Limb Malformations and Abnormal Sex Hormone Concentrations in Frogs

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Declines in amphibian populations, and amphibians with gross malformations, have prompted concern regarding the biological status of many anuran species. A survey of bullfrogs, *Rana catesbeiana*, and green frogs, *Rana clamitans*, conducted in central and southern New Hampshire showed malformed frogs at 81% of the sites sampled (13 of 16 sites). Brain gonadotropin-releasing hormone (GnRH) and the synthesis of androgens and estradiol, hormones essential to reproductive processes, were measured from limb-malformed and normal (no limb malformation) frogs. Normal frogs had significantly higher concentrations (nearly 3-fold) of *in vitro* produced androgens and of brain GnRH than malformed frogs. Because most malformations are thought to occur during development, we propose that environmental factors or endocrine-disrupting chemicals that may cause developmental abnormalities also act during early development to ultimately cause abnormally reduced GnRH and androgen production in adult frogs. The consequences of reduced GnRH and androgens on anuran reproductive behavior and population dynamics are unknown but certainly may be profound and warrant further research. *Key words*: amphibians, androgens, endocrine-disrupting chemicals, environmental influences, frogs, gonadotropin-releasing hormone. *Environ Health Perspect* 108:1085–1090 (2000). [Online 25 October 2000]

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A number of factors potentially contribute to the recent increases in amphibian malformations and declines, including habitat loss, disease, parasites, environmental contaminants, climate changes, acid precipitation, and increases in ultraviolet B radiation (*1–8*). Chemicals that interfere with the endocrine system by mimicking hormones or by blocking the action of hormones have been implicated in reproductive dysfunction and abnormal development in several species, including fish and alligators (*9*). Estrogenic pollutants have also been linked to developmental and reproductive abnormalities in wildlife (*10–13*) and are implicated in the increase of human breast and testicular cancers and in the decline of human semen quality (*14,15*). The role of endocrine-disrupting chemicals (EDCs) in amphibian malformations may be of concern due to the high deformity rates associated with sites where agricultural chemicals are used (*8*). Some pesticides, herbicides, and nematocides are documented to have endocrine-disrupting effects (*9*). To date, there are no reports linking endocrine dysfunction with amphibian malformations. However, it is well established that EDCs can be major ecologic threats to fish and aquatic wildlife by diminishing productivity and fecundity (*9*).

The normal growth and development of amphibian larvae rely on functional, uncontaminated aquatic systems. Water sources are particularly at risk to contamination by EDCs because of the accumulation and distribution of contaminating substances in sediments of rivers, lakes, and ponds. Potential sources of EDCs that impact bodies of water include municipal sewage (*13*), pulp mill effluents (*16*), agricultural runoff (pesticides and herbicides) (*17*), and petroleum from bilge water and two-cycle boat motors (*18*). Thus, the EDCs that accumulate in aquatic systems may adversely effect amphibian reproductive processes.

Gonadotropin-releasing hormone (GnRH) is a regulatory neurohormone central to the control of reproduction in vertebrates. A key neuroendocrine function of the hypothalamus is the release of GnRH, which in turn acts on the pituitary, regulating the pituitary–gonadal axis for all vertebrates. The primary structure (amino acid sequence) of two forms of GnRH, mammalian GnRH and chicken GnRH-II, has been determined in the brain of the European green frog, *Rana ridibunda* (*19*). The distribution of these two forms of GnRH was determined by immunocytochemistry in three species of *Rana* (*R. pipiens, R. esculenta, and R. ridibunda*). The dominant form of GnRH in the preoptic/hypothalamic area was shown to be mammalian GnRH, while chicken GnRH-II was predominant in all other brain areas (*20*). Therefore, mammalian GnRH appears to be the major form of GnRH responsible for regulating pituitary function in *Rana* species.

Disruption of the GnRH system that directly influences pituitary function, whether by environmental or genetic influences, produces idiopathic hypothalamic hypogonadism and infertility (*21*). In fact, deliberate disruption of the GnRH system is the basis for using GnRH analogs in active immunization paradigms for contraceptive purposes (*22*). Chemicals in the environment that influence the migration and/or development of GnRH neurons could cause significant endocrine disruption (e.g., changes in steroid metabolism). To date, there are no reported studies on the effects of endocrine disruptors on the GnRH system in frogs.

