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Altered Ovarian Steroids in *Xenopus laevis* Exposed to Environmentally Relevant Concentrations of Nitrate

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ABSTRACT.—Contamination of aquatic ecosystems with anthropogenic sources of nitrate is of increasing concern. Anurans are especially at risk for overexposure to nitrate because they occupy aquatic environments during embryonic, larval, and adult stages. Whereas there have been numerous studies examining the effect of nitrate on frog eggs and tadpoles, the effects of nitrate on adult frogs have been largely neglected. In this study, we examined whether brief exposure to environmentally relevant concentrations of aquatic nitrate influenced ovarian steroid synthesis and ovarian follicle size in adult African Clawed Frogs (*Xenopus laevis*). We exposed frogs for seven days to nitrate at concentrations of 24.8 or 49.5 mg/l in order to simulate a pulse of nitrate exposure. Relative to controls, nitrate-exposed frogs exhibited suppressed *ex vivo* synthesis of testosterone and estrogen. Follicles from nitrate-exposed frogs also exhibited an increased stage-4 diameter at both nitrate concentrations and a decreased stage-5 and -6 diameter at the highest nitrate concentration. These results indicate that anuran ovarian steroidogenesis and follicle size are modified by even a brief exposure to environmentally relevant concentrations of nitrate.

Contamination of aquatic ecosystems by anthropogenic sources of nitrate has become an increasing global concern with respect to the health of humans and wildlife (Fried, 1991; Guillette and Edwards, 2005). Nitrate contamination of aquatic habitats occurs primarily through human activities in agricultural and urban areas, and in North American rivers, streams, and lakes, nitrate concentrations range from 1 mg/l to over 100 mg/l nitrate-as-nitrogen (Rouse et al., 1999). Within the United States, agricultural nitrogen contributions have increased 20-fold in the past 50 years, because of poorly regulated runoff of nitrogen-based fertilizers and animal wastes (Puckett, 1995; Rouse et al., 1999). Within urban areas, nitrate infiltrates aquatic ecosystems primarily through release of industrial and wastewater effluents from treatment plants, runoff of fertilizer applied to lawns and golf courses, and air pollution from the burning of fossil fuels (Rouse et al., 1999). In North America, fertilizers applied adjacent to waterways, coupled with spring rainstorms, contribute to a pulse of nitrate entering aquatic habitats that coincides with the breeding season of many amphibians (Rouse et al., 1999).

Nitrate is highly soluble in water and, if ingested or absorbed transdermally, is readily transported across the epithelium of the small intestine (Ellis et al., 1998; Iizua et al., 1999). Numerous investigations of tadpoles exposed to environmentally relevant concentrations of nitrate have demonstrated altered behaviors (Hecnar, 1995; Marco and Blaustein, 1999; Marco and Blaustein, 1999), movement patterns (Xu and Oldham, 1997), growth rates, development (Baker and Waights, 1993, 1994; Edwards et al., 2006), gonadal morphology (Orten et al., 2006), and mortality rates (Marco et al., 1999; Smith et al., 2005, 2006). However, the effects of nitrate on endocrine function of adult frogs remain unknown.

A growing body of evidence demonstrates that nitrate disrupts gonadal function and steroid synthesis pathways of many vertebrates (reviewed by Guillette and Edwards, 2005). For example, nitrate exposure inhibits gonadal testosterone synthesis in rodents and bulls (Zraly et al., 1997; Panesar, 1999; Panesar and Chan, 2000). Nitrate exposure is also associated with gonadal atrophy, altered sperm morphology, and decreased sperm count in rodents (Pant and Srivastava, 2002), in addition to suppressed Leydig cell activity and reduced sperm motility in bulls (Zraly et al., 1997). Larval anurans exposed to nitrate exhibit altered sperm cell ratios and ovarian follicle maturation (Orton et al., 2006). In accord with these observations, in this study we hypothesized that exposure to environmentally relevant nitrate could alter ovarian steroid synthesis and follicle size in a model frog species, *Xenopus laevis*.

