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TOXICITY OF CADMIUM, ENDOSULFAN, AND ATRAZINE IN ADRENAL STEROIDOGENIC CELLS OF TWO AMPHIBIAN SPECIES, *XENOPUS LAEVIS* AND *RANA CATESBEIANA*

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Abstract—The effects of cadmium, endosulfan, and atrazine on corticosterone secretion and viability of adrenal cells of African clawed frog (*Xenopus laevis*) and bullfrog (*Rana catesbeiana*) were assessed in vitro using a new bioassay. The bioassay relies on stimulation with adrenocorticotropic hormone (ACTH), the endogenous secretagogue for corticosterone secretion, and with dibutyryl cyclic adenosine monophosphate (dbcAMP), an analogue of cAMP, to pinpoint the site of action of the xenobiotics within the steroidogenic cell. To compare the test toxicants according to their endocrine-disrupting potential, the lethal concentration needed to kill 50% of the cells:effective concentration of 50% (LC50:EC50) ratio was calculated, with LC50 as the concentration that kills 50% of the steroidogenic cells and the EC50 as the concentration that impairs corticosterone secretion by 50%. The higher the ratio, the higher the potential for endocrine disruption. Atrazine had no affect on cell viability and on corticosterone secretion in *X. laevis*, but its endocrine-disrupting potential was high in *R. catesbeiana*. The LC50:EC50 ratio for cadmium and endosulfan in *X. laevis* was 26.07 and 1.23, respectively, and for atrazine, cadmium, and endosulfan in *R. catesbeiana* it was 909, 41, and 3, respectively. The dbcAMP did not restore corticosterone secretion in the cells exposed to the test toxicants in both species. Our study suggests that the secretory capacity of adrenal cells of amphibians can be impaired by environmental chemicals, especially atrazine in the bullfrog, and that these adrenotoxicants disrupt the enzymatic pathways leading to corticosterone secretion downstream from the step-generating cAMP.

Keywords—Amphibians   Adrenal   Corticosterone secretion   Endocrine disruptor   In vitro bioassay

INTRODUCTION

The decline of world populations of amphibians is a major environmental issue, and pollution is one of the factors that has been linked to this phenomenon [1,2]. It has been suggested that some toxicants discharged into the environment impair development and normal functions of the hormone-dependent processes in wildlife and humans [3,4]. Recent studies provided evidence that the adrenal gland and processes leading to the secretion of corticosteroid hormones are targeted by environmental xenobiotics in teleost fish [5], birds [6], and mammals [7]. The hypothalamo-pituitary-adrenal (HPA) axis mediates the physiological response to physical or chemical stressors in all vertebrates, including amphibians [8,9] and teleost fish [5]. Following stimulation by pituitary adrenocorticotropic hormone and activation of intracellular signaling pathways and cyclic adenosine monophosphate (cAMP) production, adrenal cells synthesize corticosterone and aldosterone, corticosteroid hormones important for the maintenance of homeostasis and normal development in amphibians [10]. Thus ACTH binds to a membrane receptor, adenylate cyclase is activated, and cAMP is produced as a second messenger. Corticosterone helps maintain blood glucose levels and liver glycogen reserves and controls osmoionic balance [11,12]. Moreover, the HPA axis and corticosterone are involved in regulating vital processes such as satiety, mating, and metamorphosis of amphibian larvae [13–15].

Few studies have investigated the effects of environmental pollutants on the adrenal function in amphibians, despite the importance of corticosteroid hormones in amphibian physiology. In a field study, Gendron et al. [16] assessed the functional integrity of the corticosterone-producing axis in mudpuppy, *Necturus maculosus*, exposed to mixtures of metals and chlorinated hydrocarbons, including polychlorinated biphenyls (PCBs) and DDT in the field. Animals from PCB-contaminated sites were less able to produce as high levels of plasma corticosterone in response to standardized confinement stress or injection of ACTH compared with animals from the reference sites. A study by Hopkins et al. [9] reported that toads (*Bufo terrestris*) exposed to coal combustion wastes containing aromatic hydrocarbons, Cd, As, Cu, and Se did not increase plasma corticosterone levels in response to an injection of ACTH, whereas a similar treatment stimulated a marked increase in plasma corticosterone in nonexposed toads. *Rana pipiens* tadpoles exposed to 77-TCB, a PCB congener, had lower whole-body levels of corticosterone compared with controls, both before and after injection with ACTH [17].

Although some evidence indicates that the adrenal gland and secretion of corticosterone in amphibians may be impaired by sublethal exposures to pollutants, rapid and inexpensive amphibian bioassays are not presently available for assessing the adrenal toxicity of environmental pollutants in vitro. However, amphibian adrenal anatomy and physiology is similar to teleost fish [18], and a teleost adrenal bioassay has been developed for in vitro detection of adrenal toxicants [19,20]. Frog adrenals have been used in vitro previously in endocrinological studies as a whole gland [21] or as individualized cells [22], but neither of these in vitro studies was oriented toward toxicology.