Temperate amphibians, in general, have discontinuous spermatogenetic patterns (*23*). In northern hemisphere populations of amphibians, reproduction occurs from June to July in bullfrogs and from late May through mid-August in green frogs (*24*). During the winter months, the cycle is interrupted, and germinal cysts do not develop further than primary spermatocytes. In *R. temporaria*, for example, a refractory phase of 3 months occurs after spawning, which delays the initiation of spermatogenesis (*23*). Androgens are known to play an important role in male amphibian reproduction. Seasonal variations in plasma testosterone concentrations have been determined in many different species including *R. catesbeiana* (*23,25–27*). However, the major androgen has not been identified in *Rana clamitans*. In *R. catesbeiana*, there are conflicting data as to the major androgen produced by the testes. Callard et al. (*28*) demonstrated that testosterone was the primary metabolite in the

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testis of *R. catesbeiana*, but Muller (*29,30*) identified dihydrotestosterone as the major metabolite from testicular tissue.

Studies on wildlife species have shown a relationship between exposure to EDCs and a variety of malformations including sex alteration, reproductive impairment, abnormal thyroid function, and morphological and behavioral abnormalities (*31*). Crain and Guillette (*9*) postulated that many of the reproductive abnormalities seen in vertebrates are due, at least in part, to the alteration of normal hormonal steroidogenesis. It is possible that EDCs interact with multiple complex biochemical and molecular pathways within an organism. Because the effects of EDCs can be manifested in a variety of systems, we hypothesized that, if limb malformations are due to EDCs, such effects might also be simultaneously manifested in other systems, including reproduction.

Materials and Methods

Frog collection and maintenance. We examined newly metamorphosed green frogs (*R. clamitans*) and bullfrogs (*R. catesbeiana*) for the presence of malformations at 24 sites in southern New Hampshire in July and August 1998. The standards and protocols for estimating rates of malformations and descriptions of malformations were developed at a meeting convened in the Shenendoah National Park in April 1997 (*32*). Sampling conducted for this project followed the "Shenendoah protocols." The sites where most metamorphs were obtained are in the southern third of New Hampshire (Figure 1), and the population density proximal to these areas ranged from 4 to 650 people per square mile (*33*). Land use near sampled sites was low-density housing with mixed deciduous-pine forest.

The capture goal for each sampling site was 100 recently metamorphosed individuals of one species. This goal is often unattainable, and so as not to exclude a large number of sites, a secondary target of a minimum of 50 individuals was established. Sixteen of the 24 collection sites yielded 50 or more individuals of one species (Figure 1). Some collection sites were locations where malformed frogs had been found the previous year; however, most sites were selected with the objective of sampling a broad range of sites within southern and central New Hampshire. Frogs that had recently undergone metamorphic climax [snout–urostyle length = 23–38 mm for green frogs and 31–59 mm for bullfrogs (*24*)] were captured in the field with dip nets or by hand and were held in 12-gallon coolers containing water from the site and an ice pack. We examined individuals in the field for malformations and returned most frogs to their site of capture within 2 hr. In August 1998, frogs with external malformations and normal frogs (n = 53 frogs total) were collected from a subset of sites and transported immediately to the University of New Hampshire in coolers, where they were held in 20-gallon aquaria for 7–10 days. Frogs from different sites were held in separate aquaria. Additionally, normal and malformed frogs from the same site were housed separately. Frogs were held under natural photoperiod and air temperatures of 21°C. Each aquaria had several rocks as perching sites and approximately 3 cm of filtered, nonchlorinated well water. Water was changed daily, and frogs were fed live pin crickets (approximately two crickets per individual frog per day). There were 10 mortalities of housed frogs.

In vitro *steroid assays.* A total of 43 frogs were sampled and assayed for the ability to produce androgens and estradiol *in vitro*; 20 normal (9 male and 2 female green frogs, 8 male and 1 female bullfrogs) and 23 with malformed limbs (2 male green frogs, 3 female green frogs, 7 male bullfrogs, and 11 female bullfrogs) (Table 1). Sex determination based on external characteristics is not possible in recently metamorphosed green frogs and bullfrogs; therefore, we were unable to determine sex until the gonads were dissected and removed. After weighing, each frog was decerebrated by rapid decapitation at the first cervical vertebra. Brains were collected from 24 male and female bullfrogs. Eight and 6 brains from male bullfrogs were removed from normal and malformed frogs, respectively. One and nine brains from female bullfrogs were removed from normal and malformed frogs, respectively. Brains were immediately removed, immersed in liquid N_2, and stored at –80°C until extraction (two brains of either male or female were pooled for each sample), HPLC purification, and subsequent radioimmunoassays for mammalian and chicken-II GnRH were performed as previously described (*19*).