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Materials and Methods

We purchased adult female *X. laevis* (Xenopus Express, Plant City, Florida) and allowed them to acclimate for one week in a climate-controlled animal room under a 12 : 12 L : D cycle, in 38-liter tanks filled with 18 liters of dechlorinated water. We fed animals Spirulina pellets (Aquatic Eco-systems, Inc., Apopka, Florida) every other day and removed uneaten food to minimize the effects of bacterial activity and the buildup of nitrogenous wastes. Daily air and water temperatures averaged 21 ± 1°C and 23 ± 1°C, respectively, and water pH ranged between 7.0 and 7.4. We performed a complete water change daily on each tank and recorded water concentrations of ammonia and nitrate below 1 mg/l as measured by an Orion Ammonia Electrode and Orion Nitrate Test Kit (Model 95-12 and Kit 700005; Orion Research, Inc., Boston, Massachusetts).

After acclimation, we injected frogs with 50 IU of pregnant mare serum gonadotropin, followed three days later by 750 IU of human chorionic gonadotropin (Sigma-Aldrich, St. Louis, Missouri) into the dorsal lymph sac. These treatments stimulated ovulation and formation of new ovarian follicles over the course of five to six weeks (Dumont, 1971; Fortune and Tsang, 1981; Fortune, 1983). By synchronizing the size and maturity of ovarian follicles, we minimized potential variation among frogs in their gonadal responses to nitrate treatment. After ovulation, frogs were maintained for six more weeks to allow sufficient time for the formation of new ovarian follicles.

At the end of six weeks, we exposed the frogs to experimental treatments. Four frogs were randomly assigned to one of three replicate tanks per treatment, for a total of 12 frogs per treatment. Sodium nitrate ($NaNO_3$, 99% purity; Fisher Scientific, Orlando, Florida) was dissolved in aged tap water to create nominal concentrations of 24.8 and 49.5 mg/l NO_3-N. A control (0 mg/l) of aged tap water was also used, creating three treatment groups (i.e., 0, 24.8, and 49.5 mg/l NO_3-N). Every 24 h, we performed a complete change of water or nitrate solution of each tank. We monitored the frogs daily and no outward signs of abnormal behavior or stress were observed.

After a continuous seven-day nitrate exposure, we anesthetized frogs by submersion in 1.5% 3-aminobenzoic acid ethyl ether (Aquatic Eco-systems, Inc. Apopka, Florida). Blood was collected by cardiac puncture using heparinized syringes, placed into heparinized tubes, and centrifuged at 2,500 × G for 15 min. We stored the resultant plasma at −70°C for subsequent testosterone (T) and estrogen (E_2 or 17β-estradiol) radioimmunoassay (RIA) analyses. We could not collect sufficient blood volume for steroid analyses from two frogs in the control groups; thus, final sample sizes for plasma steroid analyses were as follows: control (N = 10), 24.8 mg/l (N = 12), and 49.5 mg/l (N =12).

After blood collection, we harvested the ovaries, livers, and oviducts and recorded their masses to contrast hepatosomatic and gonadosomatic indexes among treatment groups. We dissected ovarian follicles for *ex vivo* culture and selected follicles of specific maturation stages because stage-4 follicles synthesize predominantly E_2, whereas stage-5 and -6 follicles synthesize predominantly T (Fortune and Tsang, 1981). From each frog, we incubated, for 5 and 10 h, 33 follicles each of stages 4, 5, and 6, for a total of 99 follicles per culture, in duplicate 35 × 10 mm sterile culture dishes at 23°C with 2 ml of sterile, phenol-free culture media (1 L M199 HBSS, 3.4 ml 200 mM L-glutamine, 5.96 g/l HEPES, 0.35 g/l sodium bicarbonate, 8.0 ml 0.1 mM IBMX; pH 6.9; Sigma-Aldrich, St. Louis, Missouri). We excluded two frogs from the *ex vivo* study because there were insufficient follicle numbers of stage 4, 5, and 6 for culture. Sample sizes for *ex vivo* follicle culture were as follows: control (N = 11), 24.8 mg/l (N = 12), and 49.5 mg/l (N = 11). After incubation, culture media was decanted, flash-frozen, and stored at −70°C for RIA analyses.

To evaluate the effects of nitrate on follicle size, we measured the diameter of the remaining uncultured follicles at the widest point using a dissecting microscope and ocular micrometer. Five follicles per stage were measured from frogs for the following sample sizes: control (N = 8), 24.8 mg/l (N = 10), and 49.5 mg/l (N = 8).

