Thyroid Endocrine Disruption in Stonerollers and Cricket Frogs from Perchlorate-Contaminated Streams in East-Central Texas

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Abstract. In October 2001 and March 2002, a field survey of central stonerollers (*Campostoma anomalum*) from perchlorate-contaminated streams in central Texas was conducted to assess thyroid endocrine disruption. A survey of adult male and female cricket frogs (*Acris crepitans*) was performed at the same site between 2001 and 2003. Perchlorate is an oxidizer primarily used in solid-fuel rockets, and many sites that processed or used perchlorate are now contaminated. Histological analysis revealed that the fish from contaminated sites had increased thyroid follicular hyperplasia, hypertrophy, and colloid depletion. Multivariate analysis was generally found to be more powerful than univariate analysis. Seasonal differences existed in the degree of thyroidal perturbation were discovered, and fish were generally less sensitive to thyroidal perturbations in March compared to October. Thyroidal histological indicators were also correlated to levels of perchlorate in the fish, water, and periphyton. Periphyton was frequently most strongly correlated to thyroidal indices, suggesting that exposure through the food chain may be of import. In addition, one of the presumed reference sites turned out to be contaminated with perchlorate, and this was reflected by thyroidal biomarkers before perchlorate was detected in the stream water or biota. There was no evidence of colloid depletion or hyperplasia in frogs from any of the sites, although frogs from two sites with greatest mean water perchlorate concentrations exhibited significantly greater follicle cell hypertrophy. Furthermore, there was a significant positive correlation between follicle cell height and mean water perchlorate concentrations for frogs collected from all sites. This is the first known published account of perchlorate-induced thyroid disruption in fish under field situations, only the second known published account for amphibians, and also points out the value of biomarkers for contaminant biomonitoring.

Introduction

Perchlorate is an oxidizer used routinely in explosives, flares, solid fuel rockets and missiles, fireworks, and automobile airbags (Urbansky,

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2002). Recent analyses have suggested widespread contamination of ground and surface waters with perchlorate in many states, particularly in the western U.S. or near military and industrial installations where perchlorate has been used, manufactured, or processed (Urbansky, 2000; Motzer, 2001; Smith et al., 2001). The perchlorate anion is also relatively stable and highly water soluble. These factors illustrate the vulnerability of aquatic ecosystems to perchlorate contamination.

Concern over environmental contamination by perchlorate stems from the fact that it affects thyroid function by inhibition of iodine uptake (Wolff, 1998; Clark, 2000). This is significant because, in fishes and amphibians, thyroid hormones mediate a variety of physiological processes and fitness traits such as lipid and carbohydrate metabolism, mitochondrial function, osmoregulation, immune competence, embryo-larval survival and development, reproduction, growth, and metamorphosis (Leatherland, 1994; Cyr and Eales, 1996; Shi, 2000; Power et al., 2001; Brown et al., 2004a). Thyroid hormones also interact with other endocrine systems such as sex steroids, retinoids, and growth hormones (Cyr and Eales, 1996). Thus, disruption of thyroid function may affect bioenergetic, health, and fitness endpoints in affected organisms.

The functional unit of thyroid tissues is the thyroid follicle: a spheroid or ovoid capsule consisting of an outer basement membrane lined with a single layer of simple epithelium, which surrounds a central lumen. The follicular epithelial cells take up iodide from the Na^+/I^- symporter and incorporate it into thyroglobulin, a glycoprotein from which thyroid hormones are synthesized. Thyroglobulin is secreted from the thyrocytes into the follicle lumen to form the colloid, which serves as a repository for thyroid hormone (TH) synthesis. Secretion of TH is controlled by the pituitary via thyroid-stimulating hormone (TSH). TSH stimulates thyrocytes to synthesize and secrete TH. Circulating TH inhibit release of TSH via a negative feedback mechanism (Leatherland, 1994; Brown et al., 2004a).

Histological examination of thyroid follicles can provide an indication of disruption of thyroid hormone production by xenobiotic chemicals, and has been used to examine thyroid-disrupting effects of a number of different chemicals in fishes (Lanno and Dixon, 1994, 1996; Bhattacharya et al., 1995; Ram, 1998; Ricard et al., 1998; Grinwis et al., 2000; Cooley et al., 2001; Palace et al., 2001; Brown et al., 2004b; Park et al., 2004) and amphibians (Goleman et al., 2002a; Carr et al., 2003). Such toxicant-induced changes are based upon the physiology of the pituitary-thyroid axis. For example, inhibition of THs secretion abrogates the negative feed-back on TSH secretion, leading to an excess of this hormone (Eales and Brown, 1993). The resultant prolonged stimulation of thyroid by TSH results in follicular hypertrophy – an increase of the thyrocyte size - and hyperplasia – a proliferation of the epithelial cells. Also, prolonged inhibition of thyroglobulin synthesis may lead to a decrease in the amount of follicular colloid (colloid depletion) (Eales and Brown, 1993; Brown et al., 2004a). Histologically, these effects are evidenced by smaller follicle lumens and colloid, an increased follicle epithelial cell height, and increased number of hyperplastic follicles. Patiño et al. (2003) and Bradford et al. (2005) found that growth of capillaries into the follicles ("angiogenesis") was also an indicator of perchlorate-induced thyroidal perturbation.

By using such histopathological indices, it has been previously demonstrated that perchlorate can disrupt thyroid function in laboratory fish (Bradford et al., 2005, Patiño et al., 2003), but such effects have not yet been demonstrated in perchlorate-exposed fish in the field. Because of the involvement of thyroid hormone in vital physiological processes and fitness components, thyroid endocrine disruption could ultimately be manifested at the population, community or ecosystem level of biological organization. Consequently, determining the effects of perchlorate on thyroid function in field populations would be of principle importance in determining the ecological risk of perchlorate contamination. Thus, the objective of this study is to determine the extent of disruption of the thyroid hormone axis – via thyroid histopathology – in fishes and amphibians inhabiting perchlorate-contaminated waters. The hypotheses are that fishes and amphibians in contaminated waters would show a greater level of perchlorate-induced effects, and that these effects would be correlated with the degree of environmental perchlorate contamination.

Methods and materials

Study site and fish collection

The source of perchlorate contamination in this study was the Naval Weapons Industrial Reserve Plant McGregor (NWIRP), a facility located near McGregor, Texas, located southwest of Waco, TX (Fig. 1.). From 1952 to 1995 (upon its closure), jet-assisted take-off rocket motors and solid-fuel rocket motors were produced at the site. Perchlorate has been detected at multiple locations in the streams originating from NWIRP since 1998 (USACE, 2004). Presumably, the rocket motor production activities were the source of the contamination.

Adult female central stonerollers (*Campostoma anomalum;* 2.1–4.8 g) were collected from Harris Creek, Station Creek, and the South Bosque River (known to be contaminated with perchlorate) and Wasp Creek and Coryell Creek (chosen as reference sites; Fig. 1) in October 2001 and March 2002 with a backpack electroshocking device. The Harris Creek (H1) and Coryell Creek sites were just west of McGregor, TX; the South Bosque River Site is just south of McGregor (SB1), and the Wasp Creek site was just south of Crawford, TX. No stonerollers were found in the South Bosque River in October 2001 or at Harris Creek in March 2002. Fish were anesthetized on site, and the heads were preserved in Bouin's fixative for histological analysis. The sample sizes are as follows: for October – Harris Creek $n = 8$; Wasp Creek $n = 28$, Coryell Creek $n = 10$, Station Creek $n = 29$; for March – South Bosque $n = 12$, for all others, $n = 15$.

Adult male and female cricket frogs (*Acris crepitans*) were collected by hand in the vicinity of nearby streams at several sites between June 2001 and 2003. Frogs were rapidly anesthetized in MS-222 (buffered with $NaHCO_3^-$), the abdominal cavity opened, and whole bodies preserved in 10% neutral-buffered formalin for subsequent histology. The collection sites used for this analysis were
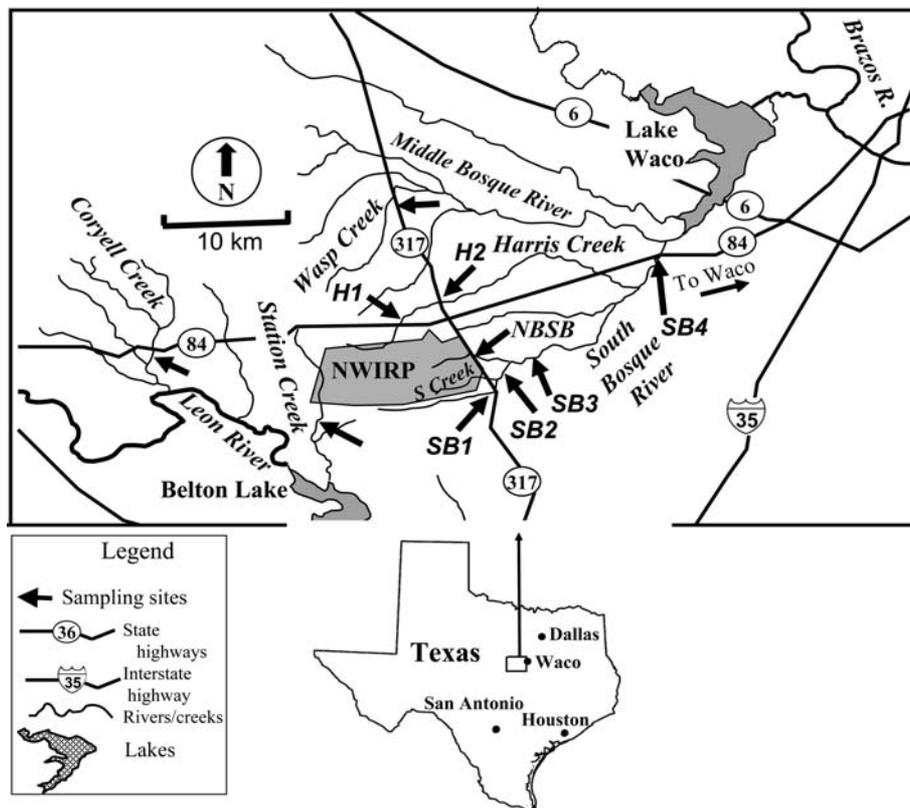

