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Ammonium Perchlorate Disruption of Thyroid Function in Natural Amphibian Populations: Assessment and Potential Impact

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Abstract: We examined indices of thyroid development in tadpoles from ammonium perchlorate (AP)-exposed sites. Bullfrog (*Rana catesbeiana*) tadpoles collected from a reference site exhibited normal developmental features, with many completing metamorphoses. In contrast, tadpoles collected from the AP contaminated site exhibited a 5-fold lower hindlimb/snout-vent length ratio than tadpoles from the reference site. The volume of the thyroid gland was 2.5-fold larger in the tadpoles from the reference site, presumably because they had progressed to late prometamorphosis and early metamorphic climax. Premetamorphic western chorus frog tadpoles (*Pseudacris triseriata*) inhabiting an ephemeral pond contaminated with AP exhibited gross morphological abnormalities of the thyroid including colloid depletion and follicle cell hypertrophy. We conclude that tadpoles exposed to AP-contaminated pond water early in larval life exhibit delayed development of thyroid-hormone sensitive structures. Additionally, there are abnormalities in the developing thyroid gland that seem to depend upon the window of AP exposure. The potential impact of thyroid disruption on development and reproduction in amphibian populations will be discussed.

Keywords: perchlorate, thyroid, metamorphosis, amphibian

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Amphibian metamorphosis is the end result of a complex set of biochemical, morphological, and behavioral changes that prepare an aquatic larval form to survive in a semi-terrestrial environment. Initiation and coordination of these events is determined in part by the onset of thyroid hormone (TH) secretion (LeLoup and Buscaglia 1977, Suzuki and Suzuki 1981, Norman et al. 1987), the timing of thyroid hormone receptor gene expression (Yaoita and Brown 1990, Kawahara et al. 1991), and tissue-specific expression of genes encoding iodothyronine deiodinase enzymes that ensure adequate conversion of tetriodothyronine (T_4) to triiodothyronine (T_3) in target tissues (Becker et al. 1997). For example, the hindlimbs respond to TH relatively early in development whereas other TH-dependent events, such as programmed cell death in tail structures, do not occur until the final stages of metamorphosis. The increase in TH secretion during metamorphic climax is generally accompanied by corresponding changes in the size and appearance of the thyroid gland, and, in some instances, an increase in the height of the thyroid follicular epithelium (Norman et al. 1987, Goleman et al. 2002b).

Reported worldwide declines in frogs species (Houlahan et al. 2000, Alford et al. 2001, Pounds 2001, Kiesecker et al. 2001) have focused attention on environmental factors that may interfere with amphibian development and reproduction. One environmental contaminant of particular concern is ammonium perchlorate (AP), a compound widely used in the aerospace industry and by the military as an oxidizer in rocket fuels. Perchlorate salts have been used for decades to experimentally inhibit amphibian development and metamorphosis (Miranda et al. 1996, Brown 1997, Rollins-Smith et al. 1997). Recently, perchlorate has been detected in surface and ground waters at various places in the Western US at levels ranging from 8 μg/L to 3.7 g/L (Urbansky 1998). Perchlorate levels as high as 31.2 ± 0.21 mg/L were recently found in surface waters at Longhorn Army Ammunition Plant (LHAAP) in Karnack, Texas (Smith et al. 2001). These concentrations fall within a wide range of sublethal perchlorate concentrations that inhibit amphibian metamorphosis as evidenced by concentration-dependent reductions in forelimb emergence, tail resorption, and hindlimb growth (Goleman et al. 2002a, 2002b). These effects are accompanied by an increase in thyroid follicle cell height and reduced whole-body thyroid hormone content in tadpoles exposed to perchlorate (Goleman et al. 2002b).

Although environmentally relevant concentrations of perchlorate inhibit metamorphosis in *Xenopus laevis*, these frogs are not native to the US and the effects of AP on native species are largely unknown. In this report we provide evidence of thyroid disruption in American bullfrog (*Rana catesbeiana*) and western chorus frog (*Pseudacris triseriata*) tadpoles collected from AP-contaminated sites at the LHAAP in East Texas.

