SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 24, No. 8, pp. 2027–2036, 2005
© 2005 SETAC
Printed in the USA
0730-7268/05 $12.00 + .00

EFFECTS OF THE SYNTHETIC ESTROGEN ETHINYLESTRADIOL ON EARLY LIFE STAGES OF MINK FROGS AND GREEN FROGS IN THE WILD AND IN SITU

BRADLEY J. PARK*†‡ and KAREN KIDD†‡§
†Department of Zoology, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada
‡Freshwater Institute, Fisheries & Oceans Canada, Winnipeg, Manitoba, R3T 2N6, Canada
§Biology Department, University of New Brunswick, Saint John, New Brunswick E2L 4L5, Canada

(*Received* 3 *May* 2004; *Accepted* 3 *February* 2005)

Abstract—Estrogenic contaminants are known to disrupt growth and development in amphibians. Field-based research is needed to elucidate their potential impacts on wild populations. Hatch success, larval growth and development rates, and gonad development were examined in native amphibians exposed to low ng/L concentrations of 17α-ethinylestradiol (EE2) in a whole-lake addition experiment at the Experimental Lakes Area, northwestern Ontario, Canada. Egg masses were reared in situ in the EE2-amended lake and in two reference lakes in 2001 and 2002. Hatching success was reduced significantly in green frogs (*Rana clamitans*) but not in mink frogs (*Rana septentrionalis*) exposed to EE2. Ethinylestradiol had no consistent effect on mass or development stage of hatchlings in the early larval stages of the caging study. Ethinylestradiol had no effect on sex ratios of either species in situ, and no intersex gonads were observed in exposed or reference green frog tadpoles or in reference mink frog tadpoles. However, 5.6% (total $n = 18$) and 12.5% (total $n = 56$) of EE2-exposed mink frog tadpoles were intersex in the 2001 and 2002 caging studies, respectively. Wild mink frog tadpoles also were examined, and EE2 had no effect on sex ratios. No intersex gonads were observed in reference lake tadpoles or in tadpoles from the experimental lake prior to EE2 additions; however, 2.4, 0, and 28.6% of wild EE2-exposed first-year tadpoles had intersex gonads (2001, 2002, and 2003, respectively). These results indicate that exposure to EE2 in the wild and in situ at concentrations comparable to those detected in effluents and, occasionally, in surface waters can impact gonad development and hatch success in native amphibians.

Keywords—Amphibian Endocrine disruption Gonad differentiation Ethinylestradiol

INTRODUCTION

Declines, extinctions, and range reductions of amphibian populations are occurring at alarming rates in many parts of the world [1]. Numerous stressors contribute to this phenomenon, including the presence of anthropogenic chemicals in the environment [2]. Some contaminants can disrupt critical hormone-dependant development processes in early life stages causing mortality and reduced fitness and fecundity [3]. Environmental estrogens are of particular concern because they can disrupt sexual development and also may interfere with metamorphosis. Natural and synthetic estrogens have been detected in sewage treatment effluents and occasionally in receiving waters due to their incomplete removal in the treatment process [4,5]. Other aquatic contaminants such as organochlorine pesticides, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons also are estrogenic [6]. Thus, amphibians in polluted areas can be exposed directly to estrogenic contaminants for a significant portion of the life cycle.

Amphibian embryos are exposed to contaminants through maternal transfer or direct uptake during or after egg coat formation [7]. Previously it has been demonstrated that exposure of embryos to an estrogenic phthalate ester reduces hatch success [8]. Field studies have demonstrated reduced hatching of amphibian eggs reared in situ in municipal wastewater–irrigated ponds [9] and in storm water detention ponds [10], although mixtures of contaminants were present in both cases and the effects were not attributed to the presence of estrogenic chemicals. The role of steroids in normal embryonic development is understood poorly and potential disruption from estrogenic contaminants has received little attention. Reduced hatch success ultimately could impair population recovery after natural or human-induced decline events.

Rates of growth and development in the larval phase have important implications for amphibians in the wild, i.e., a critical minimum body size must be attained before tadpoles can metamorphose [11] and greater size improves survival and predator avoidance in larvae and fecundity in adults [12]. Larval development primarily is controlled by the thyroid hormone system, which in turn can be modulated by estrogens [13]. Most evidence suggests that estrogens depress thyroid status, thereby inhibiting metamorphosis. Administration of estradiol reduced plasma thyroid hormone concentrations in frogs [14] and inhibited growth and development [13,15,16]. Laboratory studies demonstrate that the synthetic estrogen diethylstilbestrol delays organogenesis, induces developmental abnormalities in tadpoles [17], and can compete with endogenous thyroid hormone to bind plasma thyroid hormone–binding proteins [18]. Exposure to environmental chemicals with estrogenic activity, therefore, may disrupt thyroid hormone dynamics and inhibit development of larvae in the wild.

Sex hormones are crucial in directing normal reproductive development in amphibians, including the process of gonad differentiation [19]. Despite being predetermined genetically, the sexes are morphologically indistinct in early development until the bipotential gonad develops into an ovary or a testis, likely under the influence of endogenous sex steroids [20]. Laboratory studies indicate that exposure to hormonally active contaminants can disrupt this process. Amphibians exposed to natural and synthetic hormones exhibited full or partial sex

* To whom correspondence may be addressed
(parkb@dfo-mpo.gc.ca).

reversal (i.e., skewed sex ratios), intersex gonads, or no effect [20–22]. *Xenopus leavis* tadpoles exposed to nonylphenol, octylphenol, bisphenol A, or butylhydroxyanisol exhibited increased percentages of female phenotypes [23]. Atrazine exposure reduced testicular volume, number of spermatogonial cell nests and nurse cells [24], and caused hermaphroditism and multiple gonad development in *X. laevis* tadpoles [25]. Oocyte development was inhibited by the estrogenic pesticide methoxychlor, though exposure to natural and synthetic estrogens had varying results [26]. Furthermore, exposure to organochlorine pesticides disrupted gonaduct development in *Ambystoma tigrinum* [27]. In field studies, skewed sex ratios and intersex gonads were detected in frogs collected from contaminated sites [28,29]. Evidence indicates that hormonally active toxicants impact gonad differentiation, which in turn may threaten the viability of wild populations. Furthermore, such contaminants may be affecting other critical aspects of reproduction such as secondary sex characteristics and breeding behavior in wild populations.

Current evidence from laboratory studies indicates that environmental estrogens disrupt various aspects of amphibian development; however, understanding their impacts on wild populations requires further research. A whole-lake estrogen addition study was conducted at the Experimental Lakes Area (ELA, Ontario, Canada) to elucidate the effects of a known, potent endocrine disruptor on otherwise undisturbed aquatic biota. The 17α-ethinylestradiol (EE2), a synthetic estrogen used in oral contraceptives, has garnered some attention because it is a potent estrogen and has been detected in aquatic environments in which feminized fish populations occur [4,6,30]. Ethinylestradiol was added to Lake 260 from May to October in each of 2001 through 2003 at concentrations comparable to some previously reported environmental levels. As part of this research effort, the current study was conducted to assess impacts of EE2 on hatching, growth, development, and gonad differentiation in tadpoles of two native species, the mink frog (*Rana septentrionalis*) and the green frog (*Rana clamitans*). Both species breed in late June to early July locally, and the larvae typically transform into adults after overwintering at least once; therefore, the exposure to EE2 in this study was prolonged and occurred during potentially sensitive thyroid hormone– and steroid hormone–dependant development stages.