Figure 1. Map of streams in east-central Texas where stonerollers were collected.

an unnamed tributary of the South Bosque River, referred to by the authors as ''North Branch of the South Bosque (NBSB)'', the South Bosque east of NWIRP where it crosses highway 84 (SB4), Harris Creek just north of McGregor, TX, where it crosses highway 317 (H2), and three sites along the South Bosque River south and southeast of NWIRP: (1) where the South Bosque contacts Highway 317, 4 miles south of Hwy 84 (SB1), and immediately (2) west (SB2) and (3) east (SB3) of the confluence of the South Bosque River and the NBSB tributary (Fig. 1). Sample sizes are as follows: NBSB, $n = 14$; H2, $n = 7$; SB1, $n = 32$; SB2, $n = 3$; SB3, $n = 5$; SB4, $n = 25$.

Sample preparation and histological analysis

Fish heads were left in Bouin's fixative for 2 d to decalcify and fix tissues, after which the fixative was removed by rinsing in water and soaking in 70% ethanol. Tissues were processed with the Tissue-Tek V.I.P. 2000 Processor (Miles Laboratories, Elkhart, IN) and then embedded in paraffin. Sections were cut with a microtome (5 μm for fish, 7 μm for frogs) and mounted on microscope slides. The slides were then stained with hematoxylin and Eosin Y for observation and observed with a light microscope.

Individual sections of the preserved fish specimens were randomly chosen based on the overall quality (whole follicles, no folds or tears) of the section. For each follicle, epithelial cell height was measured using a micrometer (a total of 10 follicles measured from two different sections). For each fish, the percent hyperplastic follicles and follicles with depleted colloid were also noted. Follicles that were totally and partially depleted of colloid, and their sum, and the presence or absence of angiogenic follicles (capillaries in the follicle, interior to the basement membrane) were recorded for October 2001 fish. Percent fish with angiogenic follicles was calculated on a per site (population) basis. Because of limitations on time and manpower available for the analysis, only the total number of depleted follicles (both partial and total depletion) was measured and angiogenesis was not recorded in the March 2002 fish.

All frogs were measured for snout-vent length, hindlimb length, total length, and weight. Sex was determined by direct visual inspection of the gonads. The lower jaws of frogs were dehydrated in a graded series of alcohols and processed for routine paraffin embedding. Serial transverse sections (10 μm) through the jaws were mounted on glass slides and stained using Harris's progressive hematoxylin and eosin procedure and coverslips were mounted. The right thyroid was located for each animal and the beginning and end sections were marked. The fifth, middle, and fifth from the last sections were examined for colloid depletion, follicular cell hypertrophy, and follicular cell hyperplasia using a semiquantitative scale as described by Carr et al. (2003).

Collection of environmental matrices for perchlorate determination

At each location where fish or frogs were captured, at least two water samples were collected in precleaned glass vials (Wheaton) from just under the water surface. Water samples were kept at 4 °C until analysis. A subsample of fish were frozen whole in liquid nitrogen and kept at −20 °C until perchlorate analysis. Samples of periphyton (filamentous green algae) were collected by grab sampling. Periphyton represents a possible route of exposure for stonerollers via food. Grab samples of algae were collected at 3 locations per sampling site, with each location at least 10 m apart. Although the species was not identified, it was assumed that the species was the same at all sites, based upon micro- and macroscopic morphology. Periphyton samples were kept at −20 °C until analysis.

At each site where samples were taken for perchlorate analysis, water temperature, dissolved oxygen, pH and conductivity were also measured using a Horiba U-30 multiparameter water quality meter (Horiba, Ltd., Osaka, Japan). Temperature and pH were measured because they may affect thyroid status in fish (Castonguay and Cyr, 1998; Brown et al., 2004a). Thyroid hormones may also play a part in osmoregulatory balance in fish (Peter et al., 2000; Aas-Hansen et al., 2003), so ionic composition of the water could affect thyroid hormone status as well. In this regard, specific conductivity was taken as a covariate of total ionic composition of the water. Finally, thyroid hormones influence oxidative metabolism in fish (Peter

and Oommen, 1993), so it is possible that dissolved oxygen content of the water could additionally affect thyroid homeostasis. Thus, these environmental variables were identified as possible confounding factors influencing the responses of the fish thyroid histopathology in these streams.

Perchlorate determination

Samples were extracted and analyzed according to Smith et al. (2001) and Anderson and Wu (2002). Water samples were filtered (0.45 μm) and analyzed for perchlorate directly. Algae and fish tissue samples were weighed, rinsed with ultra-pure water, air-dried and extracted with distilled, deionized water using an Accelerated Solvent Extractor (ASE 200, Dionex Corp., Sunnyvale, CA). Analysis of perchlorate ion was carried out using a Dionex DX-500 Ion Chromatography System equipped with a GP50 gradient pump, a CD20 conductivity detector, and an AS40 automated sampler (Dionex Corp.). An eight-point standard curve was constructed from constant volume injections of calibration standards of 2.5, 5, 10, 20, 50, 100, 200, 500 ppb (ng/ml). Computer-generated peak areas were used to measure sample concentrations in an external standard mode.

Statistical analyses

Non-parametric tests were conducted on the data due to the non-normality and heteroscedascity of the data. In cases where an appropriate non-parametric test was not available (i.e., 2-way ANOVA and multivariate statistics), the parametric tests were performed on rank-transformed data.

Comparisons among sites for fish

In order to test for differences among sites, the Kruskall–Wallis test was used for individual variates. MANOVA was performed on ranked multivariate data, and multiple comparisons were conducted using pair-wise multivariate t-tests with Bonferoni correction. In order to visualize relative differences for the multivariate datasets, discriminant analysis and cluster analysis were performed on the thyroid data. For discriminant analysis, the data were plotted against the first two discriminant axes, and the distances between the group centroids on these plots were calculated. For the cluster analysis, cophenetic correlation was performed to assess fit of the dendrogram with the actual data matrix.

Comparisons between seasons for fish

Differences between samples collected in October 2001 and March 2002 for each variable were calculated using Wilcoxon Rank–Sum tests. These differences were tests only for those populations for which data was collected in both seasons (i.e., Station Creek, Coryell Creek, and Wasp Creek). Differences between October 2001 and March 2002 (Δ = March variable–October variable), as well as their confidence intervals, were estimated for each variable using the Hodges–Lehman statistic (Δ; Hollander and Wolfe, 1973). Statistically significant differences in Δ among sites were assumed when the 95% CI of the respective Δs did not overlap. Multivariate tests of the differences between October and March were also calculated by ranking the data and using a multivariate t-test for each site.

Combinations of season and site differences were analyzed by a variety of methodologies. First, differences among sites and seasons, and their interaction term, were tested using 2-way ANOVA and 2-way MANOVA, with location and season as the factors. Second, discriminant and cluster analysis were run using each combination of site and season as a factor. For discriminant analysis, Euclidian distances between October and March group centroids for each site were used as a relative indication of the degree of seasonal differences. Cluster analysis was used to visualize relative similarity among the season-site combinations.

Correlation between histopathological responses and perchlorate concentrations for fishes and frogs

Non-parametric rank correlations were calculated between indices of exposure (concentrations in water, fish, and periphyton) and the medians of each thyroidal parameter for each population. Because perchlorate concentrations in the water can be quite variable over time, and because thyroidal responses to perchlorate may require chronic exposures in order to become manifested, average water concentrations measured during 2001–2002 (for fishes) or 2001–2003 (for frogs) were calculated. Correlation analysis was then performed both with average water concentrations

and with water concentrations measured at the time of fish sample collection. In order to perform correlations: between exposure variables and a composite index for all thyroid metrics, principal component analysis (PCA) was performed on the ranks of all thyroid variables, and the first principal component score was used as the composite thyroid index. To determine if there was any association between thyroidal metrics and confounding environmental variables, correlation between these two sets of variables was performed as well.