Methods

Field Collections

All animals were collected from ponds located on site at the LHAAP in Karnack, TX. The LHAAP is located in the watershed of Caddo Lake, the largest natural lake in Texas. In order to decommission this military base, groundwater on site has been treated

to precipitate metals and with air stripping and carbon polishing to remove volatile organics. Until the spring of 2001, perchlorate in the ground water was not removed. For a more complete description of site characteristics refer to Smith et al. (2001).

Sampling of each site took place for approximately 1-2 hr by a minimum of two personnel. Tadpoles were dip-netted from around the bank around the entire circumference of each pond. Tadpoles were rapidly euthanized in MS-222 and then stored in 10% neutral-buffered formalin for subsequent histological analysis. Bullfrog (*Rana catesbeiana*) tadpoles were collected in April 2000 from one control (site A) and one AP-exposed site (site B). Tadpoles from both sites were of the same age class based on the fact that they were identical in snout-vent length and were collected in early spring, indicating that they had over-wintered at least one season. Western chorus frog (*Pseudacris triseriata*) tadpoles were collected in April 2001 from one control (site C) and one exposed (site D) site. Species identification was aided using the key developed by Altig (1970). Bullfrog developmental stages were identified using the methodology of Taylor and Kollros (TK, 1946). Gosner staging (1960) was used for chorus frog tadpoles. All animals were collected under Texas Parks and Wildlife scientific permit no. SPR1098-984. All procedures were approved by the Texas Tech University Animal Care and Use Committee.

Field sites were selected based on their history of perchlorate contamination (Smith et al. 2001). Exposed and control sites were selected based on their close proximity to one another to control for differences in rainfall and temperature that may affect development. Control sites had no detectable perchlorate at the time of animal collection. At each visit to the field site, we attempted to measure various compositional and physical parameters, including: dissolved oxygen, air and water temperature, conductivity, salinity, and pH. Perchlorate content of field water samples was determined as previously described (Smith et al. 2001). Detection and quantitation limits for perchlorate were 1 μg/L and 2.5 μg/L, respectively.

Histological Assessment of Thyroid Activity

Tadpole heads were dehydrated in a graded series of alcohols and processed for routine paraffin embedding. Serial transverse sections (10 μm) through the head were mounted on glass slides and stained using Harris' progressive hematoxylin and eosin procedure and coverslips mounted. For bullfrog tadpoles, follicle epithelial cell height and right thyroid gland volume were measured as described previously (Goleman et al. 2002b) using an Olympus BH-2 compound microscope equipped with a Sony CCD/RGB video camera and monitor and a CompuAdd 450DX2 computer with Image Pro (Media Cybernetics, Silver Spring, MD, USA) imaging software. The software was calibrated using a Bausch & Lomb calibration slide prior to recording. The calibration was saved to the computer hard drive to ensure consistent measurements over time. Epithelial cell height measurements were taken from 5 arbitrarily chosen cells in 5 sections of the right thyroid gland for each specimen (n = 25 observations/animal). A single mean value for epithelial cell height was then calculated for each animal. The cross-sectional area of all serial sections through the right thyroid gland for each animal was measured and

summed. Total cross-sectional area (mm^2) x section thickness (10 μm) was used to calculate the right thyroid gland volume for each animal.

Although quantitative image analysis yielded useful data, the method proved too inefficient to serve as a high throughput means for assessing thyroid disruption in large numbers of samples. For analysis of thyroid disruption in chorus frog tadpoles we employed a semi-quantitative approach to determine colloid depletion, follicle cell hypertrophy, and follicle cell hyperplasia using methods based on Hardisty and Boorman (1990) as modified by Hooth et al. (2001) and by the US EPA Pathology Working Group of the Effects of Ammonium Perchlorate on Thyroids (Mann 2000). Serial sections through each animal were scanned and colloid depletion, follicle cell hypertrophy, and follicle cell hyperplasia determined for all follicles in one section each from the rostral, middle, and caudal region of the thyroid gland. Values were averaged for each section and a mean calculated for all three combined sections per animal. Colloid depletion, follicle cell hypertrophy, and follicle cell hyperplasia were scored by a naïve rater on a scale of 0, 1, or, 2 in order of increasing severity as shown below:

Colloid Depletion

0 No reduction
1 Pale, lacy, vacuolated, or granular appearance; slight to moderate reduction
2 Large reduction or absence

Follicle Cell Hypertrophy

0 No hypertrophy – follicles lined by squamous to cuboidal epithelium
1 Follicles lined by tall cuboidal to columnar epithelium; cytoplasm: nucleus ratio increased
2 Follicular epithelial cells distinctly larger than normal; follicular lumen severely decreased or obliterated

Follicle Cell Hyperplasia

0 No hyperplasia – follicles lined by a single layer of normal appearing, squamous to short cuboidal epithelium
1 Two or more follicles exhibiting stratification of follicular epithelium, usually 2-3 cells thick, protruding into lumen
(**Note:** follicles exhibiting stratification near gland periphery are not counted)
2 Greater number of affected follicles exhibiting stratification of follicular epithelium, usually more than 3 layers thick, protruding into lumen; areas of hyperplasia may also have microfollicular formation within them

Data Analysis

Mean differences were compared by two-tailed Student's t-test.

Results

Perchlorate contamination of surface waters at LHAAP in 2000 and 2001 was principally confined to two areas associated with the manufacture of AP and the demilitarization of munitions in accordance with the Intermediate-range Nuclear Forces (INF) treaty; the INF pond as described by Smith et al. (2001) (site B in the present study) and temporary ponds in the vicinity of Building 25C (site D in the present study). Two cases of apparent thyroid disruption in animals collected from these AP-contaminated sites are described below.

Comparison of Bullfrog Tadpoles from Site A and Site B

Water Quality Parameters- Water quality parameters at sites A and B are shown in Table 1. Site B tended to be slightly more alkaline than site A. Perchlorate concentrations at site B have historically reached levels as high as 31 ppm (November 1999, Smith et al. 2001). At the time that bullfrog tadpoles were collected for analysis perchlorate levels at site B were close to 2 ppm but were non-detectable at the reference (site A).

Developmental Endpoints- The animals collected from the reference site (site A) were in the later stages of prometamorphosis or early metamorphic climax (TK stages XVII-XXXI). In contrast, tadpoles collected from the contaminated site had only reached premetamorphosis and early prometamorphosis (TK stages IX-XIII). Snout-vent lengths were identical between tadpoles from sites A and B (Table 2), suggesting that these animals were roughly the same age, as age and body length are generally correlated in ranids (Sagor et al. 1998, Miaud et al. 1999). In contrast, hindlimb length was 5.2x smaller in the animals from the contaminated site compared to the reference site (Table 1). To directly compare relative hindlimb lengths in the size-matched animals, we calculated the hindlimb length to snout-vent length ratio. This ratio was 5.3-fold lower in the animals collected from the contaminated site compared to animals from the reference site. This is not an insignificant finding as hindlimb length/snout-vent length ratio is an accurate predictor of perchlorate exposure in laboratory studies on developing *X. laevis* (Figure 1). Approximately 93% of the variation in hindlimb length/snout-vent length ratio in *Xenopus laevis* can be explained by perchlorate exposure (Figure 1) when exposure begins in early embryonic life and continues for at least 70 d post-hatching.

*Thyroid Histopathology-*The quantitative assessment of thyroid gland volume and follicle cell height is presented in Table 3. There were no differences in follicle cell height between the collection sites. In contrast, thyroid gland volume was 2.5-fold larger in tadpoles from site A compared to tadpoles from site B.