In vitro *steroid synthesis studies and histological examination of gonads.* The left gonad from each frog was sampled, fixed in Bouin's solution, and prepared for histological examination by imbedding in paraffin followed by hematoxylin–eosin staining and evaluation as described by Gray (*34*), Muller (*35*), Taylor and Kollros (*36*), and Hsu et al. (*37*).

The right gonadal tissue (either ovarian or testicular) was removed for *in vitro* bioassay and weighed. Each individual gonad was placed in a well of a 24-well plate containing 500 µL media (Krebs-Ringer bicarbonate solution at pH 7.3 with penicillin/streptomycin) per well. The tissues were preincubated for 30 min at 18°C. The preincubation medium was removed and the tissue was then incubated in 500 µL of culture medium supplemented with pregnenolone (127 ng/mL media) at ANOTC. Three additional normal testes were incubated without pregnenolone. The culture media were collected 5 hr later, stored at –20°C until extracted, and assayed for androgens and estradiol by radioimmunoassay (RIA) following the procedures

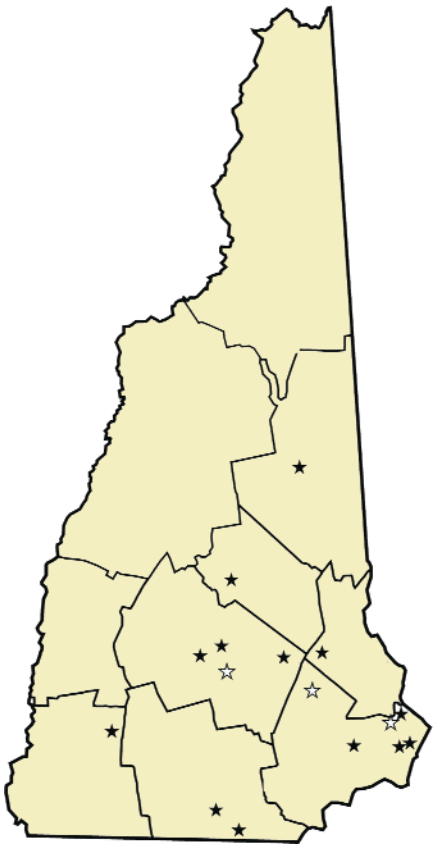

Figure 1. Location of sites in New Hampshire surveyed for malformed amphibians in 1998. Only sites where at least 50 individuals were captured are shown (n = 16). Shaded stars indicate sites where malformed amphibians were found (n = 13); open stars indicate sites in which no malformed amphibians were found (n = 3). Solid lines demarcate county boundary lines.

Table 1. Number of bullfrogs (*R. catesbeiana*) and green frogs (*R. clamitans*) captured in New Hampshire that were examined for malformations and held for further investigation.

	Bullfrogs			Green frogs		
	Normal	Malformed	Total	Normal	Malformed	Total
Male	8	7	15	9	2	11
Female	1	11	12	2	3	5
Intersex	0	2	2	0	0	0

described by Sower and Schreck (*38*) and Sower et al. (*39*). For androgens we used the antisera 11-BSA (antitestosterone) and for estradiol-17β we used antiestradiol-17β (S-244), both obtained from G. Niswender (Colorado State University, Fort Collins, CO). The androgen and estradiol antisera were used at dilutions of 1:40,000 and 1:85,000, respectively. The testosterone antibody cross-reacts with testosterone (100%) and dihydrotestosterone (69%), thus the concentrations are reported as total androgens. The lower limit of detection in both assays was 7.8 pg/0.1 mL. The intraassay and interassay coefficients of variation for the androgen and estradiol RIA were 3.2% (n = 9) and 5.7% (n = 6) and 3.6% (n = 9) and 5.4% (n = 6), respectively. The antibody efficiency ranged from 26 to 28% and from 44 to 50% in the androgen and estradiol assays, respectively.