MATERIALS AND METHODS

Study lakes and EE2 additions

The lakes used in this study are located within the Experimental Lakes Area (49°34′–47′N, 93°36′–52′W, www.umanitoba.ca/institutes/fisheries), a Fisheries and Oceans Canada research facility, and are not exposed to direct industrial, agricultural, or domestic chemical inputs. The EE2 (17 α-ethinylestradiol, Schering AG, Berlin, Germany) was added to Lake 260 in 2001, 2002, and 2003 throughout the period of thermal stratification during the open water seasons to maintain a relatively constant target concentration of 5 ng/L in the epilimnion. Three times per week from May to October of each year EE2 was dissolved in methanol, and the solution was pumped into the propeller wash of a boat while driving around the lake. Additions were modified weekly based on the depth of the epilimnion and the results of the previous week's sampling, and were done for a total of 20 to 21 weeks each year. Epilimnion depths averaged 4.3, 4.9, and 5.0 m (~57, 65, and 67% of the lakes volume) in 2001, 2002, and 2003, respectively, during the additions, and represented an average volume of 1.04×10^9, 1.06×10^9, and 1.2×10^9 L, respectively, in each of the three years.

To quantify the concentrations of EE2 in the epilimnion of Lake 260, we collected integrated replicate water samples each week from five sites around the lake (n = 10/week). These samples were extracted on C-18 solid phase extraction cartridges (Sigma Aldrich, Oakville, ON, Canada) and analyzed by radioimmunoassay using the methods and quality assurance procedures given in Palace et al. [31]. Mean concentrations of EE2 (± standard deviation [SD]) in the epilimnion of Lake 260 during the addition periods were as follows: 2001, 6.1 ± 2.8 ng/L (n = 189); 2002, 5.0 ± 1.8 ng/L (n = 169); 2003, 4.8 ± 1.0 ng/L (n = 170). Weekly means of EE2 ranged from 4.3 to 9.1, 2.9 to 8.9, and 3.3 to 7.3 ng/L in 2001, 2002, and 2003, respectively. Within weeks during the additions, EE2 concentrations varied across sampling sites by 2 to 5 ng/L. Samples also were collected in January of 2002 and 2003 from under the ice, and EE2 was present at 2.4 ± 1.1 (n = 6) and 1.6 ± 0.4 ng/L (n = 6), respectively. The EE2 concentrations in Lake 260 were comparable to those detected in various sewage treatment effluents and occasionally in surface waters [4,5], and exposure in this range has been shown to induce an estrogen receptor-mediated response in fish [32].

Lakes 114, 224, and 442 were used as reference systems in the studies described below; mink frogs and green frogs are indigenous to these lakes as well as to Lake 260. Surface water quality data were collected continually during the open water seasons on all of these lakes (Table 1). Experimental procedures were in accordance Animal Care Committee guidelines of the Freshwater Institute (Fisheries and Oceans Canada, Winnipeg, MB, Canada).

In situ exposures

In situ studies were conducted in 2001 and 2002 with naturally fertilized eggs collected from a reference site (Lake 442). Subsamples from each egg mass were distributed to prepositioned cages in Lake 260 and in the two reference lakes. Lakes 114 and 224 were used as reference lakes because of their close proximity and their chemical similarity to Lake 260. The species and sample sizes used in each year were based solely on the availability of egg masses at Lake 442. Species identification of the egg masses was confirmed after hatching. As described below, hatching success was determined for all of the egg masses. After hatching, tadpoles were reared in the cages for several months, and weight and development stage of tadpoles was assessed on several sampling dates. Gonad development was assessed in all remaining tadpoles at the end of the exposure. Surface water quality data for the embryonic exposure periods in 2001 and 2002 are in Table 2.

In 2001, four green frog (*R. clamitans*) and two mink frog (*R. septentrionalis*) masses were collected on June 25 and July 4, 2001, respectively. The embryos ranged from stage 8 to 12 at time of collection [33]. They were transported to the ELA laboratory in aerated lake water, where they were held for up to 48 h. Each egg mass was separated into smaller clumps to ensure adequate oxygen supply to developing embryos, and handling of the eggs was kept to a minimum to avoid unnecessary stress. Three subsamples of 200 eggs were taken from each mass, and a subsample from each mass was transported to each of the study lakes where it was placed in an individual cage designed to accommodate developmental requirements

Table 1. Surface water–quality data for Lake 260 (unmanipulated in 2000, ethinylestradiol-amended in 2001 to 2003) and for reference Lakes 114, 224, and 442 at the Experimental Lakes Area (ON, Canada) in 2000 to 2003. Cumulative degree days are for May 2 to September 12 of each year. Water chemistry values are means (standard deviation [SD]) based on 12 to 14 measurements taken at regular intervals throughout the ice-free season in each year

Year	Lake	Cumulative degree days	pH	Conductivity (μS/cm)	DOC[a] (μmol/)	TDN[b] (μg/L)	TDP[c] (μg/L)	NH_4 (μg/L)	NO_3 (μg/L)
2000	114	1,924.4	6.33 (0.20)	15 (2.0)	636 (148.8)	294 (26.3)	3 (2.8)	22 (18.6)	3 (5.0)
	224	1,819.6	6.98 (0.15)	20 (1.2)	286 (23.1)	190 (23.4)	3 (4.6)	18 (7.5)	2 (1.7)
	442	1,863.2	7.00 (0.21)	24 (1.3)	583 (56.5)	256 (88.1)	5 (8.0)	18 (10.4)	6 (17.5)
	260	1,858.4	7.06 (0.15)	23 (1.2)	468 (30.5)	258 (74.1)	3 (3.6)	20 (16.8)	3 (2.9)
2001	114	1,973	6.30 (0.25)	13 (1.1)	564 (69.9)	306 (35.5)	5 (4.9)	29 (44.9)	5 (8.2)
	224	1,862.4	6.84 (0.29)	19 (1.1)	265 (17.6)	184 (16.3)	2 (2.3)	19 (21.9)	4 (6.6)
	442	1,953.4	6.91 (0.31)	23 (2.3)	564 (37.3)	316 (36.8)	3 (1.4)	19 (10.0)	31 (43.9)
	260	1,890.4	7.00 (0.17)	21 (0.9)	437 (33.4)	242 (64.3)	2 (2.4)	16 (9.5)	16 (33.4)
2002	114	1,911.5	6.47 (0.24)	16 (3.0)	576 (47.1)	859 (431.8)	4 (1.6)	417 (342.2)	21 (10.2)
	224	1,757.4	6.93 (0.27)	19 (0.5)	289 (30.9)	268 (132.2)	2 (1.4)	22 (25.9)	8 (11.3)
	442	1,834	7.00 (0.29)	23 (1.2)	585 (57.3)	326 (42.7)	3 (1.2)	24 (16.8)	21 (33.2)
	260	1,808.3	7.08 (0.15)	22 (0.9)	475 (43.0)	292 (29.9)	4 (4.3)	28 (17.4)	11 (22.5)
2003	114	2,048.4	6.55 (0.23)	17 (2.9)	591 (41.8)	638 (308.7)	3 (0.7)	296 (530.4)	13 (13.6)
	224	1,952.2	7.13 (0.12)	18 (0.6)	281 (17.4)	214 (27.5)	2 (0.5)	19 (18.4)	5 (8.2)
	442	2,032.4	7.14 (0.25)	24 (1.0)	512 (16.8)	311 (52.8)	2 (0.9)	33 (39.0)	14 (23.5)
	260	2,006.1	7.16 (0.18)	23 (2.2)	449 (21.1)	303 (36.8)	3 (1.3)	23 (18.1)	12 (20.5)

[a] DOC = dissolved organic carbon.
[b] TDN = total dissolved nitrogen.
[c] TDP = total dissolved phosphorus.

of anuran embryos and larvae and prevent predation (modified from Harris and Bogart [34]). Cages were anchored in a line approximately 5 m apart from each other and approximately 5 m from shore in one area of each lake. Briefly, the cages consisted of a cylinder of white Nitex™ (Sefar America, Depew, NY, USA) nylon mesh (500-μm pore size, 60-cm depth, 35-cm diameter) with a permanent bottom and a removable lid attached with a continuous strip of Velcro™ (Canadian Drapery Hardware, Toronto, ON, Canada). The cages were suspended from unfinished wooden frames (with floats attached) such that the tops of the cages sat approximately 10 cm above the waterline, thus providing tadpoles access to air during lung development. Eggs were placed in a small floating basket within each cage to maintain their natural position near the water surface. The baskets consisted of a pocket of window screen mesh suspended from a 15- × 15-cm unfinished wooden frame.