We performed T and E_2 RIAs on plasma samples and on culture media using validated procedures (Guillette et al., 1994, 1996). Duplicate media samples or plasma (50 µl for T and E_2) were twice extracted with ethyl ether, air-dried, and reconstituted in borate buffer (0.05 M; pH 8.0). Antibodies (Endocrine Sciences, Calabasas Hills, California) were added at final concentrations of 1 : 25,000 for T and 1 : 55,000 for E_2. Radiolabeled steroids (1,2,6,7-^{3}H) testosterone or (2,4,6,7,16,17-^{3}H) estradiol at 1 mCi/ml (Amersham Biosciences, Arlington Heights, Illinois) were added at 12,000 cpm per 100 µl for a final assay volume of 500 µl. We prepared interassay variance tubes from two separate pools of media and plasma for T and E_2. We prepared standards for T and E_2 in duplicate at 0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg/tube. Assay

tubes were vortexed and incubated overnight at 4°C.

We added 500 µl of a 5% charcoal and 0.5% dextran mixture and pulse-vortexed and centrifuged (1500 × G, 4°C, 30 min) the tubes to separate bound-free hormone. Of the resulting supernatant, 500 µl were mixed with 5 ml of scintillation cocktail and counted on a Beckman scintillation counter.

Validation of steroid assays included media and plasma dilutions (20, 50, and 100 µl for T and 50, 100, and 200 µl for E_2) compared with T and E_2 standards. Media dilutions and standards gave parallel displacement curves, and an average media recovery of 98.9% for T and 91.5% for E_2 was used to correct for sample losses. Media sample intraassay and interassay variance averaged 4.19% and 8.15% for T and 2.50% and 3.86% for E_2, respectively.

Plasma dilutions and standards exhibited parallel displacement, with an average recovery of 93.8% for T and 81.0% for E_2, and were used to correct sample losses. Plasma intraassay variance for T and E_2 averaged 4.61% and 4.23%, respectively. Interassay variance for T and E_2 plasma samples are not reported because samples were run in one assay.

Statistical analyses.—We determined plasma and media concentrations of T and E_2 from raw data using Microplate Manager software (Microplate Manager III, BioRad Laboratories, Inc., Hercules, California). Statistical analyses were performed using SPSS software (v. 10, SPSS, Inc., Chicago, Illinois) with $\alpha = 0.05$.

For RIA validation, we log10-transformed pooled media dilutions (T and E_2) and pooled plasma (T and E_2) hormone concentrations before testing for homogeneity of slopes by ANCOVA. We compared concentrations of T and E_2 among replicate tanks within each treatment group using one-way ANOVA. Where no significant differences existed among replicate tanks, within treatment groups, we compared mean steroid concentrations among treatment groups with one-way ANOVA (Simmons, 2004). We compared ovarian follicle diameters for each stage among treatments with one-way ANOVA. Where significant differences were detected for one-way ANOVA analyses, we used Scheffe post-hoc contrasts. We compared wet mass of total ovary (left and right ovaries combined), total oviduct (left and right oviducts combined), and liver among treatments with body mass as a covariate using ANCOVA followed by Sidak post-hoc contrasts.

In this study, nitrate concentrations are expressed as equivalent to nitrate-as-nitrogen (NO_3-N). This represents the concentration of nitrogen (N) present in a given concentration of nitrate (NO_3) and is the standard measure used in limnology and the water quality literature. Aquatic NO_3-N concentrations can also be reported as milligrams per liter (mg/l) or millimolar (mM) units. Equivalent measures of nitrate under all of these conventions are provided in Table 1 to facilitate comparison among studies.

TABLE 1. Equivalent concentrations of various forms of nitrate as those used in this study.

