor. With the sample sizes available in this study, the statistical power ($1-\beta$) was approximately 0.065. A sample size of more than 1,000 male individuals would have been necessary to test for statistical significance ($p < 0.05$) of the observed difference with a power of 0.80. The greatest median T concentrations in plasma of males were 39 ng/ml (CGR4), 23 ng/ml (NCGR3), and 19 ng/ml (NCGR6) (Fig. 2). Similar plasma T concentrations in females were observed only at NCGR1 (20 ng/ml) and NCGR6 (16 ng/ml). At all other sites, median plasma T concentrations of females were less than 6 ng/ml (Fig. 2). Plasma T concentrations of females were negatively correlated with the logarithm of the concentrations of atrazine ($r = -0.934$; $p = 0.001$), tertbutylazine ($r = -0.736$; $p = 0.028$),

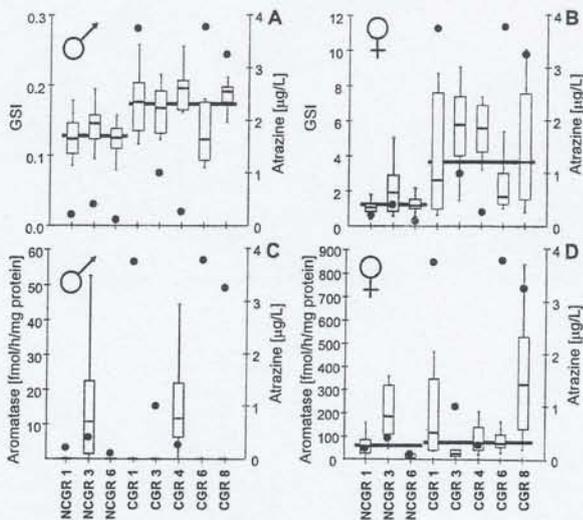


Fig. 1. Gonadosomatic index (GSI) (A and B) and aromatase activity (C and D) of male (A and C) and female (B and D) *Xenopus laevis* from the Potchefstroom region of South Africa. Solid dots = atrazine concentrations in water as four-week means. Bold horizontal lines = group medians of medians for non-corn-growing region (NCGR) sites and corn-growing region (CGR) sites. Box plots: The center horizontal line marks the median of the sample. The length of each box shows the range within which the central 50% of the values fall, with the top and bottom of the box at the first and third quartiles. The vertical T-lines represent intervals in which 90% of the values fall.

DIA ($r = -0.778$; $p = 0.023$), and DEA ($r = -0.880$; $p = 0.004$) but not DACT concentrations. Plasma T concentration in males was not correlated with the 4WM concentration of atrazine ($r = -0.554$; $p = 0.154$). However, a significant negative correlation was observed between the log of plasma T concentrations in males and the log of the DACT concentration ($r = -0.831$; $p = 0.011$). In male *X. laevis*, a significant negative correlation was observed between the pH and log T ($r = -0.823$; $p = 0.012$). No significant correlations with other cofactors were observed for males or females. Plasma T concentrations in both males and females were negatively related to a variety of ions, such as SO_4 and S, in water and Fe in sediment (Table 2).

Estradiol. In general, median concentrations of E2 in the plasma of females from the CGR were significantly less than those of females from the NCGR (t test: $p = 0.002$; Mann-Whitney U test: $p = 0.025$), but no significant difference was observed for males (Fig. 2). However, for male frogs the variability between and within sites was such that only a true difference as great as 9.3-fold could have been detected with a power of 0.80 (Table 3). In contrast, the observed difference was 2.9-fold. The greatest median E2 concentrations occurred in males from sampling sites NCGR6 (48 ng/ml) and CGR4 (34 ng/ml). However, these sites were characterized by a relatively great amount of variation among individuals. Concentrations in the lower (first) quartiles were considerably less (NCGR6 = 0.18 ng/ml; CGR4 = 0.8 ng/ml) than the medians. At all other sites, plasma E2 concentrations in males were 3 ng/ml or less. In females, the greatest plasma E2 concentrations occurred in *X. laevis* from the NCGR (27–44 ng/ml) (Fig. 2). In both males and females, plasma E2 concentrations at the sites CGR4 were similar to those in the NCGR, while at all other CGR sites, E2 concentrations were less than in the NCGR (Fig. 2). Concentrations of E2 in the plasma of both males