Eggs were monitored daily and, when the eggs hatched, the tadpoles were tipped from the baskets into the cages, at which time feeding was initiated. Tadpoles were fed boiled romaine lettuce and Tetramin™ (Tetra Werke, Melle, Germany) flake fish food to excess every 2 to 3 d. Hatch success was determined two weeks after hatching to avoid unnecessary stress on the tadpoles. At this point, tadpoles were culled to 100 individuals per cage to standardize the densities across cages. On three sampling dates (61, 82, and 96 d posthatch), 10 tadpoles were removed from each of the green frog cages, euthanized, and preserved in Davidson's solution [35]. The mass and development stage of the preserved tadpoles were determined. Mink frog tadpoles were sampled in a similar manner, but the data were not statistically analyzed because of the small number of egg masses collected ($n = 2$, data not shown). At the end of the exposure, all remaining tadpoles were euthanized and processed for histological analysis of the developing gonads. The mean Gosner developmental stages (±SD) of these histology samples were as follows: Green frogs Lake 114, 34 ± 2.0; Lake 224, 33 ± 1.7; Lake 260, 34 ± 1.8; mink frogs Lake 114, 29 ± 1.3; Lake 224, 29 ± 1.4; Lake 260, 31 ± 2.0. The total caging time was 105 and 95 d for green frogs and mink frogs, respectively. The number of degree days for the 2001 exposure period (105 d) was as follows for Lakes 114, 224, and 260 respectively: 1,571, 1,601, and 1,597.

In 2002, the study was repeated using eight mink frog egg masses that were collected from June 26 to 29 and on July 9. These egg masses typically were smaller than those collected in the previous year and did not allow us to use the same initial numbers to determine hatch success. Two of these masses were small and were combined to make one mass, for a total of

Table 2. Surface water–quality data for Lake 260 (ethinylestradiol-amended) and for reference Lakes 114 and 224 at the Experimental Lakes Area (ON, Canada) during the in situ embryonic (prehatching) development periods in 2001 and 2002 (each value is a single observation)

Year	Lake	pH	Conductivity (μS/cm)	DOC[a] (μmol/L)	TDN[b] (μg/L)	TDP[c] (μg/L)	NH_4 (μg/L)	NO_3 (μg/L)
2001	114	6.21	11	580	295	1	7	1
	224	6.94	18	290	195	1	8	1
	260	7.04	20	440	245	3	25	7
202	114	6.23	12	550	390	2	66	9
	224	7.11	18	290	210	1	18	4
	260	7.11	20	530	310	4	30	1

[a] DOC = dissolved organic carbon.
[b] TDN = total dissolved nitrogen.
[c] TDP = total dissolved phosphorus.

seven egg masses for this study. The eggs ranged from stage 16 to 20 at time of collection [33]. Three equal-sized subsamples (ranging from 133–200 eggs) were taken from each mass (the subsample size did not appear to affect the endpoints measured) and placed in individual cages. After hatching success was determined, the tadpoles were culled to 39 to 65 individuals per cage (sample sizes were equal across lakes for each egg mass). On three sampling dates (40, 61, and 87 d posthatch), 10 tadpoles were removed from each cage, euthanized, preserved, and measured as in 2001. Near the end of the exposure in 2002, all tadpoles from Lake 114 were lost due to damaged cages, therefore, data were available for hatch success and growth and development, but not for histological analysis. The mean developmental stages (±SD) of the histology samples at the end of the exposure were Lake 224, 30 ± 0.7 and Lake 260, 30 ± 0.9. Total caging time for the mink frog tadpoles was 119 d for six of the masses and 105 d for the seventh mass. The number of degree days for the 2002 exposure period (119 d) was as follows for Lakes 114, 224, and 260, respectively: 1,570, 1,680, and 1,652.

Wild exposure

Wild mink frog tadpoles were collected from Lake 260 and from reference Lakes 442 and 114 in 2000 to 2003 for histological analysis of the gonads. Lakes 442 and 114 were used as reference lakes for this part of the study because wild mink frog tadpoles were most abundant in these systems. This species grazes on epilithon in near shore areas, and typically inhabits the epilimnion (B. Park, personal observation). Tadpoles were captured using modified Beamish trap nets that were set near shore at various locations on each lake and checked the following day. Most samples were caught in the spring and fall of each year with the exception of 2003, in which only fall sampling was done. Netting effort and timing were similar across lakes. Mass and development stage were recorded, and the tadpoles were euthanized for histological analysis. Samples from Lake 260 in 2000 served as reference samples because the lake was not manipulated prior to the start of the estrogen additions in 2001. Samples ranged from stage 27 to 46 (recent metamorphs) in 2000, from stage 26 to 44 in both 2001 and 2002, and from stage 27 to 37 in 2003. Because tadpoles of this species typically overwinter for one year and transform to adults in early July, samples caught in May and June were considered second-year tadpoles; whereas, those caught after the beginning of July (i.e., the typical reproductive period) were considered first-year tadpoles.

Histology

Tadpoles were euthanized by overdose of benzocaine, each was given a unique identification number, and the mass, snout-to-vent length, and development stage were recorded. A mid-ventral incision was made through the body wall to expose the internal organs to fixative; the tadpoles then were placed in Bouin's tissue fixative (10–12 individuals/L) for 3 to 4 d and then transferred to 70% ethanol for storage. The kidney–gonad complex was excised, put through a butanol series (MVP I Tissue Processor, Fisher Scientific, Toronto, ON, Canada), and embedded in paraffin oriented for transverse sectioning. All samples were analyzed by the same person, and they were not analyzed as blind samples. Serial sections (7 μm) were made through the kidney–gonad complex, working anteriorly. The first set of 8 to 10 consecutive sections was taken at the posterior tip of the gonad and mounted on a glass microscope slide, and then three more sets were taken at 350 μm intervals. Slides were stained with Harris's haematoxylin and eosin [36]. All gonad sections were examined under a Leitz compound microscope, and each sample was classified as male, female, or intersex (i.e., both male and female tissues present in the same gonad).

Statistical analysis

Analysis of variance was used to test for significant differences among lakes in hatch success of green frog egg masses in 2001 ($n = 4$ cages) and mink frog egg masses in 2002 ($n = 7$ cages). Each cage, and not each tadpole within the cage, was considered to be and analyzed as an independent replicate. Prior to analysis, proportions of eggs hatched were transformed by taking the arcsine of the square root of the proportion. Analysis of variance also was used to test for significant differences among lakes in the mass and development stage of green frogs in situ on each of the three sample dates in 2001 ($n = 4$ cages with 10 animals sampled per cage per date). The same analysis was performed for mink frogs in 2002 ($n = 3$ cages with 10 animals sampled per cage per date). Only three of the seven cages were analyzed in 2002 because there was low hatch success in the other cages and, therefore, insufficient numbers of tadpoles available for lethal sampling. Finally, analysis of variance was used to test for significant differences among lakes in the proportion of females in the cages at the end of the exposure. The proportion of females was transformed (arcsine of the square root) prior to analysis. For each analysis, a preplanned contrast was used to determine whether the Lake 260 tadpoles were significantly different from those of the two reference lakes. All analyses were conducted using SAS® Version 8.00 (SAS Institute, Cary, NC, USA) and the level of significance was set at $\alpha = 0.05$. Sex ratio data from the wild exposure were interpreted without statistical analysis. Wild tadpoles caught in May and June of a given year (i.e., second-year tadpoles) were examined separately from those caught after July 1 (i.e., first-year tadpoles).