Comparison of Chorus Frog Tadpoles from Site C and Site D

These sites were small temporary ponds that were too shallow to record from using our analytical water quality probe. Perchlorate in the water at site C was not detectable but was close to 10 ppm at site D at the time tadpoles were collected in April 2001. We

Table 1- *Site Characteristics*

Parameter	Site A	Site B
Source of water	Rainfall	Treatment plant effluent; rainfall
Conductivity[1]	70-560 µS/cm	80-2,052 µS/cm
pH[1]	6.2-7.1	7.8-9.3
Dissolved oxygen[1]	2.6-7.8 mg/L	7.3 mg/L
Temperature range[1]	16.9-21.1 °C	15.8-31.4 °C
Perchlorate[2]	ND	1,970 µg perchlorate/L

[1]Range of values between April 2000 and July 2001.
[2]Measured in water samples collected on the day that the tadpole specimens were collected in April, 2000.
ND, Not detectable.

Table 2- *Length Measurements in Bullfrog Tadpoles*

Site	Tail Length (mm)	SVL[1] (mm)	HLL[2] (mm)	HLL/SVL
A	48.3 ± 3.52	24.3 ± 1.75	26.2 ± 2.36	1.12 ± 0.09
B	41.2 ± 1.74	24.9 ± 0.75	5.02 ± 0.60[3]	0.21 ± 0.02[3]

[1]SVL, Snout-vent length.
[2]HLL, Hindlimb length.
[3]Statistically different from control site (site A) based on Student's *t*-test, $P < 0.05$.
Values are mean ± standard error of 11-13 animals per group.

Table 3- *Thyroid Histology in Bullfrog Tadpoles*

Site	Cell Height[1] (µM)	Thyroid Gland Volume[2] (mm^3)
A	4.53 ± 0.33 (n=11)	0.035 ± 0.004 (n=10)
B	4.53 ± 0.32 (n=13)	0.014 ± 0.002[3] (n=13)

[1]Mean ± standard error of measurements from 25-30 cells per animal from the right thyroid gland.
[2]Mean ± standard error of measurements from consecutive serial sections through the right thyroid gland.
[3]Statistically different from control site (site A) based on Student's *t*-test, $P < 0.05$.

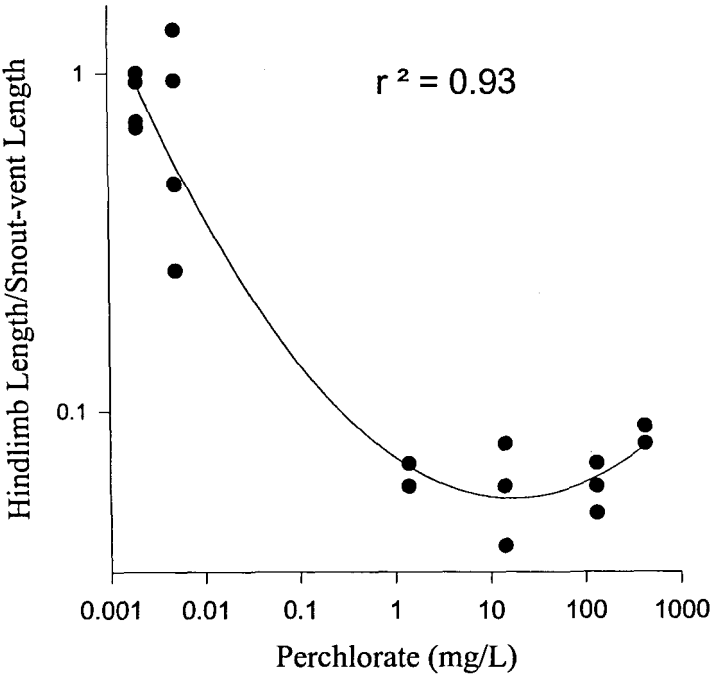

Figure 1- *Perchlorate concentration explains greater than 90% of the variation in hindlimb length/snout-vent length ratio in developing* X. laevis. *Measurements made after a 70-d exposure to AP beginning < 24 h after fertilization. Each point represents the mean of four replicates pooled from two independent trials. Sample size per replicate was 25-50. Data are re-graphed from Goleman et al. 2002a.*

Table 4- *Length Measurements in Chorus Frog Tadpoles*

Site	Tail Length (mm)	SVL (mm)	HLL (mm)	HLL/SVL
C	14.6 ± 0.41	8.72 ± 0.22	1.30 ± 0.08	0.15 ± 0.01
D	15.2 ± 0.27	9.80 ± 0.14	1.50 ± 0.06	0.15 ± 0.01

[1]SVL, Snout-vent length.
[2]HLL, Hindlimb length.
ND, Not detectable.