Results

Perchlorate exposure and stonerollers

Perchlorate analysis and water quality
The results for perchlorate analysis are reported in Table 1. For the analysis of samples from October, Harris Creek had the highest perchlorate concentration in the water, while Station Creek had the highest concentration in the fish. Harris and Station Creeks had similar levels of perchlorate. For the analysis of samples from March, Station Creek had the highest concentrations of perchlorate in water and algae. Concentrations in fish were similar for both Wasp

Creek and Station Creek. For the water concentrations averaged over 2001–2002, Station Creek had the highest concentrations of perchlorate, followed by Harris Creek and South Bosque River (Table 1). Examination of water concentrations of perchlorate over time indicates that perchlorate concentrations can be temporally variable (Fig. 2). Surprisingly, perchlorate was detected in Wasp Creek fish and periphyton; based on preliminary analysis of water, and the fact that Wasp Creek does not originate on the TNWIRP grounds, it was assumed *a priori* that Wasp Creek was a reference site.

Water quality parameters ("confounding environmental variables) are reported in Table 2. In general, these parameters are similar among sites and between seasons. The greatest variation among sites occurs for temperature in October and both temperature and dissolved oxygen in March.

Comparisons among sites

October. An illustration of the types of histological effects is depicted in Fig. 3. The percentage of follicles with partially and totally depleted colloid, and their sum, is depicted in Fig. 4. Station Creek had the highest value for partially depleted colloid, while Harris Creek

Table 1. Perchlorate concentrations[a] in water and fish collected from streams in and around TNWIRP in October 2001 and March 2002

| Sampling site | Matrix | | | |
	Water average[b]	Water	Fish[c]	Periphyton[c]
A. October 2001				
Coryell Creek	ND[d]	ND[d]	ND[e] ($n = 1$)[f]	ND[d]
Wasp Creek	ND[d]	ND[d]	ND[e] ($n = 3$)[f]	ND[d]
Harris Creek	11.31 ± 11.4	28.76 ± 4.38	ND[e] ($n = 3$)[f]	202 ± 78
Station Creek	23.09 ± 33.7	23.61 ± 0.89	79 ± 136 ($n = 7$)[f]	234 ± 59
South Bosque R.	1.45 ± 3.8	10.78 ± 9.34	–	316 ± 24
B. March 2002				
Coryell Creek	ND[d]	ND[d]	ND[e] ($n = 5$)[f]	ND[d]
Wasp Creek	ND[d]	ND[d]	637.4 ± 888.7 ($n = 5$)[f]	10 ± 7.0
Harris Creek	11.31 ± 11.4	24.23 ± 0.05	–	–
Station Creek	23.09 ± 33.7	70.23 ± 7.17	630.6 1410.1 ($n = 5$)[f]	25.81 ± 12.4
South Bosque R.	1.45 ± 3.8	ND[c]	ND[e] ($n = 5$)[f]	ND[d]

[a]Mean ± standard deviation. Concentration expressed as µg/l (perchlorate ion) for water and ng/g for tissues. "–" = not collected.
[b]Averaged over all measurements taken from 2001–2002. Data were taken from Anderson et al. (2004).
[c]Concentration expressed as µg/kg, dry weight basis.
[d]Not detected; detection limit = 1 µg/l
[e]Not detected; detection limit = 0.125 µg perchlorate per sample.
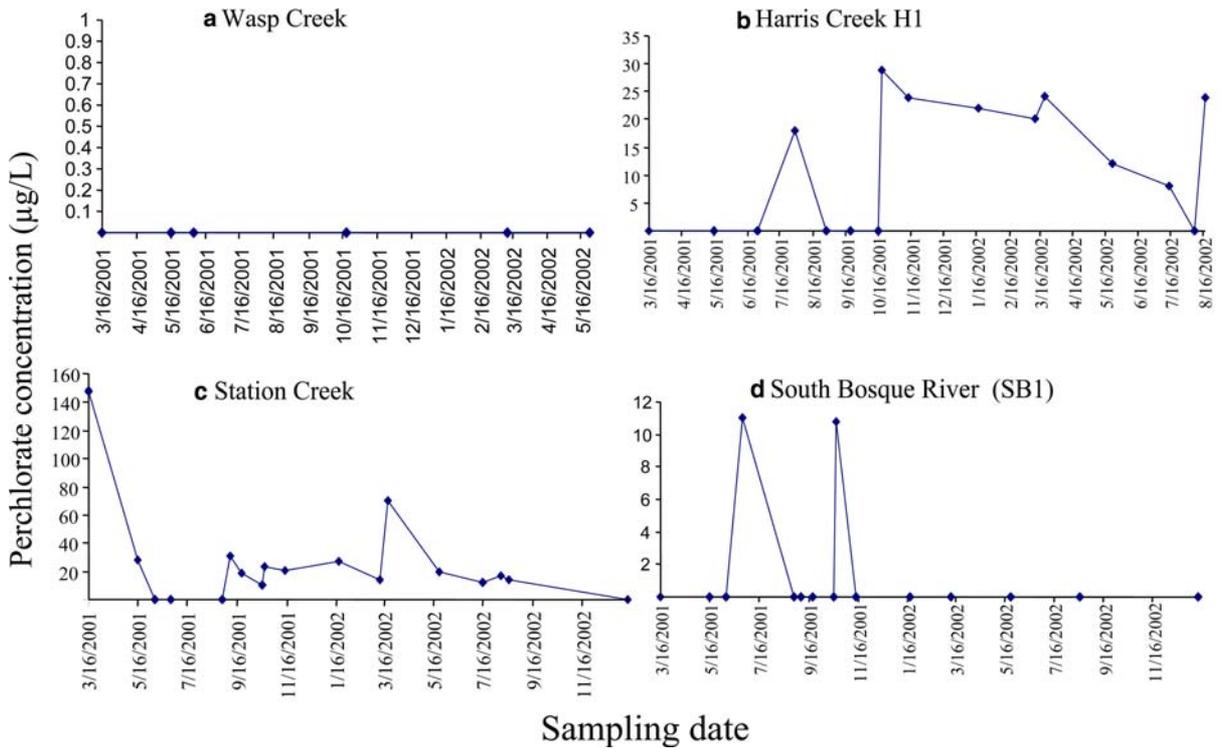[f]n = number of composite samples collected; composite samples composed of ≥5 fish.

Figure 2. Water concentrations of perchlorate during 2001–2002 in four streams in east-central Texas from which stonerollers were collected for histological analysis of thyroid tissues. Data were taken from Anderson et al. (2004).

Table 2. Physiochemical parameters for five streams from which stonerollers were collected in October 2001 and March 2002

Location	Temp. (°C)	Dissolved Oxygen (mg/l)	pH	Conductivity (µS)
A. October 2001				
South Bosque River	17.6	7.6	7.54	49
Coryell Creek	18.7	7.3	7.50	68
Wasp Creek	16.6	6.1	7.45	46
Station Creek	16.9	6.9	7.74	35
Harris Creek	20.0	8.0	7.46	49
B. March 2002				
South Bosque River	19.0	9.3	7.15	63
Coryell Creek	17.8	8.1	6.94	46
Wasp Creek	18.7	7.6	7.50	47
Station Creek	18.6	8.7	7.23	55
Harris Creek	21.1	11.0	7.09	56

Creeks had the highest values. Harris Creek also had the highest value for epithelial cell height, followed by Wasp, Station, and Coryell Creek, in that order (Fig. 4). MANOVA analysis indicated that there were differences between all sites (Table 3). Cluster analysis indicated that Wasp Creek clustered with Coryell, and Station clustered with Wasp Creek (Fig. 5a). The cophenetic correlation was high, indicating a good fit of the dendrogram with the data. The distance between the group centroids for each site and Coryell Creek (the least impacted site) – as calculated by discriminant analysis – was in the order of Station Creek > Harris Creek > Wasp Creek (Fig 6a).