Extraction and HPLC and GnRH RIA. Frozen brains were extracted as described by Yu et al. (*40*) and Fahien and Sower (*41*) and eluted on an HPLC system following the methods of Conlon et al. (*19*), Fahien and Sower (*41*), and Calvin et al. (*42*). Briefly, the extract was filtered using an ACRO LC 13 (0.45 µm) filter and then injected into a 20-µL loop on a Perkin-Elmer HPLC system with a Pecosphere 3CR C18 (0.46 × 8.3 cm) reverse-phase column. The isocratic phase consisted of 7.40 g ammonium acetate and 3.04 g citric acid in 1 L of 19% acetonitrile/water (final pH adjusted to 4.6 with phosphoric acid) (*43*). The flow rate was 2 mL/min, with fractions collected every 18 sec for the first 34 fractions and then every minute for subsequent fractions.

We determined GnRH by RIA as described by Conlon et al. (*19*), Stopa et al. (*43*), and Fahien and Sower (*41*) using synthetic mammalian GnRH as the radioiodinated tracer (New England Nuclear, Boston, MA) and standard (Peninsula Laboratories, Belmont, CA). The antiserum was used at a dilution of 1:100,000 for mammal RIA (R1245; from T. Nett, Colorado State University, Fort Collins, CO). The antibody binding ranged between 38 and 45%.

Statistics. We evaluated data for hormone concentrations (androgens, estradiol, GnRH) using one-way analysis of variance with status (normal vs. malformed) as the main effect. When significant effects ($p < 0.05$) were detected, specific means were analyzed by the Fisher's PLSD (*44*). Because of the low numbers of frogs, we did not consider the site of capture in the analysis.

Results

Malformation rates. We observed a total of 1,436 frogs in the field. Malformed amphibians were observed at 13 of the 16 sites (81% of sites). Malformation rates ranged from 0 to 9.3% at a given site, and the total malformation rate was 3.9%: 4.3% for bullfrogs (42 of 983 individuals) and 2.4% for green frogs (11/453). Most malformations (47%) involved ectromelia (absence of all or part of a limb), ectrodactyly (absence of all or part of a digit) of the hindlimb, or asymmetrical development of hindlimbs (Figure 2, Table 2). Ectrodactyly or other malformations of the front limb was found in 34% of malformed individuals, and eye and other malformations accounted for 24% of the malformations. (The sum of percentages is greater than 100% due to 3 frogs with more than one type of malformation.) Although we did not observe any visible trauma to frogs that we considered malformed, it is possible that a few hindlimb malformations were due to injury. However, it is unlikely that injury accounts for any front limb malformations because the front limbs grow protected within the branchial chamber until metamorphosis, when they emerge fully formed. The occurrence of malformation correlated moderately with human density estimates (Pearson product moment correlation = 0.29); the 3 sites with the highest malformation rates were in towns with densities ranging from 63 to 224 people per square mile.

The mean body weights for bullfrogs were 7.5 ± 0.7 g for normal (n = 9) and 7.8 ± 0.6 g (n = 18) for malformed frogs. The mean body weights for green frogs were 3.4 ± 0.2 g (n = 11) and 2.2 ± 0.1 g (n = 5) for normal and malformed green frogs, respectively. The body weights for bullfrogs did not differ significantly between malformed and control frogs (p = 0.38) or between male and female frogs (p = 0.62). However, while body weight did not differ between male and female green frogs (p = 0.20), there was a significant difference between the normal and malformed green frogs (p = 0.007).

Brain GnRH concentrations. Mammalian GnRH concentrations in normal male bullfrogs (60.0 ± 15.7 pg GnRH/brain, mean ± SE; n = 4) were higher (p = 0.08) than in malformed male bullfrogs (19.3 ± 3.4 pg/brain; n = 3); (Figure 3). The concentrations of mammalian GnRH in normal and malformed female bullfrogs were 38.0 pg GnRH/brain (n = 1) and 62.0 ± 13.7 pg GnRH/brain (n = 5), respectively. Unfortunately, there was only 1 malformed female bullfrog available for study, and thus statistical analysis could not be done on