Table 5- *Thyroid Histology in Chorus Frog Tadpoles*

Site	Perchlorate (μg/L)	Gosner Stage[1]	Colloid Depletion[2]	Hypertrophy[2]
C	ND	33-34	0.02 ± 0.02 (n=8)	0.00 ± 0.00 (n=8)
D	9,802	33-34	0.71 ± 0.22[3] (n=15)	0.79 ± 0.20[3] (n=15)

[1]After Gosner (1960).
[2]Mean ± standard error of scores made from all follicles present in three sections from the rostral, middle, and caudal regions of the thyroid gland.
[3]Statistically different from reference site (site C) based on Student's *t*-test, $P < 0.05$.
ND, Not detectable.

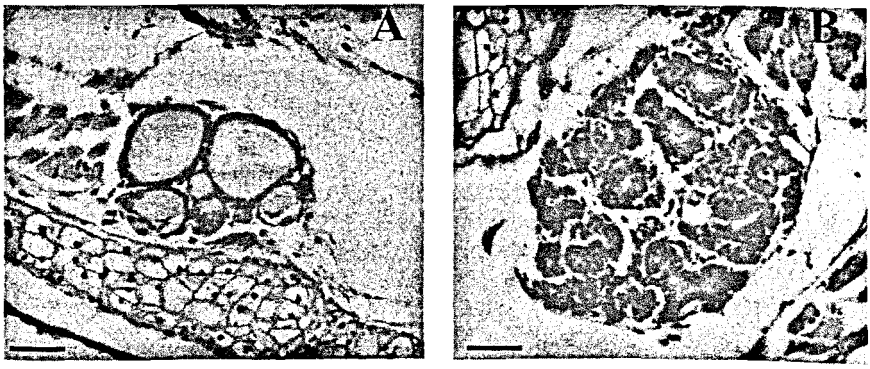

Figure 2 - *Photomicrographs of thyroid tissue from western chorus frog tadpoles (Gosner stages 33-34) collected from a reference site (Figure 2A) or an AP-contaminated site (Figure 2B). Figure 2A. Note the squamous shape of the follicle cells and the abundance of colloid. Figure 2B. Note the marked hypertrophy of the follicle cells and the reduction in follicle lumen and absence of colloid. Also note the apparent increase in size of the thyroid gland from the AP-exposed animal (Figure 2B, compare to Figure 2A). Both photomicrographs were taken at the same magnification. Bar = 50 μm.*

immediately noticed distinct hypertrophy and colloid depletion in thyroids from the animals collected at D (Figure 2). Semi-quantitative assessment of the thyroids from these animals revealed statistically significant colloid depletion and follicle cell hypertrophy compared to stage-matched animals from site C, where perchlorate was not detected. Follicle cell hyperplasia was not detected in tadpoles collected from either site.