Table 1. Toxicological characteristics of atrazine and Cd in *Xenopus laevis* and *Rana catesbeiana*

Toxicant	Xenopus laevis (FETAX)[a]			Rana catesbeiana		
	LC50[b] (μM)	EC50[c] (μM)	TI[d]	LC50 (μM)	EC50 (μM)	TI
CdCl$_2$	32	3.7	8.6[e]	17.1[f]	NA	—
Atrazine	462	152	3.04[g]	1.9[h]	NA	—

[a] Frog embryo teratogenesis assay-*Xenopus*
[b] Concentration that kills 50% of the organisms.
[c] Concentration that inhibits growth by 50%.
[d] Teratogenic Index = Ratio LC50:EC50.
[e] [32].
[f] LC50 (96-h) value for injected tadpoles [33].
[g] [31].
[h] LC50 (24-h) value for embryos by immersion [34]. NA = not available.

This project had two main objectives. The first was to develop and validate an in vitro bioassay for use with amphibian adrenal cells. The second objective was to assess in vitro and compare the adrenal toxicity of three environmental toxicants—cadmium (Cd), endosulfan, and atrazine. Cadmium is widely distributed in the environment and is readily bioaccumulated by various organisms, including amphibians [2,23]. This metal can have adverse effects on growth, survival, and the capacity to regenerate limbs in the salamander *Ambystoma gracile* [24]. However, the effects of Cd on corticosterone secretion in amphibians have not been investigated previously. Endosulfan, a widely used organochlorine pesticide, is considered highly toxic to amphibians [25]. Berrill et al. [26] observed paralysis and a high mortality rate in *Rana sylvatica*, *Bufo americanus*, and *Rana clamitans* exposed to endosulfan at tadpole stages. Adrenotoxicity of endosulfan has not been assessed in amphibians, but in rainbow trout (*Oncorhynchus mykiss*) Leblond et al. [20] reported dose-dependent loss of ACTH- or dbcAMP-stimulated cortisol secretion by adrenal cells exposed in vitro to endosulfan. Atrazine is a herbicide commonly used in corn fields. Tadpoles of *R. catesbeiana* exposed to atrazine in vivo have exhibited significant DNA damage compared with nonexposed control animals [27]. Hayes et al. [28] reported recently that atrazine at low but ecologically relevant concentrations interfered with metamorphosis and sex differentiation in *Xenopus laevis*. Larson et al. [29] noted that plasma corticosterone levels declined in larval tiger salamanders (*Ambistoma tigrinum*) exposed in vivo to atrazine.

In this study, we assessed adrenal toxicity of Cd, endosulfan, and atrazine in vitro using ACTH and dbcAMP stimulation to determine if the xenobiotics act at the level of the adrenal cell membrane or at postmembrane steps. The adrenal sensitivity was compared in two amphibian species of different sensitivities to pollutants (Table 1), *X. laevis*, the African clawed frog, an amphibian model widely used in laboratory studies, and the bullfrog *Rana catesbeiana*, an indigenous species to eastern and central Canada and the United States.

MATERIALS AND METHODS

Experimental animals

Male and female African clawed frogs (body weight 20–40 g) were obtained from a commercial supplier (Xenopus One, Dexter, MI, USA). They were kept in a 600-L flow-
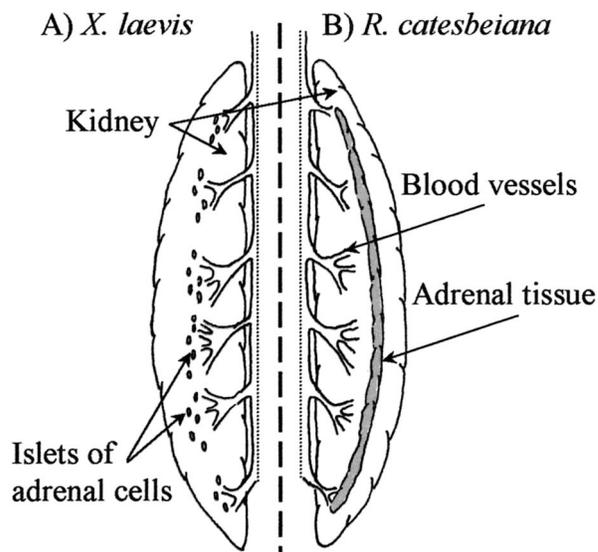

Fig. 1. Anatomy of adrenal gland of *Xenopus laevis* (**A**) and *Rana catesbeiana* (**B**).

through tank. Water was supplied to the tank at a rate of 400 ml/min, and water level in the tank was 35 cm deep. The temperature, hardness, and pH levels of the water were 22 ± 2°C, 100 to 114 mg/L (as CaCO$_3$), and 7.1 to 7.2, respectively. The study used a photoperiod of 12:12-h light:dark. The frogs were fed daily with Ward's biology adult Xenopus food ad libitum. Male and female bullfrogs (*R. catesbeiana*) (body weight 100 to 170 g), were obtained from a commercial supplier (Rana Ranch Bullfrog Farm, Twin Falls, ID, USA). They were kept under the same conditions as *X. laevis*, except the water level in the tank was maintained at 10 cm deep. They were fed daily ad libitum with AquaMax Grower 600 food (PMI Nutrition International, Brentwood, MO, USA) for *R. catesbeiana*. Two weeks of acclimation were allowed before beginning the experiments.