NO_3-N (mg/l)	$NaNO_3$ (mg/l)	NO_3 (mg/l)	NO_3 (mM)	NO_3-N (mM)
24.8	150	109.5	1.3	0.3
49.5	300	219.0	2.6	0.6

RESULTS

There were no significant differences among treatment groups for oviduct mass (ANCOVA; $F_{2,\ 30} = 0.28$, $P = 0.86$), gonadosomatic index (ANCOVA; $F_{2,\ 26} = 0.57$, $P = 0.75$), and hepatosomatic index (ANCOVA; $F_{2,\ 28} = 1.13$, $P = 0.40$). However, the nitrate-exposed frogs exhibited larger stage-4 follicle diameters than control frogs (ANOVA; $F_{2,\ 107} = 4.77$, $P = 0.01$; Table 2). Frogs exposed to the 49.5 mg/l NO_3-N nitrate had smaller stage-5 (ANOVA; $F_{2,\ 107} = 3.33$, $P = 0.04$) and stage-6 (ANOVA; $F_{2,\ 107} = 5.54$, $P = 0.005$) follicle diameters than did controls and frogs exposed to 24.8 mg/l NO_3-N.

Plasma T or E_2 concentrations were similar among the three replicate groups per treatment; thus, data from replicate animals were combined within treatments. Plasma T concentrations were not different among treatment groups (ANOVA; $F_{2,\ 31} = 0.37$, $P = 0.37$; Table 2). Plasma E_2 concentrations were not different among treatment groups, although the P-value was marginal (ANOVA; $F_{2,\ 31} = 2.81$, $P = 0.08$) and nitrate-exposed frogs had lower mean plasma E_2 concentrations (Table 2).

Ovarian *ex vivo* T and E_2 culture media concentrations were similar among replicate groups per treatment; thus, data from replicate animals were combined within treatments. After 5 h of incubation, ovarian *ex vivo* T synthesis, as detected by media concentrations, was similar among treatments (ANOVA; $F_{2,\ 31} = 2.39$, $P = 0.11$; Table 2); however, after 10 h of incubation, T synthesis was lower in nitrate-exposed frogs (ANOVA; $F_{2,\ 31} = 11.43$, $P < 0.001$). After 5 h of incubation, ovarian E_2 synthesis was lower in the 49.5 mg/l nitrate group compared to the 24.8 mg/l NO_3-N and control groups (ANOVA; $F_{2,\ 31} = 19.50$, $P < 0.001$). After 10 h of incubation, there was reduced ovarian E_2 synthesis for both groups of nitrate-exposed

TABLE 2. Summary of mean (± SE) plasma and ovarian *ex vivo* steroid concentrations (pg/ml), and ovarian follicle diameters (mm) measured from female *Xenopus laevis* after continuous seven-day immersion in various concentrations of aqueous sodium nitrate. Nitrate concentrations (mg/l) are reported as nitrate-as-nitrogen (NO_3-N). Significant differences within a row are indicated by different superscripts.

	NO_3-N		
Analysis	0 (Control)	24.8	49.5
Plasma testosterone (T)	1123.7 ± 361.6 [a]	1067.7 ± 601.6 [a]	1242.6 ± 516.1 [a]
Plasma estrogen (E_2)	611.7 ± 103.0 [a]	428.9 ± 43.0 [a]	412.4 ± 41.0 [a]
Ex vivo T 5 h	657.3 ± 105.4 [a]	553.4 ± 87.5 [a]	494.7 ± 53.3 [a]
Ex vivo T 10 h	1378.8 ± 281.8 [a]	795.7 ± 102.3 [b]	545.8 ± 57.7 [b]
Ex vivo E_2 5 h	208.8 ± 8.9 [a]	194.7 ± 8.9 [a]	136.9 ± 7.5 [b]
Ex vivo E_2 10 h	314.7 ± 8.2 [a]	247.6 ± 219.4 [b]	219.4 ± 16.3 [b]
Stage-4 follicle diameter	0.923 ± 0.015 [a]	0.982 ± 0.014 [b]	0.977 ± 0.016 [b]
Stage-5 follicle diameter	1.250 ± 0.023 [a]	1.279 ± 0.010 [a]	1.215 ± 0.010 [b]
Stage-6 follicle diameter	1.217 ± 0.022 [a]	1.256 ± 0.011 [a]	1.176 ± 0.010 [b]

frogs relative to controls (ANOVA; $F_{2, 31} = 16.70$, $P < 0.001$).