March. Wasp Creek, Station Creek, and South Bosque River all had a higher percentage of follicles with depleted colloid (both partial and total; Fig. 7). There were no differences among sites in terms of percent occurrence of hyper-
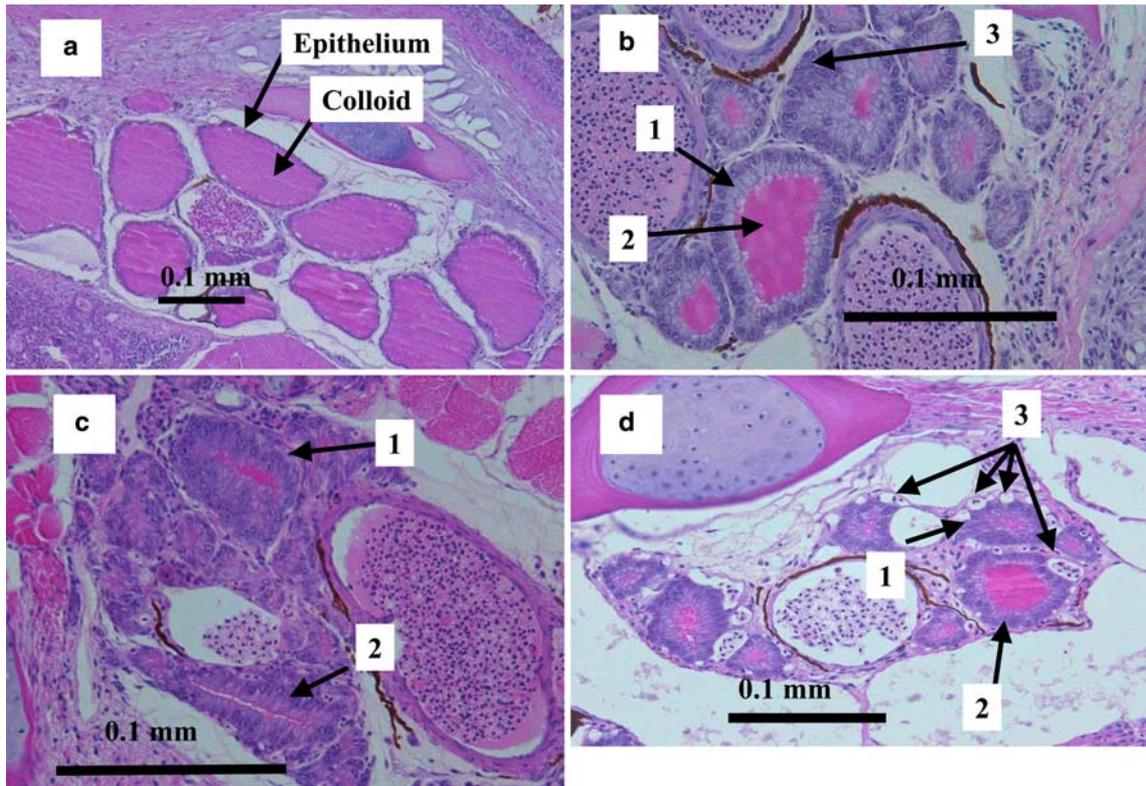
had the highest value for totally depleted and the sum. In terms of percent occurrence of hyperplasia and angiogenesis, Station and Wasp

Figure 3. Histological sections illustrating effects of perchlorate on thyroid follicles. (a) Follicles from a non-impacted fish, with simple squamous follicular epithelia; (b) 1 – hypertrophy (increased cell height; columnar epithelium), 2 – mild colloid depletion (increased vacuoles and scalloping around the colloid margin), 3 – follicles cut through the follicle wall: caution should be used not to score these as depleted follicles. When this type of cut is made, transversely cut cell outlines form a characteristic ''honeycomb'' pattern; (c) 1 – Severe colloid depletion with hyperplasia, 2 – colloid depletion with follicle collapse and hypertrophy; (d) 1 – Follicular epithelium hypertrophy and hyperplasia , 2 – angiogenesis (capillary infiltration inside the follicle basement membrane).

plasia. The values for epithelial cell height for Wasp and Station Creeks were lower than for Coryell Creek and South Bosque River (Fig. 7). MANOVA analysis indicated that there were significant differences between all sites except Wasp and Station Creeks (Table 3). Cluster analysis indicated that Station clustered with Wasp Creek, South Bosque clustered with the Station/Wasp cluster, and Coryell was the outgroup (Fig. 5b). The cophenetic correlation was high, indicating a good fit of the dendrogram with the data. The distance between the group centroids for each site and Coryell Creek (the least impacted site) – as calculated by discriminant analysis – was in the order of Station Creek > Wasp Creek > South Bosque > Coryell (Fig. 6b).

Comparisons between seasons
The percent occurrence of hyperplastic follicles and follicle depletion decreased in March relative to October, although this decrease was only significant for Coryell and Wasp Creeks ($p < 0.05$, Wilcoxon Rank–Sum Statistic, Fig. 8). The absolute difference (Hodges–Lehman Δ) between October and March hyperplasia values was significantly different between Coryell and Station Creeks, but not between Wasp and Coryell Creeks (as judged by overlap of 95% confidence intervals; Fig. 8). For colloid depletion, there were significant differences between Wasp Creek and the other two creeks, but not between Station and Coryell Creeks. There were increases in epithelial cell height in March compared to October, and these differences were statistically significant for all three sites
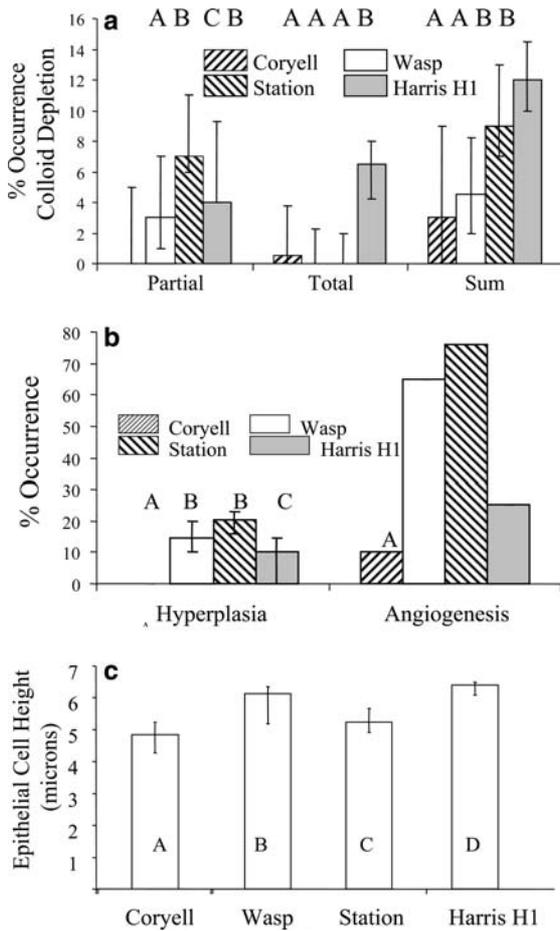
Figure 4. Percent thyroid follicles that exhibited colloid depletion (partially depleted, totally depleted, and their sum) (a), percent thyroid follicles with hyperplastic epithelia and percent individuals exhibiting thyroid follicular angiogenesis (capillaries growing within the follicle) (b), and thyroid follicular cell height (c) in stonerollers collected in October 2001 from perchlorate contaminated streams in east-central Texas. Bars are medians and error bars are first and third quartiles. Bars labeled with different letters are statistically significantly different ($p < 0.05$, Kruskall–Wallis test).

($p > 0,05$, Wilcoxon Rank–Sum Statistic; Fig. 8). The magnitude of the difference was significantly different between all three sites (lack of overlap of Hodges–Lehman Δ 95% CI), and the magnitude of the difference increased in the order of Coryell Creek > Wasp Creek > Station Creek (Fig. 8). When the relative % change was calculated ([October–March]/October), the results were generally the same as for the absolute differences, only that there were no differences among the three sites for hyperplasia (overlap of the 95% CI; Fig. 8).

Table 3. Results from 1-way and 2-way MANOVA of ranked thyroid histological data for stonerollers from perchlorate-contaminated streams in October 2001 and March 2002

Test	Wilk's Λ[a]	Prob.[b]	Non-significant differences[c]
1-Way October	0.390	< 0.001	None
1-Way March	0.524	0.001	Wasp–Station
2-Way, Factor = Location	0.695	< 0.001	–
2-Way, Factor = Seasons	0.287	< 0.001	–
2-Way Location*Season	0.535	< 0.001	–

[a] A multivariate analog of an F-statistic calculated from the eigenvalues of the data matrices.
[b] Probability of Type I error: H_o = no differences among factors (sampling sites and/or seasons).
[c] Sites between which there was no statistically significant differences, $p > 0.05$; pair-wise multivariate t-tests with Bonferoni correction. i.e., None = all sites were statistically significantly different; Wasp–Station = all sites except Wasp and Station Creeks were statistically significantly different.

The results from both univariate 2-way ANOVA indicated that there were no effects of location for hyperplasia, but there were statistically significant effects for all other factors and variables and the interaction terms (Table 4). The results for 2-way MANOVA indicated that there were significant differences for both location and season and the interaction term. For both univariate and multivariate 2-way ANOVA, multiple comparisons could not be done because of the significant interaction term. Multivariate t-tests indicated that there were differences between October and March for all sites ($p < 0.05$). Plots of group centroids on discriminant function axes indicated that (1) the amount of difference between group centroids in October versus March increased in the order of Coryell Creek < Station Creek < Wasp Creek, and (2) in agreement with the cluster analysis, the Wasp Creek centroid is closer to Coryell Creek in October (when no perchlorate was detected in Wasp Creek fish), and closer to Station Creek in March (when perchlorate was detected in Wasp Creek fish; Fig. 6c).