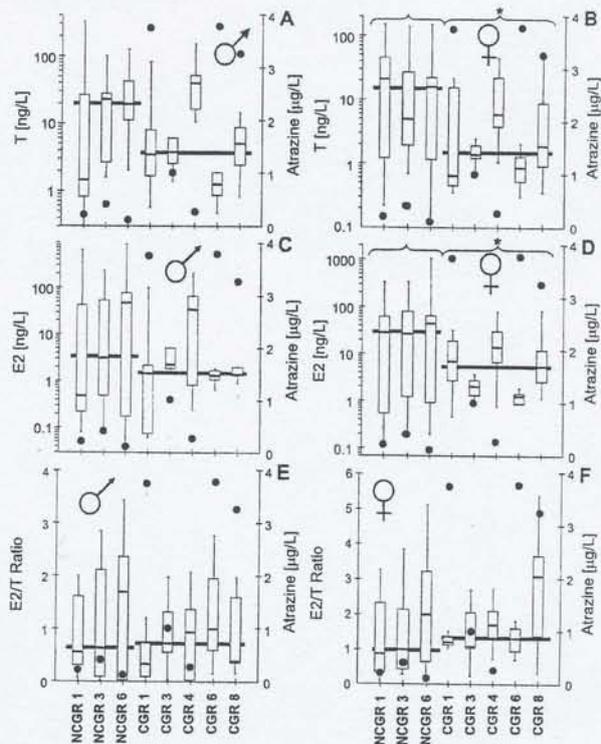


Fig. 2. Plasma T concentrations (A and B), plasma estradiol (E2) concentrations (C and D), and E2/T ratios (E and F) in male (A, C, and E) and female (B, D, and F) *Xenopus laevis* from the Potchefstroom region in South Africa. Solid dots = water atrazine concentrations at the sampling sites as four-week means. Bold horizontal lines = group medians of medians for non-corn-growing region sites (NCGR) and corn-growing region (CGR) sites. See legend of Figure 1 for an explanation of the box plots. * = group median at CGR sites significantly different from that at C sites (NCGR) (T females [B]: $p = 0.018$; E2 females [D]: $p = 0.0018$).

Table 3. Statistical power ($1-\beta$) to detect significant differences for the investigated parameters between the non-corn-growing region and the corn-growing region using a linear mixed-effect model. The model was set to detect differences between the geometric means (GM) at a significance level of <0.05 and at a target power of 0.80. The magnitude of difference that could be detected with the available sample size and inherent variance as well as the theoretical sample size required to give a statistical power of 0.80 with observed variance are also given. n = number of individuals^a

Sex	Parameter	Detectable fold difference between GM (0.80 power)	Observed fold difference between GM	Necessary n/site to achieve 0.80 power
Females	GSI	2.51	2.48	15
	T	3.9	3.54	15
	E2	8.62	2.88	>1,000
	E2/T	2.44	1.33	>1,000
Males	CYP	43.18	1.09	>1,000
	GSI	1.4	1.32	35
	T	11.39	1.65	>1,000
	E2	9.31	2.88	41
	E2/T	4.57	1.34	250
	CYP			

^a GSI = gonadosomatic index; E2 = estradiol; T = testosterone; E2/T = estradiol/testosterone ratio; CYP = aromatase activity.

and females were more variable in sites of the NCGR as compared to the CGR. A similar degree of variability as for females at the NCGR sites was observed in male *X. laevis* collected at locations CGR1 and CGR4. In females, plasma E2 was negatively correlated with 4WM atrazine concentration ($r = -0.851$; $p = 0.007$) and DEA ($r = -0.821$; $p = 0.012$). Plasma E2 concentrations in males and females were negatively correlated with some of the ions measured during this program, including SO_4 , S, Sr, Mg, and Sr, in water (Table 2).

E2/T ratio. The E2/T ratios of both males and females were not significantly different between the CGR and NCGR regardless of whether the comparisons were made based on medians or means. At the given variabilities (within and between regions), a true difference of 2.4-fold and 4.6-fold for females and males, respectively, could have been detected with a power of 0.80 (Table 3). The observed differences between the NCGR and CGR were low (1.3-fold) for frogs of both sexes (Table 3). Except for males and females at site NCGR6 and females at CGR8, no significant differences were observed in the median E2/T ratios (Fig. 2). In general, E2/T ratios were slightly greater in females than in males. In comparison with other locations, within-population variation was greater at NCGR6 (both sexes), NCGR3 (males), and CGR8 (females). The distribution of E2/T ratios of males from CGR8 was skewed with greater values within the upper quartile. The only significant correlation between the E2/T ratio for males was with DIA ($r = -0.830$; $p = 0.011$). No significant relationships were observed for the E2/T ratio for females and any of the triazine residues.

Significant negative correlations were observed between log E2/T ratio of male *X. laevis* and both log temperature ($r = 0.810$; $p = 0.015$) and dissolved oxygen ($r = 0.717$; $p = 0.045$). No such correlations were observed for the E2/T in females. When compared to ion concentrations in water and sediment, no significant negative relationships were observed for either males or females. However, the E2/T ratio in frogs of both sexes was positively correlated with concentrations of Al in the water (Table 2).

Aromatase activity. In most testes from most sites (except NCGR3 and CGR4), no gonadal aromatase activity was detectable (Fig. 1). No significant difference was observed in the median aromatase activity in ovaries between the CGR and the NCGR. The power to detect significant differences between the two regions was such that a difference of 43-fold and higher could have been detected with a power of 0.80 (Table 3). In comparison, the observed difference was small (1.1-fold) (Table 3). No power analyses could be conducted for aromatase activity in males since most of the measured values were below the detection limit. When aromatase activity was measurable in males, values ranged from 7 to 53 fmol/h/mg protein. In males from these locations, median aromatase activities in testes were 7-fold (CGR4) to 14-fold (NCGR3) less than that in ovaries of females collected at the same sites. The greatest aromatase activities in ovaries were observed at NCGR3 (1.9×10^2 fmol/h/mg protein), CGR1 (1.2×10^2 fmol/h/mg protein), and CGR8 (3.2×10^2 fmol/h/mg protein) (Fig. 1). In both testes and ovaries, greater median activities were characterized by proportionately greater variabilities (Fig. 1). The greatest variability was observed in ovaries of *X. laevis* from CGR8 where sampling occurred over an extended time period (April through September). The accessory factors with which log ovarian aromatase activity was correlated were the water concentrations of several ions (negative correlations) such as

NO_3 , NO_2 , Si, Ti, and V (Table 2). Aromatase activity was not correlated with any of the pesticides measured. No statistical tests were run on the aromatase data in males because in over 90% of the analyzed testes, aromatase activity was not detectable.