RESULTS

In situ hatch success

In 2001, the mean hatch success of green frog eggs ($n = 4$ egg masses) was significantly lower on Lake 260 (53.4%) than on Lakes 114 and 224 (77 and 75%, $p = 0.01$, Fig. 1). The median hatching success of mink frog eggs ($n = 2$ egg masses) was 81% for Lakes 260 and 224 and 82% for Lake 114. In 2002, the mean hatch success for Lake 260 (47.4%, $n = 7$) was the lowest of the three lakes (Lake 114: 83.7%, $n = 4$ and Lake 224: 65.4%, $n = 7$ egg masses), but the difference was not statistically significant ($p = 0.09$). The sample size was reduced in Lake 114 due to near-complete hatching failure in three of the cages, i.e., it appears that the subsamples were damaged in transport.

In situ growth and development

In 2001, the mean mass of green frog tadpoles from Lake 260 was not significantly different from that of reference tadpoles on any of the sampling dates ($p = 0.39$, 0.09, and 0.56 for 61, 82, and 96 dph, respectively, Table 3). The mean development stage of Lake 260 tadpoles was significantly higher than that of reference lake tadpoles at 61 and 82 dph ($p = 0.047$ and 0.01), whereas there was no significant difference at 96 dph ($p = 0.12$).

In 2002, the mean mass of mink frog tadpoles from Lake

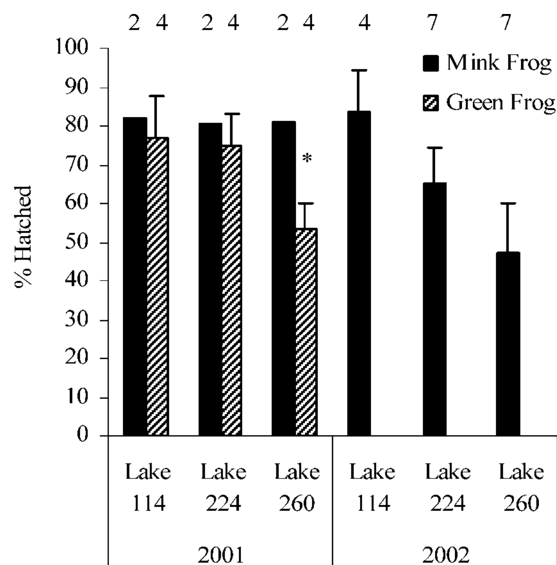

Fig. 1. Mean hatch success (+SE) of green frog and mink frog eggs reared in situ on reference lakes and on Lake 260 in 2001 and in 2002 (Experimental Lakes Area, ON, Canada). Arithmetic means are presented in figure; statistical analysis was performed with arcsine transformed data and an * indicates significant difference. Sample sizes are shown above bars.

260 was not significantly different from that of reference lake tadpoles at 40 and 61 dph (p = 0.99 and 0.17, Table 4), whereas the mean mass of Lake 260 tadpoles was significantly lower than that of reference lake tadpoles at 87 dph (p = 0.0004). Similarly, the mean development stage of Lake 260 tadpoles was not significantly different from the reference tadpoles on the first two sample dates (p = 0.32 and 0.96), whereas the mean development stage of Lake 260 tadpoles was significantly lower than that of the reference tadpoles on the final date (p = 0.0009). It should be noted that, although the stage of development was statistically lower for Lake 260 tadpoles, the mean values were equal across lakes when expressed as a whole number.

In situ gonad histology

In both years, all reference samples had clearly differentiated gonads at the earliest stages examined and there were no signs of natural intersex or transitory periods at any stage of development in either species (2001: n = 85 mink frogs and n = 155 green frogs; 2002: n = 55 mink frogs). Ovaries were composed of abundant primary growth–phase oocytes while testes contained polymorphonuclear spermatogonia (Fig. 2). Therefore, it appears that, in both species, primary sex differentiation naturally occurs early in larval development as reported for some other Ranid species [37], and that ovarian or testicular tissue normally develop directly from the indifferent gonad tissue.

Sex ratios of EE2-exposed and reference tadpoles showed no significant differences in 2001 (p = 0.59) or in 2002 (p = 0.89, Fig. 3). In 2001, the percentage of female green frog tadpoles was lower in Lake 260 cages (47%) than in the reference lake cages (54 and 51% for Lakes 114 and 224, respectively). In the same year, 56% of mink frog tadpoles were females in Lake 260 cages, and 51 and 57% were females in the Lake 114 and 224 cages, respectively. In 2002, 52% of mink frog tadpoles were females in Lake 260 cages, and 60% were females in Lake 224 cages.

We did not observe intersex gonads in any of the EE2-treated green frog tadpoles examined. However, one out of 18 and seven out of 56 EE2-exposed mink frog tadpoles showed intersex in 2001 and 2002, respectively. The intersex individual from 2001 had a distinct region containing only primary growth-phase oocytes at the posterior end of one testis, while the remainder of the gonad had the appearance of a normal male testis. Two of the intersex individuals from 2002 showed the same pattern. In the other five intersex individuals from 2002, the gonads appeared to be testes with one or several primary growth-phase oocytes in the posterior region of one or both gonads (Fig. 4).

Wild exposure gonad histology

As in the in situ study, all reference tadpoles from the wild clearly were differentiated as male or female at all stages of development (2000, n = 92; 2001, n = 40; 2002, n = 126; 2003, n = 19). Sex ratios of first- and second-year wild tadpoles (irrespective of intersexes) varied among lakes and among years and showed no treatment-related effects (Fig. 5). In second-year tadpoles exposed to EE2 the previous summer, the proportion of females from Lake 260 in 2002 (59.7%) did not differ markedly from that of the pre-addition samples (spring of 2001; 54.3%). The most skewed sex ratios for second-year tadpoles was observed in Lake 114 in 2002 (25% females). In EE2-exposed first-year tadpoles, the proportion of females varied among years from 36.4 to 53.3% and showed no treatment-related effect when compared to reference data. The most skewed sex ratio was in Lake 442 in 2001 (66.7% females).

No intersex gonads were observed in first- or second-year wild mink frog tadpoles from the reference lakes (n = 92) or from Lake 260 (n = 76) in 2000. However, some gonadal abnormalities were detected in EE2-exposed samples in 2001. Of the 42 EE2-exposed first-year mink frog tadpoles examined from Lake 260 in 2001, one was intersex and a second (classified as a male) showed abnormal gonad development. The intersex individual had a distinct region of primary growth-phase oocytes at the posterior end of one gonad, while the

Table 3. Mean mass (standard error) and Gosner development stage (standard error) of green frog tadpoles reared in situ on Lake 260 and two reference lakes in 2001 (n = 4 cages, 10 tadpoles were sampled per cage on each date.) The letter A indicates significant difference from the other reference lakes (ON, Canada)

	61 dph[a]		82 dph		96 dph	
Lake	Mass (g)	Stage	Mass (g)	Stage	Mass (g)	Stage
260	0.56 (0.21)	33 (2.0) A	0.78 (0.20)	34 (1.1) A	0.96 (0.27)	34 (1.8)
114	0.64 (0.24)	33 (2.0)	0.80 (0.25)	34 (1.6)	0.98 (0.40)	34 (2.0)
224	0.41 (0.20)	31 (2.2)	0.70 (0.22)	33 (1.7)	0.91 (0.25)	33 (1.7)

[a] dph = days post-hatch.

Table 4. Mean mass (standard error) and Gosner development stage (standard error) of mink frog tadpoles reared in situ on Lake 260 and two reference lakes in 2002 (Experimental Lakes Area, ON, Canada, n = 3 cages, 10 tadpoles were sampled per cage on each date.) The letter A indicates significant difference from the other reference lakes

Lake	40 dph[a]		61 dph		87 dph	
	Mass (g)	Stage	Mass (g)	Stage	Mass (g)	Stage
260	0.20 (0.19)	27 (1.6)	0.50 (0.20)	29 (1.5)	0.78 (0.25) A	30 (0.9) A
114	0.15 (0.13)	26 (0.9)	0.46 (0.13)	28 (1.1)	1.00 (0.25)	30 (1.1)
224	0.26 (0.10)	27 (0.9)	0.64 (0.21)	30 (1.6)	0.99 (0.28)	30 (0.7)

[a] dph = days post-hatch.

remainder of the gonad had the appearance of a normal testis. The second individual had no evidence of intersex; however, one testis appeared normal while the other was almost completely devoid of germ cells. Both individuals were caught in the fall, after approximately three months of EE2 exposure. No intersexes were observed in first- or second-year EE2-exposed tadpoles in 2002. In the 2003 first-year tadpoles, 28.6% of EE2-exposed samples were intersex, whereas all reference samples clearly were differentiated. These intersex samples also were caught in the fall, after approximately three months of EE2 exposure. The intersex gonads were testes with one or several primary growth-phase oocytes typically scattered in the posterior portion of one or both gonads (Fig. 6.)