Preparation of adrenal cell suspensions

Frogs were anesthetized deeply by immersing them in a solution of MS-222 (4 g/L; ICN Biomedicals, Irvine, CA, USA); they were then euthanized by transection of the spinal cord. A total of 100 *Xenopus* and 11 *Rana* were used in the experiments. Whole kidney (*X. laevis*, Fig. 1A) or the adrenal tissue (*R. catesbeiana*, Fig. 1B) on both sides were dissected out and deposited into a tube containing minimum essential medium (MEM) supplemented with 5 g/L bovine serum albumin and 2.2 g/L NaHCO$_3$ (Sigma Chemical, St. Louis, MO, USA), pH adjusted to 7.4. The tissues were washed twice to remove blood cells and cut into pieces (~1 mm^3). The tissue pieces were resuspended in 1 ml of MEM containing 10 mg/ml collagenase/dispase mixture (Boenhringer Mannheim, Montréal, Canada) and incubated for 60 min with agitation at room temperature. Following enzymatic digestion, the preparation was allowed to settle and the supernatant, containing individualized cells, was centrifuged at 1500 rpm for 5 min. The pellet was resuspended in 1 ml of fresh MEM (without enzymes). Cell density was determined using a hemacytometer and adjusted to 10^7 cells/ml for *X. laevis* and to 10^5 cells/ml for *R. catesbeiana*.

Table 2. Corticosterone secretion (mean ± SE) by kidney cells[a] of *Xenopus laevis* in response to adrenocorticotropic hormone (ACTH) and dibutyryl cyclic adenosine monophosphate (dbcAMP)

	Concn. of ACTH (IU/ml)				
	0	1	2	3	—
Corticosterone (ng/ml)[b]	0.16 ± 0.017	0.35 ± 0.010	0.36 ± 0.028[c]	0.34 ± 0.005	—
	Concn. of dbcAMP (mM)				
	0	2	4	6	10
Corticosterone (ng/ml)	0.21 ± 0.013	0.25 ± 0.031	0.33 ± 0.024	0.43 ± 0.018	0.46 ± 0.021[d]

[a] Cell density 10^7 cells/ml.
[b] Number of replicates = 3 in each test.
[c] ACTH- and [d] dbcAMP-stimulated corticosterone secretion (ng/ml) by control cells (not exposed to toxicant) were 0.347 ± 0.067 and 0.234 ± 0.069 (n = 11) in the experiment with Cd, 0.316 ± 0.044 and 0.29 ± 0.029 (n = 25) with atrazine, and 0.26 ± 0.027 and 0.299 ± 0.085 (n = 11) in the experiment with endosulfan.

Exposure to chemicals

Cells were plated in a 96-well microplate (Fisher Scientific, Nepean, ON, Canada) at 150 µl of cell suspension per well. For each *Xenopus* kidney, two control wells (without toxicant) and two or more (depending on kidney size) wells exposed to the toxicant were prepared. A total of 27, 43, and 30 animals were used for the experiment with Cd, atrazine, and endosulfan, respectively, with 5 to 13 replicates for each concentration of toxicant and for controls. For *Rana*, control and toxicant-exposed wells for all concentrations tested were prepared from each adrenal tissue, with a total of 11 animals used. Given the variability in corticosterone secretion between individuals, it is important to include control and toxicant-exposed wells with each cell preparation and to express the response to the toxicant as the percentage of control.

In all experiments, a 2-h pre-incubation with slow agitation was required to reach basal corticosterone secretion level. The microplate was then centrifuged at 1500 rpm for 3 min and the supernatants were removed. The basal corticosterone levels measured in this bioassay were near the limit of the detection of the radioimmunoassay. Cell pellets were resuspended in 150 µl of Ringer solution containing NaCl 6.5 g/L, $CaCl_2$ 0.2 g/L, KCl 0.2 g/L, $NaHCO_3$ 0.2 g/L, dextrose 1 g/L, and Hepes 5.95 g/L (Sigma) with toxicants (exposed cells) or without (control cells), and incubated for 60 min at 15°C. $CdCl_2$ (Fisher) dissolved in deionized water was used at concentrations of 10^{-8} to 10^{-1} M; endosulfan and atrazine (Riedel-deHaën, Oakville, ON, Canada) dissolved in dimethyl sulfoxide (DMSO) were used at concentrations of 10^{-8} to 10^{-4} M. The final concentration of DMSO in the Ringer solution was 1% v/v. This concentration did not cause detectable loss in corticosterone secretion and cell viability (data not shown). After exposure, cells were rinsed twice with the Ringer solution before stimulation.