Relationships between physiological parameters. Statistically significant positive correlations were observed between the log T and log E2 concentrations in both males ($r = 0.757$; $p < 0.001$) and females ($r = 0.868$; $p < 0.001$). Both sexes also exhibited positive correlations between log E2 and log E2/T ratio (males: $r = 0.804$; $p < 0.001$; females: $r = 0.580$; $p < 0.001$). However, neither aromatase activity nor GSI were correlated with plasma concentrations of T or E2 in either males or females.

DISCUSSION

Atrazine concentrations measured in pond water of most of the ponds that are located in the CGR of the Viljoenskroon region were similar to those reported for a variety of waters in the Midwest (USA) that receive agricultural runoff [30,31]. Although less than peak concentrations that can be observed during extreme exposure scenarios, such as storm runoffs, these concentrations reflect a typical exposure situation for many water bodies. In the current study, trace amounts of atrazine and other triazines were also detected in water from the NCGR. This is likely due to atmospheric deposition from wet and dry fall into water bodies in nonagricultural areas [32]. Atrazine concentrations in the Potchefstroom region display typical exposure scenarios likely to occur in many different areas of the world where modern agriculture is practiced. Furthermore, the fact that most concentrations of degradation products were strongly correlated with atrazine suggests that calculation of the 4WM atrazine concentration was an appropriate and accurate way to gauge atrazine levels over time at these sites.

While several studies have been conducted in which *X. laevis* were exposed to atrazine under laboratory conditions [3,20,21], no studies have been undertaken to investigate the effects of atrazine on *X. laevis* in their natural habitat. Studies on other species, such as the leopard frog (*Rana pipiens*) and the cricket frog (*Acris crepitans*), have been conducted to investigate possible impacts of atrazine in the wild [4,7]. Although these studies found an increase in the occurrence of intersex in male frogs from areas of more intense agriculture, no significant correlation was observed between concentrations of atrazine and degree of response. Therefore, the present results may be of general importance for the evaluation of atrazine exposure in the environment.

Effects on gonad growth and development

In a recent study in which female tadpoles of the African clawed frog (*X. laevis*) were exposed to 21 $\mu\text{g/L}$ atrazine, a reduced number of primary germ cells (20%) compared to controls (2%) was observed after a 48-h exposure period [19]. The same exposure in male tadpoles resulted in a 57% reduction in testicular volume, with concomitant reductions in primary spermatogonial cell nests, nursing cells, and testicular resorption among 70% of exposed animals and failed testicular development in 10% of animals [18]. Assuming that exposure to atrazine in our study was similar to that in the studies of Tavera-Mendoza (atrazine concentration in Tavera-Mendoza study: 18 ppb; maximum average concentration of triazines in the CGR during summer 2001–2002: 17.03 ppb; data not

shown), similar effects on the gonads would be expected, namely, a decrease in the GSI. But instead an increase in the GSI was observed for both male and female frogs collected at the CGR, where they had been exposed to greater concentrations of atrazine. A possible explanation for the differences in GSI could be differences in food resources, which may allow frogs from the CGR to expend more energy on gonad growth. This could be a consequence of general eutrophication in agricultural areas that may lead to increased primary production and subsequently to an increase in aquatic invertebrate biomass. The positive correlation between conductivity and GSI would favor this hypothesis since increased conductivity is known to result in an increased buffering capacity and is likely to result in greater overall production. However, differences in food availability and production were not determined in this study. It remains to be elucidated whether the increase in gonad growth is a response to edaphic conditions and whether it has any effect on the populations of *X. laevis* at these sites [23]. In summary, no evidence from the reported data shows that exposure to atrazine or degradation products had a negative impact on gonad growth under field conditions.