DISCUSSION

In this study, brief exposure of female *X. laevis* to environmentally relevant nitrate concentrations was associated with suppressed ovarian steroid synthesis. These findings are consistent with reports of suppressed testicular T synthesis in rodents drinking nitrate-contaminated water (Panesar and Chan, 2000). Our study also demonstrates that nitrate-exposed *X. laevis* exhibit altered ovarian follicle diameters. These results are similar to altered ovarian follicles reported for larval Leopard Frogs exposed to nitrate (Orton et al., 2006).

We found that plasma steroid concentrations in nitrate-exposed *X. laevis* were apparently unaffected (although we consider the marginal [$P = 0.08$] trend of lower plasma E_2 concentrations with exposure to the highest nitrate concentration in *X. laevis* to be biologically relevant); this pattern differs from rodents exposed to nitrate (Panesar and Chan, 2000), which exhibited decreased plasma steroid concentrations (*in vivo*). This difference suggests that *in vivo* and *ex vivo* steroid synthesis, in response to nitrate, might be regulated through different physiological mechanisms. For example, plasma steroid concentrations may have remained unchanged in *X. laevis* because of compensatory responses of the hypothalamic-pituitary-gonadal axis. Inhibition of ovarian steroidogenesis might have stimulated a positive feedback for release of hypothalamic gonadotropin-releasing hormone and pituitary luteinizing hormone and follicle-stimulating hormone. Gonadotropin stimulation might have contributed to a compensatory increase in ovarian *in vivo* steroid synthesis in nitrate-exposed frogs that matched plasma steroid concentrations of unexposed frogs. Plasma gonadotropins and gonadal inhibin secretion were not examined in *X. laevis*; thus, this hypothesis remains untested.

An alternate hypothesis involves alteration in hepatic clearance of sex steroids in nitrate-exposed frogs. The liver is the site of steroid hormone biotransformation, which is the initial step of steroid excretion (Guillette and Gunderson, 2001). The liver is also the main organ for nitrate degradation (Doblander and Lackner, 1996). Steroid and nitrate metabolism require the activity of hepatic P450 enzymes, and continuous administration of nitrate has been shown to inhibit hepatic P450 enzyme activity (Minamiyama et al., 2004). Although the hepatosomatic index did not vary among treatment groups for *Xenopus*, hepatic nitrate metabolism might have inhibited hepatic P450-induced steroid metabolism leading to stasis or augmentation of circulating steroid concentrations. For this mechanism to be confirmed, hepatic P450 enzyme activity should be examined in future studies of nitrate-exposed *X. laevis*. Adrenal steroid synthesis might also have contributed to the plasma steroid concentrations. However, the contribution of adrenal steroid synthesis to the total circulating steroid concentrations is likely to be minimal. An adrenal stress response can induce a surge in plasma corticosteroids, which have been shown to inhibit steroid synthesis in reptiles and amphibians (Licht et al., 1983; Lance and Elsey, 2005); however, we observed no overt indications of stress in *X. laevis* during the experiment. Future studies should include monitoring of adrenal steroid and cortisol synthesis.

Although no difference in the gonadosomatic index was observed, follicle diameter did vary with treatment. The diameter of E_2-producing follicles (stage 4) was greater in nitrate-exposed frogs, whereas the diameter of T-producing follicles (stage 5 and 6) was smaller in the 49.5 mg/l nitrate group compared to the control

group and 24.8 mg/l nitrate group. These differences could reflect altered growth in response to reduced follicular E_2 synthesis, which drives hepatic production of vitellogenin. Follicular diameter might have varied in response to ionic and osmotic changes within the follicles. Panesar (1999) speculated that steroidogenesis in mouse Leydig tumor cells (MLTC-1) was mediated by chloride channels through activation of adenylate cyclase and cyclic adenosine monophosphate (cAMP). Nitric oxide is known to inhibit steroidogenesis in Leydig cells and granulosa-luteal cells (Van Voorhis et al., 1994; Welch et al., 1995; Punta et al., 1996). Furthermore, Panesar (1999) reported that the uptake of inorganic nitrate led to chloride depletion and a subsequent inhibition of steroidogenesis in MLTC-1. In our study, a moderately toxic effect of nitrate might have altered follicular morphology and steroidogenesis.