DISCUSSION

This study showed a significant reduction in the hatch success of green frog eggs exposed in situ to low ng/L concentrations of EE2. Although in field studies of this nature it is not possible to control for all variables that may impact the endpoints measured, we consider significant differences in exposed tadpoles to be attributable to the presence of EE2 in Lake 260. A previous study demonstrated reduced hatch success in *Rana arvalis* eggs exposed to ecologically relevant concentrations of an estrogenic phthalate ester; however, the mechanism of action was not determined [8]. Conversely, exposure to the weakly estrogenic insecticide endosulfan had no effect on hatch success of amphibian embryos [38]. Thus, the effects of known estrogen mimics on amphibian embryos are varied. Mechanisms of embryonic disruption by xenoestrogens have not been reported for amphibians, but, given the lack of a role for steroid hormones in early embryonic development [7], it is not likely that endocrine modulation has occurred. In *Rana* larvae, survival generally is less than 10% under natural conditions [39]. Thus, despite relatively large clutch sizes,

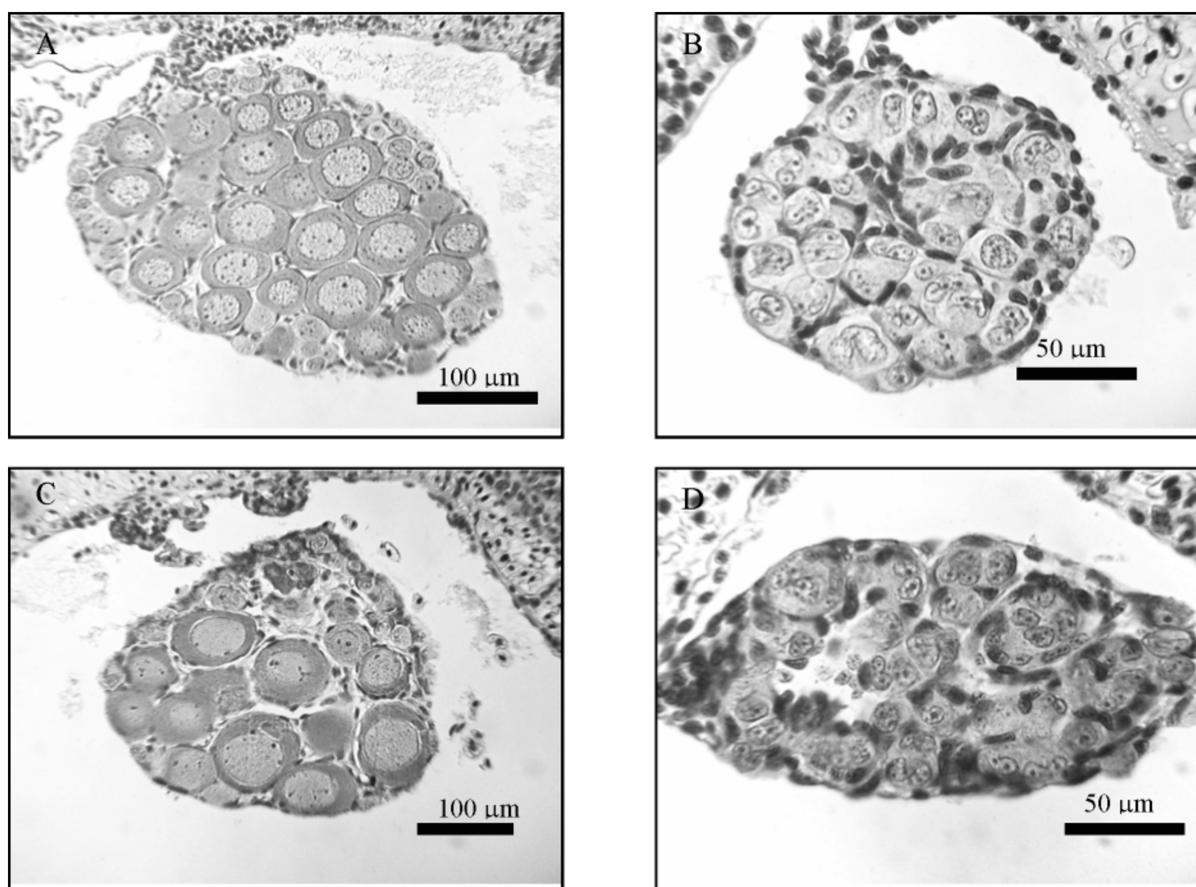

Fig. 2. Photomicrographs of transverse gonad sections of mink frog and green frog tadpoles from reference lakes. (**A**) Ovary and (**B**) testis from green frog tadpoles; (**C**) ovary and (**D**) testis from mink frog tadpoles. Ovaries consist of primary oocytes; testes contain primary spermatogonia.

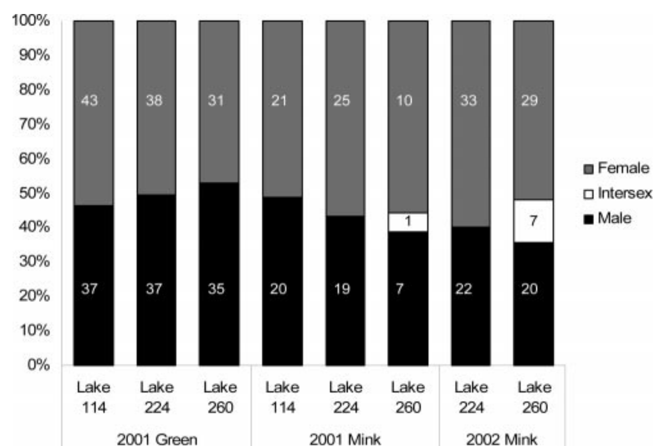

Fig. 3. Gonadal sex of green frog and mink frog tadpoles reared in situ from the midembryonic to midlarval stages on Lake 260 (ethinylestradiol [EE2] addition lake) and on reference lakes in 2001 and 2002 (Experimental Lakes Area, ON, Canada). Sample sizes are in bars.

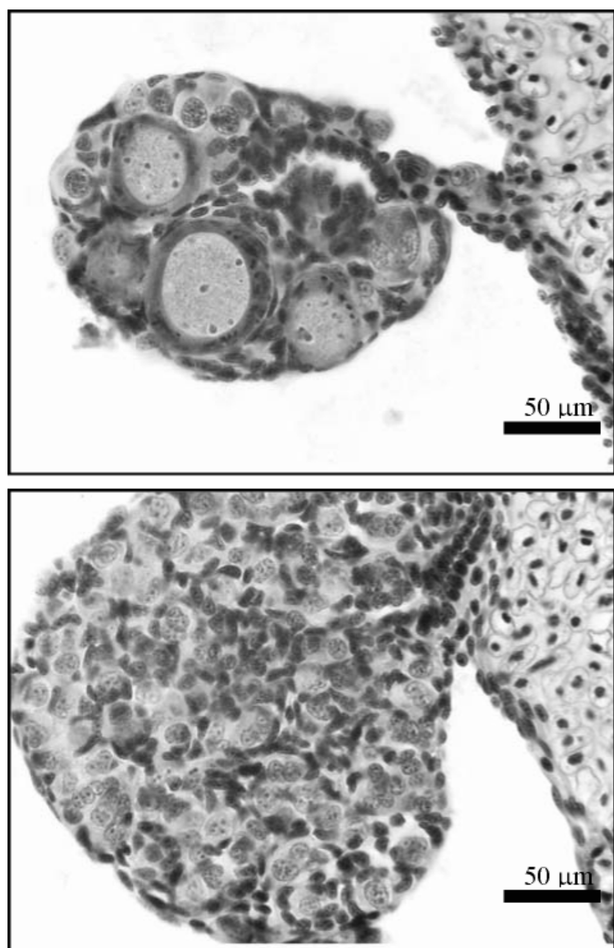

Fig. 4. Photomicrographs of transverse sections through the gonad of an intersex cage-reared mink frog tadpole from Lake 260 (2001, Experimental Lakes Area, ON, Canada). (**top**) Posterior section showing primary growth-phase oocytes. (**bottom**) Anterior section showing testicular development (primary spermatogonia) seen throughout the remainder of the gonad.