Correlation between histopathological responses and perchlorate concentrations

October. There were significant univariate correlations between the following variables: colloid depletion and recent (October) water concentrations
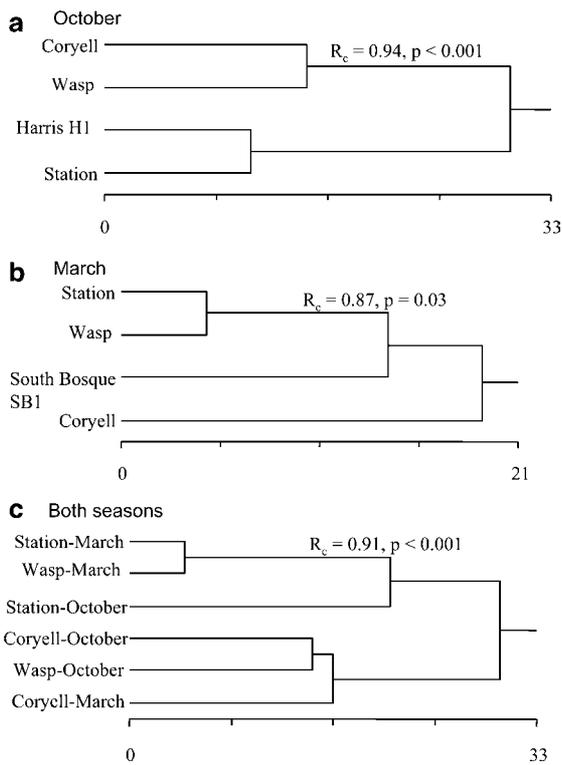
Figure 5. Cluster analysis using the thyroid follicle histopathological variates percent hyperplastic follicles, percent follicles with colloid depletion, thyroid epithelial cell height, and percent individuals exhibiting angiogenic follicles (not used for b and c) in stonerollers collected from perchlorate contaminated streams in east-central Texas in October 2001 (a), March 2002 (b), or stream-season combinations (c). R = cophenetic correlation, p = significance of cophenetic correlation.

of perchlorate; hyperplastic follicles and perchlorate fish body burdens; epithelial cell height and both average (2001–2002) water concentrations and algae; and PCA scores and recent water concentrations of perchlorate ($p < 0.05$, Spearman rank correlation; Table 5). There were no significant correlations between any thyroid metric and any confounding environmental variable.

March. There were significant correlations between the following variables: colloid depletion and recent (March) water perchlorate concentrations, fish perchlorate body burdens, and periphyton perchlorate concentrations; hyperplastic follicles and perchlorate water and periphyton concentrations; epithelial cell height and recent (March) water perchlorate concentrations, fish

perchlorate body burdens, and periphyton perchlorate concentrations; and PCA scores and concentrations of perchlorate recent water and algae samples ($p < 0.05$, Spearman rank correlation; Table 5). There were no significant correlations between any thyroid metric and any confounding environmental variable.

Perchlorate exposure and frogs

Perchlorate concentrations from surface waters were averaged from individual measurements performed between March 2001 and June 2003. Average perchlorate concentrations in surface water were statistically greater at site NBSB than all other sites (Fig. 9). Average perchlorate concentrations at site H1 were greater than SB1 and SB4. The rank order for perchlorate concentrations at the study sites was NBSB > H1 > SB1 > SB4 > SB2 > SB3. There was no evidence of colloid depletion or follicle cell hyperplasia in any of the 86 animals studied. There was evidence for moderate follicle cell hypertrophy in animals from NBSB and H1, the two sites with the greatest measured perchlorate concentrations. Follicle cell hypertrophy was statistically greater in NBSB animals than animals from all other sites (Fig. 9). Follicle cell hypertrophy was greater in animals from H1 than in animals from SB4 and SB1. The rank order for follicle cell hypertrophy based on collection sites was NBSB > H1 > SB2 > SB4 > SB1 > SB3. These findings suggested an association between follicle cell hypertrophy and average water perchlorate concentrations at the study sites. Hypertrophy data were plotted against mean water perchlorate concentration and analyzed by Spearman's rank correlation. There was a significant positive correlation ($p < 0.0001$) between hypertrophy score and average perchlorate concentration in surface water (Fig. 9), although the relationship explained only 36% of the variation in thyroid hypertrophy score amongst the animals studied.

Discussion

Comparisons among sites

Overall, the results presented herein support the hypothesis that fishes and amphibians living in or
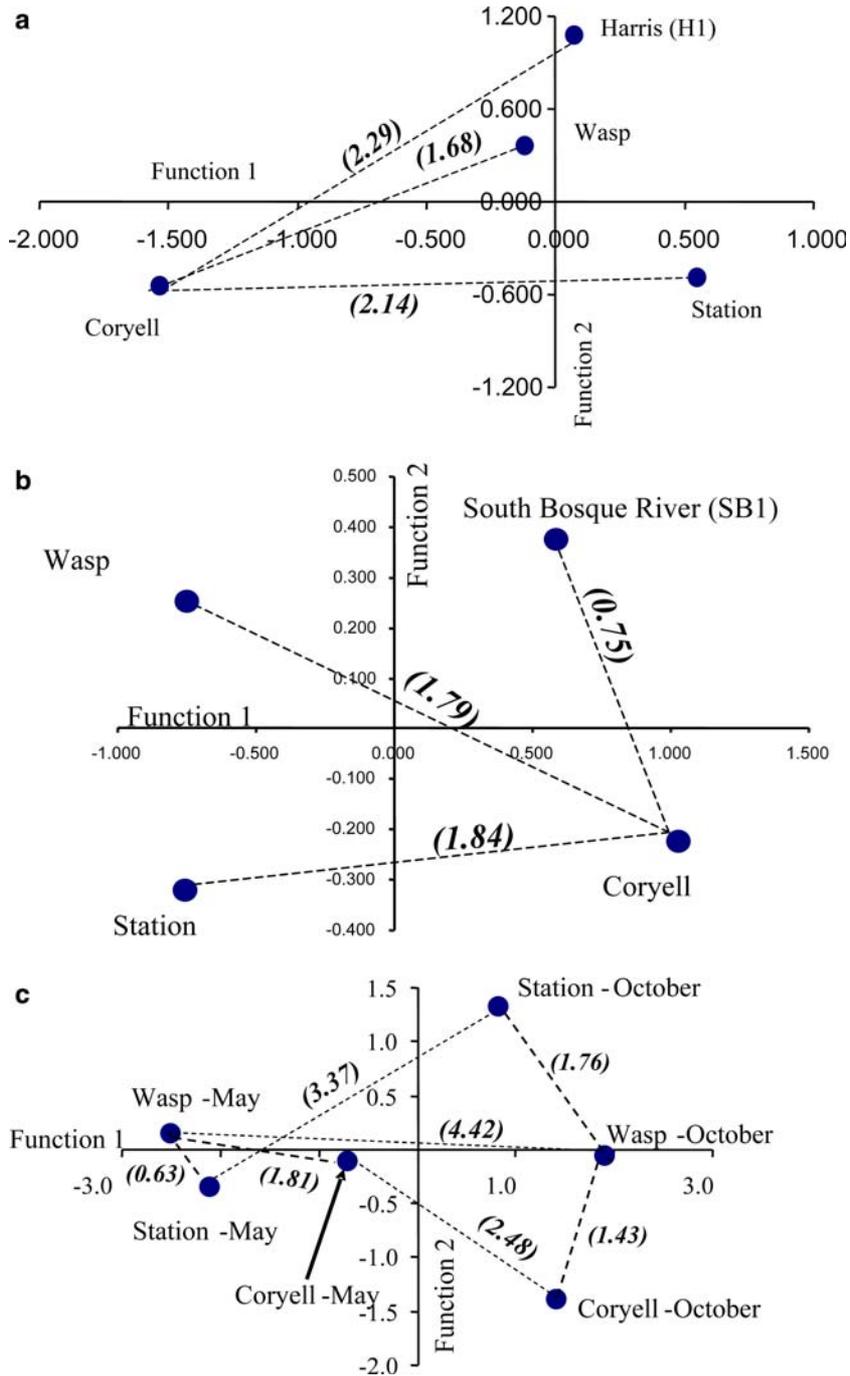
Figure 6. Group centroids using the first two discriminant functions generated by discriminant analysis using the thyroid follicle histopathological variates percent hyperplastic follicles, percent follicles with colloid depletion, thyroid epithelial cell height, and percent individuals exhibiting angiogenic follicles (not used for b and c) in stonerollers collected from perchlorate contaminated streams in east-central Texas in October 2001 (a), March 2002 (b), or stream-season combinations (c). Dotted lines and values in parentheses represent Euclidean distances (calculated from discriminant coordinates) between group centroids as indicated.
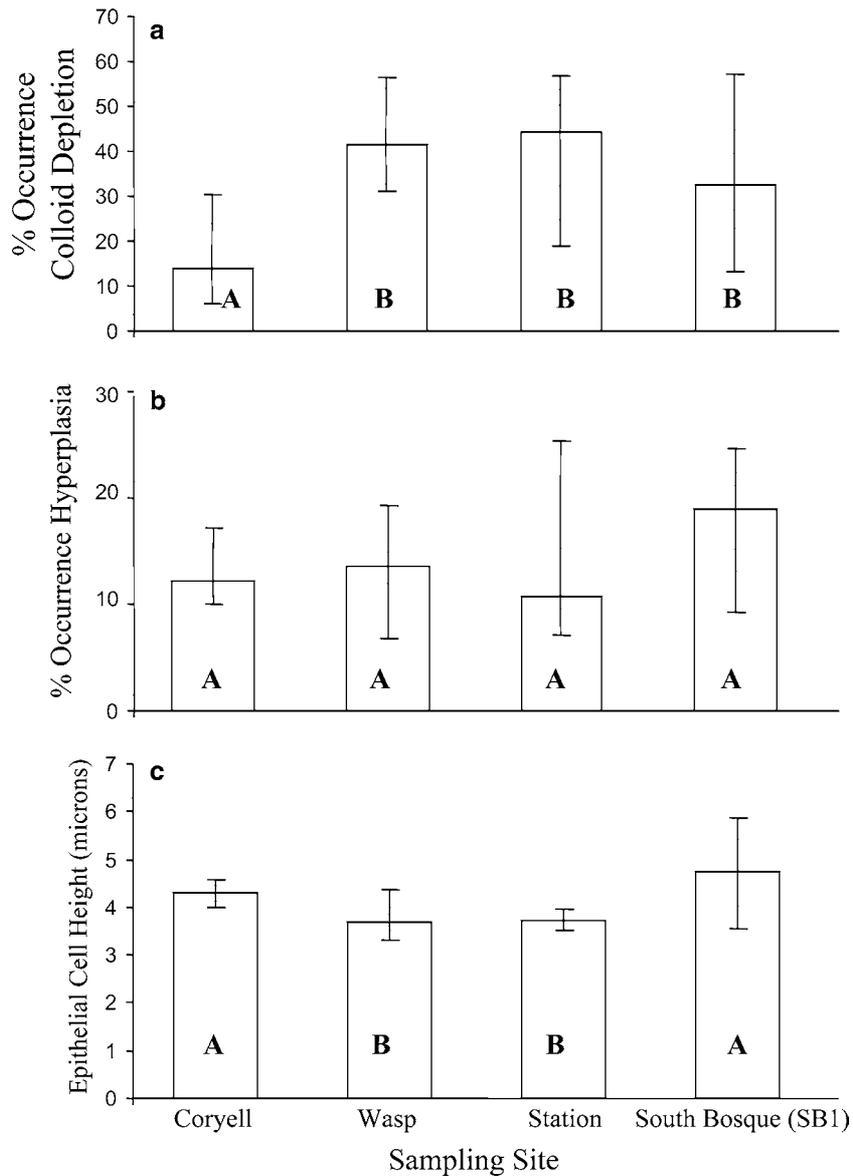
Figure 7. Percent thyroid follicles that exhibited colloid depletion (a), percent thyroid follicles with hyperplasic epithelia (b), and thyroid follicular cell height (c) in stonerollers collected in March 2002 from perchlorate contaminated streams in east-central Texas. Bars are medians and error bars are first and third quartiles. Bars labeled with different letters are statistically significantly different ($p < 0.05$, Kruskall–Wallis test).