Within body tissues, various isoforms of nitric oxide synthase (NOS) enzymes can convert nitrate into nitric oxide (NO; VanVoorhis et al., 1995; Olsen et al., 1996; Jablonka-Shariff and Olson, 1997; Srivastava et al., 1997). Studies of rodents, humans, and bulls have shown that enzyme-dependent NO synthesis inhibits T and E_2 synthesis (VanVoorhis et al., 1994; Wang and Marsden, 1995; Zraly et al., 1997; Basini et al., 1998). The mechanisms for nitrate-associated endocrine disruption, proposed by these and other studies, include nitrate and nitrite conversion to NO and inhibition of steroidogenic P450 enzymes necessary for conversion of free cholesterol into steroids (Wink et al., 1993; Snyder et al., 1996). In addition to NOS-dependent NO formation, a growing literature suggests that acidic reduction and hemoglobin mediates nonenzymatic NO synthesis from nitrite (Zweier et al., 1995, 1999; Modin et al., 2001). Our study did not test a specific mechanism by which nitrate alters steroidogenesis, but we hypothesize that modified P450 activity and NO synthesis are likely pathways leading to disruption. Future studies need to test specific mechanisms to determine the underlying cause of this altered ovarian steroidogenesis.

The ovarian responses associated with nitrate exposure observed in *X. laevis* might not reflect the responses of other anurans, because different species have been shown to have variable responses and tolerance to nitrate exposure (Johansson et al., 2001; Marco and Blaustein, 1999; Vaala et al., 2004; Smith et al., 2005). More research is required to determine whether other species exhibit altered gonadal steroid synthesis when exposed to nitrate as shown in *X. laevis*. Further studies are also needed to determine the minimum nitrate concentrations capable of altering ovarian steroidogenesis. Finally, the mechanism by which nitrate inhibits steroidogenesis in anurans merits investigation. Endpoints in future studies might include the effects of nitrate exposure on steroid regulation by the hypothalamic-pituitary-gonadal axis and on hepatic degradation and clearance of steroids and nitrate circulating in the blood.

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Literature Cited

Baker, J., and V. Waights. 1993. The effect of sodium nitrate on the growth and survival of toad tadpoles (*Bufo bufo*) in the laboratory. Herpetological Journal 3:147–148.

———. 1994. The effects of nitrate on tadpoles of the tree frog (*Litoria caerulea*). Herpetological Journal 4:106–108.

Basini, G., M. Baratta, N. Ponderato, S. Bussolati, and C. Tamanini. 1998. Is nitric oxide an autocrine regulator of bovine granulosa cell function? Reproduction, Fertility, and Development 10: 471–478.

Doblander, C., and R. Lackner. 1996. Metabolism and detoxification of nitrite by trout hepatocytes. Biochimica et Biophysica Acta-General Subjects 1289:270–274.

Dumont, J. N. 1971. Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. Journal of Morphology 136:153–180.

Edwards, T. M., K. A. McCoy, T. Barbeau, M. W. McCoy, J. M. Thro, and L. J. Guillette Jr. 2006. Environmental context determines nitrate toxicity in Southern Toad (*Bufo terrestris*) tadpoles. Aquatic Toxicology 78:50–58.

Ellis, G., I. Adatia, M. Myazdanparah, and S. K. Makela. 1998. Nitrate and nitrite analyses: a clinical and biochemistry perspective. Clinical Biochemistry 31:195–220.

Fortune, J. E. 1983. Steroid production by *Xenopus* ovarian follicles at different developmental stages. Developmental Biology 99:502–509.

Fortune, J. E., and P. C. Tsang. 1981. Production of androgen and estradiol-17-β by *Xenopus* ovaries treated with gonadotropins *in vitro*. General and Comparative Endocrinology 43:234–242.

Fried, J. J. 1991. Nitrates and their control in the EEC aquatic environment. *In* I. Bogardi, R. D. Kuzelka, and W. G. Ennenga (eds.), Nitrate Contamination: Exposure, Consequence, and Control V30, pp. 3–11. Springer-Verlag, New York.

Guillette, L. J., Jr., and T. M. Edwards. 2005. Is nitrate an ecologically relevant endocrine disruptor in vertebrates? Integrative and Comparative Biology 45:19–27.

GUILLETTE, L. J., Jr., AND M. P. GUNDERSON. 2001. Alterations in the development of the reproductive and endocrine systems of wildlife exposed to endocrine disrupting contaminants. Reproduction 122:857–864.