Discussion

Between April 2000 and July 2001 we examined approximately 97 adult and larval frogs (*Hyla cinerea*, *R. catesbeiana*, and *P. triseriata*) collected at various perchlorate-free reference sites at LHAAP and prepared as described above. There was no evidence of thyroid disruption in any of the animals from the perchlorate-free sites. In contrast, our data suggest that bullfrog tadpoles inhabiting a perchlorate-contaminated pond at LHAAP (site B) exhibited signs of thyroid disruption as evidenced by reduced hindlimb growth relative to body growth and delayed metamorphosis relative to size-matched tadpoles from a nearby reference site. The tadpoles from the control and reference sites had over-wintered at least one season and had identical snout-vent lengths, suggesting that they belonged to the same age class (Sagor et al. 1998, Miaud et al. 1999). The volume of the thyroid gland was more than two times larger in the tadpoles from the reference pond, presumably because these animals were entering the later stages of prometamorphosis and metamorphic climax, which begins at TK stage XX. Whether animals from site B had been exposed to perchlorate during their entire larval period, which can last up to 2-3 years, isn't known, although perchlorate levels at the time of capture at site B were close to 2 ppm. Certainly one factor that can determine the extent to which developing frogs can be affected by perchlorate exposure is the variability with which perchlorate is present within surface waters. This in turn can be affected by several factors including leaching of perchlorate from soil, movement of perchlorate from ground water into surface water, and runoff of perchlorate from surface sediments. Data that have been collected from LHAAP suggest that the presence of perchlorate at site B can vary significantly from month to month. Perchlorate levels at site B the November preceding capture were close to 31 ppm (Smith et al. 2001) while in the months following capture, perchlorate levels ranged from 1700 μg/L in May to a range of 0-257 μg/L in June of 2000. At site B we would expect perchlorate levels to vary with the discharge of treated groundwater into the site. During dry periods treated groundwater was historically pumped to site B, but during the wetter months the discharge was diverted to nearby bayous that emptied into Caddo Lake (Smith et al. 2001). Thus it is likely that developing bullfrog tadpoles at site B were exposed to intermittently high levels of perchlorate. The fact that the effects of perchlorate on metamorphosis are largely reversible (Goleman et al. 2002b) suggests that developing frogs that are exposed intermittently over the course of a long larval period may have low- or non-exposure recovery periods, which may explain the fact that the bullfrog tadpoles at site B had progressed to early prometamorphosis and exhibited evidence of hindlimb growth, albeit significantly less than the hindlimb growth observed in tadpoles from the reference site. We (Carr and Goleman) know from ongoing studies in our laboratory that the $t_{1/2}$ for whole-body

elimination of perchlorate anions in bullfrog tadpoles is approximately 48 hr (unpublished data). Thus it is likely that over the course of their development tadpoles from site B had non- or low-exposure recovery periods from perchlorate.

The chorus frog tadpoles collected at site D exhibited dramatic disruption of thyroid function at the histological level compared to stage-matched tadpoles from a nearby reference site (site C). To our knowledge this is the first report of thyroid disruption in an amphibian inhabiting an AP-contaminated site. Given the relatively early developmental stage (Gosner stages 33-34) of the animals we examined, we would not have expected to see measurable differences in hindlimb growth in the animals from the contaminated site. However, the thyroid histopathology data clearly indicate thyroid disruption consistent with perchlorate exposure in the animals from site D. Exposure of frogs (Goleman et al. 2002b) or rodents (Siglin et al. 2000) to perchlorate under laboratory conditions causes a specific set of histopathological changes in the thyroid gland that include follicle cell hypertrophy, colloid depletion, and, in some instances, follicle cell hyperplasia. These changes result from over secretion of TSH due to lack of negative feedback that results from reduced TH synthesis (Norris 1997). The fact that the amphibian thyroid is functionally identically to that of mammals suggests that histopathological characteristics developed for assessment in mammals are equally useful in assessing perchlorate exposure in amphibians.

Collectively, results of the present study and previous work performed in *X. laevis* (Goleman et al. 2002b) suggest that thyroid histology is a useful indicator of perchlorate disruption at earlier stages of development, prior to metamorphic climax. However, because thyroid histology changes dramatically during metamorphic climax, other endpoints, such as hindlimb length relative to snout-vent length and forelimb emergence, are useful for detecting perchlorate exposure in late developmental stage tadpoles.

The potential impact of perchlorate on amphibian development goes beyond the predictable effects on thyroid-sensitive aspects of development and metamorphosis. Disruption of thyroid function interferes with normal gonadal development in some frog species (Hayes 1997; Goleman et al. 2002b). Whether perchlorate disrupts gonadal development under field exposure scenarios remains an open question, as we did not examine species that possess fully differentiated gonads at the time of capture. However, it is an important endpoint to keep in mind for future studies, as disruption of gonadal differentiation could clearly impact reproductive fitness at the population level.

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