Stimulation of corticosterone secretion and determination of cell viability

After being rinsed, pellets were resuspended in 150 µl of MEM with 10 mM dbcAMP (N^6, $2'$-*o*-dibutyryl adenosine $3'$: $5'$-cyclic monophosphate; Sigma) or 2 IU/ml ACTH (porcine adrenocorticotropin 1–39; Sigma) and incubated at 22°C for 120 min. The microplate was then centrifuged at 1500 rpm for 3 min and the supernatants were frozen (−20°C) until corticosterone determination by a corticosterone ^{125}I radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA, USA). Cross-reaction for corticosterone is 100%. The percentages of

cross reaction for other corticosteroids are less than 0.34%. Intra- and interassay variabilities, expressed as coefficients of variation of values obtained for a pooled plasma sample assayed several times (n = 10) in one assay and in several assays (n = 10), respectively, were 6 and 9%, respectively. Parallelism between dilutions of plasma from *Xenopus* and *Rana* and standard hormone solutions was verified. Cell viability was assessed using the Trypan blue (Fisher) exclusion method after stimulation with ACTH or dbcAMP.

Data analyses

Statistical differences between responses of cells (ACTH- and dbcAMP-stimulated corticosterone secretion, viability) exposed to toxicants and their respective controls were determined using one-way analysis of variance and Dunnett test (α = 0.05). Differences between corticosterone secretion stimulated by ACTH and dbcAMP were determined using Student's *t* test (α = 0.01). The lethal concentration needed to kill 50% of the cells, the effective concentration that diminishes corticosterone secretion in response to ACTH by 50% (EC50), and their respective 95% confidence limits were determined using the best-fitting polynomial model with the JMPIN software system (Ver 4.0.3, SAS Institute, Cary, NC, USA) . The ratio LC50:EC50 was calculated to assess the capacity of the toxicants to diminish corticosterone secretion without inducing a loss in cell viability. A high ratio corresponds to a specific action of the chemicals on corticosterone secretion, whereas a low ratio (e.g., ~1.0) suggests a nonspecific action of the chemical and the loss of corticosterone secretion due to cell death.

RESULTS

ACTH- and dbcAMP-stimulated corticosterone secretion and cell viability

The optimal concentrations of ACTH and dbcAMP required to stimulate corticosterone secretion in vitro were selected in a pilot study with *X. laevis* (Table 2). Concentration of 2 IU ACTH/ml and 10 mM dbcAMP were selected for experiments with cells of both amphibian species. A pilot study with *R. catesbeiana* adrenal cells incubated at different cell densities (Table 3) demonstrated detectable corticosterone levels at 10^4 cells/ml. However, an optimal secretion was obtained with 10^5 cells/ml, a density subsequently used in all the experiments with *R. catesbeiana* cells. Density of 10^7 cells/ml was used with *X. laevis* cells, a concentration similar to the teleost bioassay using the head kidney cells [20]. Since no significant

Table 3. Corticosterone secretion (mean $\pm$ SE) by adrenal cells of *Rana catesbeiana* incubated at different cell densities and stimulated with adrenocorticotropic hormone (ACTH) and dibutyryl cyclic adenosine monophosphate (dbcAMP)

Cell density	Corticosterone (ng/ml)	
	ACTH-stimulated[a]	dbcAMP-stimulated[b]
10^4 cell/ml	0.57 ± 0.16	0.54 ± 0.10
10^5 cell/ml	2.70 ± 0.77	3.57 ± 0.5
	1.833 ± 0.468 ($n = 11$)[c]	1.985 ± 0.470 ($n = 11$)[d]
10^6 cell/ml	11.19 ± 1.04	11.13 ± 1.00

[a] Concentration of ACTH = 2 IU/ml.
[b] Concentration of dbcAMP = 10 mM.
[c] ACTH-stimulated corticosterone secretion by control cells (not exposed to toxicants) in experiments with Cd, atrazine, and endosulfan.
[d] dbcAMP-stimulated corticosterone secretion by control cells (not exposed to toxicants) in experiments with Cd, atrazine, and endosulfan.

differences were observed between ACTH-stimulated corticosterone secretion in males and females in either species, both males and females were used in subsequent experiments with toxicants. Cell viability for controls and cells stimulated with ACTH and dbcAMP was >95% in each experiment (data not shown).
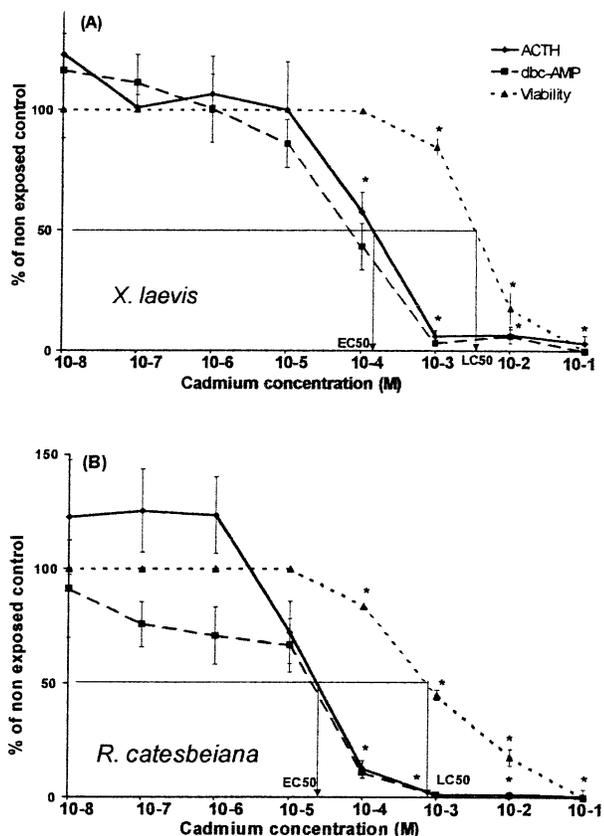