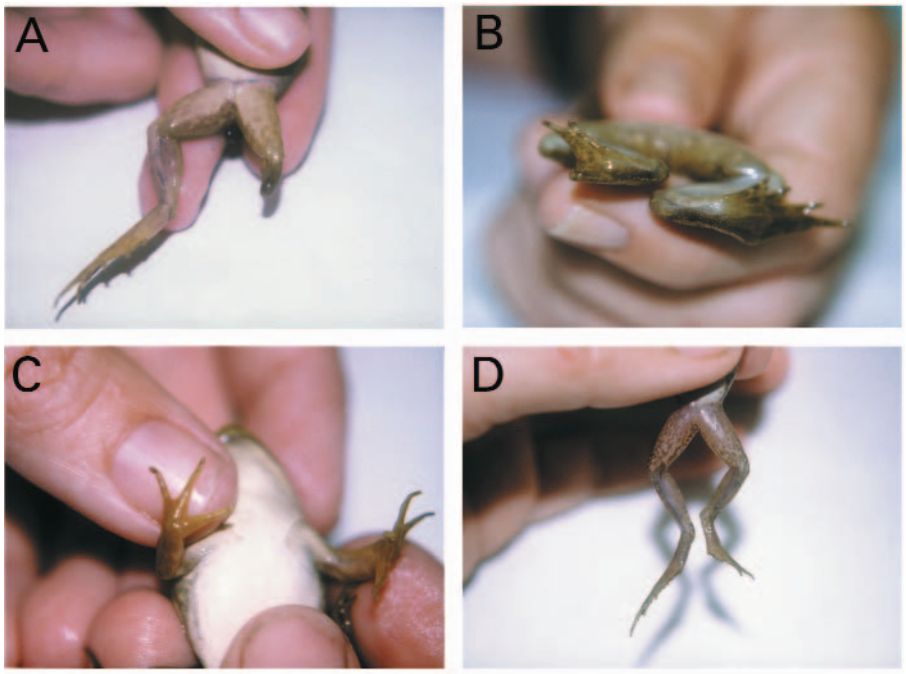

Figure 2. Examples of limb malformations from *R. catesbeiana* (*A*–*C*) and *R. clamitans* (*D*) metamorphs. Malformations are (*A*) ectromelia of left hindlimb, (*B*) ectrodactyly of right hindlimb, (*C*) ectrodactyly of right front limb, and (*D*) asymmetrical development of hindlimbs.

Table 2. Specific malformations (expressed as percentage of total malformations) for bullfrogs (*R. catesbeiana*) and green frogs (*R. clamitans*) collected from 24 sites in 1998.

Frog	Hemi- and ectromelia, hindlimb (%)	Ectrodactyly, hindlimb (%)	Hindlimb asymmetry (%)	Hindlimb atrophy (%)	Front limb (%)	Eye (%)	Other (%)
Bull	27.9	7.0	2.3	4.7	37.2	7.0	14.0
Green	7.7	23.1	7.7	15.4	15.4	7.7	23.1

these samples (Table 3). Chicken GnRH-II concentrations were undetectable in brains from bullfrogs. Brains of green frogs were not assayed because of low numbers.

In vitro *androgen concentrations.* Normal frogs had significantly higher (p = 0.03) levels of androgens compared to frogs that had limb malformations (Figure 4). The mean concentration of androgens of grossly normal male gonads without incubation with pregnenolone was 20.03 pg/mg testis (n = 3). The range of androgens in all normal male testes and in all normal female ovaries incubated with pregnenolone was 87.06–704.23 pg/mg testis (n = 12) and nondetectable to 0.96 pg/mg ovary (n = 3), respectively. The range of androgens in all malformed male testes incubated with pregnenolone was 24.75–519.44 pg/mg testis (n = 9). The range of androgens in the 13 ovaries from malformed females, incubated with pregnenolone, was nondetectable to 2.35 pg/mg ovary (Table 3). We were unable to detect estradiol concentrations in media from incubated normal or malformed frog testes or ovaries in our assay system.

Histology. Testes of apparently normal frogs were characterized by a large number of spermatogonia, whereas the testes of the malformed frogs showed a lack of development of the seminiferous tubules, with only slight development of rete, stromal, or polymorphonuclear germ cells (Figure 5). Ovaries of normal and malformed females consisted mainly of growing primary oocytes of various sizes. On the periphery of the ovaries, small primordial germ cells and synaptenes were occasionally found. There were two intersex gonads that were excluded from the steroid metabolism studies. In one case, the right gonad was primarily an ovary and the left gonad was primarily a testis. In the second case, both left and right gonads contained testicular tissue as well as primary oocytes.