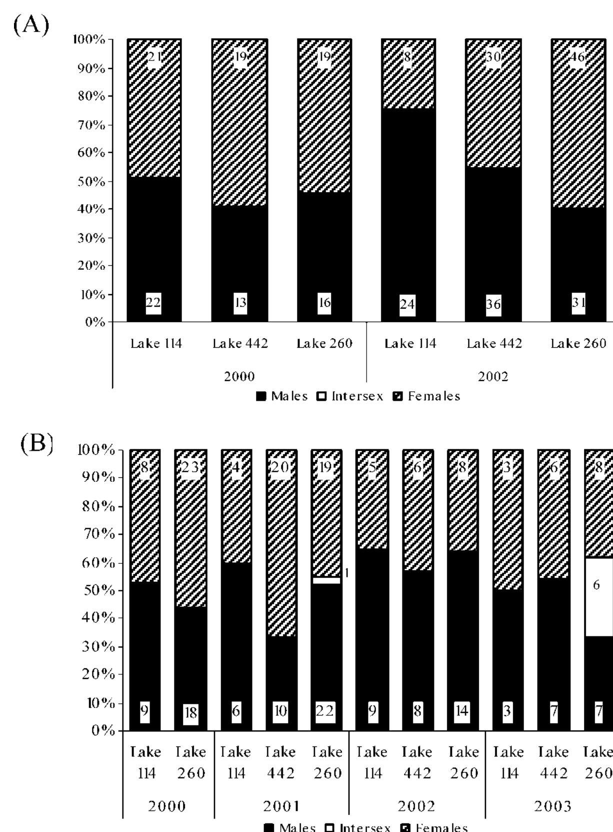

Fig. 5. Gonadal sex of wild mink frog tadpoles from Lake 260 and from reference lakes in 2000 to 2003 (Experimental Lakes Area, ON, Canada). (**A**) Second-year tadpoles caught in May and June (Lake 260 tadpoles were exposed to ethinylestradiol (EE2) for a minimum of 11 months in 2002 because of low levels of the estrogen under ice; see *Methods* section). (**B**) First-year tadpoles caught from July to October. Sample sizes are given in the bars.

reduced recruitment from the embryonic to the larval phase could impact population sustainability. In this study, eggs were exposed to EE2 after oviposition and fertilization when the egg coat was fully formed. In the wild, EE2 also could affect eggs and sperm during breeding, and a pulse of contaminants may be taken up as the jelly layer swells with water during egg coat formation. Furthermore, because the egg masses in this study were collected at a reference site, there was no parental exposure to EE2; the exposure of adult frogs to EE2 during gonad maturation may augment the effects we observed in this study.

In contrast, hatching success of mink frog eggs appeared to be unaffected by EE2 exposure in both years of the study, although there was a nonsignificant reduction in 2002, i.e., mean hatch success was 47% in EE2-exposed eggs versus 84 and 65% in reference lakes. These results indicate a species-specific difference in sensitivity of hatching to toxicants. Species-specific differences in sensitivity to pesticides have been reported previously for anuran early life stages [7,40,41]. The nature of the jelly layer surrounding anuran embryos is critical in determining the sensitivity to perturbations [9]. The number of layers, complexity, and physical and chemical characteristics of the layers vary among species, and incur varying levels of protection [3]. Therefore, the different responses seen in this study may be the result of different egg morphology.

The two species examined in situ showed different growth and development responses to chronic EE2 exposure through-

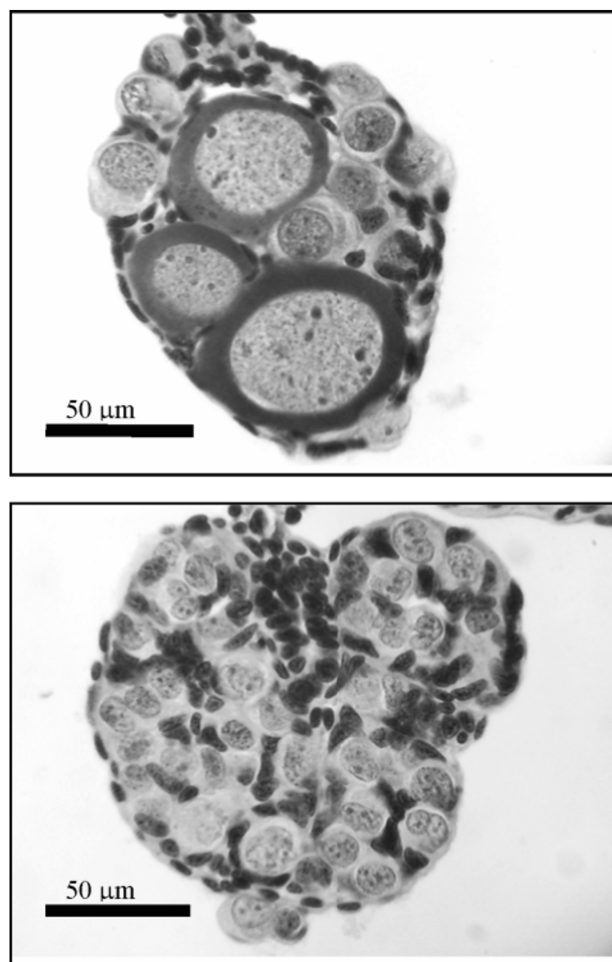

Fig. 6. Photomicrographs of transverse sections through the gonad of an intersex wild mink frog tadpole from Lake 260 (2003, Experimental Lakes Area, ON, Canada). (**top**) Posterior section showing well-developed primary oocytes at various stages of development. (**bottom**) Anterior section showing testicular development (primary spermatogonia) seen throughout the remainder of the gonad.

out a portion of the embryonic and early larval phases. Ethinylestradiol had no effect on growth in green frog tadpoles, whereas development was accelerated in the early sampling dates. It is unlikely that accelerated development would have adverse effects on wild tadpoles in the early posthatch phase. On the final sampling date, there was no difference in development stage of EE2-exposed tadpoles relative to controls; therefore, EE2 had no marked effects on green frog tadpoles. Also, there was no effect of EE2 on mink frog tadpoles for the first two sampling dates; however, both growth and development were inhibited significantly on the final sampling date. Exposure to EE2 may have impacted the function of the thyroid axis, as previously reported for other estrogenic substances. Estrogen exposure has been shown to antagonize the thyroid axis, and the vast majority of reports indicate that growth and development of larval anurans are inhibited by exogenous estrogen [13–15,17]. Retarded growth and development in anuran larvae can impair fitness, reproduction, and predator avoidance [12]; thus, estrogenic contaminants are of concern for wild anuran populations. In our study, both species were exposed for only a portion of the larval phase. Chronic exposure throughout the entire larval phase may result in greater impacts on growth and development.