Fig. 2. Adrenocorticotropic hormone (ACTH)– and dibutyryl cyclic adenosine monophosphate (dbcAMP)–stimulated corticosterone secretion and viability (percentage of control, mean $\pm$ SE) of adrenal cells in *Xenopus laevis* (**A**) and *Rana catesbeiana* (**B**) following a 60-min in vitro exposure to Cd ($n = 5$–11). Asterisks indicate significant differences from controls not exposed to Cd (100%) for corticosterone secretion and viability ($p \leq 0.05$). The concentration that kills 50% of the adrenal cells in vitro (LC50) and concentration that impairs 50% of ACTH-stimulated corticosterone secretion in vitro (EC50) values are indicated by arrows.
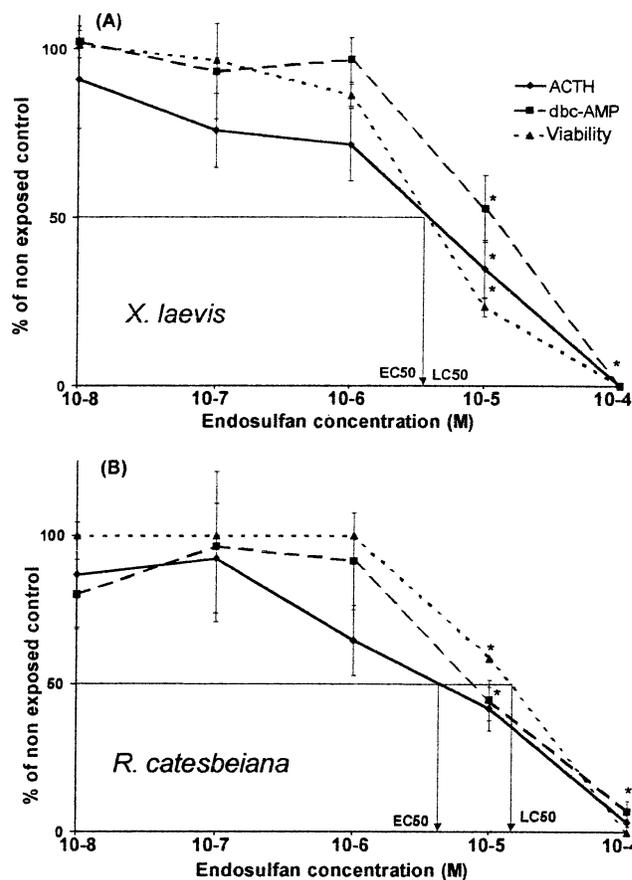
Fig. 3. Adrenocorticotropic hormone (ACTH)– and dibutyryl cyclic adenosine monophosphate (dbcAMP)–stimulated corticosterone secretion and viability (percentage of control, mean $\pm$ SE) of adrenal cells in *Xenopus laevis* (**A**) and *Rana catesbeiana* (**B**) following a 60-min in vitro exposure to endosulfan ($n = 6$–13). Asterisks indicate a significant difference from controls not exposed to endosulfan (100%) for corticosterone secretion and viability ($p \leq 0.05$). The concentration that kills 50% of the adrenal cells in vitro (LC50) and concentration that impairs 50% of ACTH-stimulated corticosterone secretion in vitro (EC50) values are indicated by arrows.

Effects of acute exposure to toxicants on ACTH- and dbcAMP-stimulated corticosterone secretion and cell viability

The responses of adrenal cells of *X. laevis* and *R. catesbeiana* exposed in vitro to Cd, endosulfan, and atrazine for 60 min are shown in Figures 2 through 4. Dose-dependent effects on the secretory capacity of adrenal cells and on their viability were observed following exposures to Cd and to endosulfan in both species, and to atrazine in the bullfrog. Since no significant differences in the responses to toxicants were observed between males and females, data from males and females were grouped together.

Exposure to Cd for 60 min (Fig. 2) significantly decreased ACTH- and dbcAMP-stimulated corticosterone secretion at concentrations of 10^{-4} M and higher in both amphibian species. However, cell viability decreased significantly at 10^{-3} M in *X. laevis* (Fig. 2A), and at 10^{-4} M in *R. catesbeiana* (Fig. 2B).