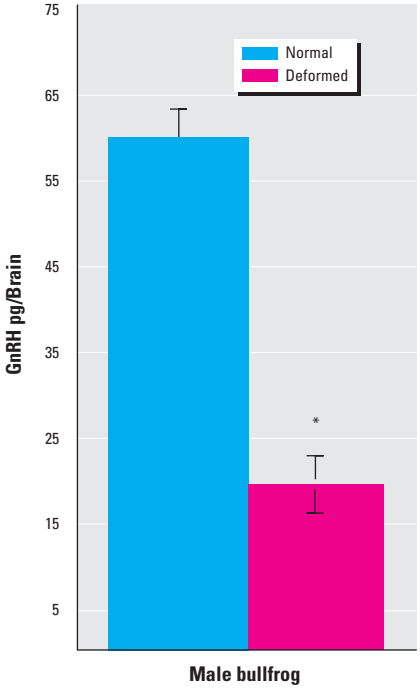

Figure 3. Mean brain mammalian GnRH (pg/brain) concentrations for male normal and malformed bullfrogs.
*Significant at $p < 0.05$.

Discussion

In the present study, a survey of newly metamorphosed bullfrogs, *R. catesbeiana*, and green frogs, *R. clamitans*, conducted in southern New Hampshire found malformed frogs at 81% of the sites sampled (13 of 16 sites). Normal frogs had significantly higher concentrations (nearly 3-fold) of *in vitro* produced androgens and of brain mammalian GnRH compared to malformed frogs. We suggest that environmental influences may play a role in producing amphibian malformations in natural frog populations. These are the first data to demonstrate a deficiency of androgen and GnRH production in malformed frogs.

Numerous etiologies have been postulated for amphibian malformations, including ultraviolet (UV) radiation, retinoid and other xenobiotic chemical exposures, and infectious agents (*1,3,5,6,45,46*). Exposure of developing anurans to UV-B radiation has generated mixed results, varying from no effect on development or mortality (*45*) to increased mortality and malformations including lateral flexure of the tail, blistering, and edema (*1*). However, there are no reports of limb or eye malformations associated with UV exposure (*46*). Retinoid exposure has been associated exclusively with mirror-image limb duplications (*6*); this was also the principal lesion documented by Sessions and Ruth (*5*) in Pacific tree frogs, *Hyla regilla*, exposed to digenetic trematodes. They reported few missing limbs in frogs, and only one forelimb abnormality was observed (*5*). Similarly, Johnson et al. (*3*) reported the experimental induction of limb abnormalities in *H. regilla* exposed to *Ribeiroia* sp. cercariae (a digenetic trematode). In that study, missing limbs and digits were documented (although lower in frequency than multiple limbs); however, the abnormalities were restricted to the hindlimbs. Although chemical mechanisms cannot be ruled out, alterations of GnRH, steroidogenesis, and gonadal histology coupled with malformations not previously attributed to trematode infestation, suggest that parasite infestation is not the causal agent in our study.

Ouellet et al. (*8*) described an increased frequency of deformity among frogs, *R. clamitans* and *R. pipiens*, living in ponds exposed to agricultural pesticide runoff. The principle lesions were ectromelia and ectrodactyly involving hindlimbs, although occassionally missing forelimbs and eyes were also noted. Although the precise etiology is unknown in this study, a study by Cooke (*47*), described kinks in the base of the tail of tadpoles and malformation of hindlimbs in newly metamorphosed frogs naturally exposed to DDT, a

Table 3. GnRH and testosterone levels for bullfrogs (*R. catesbeiana*) and green frogs (*R. clamitans*).

	GnRH (pg/mg brain)		Testosterone (pg/mg testes)	
	Normal	Malformed	Normal	Malformed
Green frogs				
Male	ND	ND	158.9 ± 23.1 (5)	60.9 ± 0.58 (2)
Female	ND	ND	0.0 (2)	0.85 ± 0.44 (5)
Total (male and female)	ND	ND	113.5 ± 33.4 (7)	18.0 ± 11.1 (7)
Bullfrogs				
Male	6.0 ± 1.6 (4)	1.9 ± 0.34 (3)	227.5 ± 82.1 (7)	159.9 ± 65.8 (7)
Female	3.8 (1)	6.24 ± 1.37 (5)	0.96 (1)	6.24 ± 1.37 (9)
Total (male and female)	5.5 ± 1.3 (5)	4.6 ± 1.1 (8)	199.2 ± 76.5 (8)	70.2 ± 34.2 (16
Green frogs/bullfrogs				
Male	ND	ND	198.9 ± 48.3 (12)	137.9 ± 52.3 (9)
Female	ND	ND	0.32 ± 0.32 (3)	0.54 ± 0.18 (14)
Total (male and female)	ND	ND	159.2 ± 43.8 (15)	54.3 ± 24.4 (23)

ND, not done. Data are presented as means ± SEM. The number of frogs analyzed (n) is in parentheses.