Ethinylestradiol had no discernable effect on sex ratios in wild or caged tadpoles in this study. In laboratory studies, exposure to estradiol and ethinylestradiol at μg/L concentrations induced female-skewed sex ratios [21,22] and estradiol (1 μM) induced complete sex reversal of genetic males [42]. The estrogen concentration in the current study (5 ng/L = 16.9 pM) was probably too low to induce full reversal of gonadal sex in the larval stage. Further, Reeder et al. [29] reported altered sex ratios of juvenile and adult frogs at sites contaminated with polychlorinated biphenyl and polychlorinated dibenzofuran; these results suggest that there is the potential in this study for the sex ratios to be impacted later in the life cycle after a prolonged exposure to EE2. In addition, to interpret data from contaminated field sites, more information is required on natural variability of sex ratios of native species.

Intersex gonads and hermaphroditism are known to occur spontaneously, though infrequently, in amphibians [43,44]. However, in this study, intersex gonads occurred exclusively in mink frog tadpoles exposed to EE2 in the wild and in situ; no abnormal gonads were detected in a total of 353 wild and 140 caged reference samples of the same species. Male tadpoles partially were feminized by estrogen exposure during larval gonad development, as has been reported in other laboratory studies [42,45]. Few studies have assessed rates of intersex in amphibians at field sites. Intersex wild adult cricket frogs (*Acris crepitans*) were detected at sites with multiple contaminants [29]. Furthermore, male *Rana pipiens* with testicular oocytes consistently were found at field sites associated with atrazine contamination [28]. Overall there is mounting evidence that hormonally active contaminants can disrupt gonad development in amphibians.

Ethinylestradiol had no apparent effect on green frog gonad development in the in situ exposure, whereas mink frogs had 5.6 and 12.5% intersex in 2001 and 2002, respectively. This indicates a species-specific difference in sensitivity to EE2. Gonadal responses to sex steroids vary greatly among anuran species [20]. Impacts on mink frog tadpoles in both years of the study indicate that the midembryonic to midlarval stages of this species are sensitive to estrogenic aquatic contaminants. A previous study showed that the rate of feminization in 17β-estradiol (E2)–exposed male *R. catesbiana* tadpoles increased with duration of exposure [46]. Adult mink and green frogs are highly aquatic and mature within one to two years of transformation [47]. It is not known whether the impacts of xenoestrogens are greater on the offspring from individuals exposed for their entire life cycle. At sites contaminated with environmental estrogens for many years there likely would be parental exposure as well as exposure throughout the entire embryonic and larval phase. Thus, impacts on reproductive development may be even greater at chronically contaminated sites than those seen in the current study.

Intersex gonads were observed in wild EE2-exposed mink frog tadpoles in two out of three exposure years and were most prominent in the final year of estrogen additions to the lake. All of the intersex individuals were caught in the fall, i.e., first-year tadpoles that had been exposed throughout the embryonic and early larval stage. Effects were minimal or absent in the first two exposure years. A single intersex tadpole was caught in the fall of 2001, and no intersexes were detected in Lake 260 in the spring or fall of 2002. However, most of the samples collected in 2002 were second-year tadpoles caught prior to the initiation of the EE2 additions that year. The highest incidence of intersex in this study occurred in first-year tad-

poles collected in the fall of 2003 (year 3 of the whole-lake additions), and this may be due to prolonged parental exposure to EE2. Exposure of mature frogs to EE2 in the open-water seasons of 2001 and 2002 may have resulted in significant accumulation of the estrogen in the adults and maternal transfer to the yolk. The higher incidence of intersex observed in the last year of estrogen additions, therefore, may be due to exposure to EE2 in various reproductive stages.

In this study disruption of gonad development was seen in the posterior portion of the intersex gonads; the same pattern of disruption has been reported previously in *R. rugosa* tadpoles exposed to an estrogenic phthalate [42]. Chang et al. [46] documented the induction of oocyte development by E2 administration in otherwise male tadpole gonads. Low levels of endogenous steroids in early larval development could regulate the proliferation and differentiation of germ cells [48]. Experimental evidence indicates that administration of estradiol by silastic implants results in increased estradiol concentrations in the ovary; thus, environmental estrogens may accumulate in the gonad as well [14]. Primordial germ cells in amphibians have the potential to develop into either germ cell type. Thus, it is possible that the initial (genetic) determination induced male differentiation in the intersex specimens, with occasional primordial germ cells being induced into the oocyte development pathway by EE2.

It is not known whether the gonadal disruption observed in this study in the larval phase will reduce adult fecundity. Active spermatogenesis has been documented in amphibian testes containing occasional oocytes [29,44]. Therefore, a slightly feminized male may be able to mate successfully despite the presence of female gonad tissue. Alterations to sex differentiation are likely to interfere with reproductive function at some level [49], and contaminant-induced impacts on both primary and secondary sex differentiation ultimately may impair wild populations [50]. However, the population-level implications of contaminant-induced intersexuality currently remain unclear.

The current study indicates that exposure to low ng/L concentrations of an environmental estrogen disrupted hatching and gonad development of native amphibians in situ and in the wild. Chronic exposure in polluted environments may have more pronounced effects than those documented in this study, and environmental estrogens may affect the reproductive axis at other regulatory points, which ultimately may impair the ability of populations to recover from natural or human-induced decline events.

Acknowledgement—Thanks to field assistants P. Mutch, T. Hodge, K. Dsyzy, K. Aoki, and J. Hare; R. Evans for providing valuable assistance with histology; L. Vandenbyllardt for conducting water chemistry analyses; K. Londry for providing technical assistance in confirmation of the ethinylestradiol concentrations; and S. Kasian for providing water-quality data from the Experimental Lakes Area lakes. This work was supported by Schering AG (Berlin, Germany), the American Chemistry Council, the Canadian Toxic Substances Research Initiative 94, the Canadian Network of Toxicology Centres, Fisheries and Oceans Canada's Environmental Science Strategic Research Fund, and the Experimental Lakes Area Graduate Fellowship Program.