Plasma sex steroid titers in ranids: Natural versus laboratory backgrounds

To our knowledge, no published studies report plasma sex steroid concentrations in wild *X. laevis*. However, when males from the NCGR were compared to a laboratory study sampled during summer (June and August) [33], plasma concentrations of T were similar. In contrast, plasma T concentrations in females from the NCGR were greater than those reported by Kang et al. [33] but similar to those reported for female *X. laevis* by Pickford and Morris that were stimulated to breed by injection with human chorionic gonadotropin (hCG) [34]. It is known that plasma sex steroid titers in anurans change significantly during the reproductive season [35,36]. Given that the females analyzed in the Kang et al. [33] study were sampled only twice within a year at unknown time points during their reproductive cycle (three-month sample interval, total $n = 8$), it is unlikely that their reproductive status was similar to that of frogs that were collected in the late breeding season in our study. In contrast, female *X. laevis* from the Pickford et al. [34] study were induced to breed and were most likely at a similar reproductive stage as females collected from the NCGR. This hypothesis is supported by findings in other anuran species, the European green frog (*Rana esculenta*) and the bull frog (*Rana catesbeiana*), which were collected from the wild. In these studies, frogs at the end of their spawning season had plasma T levels that were similar to frogs from our study [35,36]. Furthermore, these findings are consistent with observations of seasonal profiles for plasma T concentrations in females of other vertebrate species such as fish [37,38,39]. Another reason for the discrepancy between Kang et al. [33] and our study is that frogs from our study were collected from the wild, while *X. laevis* used in the Kang et al. study [33] were commercially supplied and kept under artificial conditions in the laboratory. Distinct differences have been reported for the concentrations of plasma sex steroids in another ranid species, *R. catesbeiana*, held under laboratory conditions [36]. These authors hypothesized that commercially supplied frogs were not likely to provide useful information on normal seasonal changes in plasma sex hormone profiles. They based this on the fact that these animals were maintained in an artificial environment and may have been subjected to

long-term stress from both transport and/or confinement and that effects on hormone profiles could persist for many weeks even though the frogs appear to be well.

Estradiol concentrations in plasma of NCGR females were similar to those observed for *X. laevis* females that were injected with hCG [34,40] but were slightly greater than those reported for feral *R. esculenta* and *R. catesbeiana* during their breeding period [35,36]. These results indicate that NCGR frogs were still at the peak of their reproductive activity, and thus the differences in the absolute concentrations of plasma sex steroids measured in the study by Paolucci et al. [35] and our study may be due to differences among species. In our study, males from the NCGR exhibited greater E2 concentrations than those reported for males from another study [41], but were similar to those in *R. clamitans* (control group) that were confined under laboratory conditions [42]. The reasons for these discrepancies are unknown. However, a study with the European green frog (*R. esculenta*) found that plasma E2 concentrations in males at the end of their reproductive period were similar to the results of our study [35]. It has been hypothesized that E2 might be involved with the interruption of reproductive processes in males and its increase could be responsible for the inhibition of androgen biosynthesis at the end of the spawning season (summarized in Paolucci et al. [35]). It has been shown in mammalian studies that estrogens derived from the testis play an important role in the negative feedback regulation of follicle-stimulating hormone [43,44]. In our study, frogs were collected in the late or postspawning period when follicle-stimulating hormone would be expected to be down-regulated. It is therefore possible that the elevated E2 concentrations measured in males resulted from the negative feedback control of gonadotropin secretion. While plasma E2 concentrations reported by Tobias et al. [41] were measured at a different time during the reproductive season, none of the frogs in that study had completed yet a breeding cycle in their life history. Since different stages in the reproductive cycle are characterized by dramatic changes in plasma sex steroid titers and frogs in our study were investigated at a different time than those in the Tobias et al. [41] study, it is difficult to directly compare these results. However, no direct evidence exists for this type of regulatory mechanism from the data presented, and it would require further research to test for this hypothesis.

The variability observed in plasma T and E2 concentrations was likely due to the fact that frogs were collected at the end of the breeding season, when physiological processes are known to undergo massive changes. This large variability reduced the power to demonstrate differences among locations. However, it was still possible to discriminate statistically between concentrations in females from the two regions. In contrast, the power to detect statistically significant differences for T in males was poor. To detect such differences with a power of 0.80 or greater would have required a sample size of more than 1,000 individuals. It was logistically impossible to sample the frogs over a shorter period of time, let alone to collect the required number of male frogs. Also, the sample sizes were such that it was impossible to stratify the frogs by season or age. The variability in concentrations of plasma T and E2 observed in our study is comparable to the variability in two other laboratory studies [33,41].

Effects on plasma sex steroids and gonadal aromatase activity

The reason for the differences in plasma concentrations of T and E2 in female *X. laevis* between the CGR and NCGR is

unknown at this time. Statistically significant differences were observed between the CGR and NCGR, and a statistically significant negative correlation was observed between both T and E2 concentrations in females and atrazine as well as most of its degradation products and overall chlorotriazine concentrations. Although a similar trend was observed in males between plasma T and E2 concentrations at the NCGR and CGR sites, these differences were not significant. This may have been due to the fact that the power to detect significant differences for T was poor in males compared to that for females, resulting in an increase of the chance of a type II error. Therefore, it cannot be totally excluded that any significant effects on plasma T concentrations existed. However, the differences in the concentrations of T between the different regions were small for males (1.7-fold) as compared to differences in females (3.5-fold), while no difference in the absolute average values in the NCGR existed. It is questionable—given the variability within and between sites—if such a difference is of any biological relevance. No significant correlation was observed between plasma sex steroids in males and atrazine. However, plasma concentrations of T in males were significantly negatively correlated with DACT ($p = 0.011$) and overall chlorotriazine concentrations ($p = 0.01$). It is not possible from the correlation alone to establish causality between exposure to atrazine and plasma concentrations of T or E2, especially since other factors, such as ions, were covariant to both triazine concentrations and biomarker responses. However, the significant relationships between plasma T in males and DACT and overall chlorotriazine concentrations indicate a possible effect of environmentally relevant triazine concentrations and *Xenopus* physiology that deserves further investigations. The CGR was characterized by intensive agriculture, and a variety of different chemicals, including herbicides, fungicides, insecticides, and fertilizers, was applied. Thus, to determine a possible causative agent, more detailed studies would be necessary to determine the effects of both chemical and edaphic factors.