nearby streams contaminated with perchlorate will show evidence of perturbations of thyroid function. The fact that similar patterns were seen for stonerollers in both October and March sampling periods argues against differences between sites being solely due to natural cyclical variations in perchlorate histopathology. To our knowledge,

this is the first reported instance of perchlorate inducing thyroidal perturbations in fish exposed to perchlorate in field situations. These results are in accordance with other studies that have found thyroid histopathological effects in fish exposed to thyrotoxic compounds (other than perchlorate) under field conditions (Sonstegard and Leatherland, 1984;
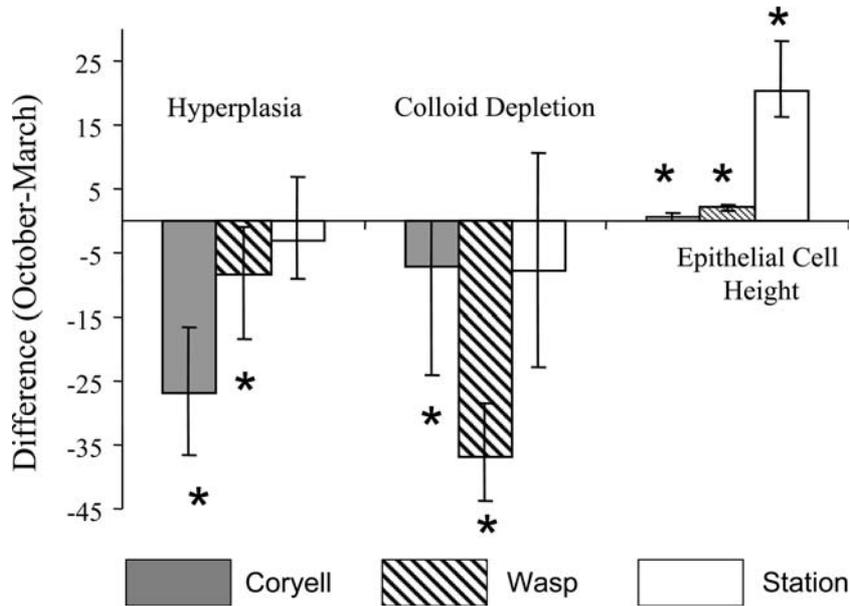
Figure 8. Estimated amount of difference between October 2001 and March 2002 values for colloid depletion, percent thyroid follicles with hyperplastic epithelia, and thyroid follicular cell height in stonerollers collected in March 2002 from perchlorate contaminated streams in east-central Texas. Bars are Hodges–Lehman estimates of the difference between medians and error bars are 95% confidence intervals. Asterisks indicate statistically significant differences between sites (i.e., 95% confidence intervals do not overlap).

Table 4. Results from 2-way ANOVA of ranked thyroid histological data for stonerollers from perchlorate-contaminated streams in October 2001 and March 2002

Variable	Factor	F	Prob.
Hyperplasia	Location	1.640	0.20
	Season	26.735	< 0.01
	Location*Season	9.633	< 0.01
Colloid depletion	Location	17.253	< 0.01
	Season	34.566	< 0.01
	Location*Season	11.761	< 0.01
Epithelial cell height	Location	1.693	0.02
	Season	94.392	< 0.01
	Location*Season	8.878	< 0.01

Zhou et al., 1999a, b; Nugegoda et al., 2000), and with laboratory studies of Bradford et al. (2005), Patiño et al. (2003), and Crouch (2003), who have found that environmentally relevant concentrations of perchlorate cause histopathological changes in fish thyroidal tissue.

Perhaps the most surprising outcome was that fish from Wasp creek showed evidence of thyroid disruption. This was wholly unexpected based upon preliminary results (taken in late 2000 and early 2001) that did not detect perchlorate in surface waters in Wasp Creek (data not shown), and the fact that Wasp Creek did not originate on the NWIRP grounds. However, subsequent analysis of other environmental matrices have also found perchlorate in green sunfish (*Lepomis cyanellus*), periphyton, detritus, invertebrates, and water from Wasp Creek (Anderson et al., 2004, and unpublished data), providing additional evidence that Wasp Creek is contaminated. Although the source of the perchlorate in this creek is unknown, other analyses found perchlorate in the groundwater around NWIRP (USACE, 2004), and there has been at least one documented case of a stream on the TNWIRP grounds being contaminated by groundwater from a spring (Anderson et al., 2004). Thus, it could be that the contaminated groundwater extends outwards toward Wasp Creek, and that this could be the source of the perchlorate contamination.