GUILLETTE, L. J., Jr., T. S. GROSS, G. R. MASSON, J. M. MATTER, H. F. PERCIVAL, AND A. R. WOODWARD. 1994. Developmental abnormalities of the gonad and abnormal sex-hormone concentration in juvenile alligators from contaminated and control lakes in Florida. Environmental Health Perspectives 102:680–688.

GUILLETTE, L. J., Jr., M. C. COX, AND D. A. CRAIN. 1996. Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. General and Comparative Endocrinology 104:116–122.

HECNAR, S. J. 1995. Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from Southern Ontario. Environmental Toxicology and Chemistry 14:2131–2137.

IIZUA, T., M. SASAKI, K. OISHI, S. UEMURA, M. KOIKE, AND M. SHINOZAKI. 1999. Non-enzymatic nitric oxide generation in the stomachs of breastfed neonates. Acta Paediatric 88:1053–1055.

JABLONKA-SHARIFF, A., AND L. M. OLSON. 1997. Hormonal regulation of nitric oxide synthases and their cell-specific expression during follicular development in the rat ovary. Endocrinology 138:460–468.

JOHANSSON, M., K. RASANEN, AND J. MERILA. 2001. Comparison of nitrate tolerance between different populations of the common frog, *Rana temporaria*. Aquatic Toxicology 54:1–14.

LANCE, V. A., AND R. M. ELSEY. 2005. Stress-induced suppression of testosterone secretion in male alligators. Endocrinology 239:241–246.

LICHT, P., B. R. MCCREERY, R. BARNES, AND R. PANG. 1983. Seasonal and stress related changes in plasma gonadotropins, sex steroids, and corticosterone in the bullfrog, *Rana catesbeiana*. General and Comparative Endocrinology 50:124–145.

MARCO, A., AND A. R. BLAUSTEIN. 1999. The effects of nitrate on behavior and metamorphosis in Cascades Frogs (*Rana cascadae*). Environmental Toxicology and Chemistry 18:946–949.

MARCO, A., C. QUILCHANO, AND A. R. BLAUSTEIN. 1999. Sensitivity to nitrate and nitrite in pond-breeding amphibians from the Pacific Northwest, USA. Environmental Toxicology and Chemistry 18:2836–2839.

MINAMIYAMA, Y., S. TAKEMURA, K. YAMASAKI, S. HAI, K. HIROHASHI, Y. FUNAE, AND S. OKADA. 2004. Continuous administration of organic nitrate decreases hepatic cytochrome P450. Journal of Pharmacology and Experimental Therapeutics 308:729–735.

MODIN, A., H. BJORNE, M. HERULF, K. ALVING, E. WEITZBERG, AND J. O. N. LUNDBERG. 2001. Nitrite-derived nitric oxide: a possible mediator of acidic-metabolic vasodilation. Acta Physiologica Scandinavica 171:9–16.

OLSEN, L. M., C. M. JONES-BURTON, AND A. JABLONKA-SHARIFF. 1996. Nitric oxide decreases estradiol synthesis of rat luteinized ovarian cells: possible role for nitric oxide in functional luteal regression. Endocrinology 137:3531–3539.

ORTON, F., J. A. CARR, AND R. D. HANDY. 2006. Effects of nitrate and atrazine on larval development and sexual differentiation in the Northern Leopard Frog *Rana pipiens*. Environmental Toxicology and Chemistry 25:211–217.

PANESAR, N. S. 1999. Role of chloride and inhibitory action of inorganic nitrate on gonadotropin-stimulated steroidogenesis in mouse Leydig tumor cells. Metabolism–Clinical and Experimental 48:693–700.

PANESAR, N. S., AND K. W. CHAN. 2000. Decreased steroid hormone synthesis from inorganic nitrite and nitrate: studies *in vitro* and *in vivo*. Toxicology and Applied Pharmacology 169:222–230.

PANT, N., AND S. P. SRIVASTAVA. 2002. Testicular and spermatotoxic effect of nitrate in mice. Human and Experimental Toxicology 21:37–41.

PUCKETT, L. J. 1995. Identifying the major sources of nutrient water pollution. Environmental Science and Technology 408:414A.