Few studies have reported effects of atrazine on plasma E2 concentrations. A study in which juvenile *X. laevis* were exposed to a range of atrazine concentrations (0.1–25 $\mu\text{g/L}$) from 72 h posthatch until two to three months postmetamorphosis found no statistically significant differences in plasma T concentrations among atrazine treatments and controls [21]. Also, no statistically significant effects of waterborne atrazine exposure were observed on plasma concentrations of E2 in females. However, in males exposed to 1.0 $\mu\text{g/L}$ atrazine, plasma concentrations of E2 were significantly less than those of controls, but not at greater or lesser doses, such that no consistent dose–response relationship was seen [21]. In summary, no evidence exists from both laboratory and field studies that atrazine leads to an increase of plasma E2 concentrations in the blood plasma of male *X. laevis* subsequently resulting in feminization.

The presence of small concentrations of atrazine in the reference ponds precluded the ability to make an absolute test of hypothesis that frogs from atrazine-exposed and unexposed areas were different using a fixed-effects model. However, the small concentrations of atrazine in water in the NCGR, which were initially selected as the reference areas, did not seem to have an effect on plasma T concentrations of males because the average concentration and variability observed in this region were both similar to those reported for adult male *X. laevis* in a different study under control conditions [33].

Induction of aromatase as a potential mode of action

It has been hypothesized that atrazine affects sexual differentiation in both sexes by inducing aromatase. Such a change in aromatase activity could result in lesser concentrations of T, which could result in demasculinization of males or, through inappropriate synthesis and secretion of E2, in feminization of males [4]. Such a mechanism was deemed plausible based on in vitro studies with human and fish cell lines [22,45], but until the studies on which we report here, the hypothesis had not been tested in vivo in amphibians under field conditions. Differences were not observed in aromatase activities among regions, nor were significant correlations between exposure to triazines and aromatase activity and increases in plasma estradiol levels. These results do not support the hypothesis that atrazine causes an increase in the production of E2 by inducing aromatase activity [3]. However, aromatase activities were rather variable in this study. Thus, the statistical power to determine significant differences at the given number of samples was poor.

Given the good reproducibility and very low intra-assay variation, it is unlikely that the test systems used are responsible for the observed variability. We believe that the time of sampling, during the late spawning season, is the major factor contributing to the variability within sites observed during this study. The frogs were caught after or during the late breeding season (April through May), when physiological processes are known to change dramatically. Although the power to detect significant differences was poor, the observed difference between the different regions was small and may not be of any biological relevance. Given the very low aromatase activities in males regardless of season, it is unlikely that such minute levels of activity would have much biological significance.

In males, no significant correlation was observed between atrazine concentrations and plasma concentrations of T or the E2/T ratio, which would be expected if a causal relationship existed between atrazine exposure and an aromatase-mediated response. However, a significant negative correlation was observed between the E2/T ratio of males and DIA. In the H295R human adenocarcinoma cell line, DIA significantly increased *CYP-19* mRNA expression as well as aromatase activity [22]. The observed relationship between E2/T ratio and DIA was not consistent with published literature or with the hypothesis that atrazine or its degradation products cause changes in plasma T concentrations through an aromatase-mediated process. The observed trend in plasma E2 concentration was in the opposite direction that would be expected if aromatase activity was up-regulated. Thus, it is unlikely that an effect on aromatase activity existed that was due to atrazine or its degradation products, and it also seems unlikely that a strong case existed for decreased plasma T in males that was associated with the presence of atrazine.

Alternative modes of action

In fact, the significant positive correlation between E2 and T in both males and females indicates that both E2 and T are equally affected, and, therefore, the observed effects may be due to a more generalized mechanism, such as increased metabolism. A study of Atlantic salmon (*Salmo salar*) hypothesized that atrazine can affect T concentrations by increasing metabolic breakdown of the hormone [46]. However, no E2 measurements were made in that study. Increased metabolic activity is a common response to exposure of environmental pollutants as well as being a natural phenomenon in the re-

crudescence cycle. Thus, it cannot be excluded as a possible cause for the observed decrease in plasma sex steroids.

Another hypothesis is that atrazine or its degradation products may affect the endocrine system at the level of the central nervous system. Atrazine has been found to affect serum-luteinizing hormone and prolactin concentrations in Sprague-Dawley and Long-Evans female rats by altering the hypothalamic control of these hormones [47]. Similar conclusions were drawn in a recent paper by Stoker et al. [48] suggesting effects of atrazine degradation products on puberty and thyroid function in male Wistar rats via actions on the central nervous system and its control of the pituitary-gonadal axis. However, the doses at which atrazine caused the observed effects were much greater than those observed in this study (100–200 mg/kg; oral administration). Because of atrazine's low bioconcentration factor, this is unlikely to reflect realistic exposure scenarios of atrazine in the environment. Thus, again, it is unlikely that atrazine would cause the observed decreases in plasma concentrations of E2 and T.