When comparing the various sites, all of the thyroid metrics for stonerollers do not show the same trends with regard to inter-population variation, e.g., for trends in epithelial cell heights, hyperplasia, and colloid depletion in October

Table 5. Results from correlation analysis[a] between median thyroid variate (dependant variable) and four measures of perchlorate exposure[b] for stonerollers collected from perchlorate contaminated streams in October 2001 and March 2002

	Histological variable									
	Colloid depletion		Percent hyper-plasic follicles		Epithelial cell height		Occurrence of angiogenesis[c]		PCS[d]	
Perchlorate variable	R	p	R	p	R	p	R	p	R	I
October										
Water average	0.717	0.167	0.000	0.542	0.956	0.042	0.598	0.208	0.717	0.16
Water recent	0.956	0.042	−0.239	0.375	0.717	0.167	0.120	0.458	0.956	0.04
Stoneroller	−0.239	0.625	0.956	0.042	0.120	0.458	0.956	0.042	−0.239	0.625
Periphyton	0.717	0.167	0.000	0.542	0.956	0.042	0.598	0.208	0.717	0.16
March										
Water average	0.598	0.208	−0.478	0.208	0.120	0.542	–	–	0.598	0.208
Water recent	0.956	0.042	−0.837	0.042	−0.956	0.042	–	–	0.956	0.042
Stoneroller	0.956	0.042	0.717	0.167	−0.956	0.042	–	–	−0.357	0.375
Periphyton	0.956	0.042	−0.837	0.042	−0.956	0.042	–	–	0.956	0.042

[a]Spearman rank correlation between median thyroid variate (determined histologically) for each of 5 stoneroller populations and average perchlorate concentrations in 4 different matrices. R = correlation coefficient, p = probability of type I error.
[b]Measures include: water average = perchlorate water concentrations averaged over all measurements taken from 2001 to 2002; water recent = perchlorate concentrations in water collected when the fish were collected; stoneroller = perchlorate whole body burden in stonerollers collected from each site; periphyton = perchlorate concentrations in periphyton (filamentous green algae) collected from each site.
[c]Number of individuals that displayed capillaries within the thyroid follicle.
[d]First principal component score (derived from principal components analysis) for all thyroid variables together.s

(Fig. 4). Other metrics do show the same trends, e.g., October hyperplasia and angiogenesis (Fig. 4). The only March metric which was consistently different between Coryell Creek and the contaminated sites is colloid depletion (Fig. 7). Differences in the trends for all these metrics may be explained by the physiology of the thyroid. Angiogenesis (capillaries within the follicle) has been associated with perchlorate-induced thyroid disruption in fish in the laboratory (Bradford, et al., 2005; Patiño et al., 2003), but the mechanism by which this occurs or its physiological consequences remain unknown. Epithelial cell height is increased in response to TSH stimulation. This is a compensatory response to low serum thyroid hormone levels: as thyroid hormone levels drop, the negative feedback on the pituitary is reduced, and TSH levels rise in an attempt to increase serum thyroid hormone levels. Thus, epithelial cell height may be a marker of exposure rather than effect. Depletion of the colloid is an indication of disruption of the balance between thyroid hormone (or iodinated thyroglobulin) synthesis and use. Therefore, colloid depletion is likely a biomarker of effect. Follicular epithelium hyperplasia is though to arise from overstimulation by TSH, again due to low thyroid hormone levels and resultant negative feedback on pituitary TSH secretion. Hyperplasia in general is often regarded as a pathological condition, and this may apply to the thyroid tissues as well. When follicular epithelial hyperplasia occurs, there is often more than one layer of hyperplastic epithelium, so that these cells are no longer in contact with the follicular lumen. Therefore, presumably they are not functional in secreting colloid into the lumen, so this is not an adaptive response. Hyperplasia typically occurs after prolonged stimulation by TSH (Eales and Brown, 1993). In addition, reversal of epithelial cell height, colloid depletion, and hyperplastic effects requires turnover and degradation of intracellular constituents, de novo synthesis and secretion of thyroglobulin, and cell destruction and resorption, respectively, so that the rates of recovery may differ among these endpoints. Thus, relative responses among endpoints would be dependant not only levels of perchlorate in the environment, but also temporal variability in concentrations and clearance of perchlorate from environmental compartments, and rate of recovery.
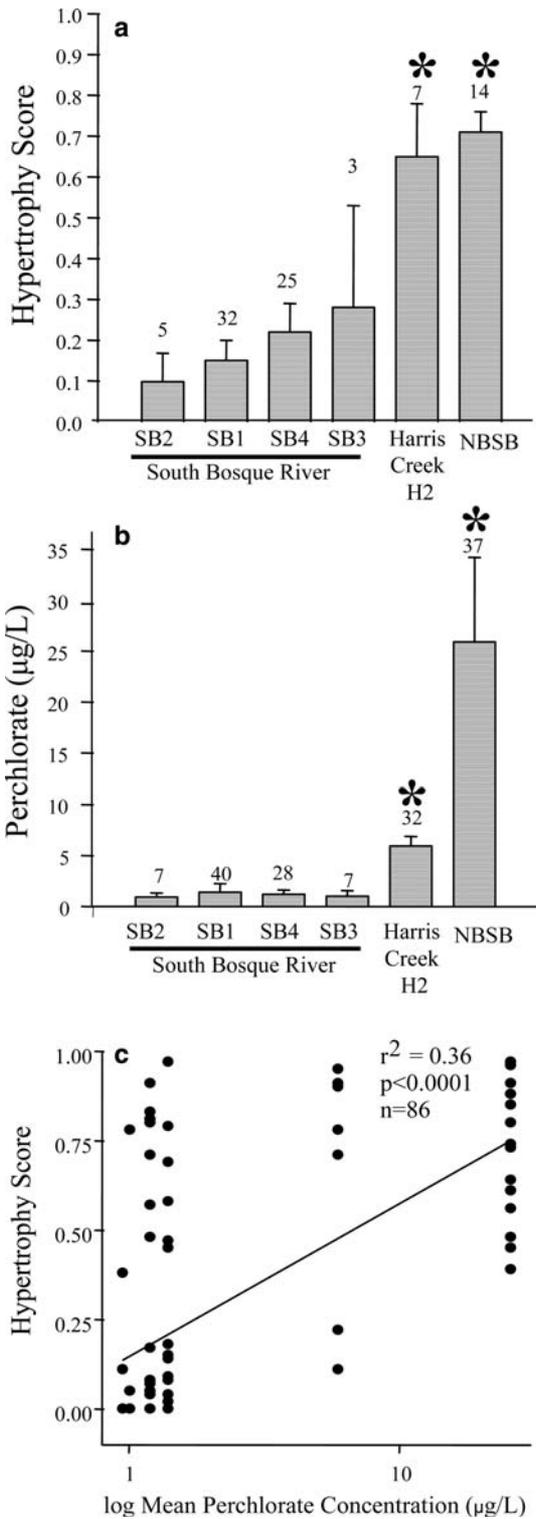
Figure 9. Thyroid follicle cell hypertrophy score (a) and mean water perchlorate concentrations (b) at sites where adult *Acris crepitans* were collected. Bars are the mean ± SEM. Numbers above the bars indicate sample size. Asterisks indicate significant difference based on ANOVA. Site NBSB was located on a tributary of the south Bosque creek on the eastern edge of the Naval Weapons Industrial Reserve Plant McGregor (NWIRP), while site H2 was located on Harris Creek north of the facility near McGregor, TX. Other collection sites were located southeast (SB1, SB2, SB3) and northeast (SB4) of NWIRP. (c) Thyroid hypertrophy score plotted against the log of mean water perchlorate concentration for sites at which *Acris crepitans* adults were collected. Each point represents the thyroid hypertrophy score for a single animal. There was a positive correlation between hypertrophy score and mean water perchlorate concentration based on Spearman's rank correlation.

Three main findings can be seen in terms of the frog responses: (1) The overall rank order of histological response (NFSB > Harris Creek > South Bosque River sites) is in accordance with the degree of perchlorate contamination in these streams. Thus, even though no reference site was sampled for cricket frogs, the decreasing responses with decreasing levels of contamination would argue that the perchlorate is the cause of the histological responses. (2) The rank order of sites within the South Bosque (SB4 > SB2 > SB1 > SB3) can be explained by drainage patterns of contaminated streams into the South Bosque River. The SB4 site is immediately below the confluence of Harris Creek, which is heavily contaminated with perchlorate. The SB2 site is also directly below the confluence of "S Creek" (Fig. 1), although this no stonerollers or cricket frogs were found in this creek when it was sampled, chemical analysis indicates that S Creek was the most heavily contaminated creek in the region, with perchlorate concentrations at least an order of magnitude above what was found in any other creek in th area (frequently in the hundreds of µg/l; Anderson et al., 2004). Thus, input of perchlorate from S Creek may have contributed to increased thyroidal effects of frogs sampled from the South Bosque River immediately below S Creek. (3) The thyroidal effects in Harris Creek were greater than those in the South Bosque River. Although the exact sampling locations for the frogs did not correspond to those for the stonerollers (except for SB1), these findings were in concordance with the

stoneroller thyroidal data, which also showed greater impacts for fish from Harris Creek.

Because different endpoints may exhibit different trends among sites, it would be best to use an integrated response when monitoring for exposure and effects of perchlorate, or any other thyroid disruptor. To this end, multivariate statistics may provide a more holistic and statistically powerful measurement of exposure and effects than would any single metric alone. The multivariate analyses in this manuscript have suggested that the overall level of effect for October was Harris Creek ≥ Station Creek > Wasp Creek > Coryell Creek, and for March it was Station Creek ≥ Wasp Creek > South Bosque River > Coryell Creek, in accordance with degree or occurrence of perchlorate-contaminated media (water, fish, and/or periphyton).