The effects of endosulfan on corticosterone secretion and cell viability were similar in both amphibian species (Fig. 3). Significant decreases, both in viability and ACTH- and dbcAMP-stimulated corticosterone secretion, were noted at endosulfan concentrations $\geq 10^{-5}$ M.
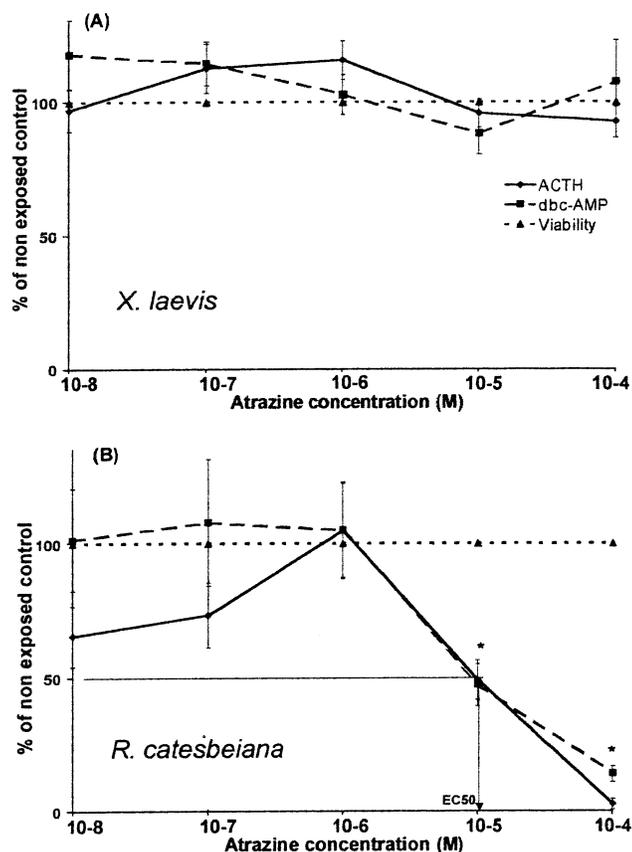
Exposure to atrazine (Fig. 4) had no effect on the corti-

Fig. 4. Adrenocorticotropic hormone (ACTH)– and dibutyryl cyclic adenosine monophosphate (dbcAMP)–stimulated corticosterone secretion and viability (percentage of control, mean ± SE) of adrenal cells in *Xenopus laevis* (**A**) and *Rana catesbeiana* (**B**) following a 60-min in vitro exposure to atrazine ($n = 7$–11). Asterisks indicate a significant difference from controls not exposed to atrazine (100%) for corticosterone secretion and viability ($p \leq 0.05$). The concentration that kills 50% of the adrenal cells in vitro and concentration that impairs 50% of ACTH-stimulated corticosterone secretion in vitro (EC50) values are indicated by arrows.

costerone secretion in *X. laevis* (Fig. 4A). However, atrazine significantly decreased corticosterone secretion at concentrations $\geq 10^{-5}$ M in *R. catesbeiana* (Fig. 4B). Cell viability was not reduced significantly in either species at any of the concentrations tested.

Toxicological characteristics of Cd, endosulfan, and atrazine

The LC50, EC50, and the ratio LC50:EC50 were determined for the xenobiotics tested in this study with corticoste-

rone secretion and cell viability (Table 4). Toxicological parameters were used to rank the chemicals according to their cytotoxicity (LC50) and their endocrine toxicity (EC50). For *X. laevis*, the sequence was endosulfan < Cd < atrazine for LC50 and EC50. For *R. catesbeiana*, the sequences were endosulfan < Cd < atrazine for LC50, and endosulfan < atrazine < Cd for EC50.

The ratio LC50:EC50 was used to compare the specificity of the xenobiotics to impair corticosterone secretion by adrenal cells in the two species. The higher the ratio, the higher was the capacity of the xenobiotic to impair corticosterone secretion without loss of cell viability. For *X. laevis*, the decreasing order of specificity was Cd > endosulfan > atrazine. For *R. catesbeiana*, the order of specificity was atrazine > Cd > endosulfan. A ratio of 26.07 for Cd in *X. laevis* indicated that the concentration of $CdCl_2$ required to kill 50% of the cells was 26 times greater than the concentration required to impair the corticosterone secretion by 50%. A ratio near 1.0 suggests that the loss of ability to respond to ACTH is due to cell death, rather than to functional impairment of secretory pathways. Toxicological characteristics of Cd, endosulfan, and atrazine in adrenal cells of rainbow trout [20,30] are included in Table 4, allowing comparison of sensitivity in a teleost versus the amphibians.

DISCUSSION

An adrenal bioassay for detection of adrenotoxic chemicals for use in amphibians has been developed, and the toxicity of Cd, endosulfan, and atrazine has been compared in steroidogenic cells of *X. laevis* and *R. catesbeiana* in vitro. Significant toxicant and species-related differences were detected. The secretory capacity of the cells was assessed by measuring corticosterone production after acute (60 min) exposure to a potential endocrine disrupter. The bioassay relies on stimulation with ACTH and dbcAMP to pinpoint the site of action of the xenobiotics within the steroidogenic cell. A loss of the capacity to secrete corticosterone in response to ACTH, but not to dbcAMP, indicates that the xenobiotic acts on the membrane receptor or the steroidogenic pathway upstream from the cAMP step. If the toxicant impairs the pathway downstream from cAMP, the response to both ACTH and dbcAMP is lost. Cytotoxicity, characterized by the loss of cell viability, was expressed as a LC50, the concentration of toxicant that kills 50% of the cells. Both LC50 and EC50 (concentration of toxicant that inhibits corticosterone ACTH-stimulated secretion by 50%) were determined in this study in order to quantitatively compare the toxicants. At cytotoxic concentrations of the test toxicants, the loss of hormone secretion was the result