REFERENCES

1. Blaustein AR, Wake DB. 1990. Declining amphibian populations: A global phenomenon? *Trends Ecol Evol* 5:203–204.
2. Cowman DF, Mazanti LE. 2000. Ecotoxicology of ''new generation'' pesticides to amphibians. In Sparling DW, Linder G, Bishop CA, eds, *Ecotoxicology of Amphibians and Reptiles.* SETAC, Pensacola, FL, USA, pp 233–268.
3. Carey C, Bryant CJ. 1995. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. *Environ Health Perspect Suppl* 103:13–17.
4. Ternes TA, Stumpf M, Mueller J, Haberer K, Wilken RD, Servos M. 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants—I. Investigations in Germany, Canada, and Brazil. *Sci Total Environ* 225:81–90.
5. Belfroid AC, Van der Horst A, Vethaak AD, Schafer AJ, Rijs GBJ, Wegener J, Cofino WP. 1999. Analysis and occurrence of estrogenic hormones and their glucoronides in surface water and wastewater in The Netherlands. *Sci Total Environ* 225:101–108.
6. Sumpter JP, Jobling S, Tyler CR. 1997. Oestrogenic substances in the aquatic environment and their potential impact on animals, particularly fish. In Taylor EW, ed, *Toxicology of Aquatic Pollution: Physiological, Cellular, and Molecular Approaches.* Cambridge University, Cambridge, UK, pp 205–224.
7. Henry PFP. 2000. Aspects of amphibian anatomy and physiology. In Sparling DW, Linder G, Bishop CA, eds, *Ecotoxicology of Amphibians and Reptiles.* SETAC, Pensacola, FL, USA, pp 71–110.
8. Larsson P, Thuren A. 1987. Di-2-ethylhexylphthalate inhibits the hatching of frog eggs and is bioaccumulated by tadpoles. *Environ Toxicol Chem* 6:417–422.
9. Laposata MM, Dunson WA. 2000. Effects of spray-irrigated wastewater effluent on temporary pond-breeding amphibians. *Ecotoxicol Environ Saf* 46:192–201.
10. Bishop CA, Struger J, Shirose LJ, Dunn L, Campbell GD. 2000. Contamination and wildlife communities in storm water detention ponds in Guelph and the greater Toronto area, Ontario, 1997 and 1998. Part II—Contamination and biological effects of contamination. *Water Qual Res J Can* 35:437–474.
11. Wilbur HM, Collins JP. 1973. Ecological aspects of amphibian metamorphosis. *Science* 182:1305–1314.
12. Denver RJ. 1997. Proximate mechanisms of phenotypic plasticity in amphibian metamorphosis. *Am Zool* 37:172–184.
13. Hayes TB. 1997. Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis. *Am Zool* 37:185–194.
14. Vandorpe G, Kuhn ER. 1989. Estradiol-17B silastic implants in female *Rana ridibunda* depress thyroid hormone concentrations in plasma and in the in vitro 5′-monodeiodination activity of kidney homogenates. *Gen Comp Endocrinol* 76:341–345.
15. Gray KM, Janssens PA. 1990. Gonadal hormones inhibit the induction of metamorphosis by thyroid hormones in *Xenopus laevis* tadpoles in vivo, but not in vitro. *Gen Comp Endocrinol* 77:202–211.
16. Hayes T, Chan R, Licht P. 1993. Interactions of temperature and steroids on larval growth, development, and metamorphosis in a toad (*Bufo boreas*). *J Exp Zool* 266:206–215.
17. Nishimura N, Fukazawa Y, Uchiyama H, Iguchi T. 1997. Effects of estrogenic hormones on early development of *Xenopus laevis*. *J Exp Zool* 287:221–233.
18. Yamauchi K, Praponpoj P, Richardson SJ. 2000. Effect of diethylstilbestrol on thyroid hormone binding to amphibian transthyretins. *Gen Comp Endocrinol* 119:329–339.
19. Wallace H, Badawy GMI, Wallace BMN. 1999. Amphibian sex determination and sex reversal. *Cell Mol Life Sci* 55:901–909.
20. Hayes TB. 1998. Sex determination and primary sex differentiation in amphibians: Genetic and developmental mechanisms. *J Exp Zool* 281:373–399.
21. Saidapur SK, Gramapurohit NP, Shanbag BA. 2001. Effect of sex steroids on gonadal differentiation and sex reversal in the frog, *Rana curtipes*. *Gen Comp Endocrinol* 124:115–123.
22. Mackenzie CA, Berrill M, Metcalfe C, Pauli BD. 2003. Gonadal differentiation in frogs exposed to estrogenic and antiestrogenic compounds. *Environ Toxicol Chem* 22:2466–2475.
23. Kloas W, Lutz I, Einspanier R. 1999. Amphibians as a model to study endocrine disrupters: II. Estrogenic activity of environmental chemicals in vitro and in vivo. *Sci Total Environ* 225:59–68.
24. 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.
25. 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* 99:5476–5480.
26. Pickford DB, Morris IA. 1999. Effects of endocrine-disrupting contaminants on amphibian oogenesis: Methoxychlor inhibits progesterone-induced maturation of *Xenopus laevis* oocytes in vitro. *Environ Health Perspect* 107:285–292.
27. Clark EJ, Norris DO, Jones RE. 1998. Interactions of gonadal steroids and pesticides (DDT, DDE) on gonaduct growth in larval tiger salamanders, *Ambystoma tigrinum*. *Gen Comp Endocrinol* 109:94–105.
28. Hayes T, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A. 2002. Feminization of male frogs in the wild. *Nature* 419:895–896.
29. Reeder AL, Foley GL, Nichols DK, Hansen LG, Wickoff B, Faeh S, Eisold J, Wheeler MB, Warner R, Murphy JE, Beasly 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.
30. Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP. 1998. Widespread sexual disruption in fish. *Environ Sci Technol* 32: 2498–2506.
31. Palace VP, Evans RE, Wautier K, Baron C, Vandenbyllardt L, Vandersteen W, Kidd K. 2002. Induction of vitellogenin and histological effects in wild fathead minnows from a lake experimentally treated with the synthetic estrogen, ethinylestradiol. *Water Qual Res J Can* 37:637–650.
32. Larsson DJG, Adolfsson-Erici M, Parkkonen J, Pettersson M, Berg AH, Olsson P-E FL. 1999. Ethynyloestradiol—an undesired fish contraceptive? *Aquat Toxicol* 45:91–97.
33. Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183–190.
34. Harris ML, Bogart JP. 1997. A cage for evaluation of in situ water quality using frog eggs and tadpoles. *Herpetol Rev* 28:134–135.
35. Moore KL, Graham MA, Barr ML. 1953. The detection of chromosomal sex in hermaphrodites from a skin biopsy. *Surgery, Gynecology, and Obstetrics* 96:641–648.
36. Edwards JE. 1967. Methods for the demonstration of intercellular substances of the connective tissues. In McClung Jones R, ed, *McClung's Handbook of Microscopical Technique,* 3rd ed. Hafner, New York, NY, USA, pp 248–268.
37. Gramapurohit NP, Shanbhag BA, Saidapur SK. 2000. Pattern of gonadal sex differentiation, development, and onset of steroidogenesis in the frog, *Rana curtipes*. *Gen Comp Endocrinol* 119: 256–264.
38. Berrill M, Coulson D, McGillivray L, Pauli B. 1998. Toxicity of endosulfan to aquatic stages of anuran amphibians. *Environ Toxicol Chem* 17:1738–1744.
39. Duellman WE, Trueb L. 1986. *Biology of Amphibians.* McGraw-Hill, New York, NY, USA.
40. Berrill M, Bertram S, McGillivray L, Kolohon M, Pauli B. 1994. Effects of low concentrations of forest-use pesticides on frog embryos and tadpoles. *Environ Toxicol Chem* 13:657–664.
41. Berrill M, Bertram S, Pauli B, Coulson D, Kolohon M, Ostrander D. 1995. Comparative sensitivity of amphibian tadpoles to single and pulsed exposures of the forest-use insecticide fenitrothion. *Environ Toxicol Chem* 14:1011–1018.
42. Ohtani H, Miura I, Ichikawa Y. 2000. Effects of dibutyl phthalate as an environmental endocrine disruptor on gonadal sex differentiation of genetic males of the frog *Rana rugosa*. *Environ Health Perspect* 108:1189–1193.
43. Grafe TU, Linsenmair KE. 1989. Protogynous sex change in the reed frog *Hyperolius viridiflavus*. *Copeia* 4:1024–1029.
44. Sullivan BK, Propper CR, Demlong MJ, Harvey LA. 1996. Natural hermaphroditic toad. Copeia 2:470–472.
45. Ohtani H, Ichikawa Y, Iwamoto E, Miura I. 2001. Effects of styrene monomer and trimer on gonadal sex differentiation of genetic males of the frog *Rana rugosa*. *Environ Res* 87:175–180.
46. Chang L, Yu N, Hsu C, Liu H. 1996. Gonadal transformation in male *Rana catesbiana* tadpoles intraperitoneally implanted with estradiol capsules. *Gen Comp Endocrinol* 102:299–306.
47. Harding JH. 1997. *Amphibians and Reptiles of the Great Lakes Region.* University of Michigan, Ann Arbor, MI, USA.
48. Petrini S, Zaccanti F. 1998. The effects of aromatase and 5 α-reductase inhibitors, antiandrogen, and sex steroids on Bidder's organ development and gonadal differentiation in *Bufo bufo* tadpoles. *J Exp Zool* 280:245–259.
49. McNabb A, Schreck C, Tyler C, Thomas P, Kramer V, Specker J, Mayes M, Selcer K. 1999. Basic physiology. In Di Giulio RT, Tillit DE, eds, *Reproductive and Developmental Effects of Contaminants in Oviparous Vertebrates.* SETAC, Pensacola, FL, USA, pp 113–223.
50. Hayes TB. 2000. Endocrine disruption in amphibians. In Sparling DW, Linder G, Bishop CA, eds, *Ecotoxicology of Amphibians and Reptiles.* SETAC, Pensacola, FL, USA, pp 573–593.