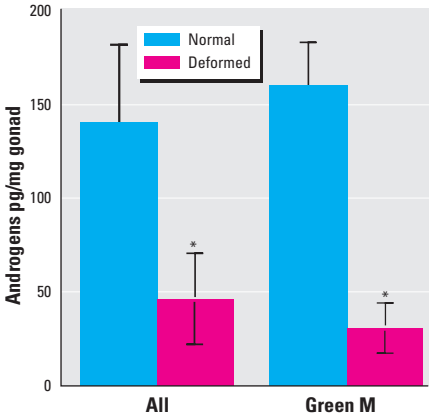

Figure 4. Mean androgen (pg/mg gonad) concentrations for normal and malformed frogs. "All" refers to all frogs tested (male and female, bullfrogs and green frogs). "Green M" refers to all male green frogs sampled.
*Significant at $p < 0.10$.

known estrogenic xenobiotic chemical associated with reproductive alterations in wildlife.

Factors regulating neuroendocrine development likely affect individuals during narrow but critical periods of life. The significant decrease in GnRH concentration correlated with depressed androgen concentrations and lack of proper testicular morphology in malformed frogs provides evidence that development of the neuroendocrine system may have been disrupted during early frog development. There are few reported studies on the effects of pollutants on the hypothalamus. In one study in catfish, 5-month exposure to lead nitrate induced degenerative changes in the hypothalamus, resulting in a failure to secrete GnRH (*48*). Thus, chemicals in the environment that influence the migration and/or development of GnRH neurons could cause significant endocrine disruption (i.e., change in steroid metabolism). However, in the present study we have not identified any one biotic or abiotic factor.

An increasing number of environmental pollutants with estrogenic activity have been reported recently. These include the alkylphenols, microbial breakdown products of alkylphenol polyethoxylate, and nonionic surfactants (*49*). EDCs such as PCBs (2′,4′,6′-trichloro-4-biphenol and 2′,3′,4′,5′-tetrachloro-4-biphenol), DDT and DDE, and alkyl phenolics (*50,51*) have been identified in southern New Hampshire and Great Bay and have been associated with domestic sewage (*52*). Guillette et al. (*10*) hypothesized that these xenobiotic compounds can modify reproductive and endocrine development based, in part, on *in vitro* experiments in alligators. In these studies, the synthesis of estrogenic steroids was significantly different *in vitro* when ovaries from alligators hatched from contaminated and uncontaminated lakes were compared. On the basis of these and other studies, Crain and Guillette (*9*) proposed that many of the reproductive abnormalities seen in vertebrates may be due to alteration in steroidogenesis upon exposure to EDCs. In the present study, androgen synthesis was significantly altered in malformed versus normal frogs. There have been some reports on the effects of pollutants resulting in intersex gonads (containing both testicular and ovarian tissues) in the medaka (*Oryzias latipes*) (*53,54*). In the present study, two malformed frogs had intersex gonads. We hypothesize that significant, and often detrimental, interactions occur among environmental and endocrinological factors necessary for development of amphibian limbs and reproductive processes.

Despite the current documentation of amphibian declines and malformations, there are few reports on the use of amphibians as models for abnormalities of reproductive processes by exposure to EDCs. In a recent study, the interactions of gonadal steroids and pesticides (DDT, DDE) on gonaduct growth in larval tiger salamanders, *Ambystoma tigrinum*, were examined (*55*). The salamanders were immersed in a solution of DDE, DDT, or injected with estradiol or dihydrotestosterone. Essentially all the compounds tested had some adverse effect on the gonaduct growth in salamanders. Clark et al. (*55*) reported that amphibians are potentially more sensitive to environmental disturbances than many other vertebrates because of their complex life histories and water-permeable skins. Treatment with various steroids in *R. clamitans* and *R. catesbeiana* has significant effects on primary sex differentiation; for example, injection of testosterone proprionate will induce 100% males (*56*). Chemicals mimicking steroids could thus have profound effects on the reproductive system. However, the underlying mechanisms associated with contaminant-induced reproductive modifications are still poorly understood and will require extensive research (*57*).