Comparisons between seasons

In general, it seems as though the fish are more susceptible to the effects of thyroid hormone disruptors in the October sampling season than in March. There were also differences between October and March for each of the three parameters, as attested by the Wilcoxon Rank–Sum tests between. Because the temperature, dissolved oxygen, pH, and conductivity were similar between October and March for all sites, this is probably not attributable to differences in water physiochemical parameters. Thus, such differences in thyroid parameters may be affected by other seasonal variates such as photoperiod, nutritional state, or breeding condition (McKenzie et al., 1987, 1989; Pavlidis et al., 1991; Reddy and Leatherland, 2003). In fact, for many fish species, production of thyroid hormones peak at or just before the spawning period. Stonerollers typically spawn from early mid spring to early summer (Burkhead, 1980), so the March sampling period corresponds to the beginning of the spawning or the prespawning period, while October is post spawning. The fact that females in the spawning/prespawning period were less sensitive to the effects of a thyroid endocrine disruptor is in accordance with Park et al. (2004), who found that mosquitofish (*Gambusia affinis*) females in the laboratory were also less sensitive to thyroidal disruption by endosulfan in the spawning period.

In examining clustering of site-season combinations (Fig. 5), it is interesting to note that Wasp Creek clusters with Station Creek (contaminated site) in March, when perchlorate was detected in the fish tissues from Wasp Creek, but clusters with Coryell Creek (reference) in October, when perchlorate was not detected in fish tissues in these fish. This is probably due to contribution from colloid depletion, for which Wasp Creek was not different from Coryell Creek in October, but was in March. This is also concordant with the fact that colloid depletion was positively correlated to fish tissue concentrations in March.

In terms of the other two metrics, epithelial cell height decreased in proportion to the amount of perchlorate present in the environment, while hyperplasia increased in inverse proportion to the amount of perchlorate present (Fig. 8). This argues that the degree of seasonal change is affected by the amount of perchlorate contamination in the environment. This is also concordant with the negative correlations between perchlorate concentrations in environmental matrices and cell height/hyperplasia in March, but positive correlations in October (Table 5).

Correlation between histopathological responses in stonerollers perchlorate concentrations

Generally, there was at least one statistically significant correlation between any index of exposure and at least one of the thyroidal variables. Differences among the various indices of exposure and among thyroidal variables may be due to temporal variability of perchlorate concentrations. From Fig. 2, it can be seen that water concentrations can vary markedly over time. Thus, thyroid metrics that are correlated with recent water concentrations may be more responsive to recent conditions than to long term trends. This is true for thyroid metrics in the fall, and colloid depletion and overall PCA score in the spring. Metrics that are correlated to average water concentrations – such as epithelial cell height – may be more responsive to longer term trends. In addition, perchlorate can be detected in periphyton and fish at levels that are higher than the water. This implies that either (1) perchlorate bioconcentrates in these organisms – which is contrary to laboratory findings (Bradford, et al., 2005), or (2) fish and

periphyton concentrations may be more stable than water concentrations, and reflect longer term trends in perchlorate concentrations that are intermediate. There were significant correlations between concentrations in periphyton and epithelial cell height in October and between periphyton and all thyroid metrics in March. Taken together with the water concentrations, this would suggest that epithelial cell heights in October are responsive to mid- to long-term trends in perchlorate levels, while thyroidal variables in March are responsive to short- to mid-term trends in perchlorate concentrations. The fact that many thyroid variables were correlated with perchlorate concentrations in periphyton suggests that the food chain may be a route of exposure for perchlorate into fish. An alternative is that concentrations in the periphyton are a reflection of average concentrations in the water over time. In either case, for monitoring exposure and effects of perchlorate on herbivorous fish, it would be advantageous to monitor perchlorate levels in algae as well as fish.

Besides perchlorate levels in environmental matrices, it is also possible that thyroid histological variables are influenced by confounding environmental variables such as temperature, pH, dissolved oxygen, and ionic concentrations, but there were no significant correlations to any of the thyroidal variables. This was not the case for perchlorate concentrations. This is in agreement with the hypothesis that it was perchlorate that most affected thyroid histopathology in these streams.

Relationship between follicle cell hypertrophy and perchlorate in frogs

In the laboratory full larval period exposures to concentration as low as 59 µg perchlorate/l result in follicle cell hypertrophy in African clawed frogs, *Xenopus laevis* (Goleman et al., 2002a). Furthermore, tadpoles (*Pseudacris triseriata*) exposed to greater than 9 mg perchlorate/l in native ponds demonstrate colloid depletion and follicle cell hypertrophy. Our data indicate that frogs collected from NBSB and MGRB showed evidence of follicle cell hypertrophy but not colloid depletion of follicle cell hyperplasia. These findings are of interest because both NBSB and MCGR contained the greatest mean perchlorate concentra-

tions of all of the sites studied. Furthermore, linear regression analysis of all of the hypertrophy scores against mean water perchlorate concentration over a 2 year period revealed that mean perchlorate concentrations accounted for only 36% of the variation in hypertrophy score, suggesting that other factors may also be involved. Dioxins and numerous other synthetic contaminants can influence thyroid function (Howdeshell et al., 2002), and the possibility that other contaminants may be involved has not been addressed.

Conclusions

Taken together, the results of this study illustrate several points. The first is that thyroid histopathology can be used as an indicator of perchlorate contamination in natural streams. Second, field studies should always employ two or more reference sites, because one never knows when a presumed reference site is found to be contaminated. Third is the fact that using biomarkers like thyroid histopathology can not only provide indication of effects, but they also can provide more information on exposure and possible contamination than can be gathered by using chemical analysis alone. This was illustrated by the point that biomarker responses indicated perchlorate contamination in Wasp Creek before chemical analysis did. This may be especially pertinent in cases where (1) biomarkers are integrated responses of variations in chemical contamination over time, (2) the levels of the chemical in the environment are highly variable, such that sampling at intervals may miss spikes of detectable levels of chemical, and/or (3) exposure routes are unknown or are different from the environmental matrix being monitored. The fourth major point of this study is that multivariate analysis of several biomarkers may provide a better integrated and more statistically powerful measurement of exposure and effects than measuring single biomarkers or performing separate univariate analyses on multiple biomarkers. Finally, the results present here provide evidence of causal relationships between perchlorate exposure and thyroidal effects.

It has been argued that, in order to establish causality between exposure and effects in field situations, several criteria must be met (Adams,

2003). These criteria may include: (1) strength of association, (2) consistency of association, (3) specificity of association, (4) time order or temporality, (5) biological gradient, (6) experimental evidence and (7) plausibility. The strength of association criterion in the present study was met by statistically significant differences between multiple contaminated sites and Coryell Creek. The consistency of association criterion was met by the finding that the perchlorate contaminated sites were significantly different from Coryell Creek, at least in some cases (most notably Station Creek colloid depletion and PCA scores). In addition, histological effects were greater in the Harris Creek than in the South Bosque River for both the stonerollers and cricket frogs, further satisfying the consistency of association criterion. The finding that there were statistically significant correlations between thyroid variables and not environmental confounding variables argues that the specificity criterion was met. In addition, there are numerous chemicals that can inhibit thyroid activity in vertebrates (Brown et al., 2004a), perchlorate was the only known thyroid-disrupting agent detected in the streams at elevated levels (USACE). This, and the fact that all sampling sites were in a rural environment, indicates that high concentrations of other potentially thyroid-disrupting chemicals were not present in these streams. The time order/temporality criterion specifies that exposure must precede effects and that when the exposure ceases the effects will dissipate. This does not exactly apply in the present case, except perhaps for the case for colloid depletion in Wasp Creek colloid depletion and clustering analysis indicated that Wasp Creek was more similar to Coryell Creek in October 2001, when no perchlorate was detected in the stonerollers, and Wasp Creek was more similar to Station Creek in March 2002, when there was detectable perchlorate in the stonerollers. The biological gradient criterion was met by the finding of statistically significant multivariate, canonical, and multiple univariate correlations between indicators of exposure and effect. The experimental evidence criterion was met because, although experiments with stoneroller minnows have not been done, laboratory exposures using other species such as zebrafish (*Danio rerio*), goldfish (*Carrasius auratus*), mosquitofish, and amphibians such as *Xenopus laevis*, *Bufo arenarum*, and *Hyla versicolor* have indicated that perchlorate salts induce thyroid histopathological effects in fish and frogs (Miranda et al., 1992, 1995; Goleman et al., 2001a, b; Patiño et al., 2003; Sparling et al., 2003; Crouch, 2003; Bradford et al., 2005). The plausibility criterion has been met because perchlorate is a well known thyroid disruptor in vertebrates (Stanbury and Wyngaarden, 1952; York et al., 2001; Goleman, et al., 2001a, b; Manzon et al., 2001; Greer et al., 2002).

Thus, this study provides evidence that environmentally relevant concentrations of perchlorate affect thyroid physiology under field conditions. However, it is still not known if the thyroidal effects seen here are translated into effects on physiology, bioenergetics, fitness parameters, populations, or communities, or if the histopathological effects seen here represent compensatory responses versus manifestations of pathological conditions. Such information is necessary before adequate ecological risk assessments of perchlorate can be completed.

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