Study design and future research needs

Both laboratory and field investigations have advantages and disadvantages. Laboratory studies allow for replication and the control of various parameters and conditions to allow the isolation of potential factors or causative agents. This often results in less variation in both the stressor of interest and the response. While this allows for efficient tests of mechanistic hypotheses, it does not represent the real-world situation in which we are ultimately interested. Alternatively, field studies, while realistic, are limited by a lack of replication, small sample sizes, and the confounding effects of many factors. Certainly that was the case in this study. Therefore, the authors feel that a combination of both types of studies would be useful for drawing conclusions about the potential effects of environmentally relevant concentrations of agricultural chemicals on frogs. The study conducted here was designed to test the hypothesis that aromatase and plasma concentrations of E2 and T were altered when exposed to agricultural chemicals under field conditions. Furthermore, the study was designed to investigate frog populations in two distinct regions, one of which was expected to have greater concentrations of triazine herbicides and one expected to have lesser concentrations. Additionally, the study was designed so that if no differences were observed between the two regions and no significant correlations were found between exposure to triazine herbicides and study parameters, it could be concluded that a causal relationship was unlikely. This information could be used along with information collected in controlled laboratory studies in a weight-of-evidence approach to make conclusions about the potential for triazine herbicides to affect populations of *X. laevis* under likely exposure scenarios along with the effects of other stressors, including chemical, physical, and biological. This was not the case; thus, a simple interpretation of the results is not possible. In regulatory terms, this study was designed to rebut the presumption of effects. While this could not be done the way the study was designed, it is impossible to determine the causes of differences in response between the two regions. The results of this study indicate that differences existed in some of the parameters among locations and between the two regions, so the simple hypothesis that modulation of plasma concentrations of E2 and T are caused by exposure to atrazine or coapplied triazines could not be rejected.

CONCLUSION

The current study provides evidence that plasma concentrations of both T and E2 in females are less in the CGR as compared to the NCGR, where concentrations of pesticides, including atrazine, were greater. While the median plasma titers of hormones were less in the CGR, the ranges of values overlapped such that it is unlikely that biologically relevant effects would have been expected to occur in these populations. For instance, the observed effects on steroid homeostasis in wild frogs did not negatively affect gonad growth as reported in two different studies [18,19]. Furthermore, while atrazine has been used in these areas for over 40 years, all the locations have robust populations of frogs with similar age distributions [49].

No evidence exists that these differences were due to induction of aromatase as has been previously suggested [3,4]. The data presented here demonstrate that, if there is an effect of atrazine on the endocrine system, it is unlikely to be via direct alteration of aromatase enzyme activity. Because frogs in the two areas were exposed to other factors, some of which (e.g., selected ions) showed significant correlations with both atrazine concentrations and biomarkers, it is impossible to determine the extent and mode of action by which atrazine may affect the endocrine systems of *X. laevis*. To adequately test this hypothesis, the critical mechanism of action of atrazine needs to be established. When this is done, an appropriate biomarker for atrazine-specific responses can be developed for use in definitive laboratory studies and field investigations. The use of larger-scale mesocosms might be one way to test for the potential effects of specific compounds like triazine herbicides [50]. These types of studies could be designed to isolate and control for a number of parameters. In addition, mesocosm studies could allow for a reasonable sample size while still allowing for more realistic exposure conditions.

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REFERENCES

1. Clark JR, Dickson KL, Giesy JP, Lackey RT, Mihaich EM, Stahl RG, Zeeman MG. 1999. Using reproductive and developmental effects data in ecological risk assessments for oviparous vertebrates exposed to contaminants. In Di Giulio R, Tillitt DE, eds. *Reproductive and Developmental Effects of Contaminants in Oviparous Vertebrates*. SETAC, Pensacola, FL, USA. pp 363–401.
2. Houlihan JE, Findlay CS, Schmidt BR, Meyer AH, Kuzmin SL. 2000. Quantitative evidence for global amphibian population declines. *Nature* 404:752–755.
3. Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide, atrazine, at low ecologically relevant doses. *Proc Natl Acad Sci USA* 99:5476–5408.
4. Hayes T, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A. 2002. Feminization of male frogs in the wild. *Nature* 419:896.
5. Kiesecker JM. 2002. Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature? *Proc Natl Acad Sci USA* 99:9900–9904.
6. Hayes TB, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A. 2003. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): Laboratory and field evidence. *Environ Health Perspect* 111:568–575.
7. Reeder AL, Foley GL, Nichols DK, Hansen LG, Wikoff B, Fach