Table 4. Adrenotoxicity (LC50, EC50, and LC50/EC50) of xenobiotics tested in *Xenopus laevis, Rana catesbeiana,* and *Oncorhynchus mykiss* steroidogenic cells in vitro

Toxicant	*Xenopus laevis*			*Rana catesbeiana*			*Oncorhynchus mykiss*		
	LC50 (μM)[a]	EC50 (μM)[b]	Ratio[c]	LC50 (μM)	EC50 (μM)	Ratio	LC50 (μM)	EC50 (μM)	Ratio
CdCl$_2$	3,312	127	26.07	907	22	41.22	10,800	168	64.29[d]
Endosulfan	4.7	3.8	1.23	11	3.6	3.05	308	17.3	17.8[e,f]
Atrazine	>100,000	>10,000	—	>10,000	11	>909	>50,000	>50,000	—[f]

[a] Concentration that kills 50% of the adrenal cells in vitro.
[b] Concentration that impairs 50% of ACTH-stimulated corticosterone secretion in vitro.
[c] LC50: EC50.
[d] [19].
[e] [20].
[f] [30].

of the declining number of living cells. The specificity of the toxicant for disruption of hormone secretion (corticosterone) was determined by the ratio LC50:EC50 [20,30]. A strong endocrine-disrupting potential was characterized by a high ratio LC50:EC50, indicating impairment of steroid secretion at noncytotoxic concentrations of the test toxicant.

The experiments we conducted to develop the adrenal bioassay allowed us to optimize the assay with respect to the number of adrenal cells per microplate well and the concentrations of ACTH and dbcAMP required to enhance corticosterone secretion. We selected 2 IU/ml of ACTH and 10 mM of dbcAMP for the experiments. The differences in adrenal anatomy between the two amphibian species (Fig. 1) were considered and influenced the number of cells used in the assay. The cell concentrations selected were 10^7 and 10^5 cells/ml for *X. laevis* and *R. catesbeiana*, respectively. The smaller number of *R. catesbeiana* cells reflects the organization of the adrenal cells into a distinct tissue that can be dissected out, in contrast to the more diffuse arrangement in the kidney of *X. laevis*. Similar modifications may be necessary when working with other amphibian species.

The cytotoxic and endocrine-disrupting potentials of Cd, endosulfan, and atrazine were assessed with the new adrenal bioassay. Atrazine was identified as the toxicant with the greatest LC50 in cells of *X. laevis*, followed by Cd and endosulfan, the most cytotoxic chemical tested with LC50 of 4.7 μM. Endosulfan was about 700 times more toxic than Cd in the adrenal bioassay with *X. laevis* cells (Table 4). Two studies using Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX), a whole embryo assay, reported a LC50 of 462 μM for atrazine [31] and 32 μM for CdCl₂ (Table 1) [32]. These concentrations are lower compared with those determined in the present acute (60 min) exposure study. However, the LC50 of atrazine was, as we reported, higher than the LC50 of Cd. The differences in sensitivity between adrenal cells and whole embryos, and differences in the exposure periods used in the tests, underlie the differences in LC50s.

The EC50s, for impairment of corticosterone secretion by the three toxicants, followed the same pattern as LC50s in *X. laevis*. Atrazine had no effect on corticosterone secretion, and the EC50 of endosulfan was lower than that of Cd (Table 4). The EC50 and LC50 for endosulfan were similar, and the ratio LC50:EC50 was near 1.0, indicating that endosulfan may be cytotoxic to adrenal cells of *X. laevis*.

To compare toxicants according to their endocrine-disrupting potential, the LC50:EC50 ratio was calculated. The LC50:EC50 ratio of atrazine could not be calculated for *X. laevis* because atrazine had no effect on adrenal cell viability or corticosterone secretion. In contrast, Morgan et al. [31] reported a LC50:EC50 ratio of 3.04 for atrazine with the FETAX test, with a EC50 value based on the inhibition of growth of the larvae. This ratio indicated that the concentration needed to inhibit growth by 50% was one third of the concentration needed to kill 50% of the frogs. The LC50:EC50 ratio of endosulfan in the present study (1.23) indicated that this toxicant was not a specific disruptor of corticosterone secretion by cells of *X. laevis* and that the loss of corticosterone secretion was due to loss of cell viability. In contrast, Cd had a relatively high LC50:EC50 ratio (26.07), indicating that Cd had a specific action on corticosterone secretion since the concentration required to kill 50% of cells was 26 times higher than the concentration required to impair 50% of the secretion. Sunderman et al. [32] observed a LC50:EC50 ratio of 8.6 for Cd with

FETAX (96-h) compared with 3.04 for atrazine with the same test (Table 1) [31]. The paucity of toxicological data and test systems for amphibians makes the comparison between our data and other studies difficult, but some similarities in relative toxicity (Cd and endosulfan) are apparent.