PUNTA, K. D., E. D. CHAREAU, AND O. P. PIGNATARO. 1996. Nitric oxide inhibits Leydig cell steroidogenesis. Endocrinology 137:5337–5343.

ROUSE, J. D., C. A. BISHOP, AND J. STRUGER. 1999. Nitrogen pollution: an assessment of its threat to amphibian survival. Environmental Health Perspectives 107:799–803.

SIMMONS, A. M. 2004. Call recognition in the bullfrog, *Rana catesbeiana*: Generalization along the duration continuum. Journal of the Acoustical Society of America 115:1345–1353.

SMITH, G. R., K. G. TEMPLE, D. A. VAALA, AND H. A. DINGFELDER. 2005. Effects of nitrate on the tadpoles of two ranids (*Rana catesbeiana and R. clamitans*). Archives of Environmental Contamination and Toxicology 49:559–562.

SMITH, G. R., K. G. TEMPLE, H. A. DINGFELDER, AND D. A. VAALA. 2006. Effects of nitrate on the interactions of the tadpoles of two ranids (*Rana clamitans* and *R. catesbeiana*). Aquatic Ecology 40:125–130.

SNYDER, G. D., R. W. HOLMES, J. N. BATES, AND B. J. VAN VOORHIS. 1996. Nitric oxide inhibits aromatase activity: mechanisms of action. Journal of Steroid Biochemistry and Molecular Biology 58:63–69.

SRIVASTAVA, V., B. J. JONES, H. DOOKWAH, J. K. HINEY, AND W. L. DEES. 1997. Ovarian nitric oxide synthase (NOS) gene expression during peripubertal development. Life Sciences 61:1507–1516.

VAALA, D. A., G. R. SMITH, K. G. TEMPLE, AND H. A. DINGFELDER. 2004. No effect of nitrate on Gray Treefrog (*Hyla versicolor*) tadpoles. Applied Herpetology 1:265–269.

VAN VOORHIS, B. J., M. S. DUNN, G. D. SNYDER, AND L. P. WEINER. 1994. Nitric oxide: an autocrine regulator of human granulosa cell steroidogenesis. Endocrinology 135:1799–1806.

VAN VOORHIS, B. J., K. MOORE, P. J. L. STRIJBOS, S. NELSON, S. A. BAYLIS, D. GRZYBICKI, AND C. P. WEINER. 1995. Expression and localization of inducible and endothelial nitric oxide synthase in the rat ovary. Journal of Clinical Investigation 96:2719–2726.

WANG, Y., AND P. A. MARSDEN. 1995. Nitric oxide synthases: gene structure and regulation. *In* L. Ignarro and F. Murad (eds.), Advances in Pharmacology V34, pp. 71–90. Academic Press, New York.

WELCH, C., M. E. WATSON, M. POTH, P. MERRILY, T. HONG, AND T. L. FRANCIS. 1995. Evidence to suggest nitric oxide is an interstitial regulator of Leydig cell steroidogenesis. Metabolism 44:234–238.

WINK, D. A., Y. OSAWA, J. R. DARBYSHIRE, C. T. JONES, S. C. ESHENAUR, AND R. W. NIMS. 1993. Inhibition of cytochrome P450 by nitric oxide and a nitric oxide-releasing agent. Archives of Biochemistry and Biophysics 300:115–123.

XU, Q., AND R. S. OLDHAM. 1997. Lethal and sublethal effects of nitrogen fertilizer ammonium nitrate on common toad (*Bufo bufo*) tadpoles. Archives of Environmental Contamination and Toxicology 32:298–303.

ZRALY, Z., J. BENDOVA, D. SVECOVA, L. FALDIKOVA, Z. VEZNIK, AND A. ZAJICOVA. 1997. Effects of oral intake of nitrates on reproductive functions of bulls. Veterinary Medicine–Czech 42:345–354.

ZWEIER, J. L., P. H. WANG, A. SAMOUILOV, AND P. KUPPUSAMY. 1995. Enzyme-independent formation of nitric-oxide in biological tissues. Nature Medicine 1:804–809.

ZWEIER, J. L., A. SAMOUILOV, AND P. KUPPUSAMY. 1999. Nonenzymatic nitric oxide synthesis in biological systems. Biochimica et Biophysica Acta 1411: 250–262.

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