- S, Eisold J, Wheeler MB, Warner R, Murphy JE, Beasley VR. 1998. Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (*Acris crepitans*). *Environ Health Perspect* 106:261–266.
8. U.S. Environmental Protection Agency. 2001. Health Effects Division's (HED's) revised preliminary Human Health Risk Assessment for the re-registration eligibility decision (RED). PC 080803. Case 0062. Health Effects Division, Washington, DC.
 9. Hopenhayn-Rich C, Stump ML, Browning SR. 2002. Regional assessment of atrazine exposure and incidence of breast and ovarian cancers in Kentucky. *Arch Environ Contam Toxicol* 42:127–136.
 10. Torres AMR, O'Flaherty LM. 1976. Influence of pesticides on *Chlorella*, *Chlorococcum*, *Stigeoclonium* (Chlorophyceae), *Tribonema*, *Vaucheria* (Xanthophyceae), and *Oscillatoria* (Cyanophyceae). *Phycologia* 15:25–36.
 11. Solomon KR, Baker DB, Richards RP, Dixon KR, Klaine SJ, LaPoint TW, Kendall RJ, Giddings JM, Giesy JP, Hall LWJ, Weiskopf CP, Williams WM. 1996. Ecological risk assessment of atrazine in North American surface waters. *Environ Toxicol Chem* 15:31–76.
 12. Detenbeck NE, Hermanutz R, Allen K, Swift MC. 1996. Fate and effects of the herbicide atrazine in flow-through wetland mesocosms. *Environ Toxicol Chem* 15:937–946.
 13. Howe GE, Gillis R, Mowbray RC. 1998. Effect of chemical synergy and larval stage on the toxicity of atrazine and alachlor to amphibian larvae. *Environ Toxicol Chem* 17:519–525.
 14. Diana SG, Resetarits WJ Jr, Schaeffer DJ, Beckmen KB, Beasley VR. 2000. Effects of atrazine on amphibian growth and survival in artificial aquatic communities. *Environ Toxicol Chem* 19:2961–2967.
 15. Allran JW, Karasov WH. 2000. Effects of atrazine and nitrate on northern leopard frog (*Rana pipiens*) larvae exposed in the laboratory from posthatch through metamorphosis. *Environ Toxicol Chem* 19:2850–2855.
 16. Giddings JM, Anderson TA, Hall LWJ, Kendall RJ, Richards RP, Solomon KR, Williams WM. 2000. Aquatic ecological risk assessment of atrazine—A tiered probabilistic approach: A report of an expert panel. Technical Report 709-00. Syngenta Crop Protection, Greensboro, NC, USA.
 17. Novartis Crop Protection. 2003. Summary of environmental fate of atrazine. Technical Report 1213-99. Greensboro, NC, USA.
 18. Tavera-Mendoza L, Ruby S, Brousseau P, Fournier M, Cyr D, Marcogliese D. 2002. Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. *Environ Toxicol Chem* 21:527–531.
 19. Tavera-Mendoza L, Ruby S, Brousseau P, Fournier M, Cyr D, Marcogliese D. 2002. Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the ovary. *Environ Toxicol Chem* 21:1264–1267.
 20. Carr J, Gentles A, Smith E, Goleman W, Urquidí L, Thuett K, Kendall R, Giesy J, Gross T, Solomon K, Van Der Kraak G. 2002. Response of larval *Xenopus laevis* to atrazine: Assessment of gonadal and laryngeal morphology. *Environ Toxicol Chem* 22:396–405.
 21. Coady KK, Murphy MB, Villeneuve DL, Hecker M, Jones PD, Carr JA, Solomon KR, Smith EE, Van Der Kraak G, Kendall RJ, Giesy JP. 2003. Effects of atrazine on metamorphosis, growth, laryngeal, and gonadal development, aromatase activity, and sex steroid concentrations in *Xenopus laevis*. *Environ Toxicol Chem* (in press).
 22. Sanderson JT, Letcher RJ, Heneweer M, Giesy JP, 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* 109:1–6.
 23. Smith EE, DuPreez LH, Solomon KR. 2003. Field exposure of *Xenopus laevis* to atrazine and other triazines in South Africa: Exposure characterization and assessment of laryngeal and gonadal responses. SA-01b. Ecorisk, Greensboro, NC, USA.
 24. Cuisset B, Pradelles P, Kime DE, Kuehn ER, Babin P, Davail S, Le Menn F. 1994. Enzyme immunoassay for 11-ketotestosterone using acetylcholinesterase as label: Application to the measurement of 11-ketotestosterone in plasma of Siberian sturgeon. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 108:229–241.
 25. Hecker M, Tyler CR, Hoffmann M, Maddix S, Karbe L. 2002. Plasma biomarkers in fish provide evidence for endocrine modulation in the Elbe river, Germany. *Environ Sci Technol* 36:2311–2321.
 26. Nash JP, Davail-Cuisset B, Bhattacharyya S, Suter HC, Le Menn F, Kime DE. 2000. An enzyme linked immunosorbant assay (ELISA) for testosterone, estradiol, and 17,20β-dihydroxy-4-pregnen-3-one using acetylcholinesterase as tracer: Application to measurement of diel patterns in rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol Biochem* 22:255–263.
 27. Lephart ED, Simpson ER. 1991. Assay for aromatase activity. In Waterman MR, Johnson EF eds, *Methods of Enzymology*. Academic, New York, NY, USA, pp 477–483.
 28. Davis CS. 2002. *Statistical Methods for the Analysis of Repeated Measurements*. Springer-Verlag, New York, NY, USA.
 29. Littell RC, Milliken GA, Stroup WW, Wolfinger RD. 1996. *SAS® System for Mixed Models*. SAS Institute, Cary, NC, USA.
 30. Brady JF, LeMasters GS, Williams RK, Pittman JH, Daubert JP, Cheung MW, Skinner DH, Turner JL, Rowland MA, Lange J, Sobek SM. 1995. Immunoassay analysis and gas chromatography confirmation of atrazine residues in water samples from a field study conducted in the state of Wisconsin. *J Agric Food Chem* 43:268–274.
 31. Richards RP, Baker DB. 1998. Triazine in waters of the Midwest: Exposure patterns. In Ballantine LG, McFarland JE, Hackett DE, eds, *Triazine Herbicides: Risk Assessment*. American Chemical Society, Washington, DC, pp 336–346.
 32. Rawn DF, Halldorson THJ, Muir DCG. 1998. Atmospheric transport and deposition, an additional input pathway for atrazine to surface waters. In Ballantine LG, McFarland JE, Hackett DE, eds, *Triazine Herbicides: Risk Assessment*. American Chemical Society, Washington, DC, pp 158–176.
 33. Kang L, Marin M, Kelley D. 1995. Androgen biosynthesis and secretion in developing *Xenopus laevis*. *Gen Comp Endocrinol* 100:293–307.
 34. Pickford DB, Morris ID. 2003. Inhibition of gonadotropin-induced oviposition and ovarian steroidogenesis in the African clawed frog (*Xenopus laevis*) by pesticide methoxychlor. *Aquat Toxicol* 62:179–194.
 35. Paolucci M, Esposito V, Di Fiore MM, Botte V. 1990. Effect of short postcapture confinement on plasma reproductive hormone and corticosterone profiles in *Rana esculenta* during the sexual cycle. *Ital J Zool* 57:253–259.
 36. Licht P, McCreery BR, Barnes R, Pang R. 1983. Seasonal and stress related changes in plasma gonadotropins, sex steroids, and corticosterone in the bullfrog, *Rana catesbeiana*. *Gen Comp Endocrinol* 50:124–145.
 37. Pasmanik M, Callard GV. 1988. Changes in brain aromatase and 5α-reductase activities correlate significantly with seasonal reproductive cycles in goldfish (*Carassius auratus*). *Endocrinology* 122:1349–1355.
 38. Hecker M. 2001. Natural variability of endocrine functions and their modulation by anthropogenic influences: Investigations of the bream (*Abramis brama* [L.]) along the Elbe river, and in a reference site. PhD thesis. University of Hamburg, Hamburg, Germany.
 39. Sen U, Mukherjee D, Bhattacharyya SP, Mukherjee D. 2002. Seasonal changes in plasma steroid levels in Indian major carp *Labeo rohita*: Influence of homologous pituitary extract of steroid production and development of oocyte maturational competence. *Gen Comp Endocrinol* 128:123–134.
 40. Wu KH, Tobias ML, Kelley DB. 2001. Estrogen and laryngeal synaptic strength in *Xenopus laevis*: Opposite effects of acute and chronic exposure. *Reproductive Neuroendocrinology* 74:22–32.
 41. Tobias ML, Tomasson J, Kelley DB. 1998. Attaining and maintaining strong vocal synapses in female *Xenopus laevis*. *J Neurobiol* 18:441–448.
 42. Mosconi G, Carnevali O, Franzoni MF, Cottone E, Lutz I, Kloas W, Yamamoto K, Kikuyama S, Polzonetti-Magni AM. 2002. Environmental estrogens and reproductive biology in amphibians. *Gen Comp Endocrinol* 126:125–129.
 43. Finkelstein JS, O'Dea LSL, Whitcomb RW, Crowley WF. 1991. Sex steroid control of gonadotropin secretion in the human male. II: Effects of estradiol administration in normal and gonadotropin-releasing hormone-deficient men. *J Clin Endocrinol Metab* 70:621–628.
 44. Bagatell CJ, Dahl KD, Bremner WJ. 1994. The direct pituitary effect of testosterone to inhibit gonadotropin secretion in men is