Our findings suggest that significant alterations in GnRH, steroidogenesis, and gonadal histology correlate with limb deformity and may reflect the endocrine nature of disruptor exposures. Growth, development, and metamorphic processes in anuran larvae are complex and involve corresponding hormonal and neuronal control (*58*). It is possible that endocrine disruptors affect androgen production either at the hypothalamo-pituitary axis or directly at the testis (*59*).

Androgens are important in male amphibian reproduction, and seasonal variations in plasma testosterone concentrations are found in many species, including *R. catesbeiana* (*23,25–27*). However, relatively few studies have examined the process of sex determination and differentiation in amphibians (*56*). Although the timing of sexual differentiation in many frogs is unknown, it is species- and temperature-dependent (*58*). For example, *Xenopus* develop gonads at the limb bud stage, but *Bufo* do not undergo sexual differentiation until after metamorphosis. The timing for sexual differentiation in *R. clamitans* and *R. catesbeiana* is unknown (*58*). Thus, the critical stage of exposure for effects on sexual differentiation is also unknown.

For this reason, further experimentation is necessary to determine the effects of EDCs on amphibian larval development and metamorphosis and to identify contaminants that may cause neuroendocrine and gonadal developmental problems such as intersexes or reduced steroidogenesis. In addition, there needs to be further baseline studies on endocrine parameters associated with development in amphibians. Based on the present data, we propose that, similar to fish (*16*), alterations in the hypothalamic–pituitary–gonadal axis may serve as potential bioindicators of endocrine disruptor exposure in amphibians.

In conclusion, this study determined a significant decrease in androgens and GnRH concentrations in malformed frogs when compared to normal frogs. This is the first

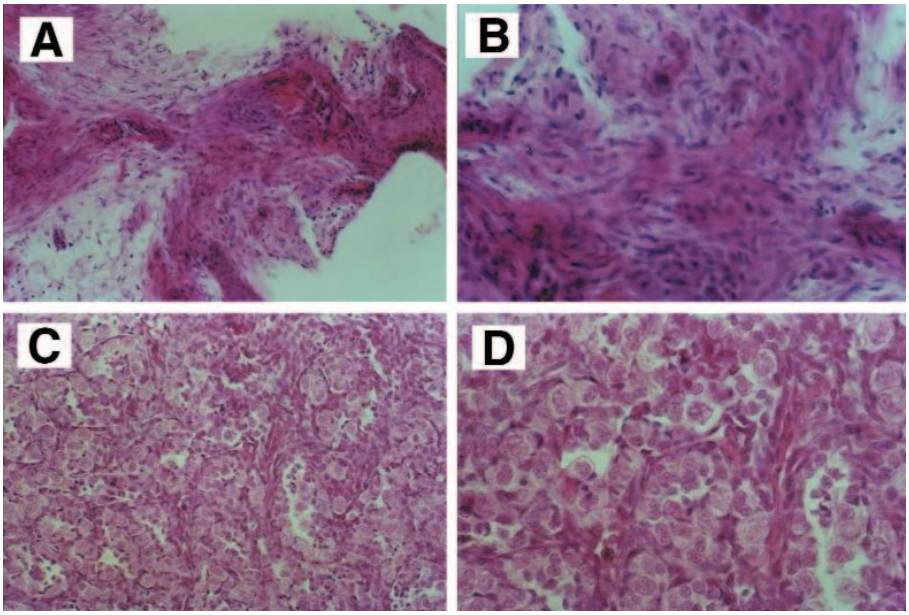

Figure 5. Histologic examination of bullfrog testes. Testes of malformed frogs (*A,B*) typically showed degeneration of the seminiferous tubules and slight development of rete and stromal cells. Testes of normal males (*C,D*) were characterized by a large number of spermatogonia. *A,C* = 20×; B,D = 40×.

report of such differences in amphibians. EDCs are proposed to be involved in these differences. However, the potential nature of EDC interaction with reproductive and developmental process in amphibians still needs to be determined.

References and Notes

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