The cytotoxicity pattern observed for *R. catesbeiana* cells was similar to that for *X. laevis* cells. Endosulfan had the lowest LC50 and was 82 times more cytotoxic than Cd. In comparison, Zettergreen et al. [33] measured a LC50 96 h of 17.1 μM of Cd for *R. catesbeiana* tadpoles. Atrazine was not cytotoxic even at 100 μM (Fig. 4B). In contrast, Birge et al. [34] reported a LC50 24 h of 1.9 μM for *R. catesbeiana* embryos by immersion in atrazine, and as for other embryonic or larval tests, the exposure periods were longer than the 60 min period used in the adrenal bioassay. The endocrine toxicity, estimated by the EC50, was remarkably different for *R. catesbeiana* compared with *X. laevis*. With each of the toxicants, the EC50s were always lower for *R. catesbeiana* than for *X. laevis* and could be ranked as Cd > atrazine > endosulfan.

Atrazine was the most specific endocrine-disrupting xenobiotic tested with *R. catesbeiana* and had the highest LC50: EC50. Thus in *R. catesbeiana* cells, atrazine impaired the corticosterone secretion in vitro without decreasing the viability of the cells. The Cd followed atrazine with a LC50:EC50 ratio of about 41. These results suggest a high specificity of atrazine and Cd in the bullfrog steroidogenic cells. They also confirm previous results with another aquatic species, the rainbow trout, *Oncorhynchus mykiss* [20] for Cd, and revealed a major species difference for atrazine (identified as nonadrenotoxic) in both *X. laevis* and rainbow trout [30]. The least endocrine-specific toxicant, compared with Cd and atrazine, was endosulfan with a LC50:EC50 ratio of 3.05 for *X. laevis*. Harris et al. [35] reported a LC50 (96 h) of 21.7 μM and a EC50 (deformities) of 11.2 μM for green frog (*Rana clamitans*) embryos exposed to endosulfan. Our results for endosulfan showed a similar range of values for LC50 and EC50 for *X. laevis* and *R. catesbeiana*, respectively. Berrill et al. [26] reported mortality even at the lowest doses of endosulfan used, 0.31, 0.19, and 0.24 μM, in *Rana sylvatica*, *Bufo americanus*, and *Rana clamitans*, respectively, at tadpole stage. These concentrations are similar to the concentrations causing a decrease in the corticosterone secretion and a decrease in the viability of the cells observed in the present study. Vonier et al. [36] demonstrated that endosulfan inhibited the binding of [³H] 17β-estradiol to the estrogen receptor in a reptile, *Alligator mississippiensis*, and atrazine had a similar effect with a IC50 of 20.7 μM, suggesting that these toxicants can impact the endocrine system. Another study [37] reported that the pheromonal system of the red-spotted newt, *Notophalmus viridescens*, was highly susceptible to low-concentration exposure to endosulfan.

This study provides evidence that corticosterone secretion was impaired by Cd and endosulfan in a similar pattern in the two amphibian species; Cd has a greater endocrine specificity, compared with endosulfan; and atrazine impaired corticosterone secretion of *R. catesbeiana* adrenal cells, but did not have an effect on *X. laevis*. It is difficult to compare the adrenal sensitivity of the two amphibian species due to differences in the cell preparations (cells from the whole kidney in *X. laevis* vs cells from adrenal tissue in *R. catesbeiana*), but our data suggest that the adrenal cells of *R. catesbeiana* were more sensitive than kidney cells of *X. laevis* to each of the xeno-

biotics we tested. Similarly, Birge et al. [38], comparing LC50s, classified different amphibian species and identified *X. laevis* as a tolerant species and *R. catesbeiana* as a very sensitive species. Our data clearly demonstrate that *Xenopus* cells are less vulnerable to Cd and do not respond to atrazine compared with cells from the bullfrog. These findings are important because they suggest that FETAX (an assay using *Xenopus* embryos) might underestimate toxicity to other amphibians and may not be adequately protective. Moreover, the much lower effective concentration (EC50) of atrazine in *Rana* compared with *Xenopus* indicates that the adrenal system of the bullfrog, a North American anuran, might be extremely vulnerable to atrazine. Our data on adrenal toxicity of atrazine and recent findings by Hayes et al. [28] that atrazine at concentrations detected in the field caused reproductive anomalies in another amphibian species constitute evidence that this pesticide has a high endocrine toxicity. Thus its use may need to be reevaluated. Compared with the teleost adrenal bioassay [19,20,30] (Table 4), amphibian adrenal cells are more sensitive to endosulfan and Cd, as indicated by the LC50 and EC50, and atrazine does not impair cortisol secretion in either rainbow trout or in *X. laevis*. The loss in secretory activity could not be restored by stimulation with dbcAMP in the present study with any of the adrenotoxic chemicals we tested. Therefore, the likely mechanism of impairment of the steroidogenic pathway is downstream from the step that generates cAMP.

In conclusion, the amphibian assay described in this study can be used to assess the adrenotoxicity of environmental pollutants following an acute (60 min) in vitro exposure. Whether the neuroendocrine stress response is also impaired in vivo, as has been reported in teleosts for Cd [39] and the amphibian mudpuppy [16] through chronic exposures to PCBs, has not been investigated in *R. catesbeiana* and *X. laevis*. The importance of pollutant-induced disruption of the synthesis of corticosteroids in amphibian decline remains to be assessed as well.

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