- partially mediated by aromatization to estradiol. *J Androl* 15:15-21.
45. Sanderson JT, Seinen W, Giesy JP, Van den Berg M. 2000. 2-chloro-s-triazine herbicides induce aromatase activity in H295R human adrenocortical carcinoma cells. A novel mechanism for estrogenicity. *Toxicol Sci* 54:127.
 46. Moore A, Waring CP. 1998. Mechanistic effects of a triazine pesticide on reproductive endocrine function in mature male atlantic salmon (*Salmo salar*). *Pestic Biochem Physiol* 62:41-50.
 47. Cooper RL, Stoker TE, Tyrey L, Goldman JM, McElroy WK. 2000. Atrazine disrupts the hypothalamic control of pituitary-ovarian function. *Toxicol Sci* 53:297-307.
 48. Stoker TE, Guidici DL, Laws SC, Cooper RL. 2002. The effects of atrazine metabolites on puberty and thyroid function in male Wistar rats. *Toxicol Sci* 67:198-206.
 49. Smith EE, DuPreez LH, Solomon KR. 2003. Field exposure of *Xenopus laevis* to atrazine and other triazines in South Africa: Exposure characterization and assessment of laryngeal and gonadal responses. SA-01a. Ecorisk, Greensboro, NC, USA.
 50. Giesy JP, Odum EP. 1980. Microcosmology: The theoretical basis. In Giesy JP, ed, *Microcosms in Ecological Research*. Department of Energy Technical Information Center, Oak Ridge, TN, USA, pp 1-13.