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INVESTIGATION OF THE EFFECTS OF PERCHLORATE ON THYROID AND REPRODUCTIVE SYSTEM FUNCTION IN GOLDFISH

A Thesis in

Wildlife and Fisheries Science

by

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With the development of new analytical detection capabilities, perchlorate contamination has been found in ground and surface waters across the United States (U.S.). Perchlorate, as ammonium perchlorate, is an oxidant used in solid rocket fuels hence, sources of contamination generally occur near military test sites and chemical manufacturing plants. The perchlorate anion (ClO4 -) is known to inhibit thyroid function in mammals. In some locations in the U.S., perchlorate has been found at part per million concentrations in surface water inhabited by fish. To test the hypothesis that exposure to environmentally relevant concentrations of perchlorate can impact fish thyroid and reproductive function, adult male and female common goldfish (Carassius auratus) were exposed in a laboratory flow-through exposure system for 30 days to four concentrations of perchlorate ranging from 14 ppb to 31 ppm, along with controls. Exposure was continued to 60 d for an additional set of controls and 31 ppm treatment groups. Gonadosomatic index (GSI), gonad histology, and plasma sex steroids (17β-estradiol testosterone, and 11-ketotestosterone) were examined to assess reproductive function. Thyroid activity was assessed by use of a nonparametric rank-order assessment method developed for fish during this study. Significant increases in head kidney thyroid activity occurred at 1200 ppb and 31 ppm in females and at 31 ppm in males. Pharyngeal thyroid activity increased in both male and female goldfish exposed to 31 ppm perchlorate. Exposure to perchlorate concentrations up to 31 ppm for 30 or 60 d had no effect on GSI. Some statistically significant effects on plasma sex steroid concentrations were observed for both sexes, but the differences were small and concentrations remained within normal ranges previously reported for goldfish in this reproductive state.

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Abstract

With the development of new analytical detection capabilities, perchlorate contamination has been found in ground and surface waters across the United States (U.S.). Perchlorate, as ammonium perchlorate, is an oxidant used in solid rocket fuels; hence, sources of contamination generally occur near military test sites and chemical manufacturing plants. The perchlorate anion (ClO_4^-) is known to inhibit thyroid function in mammals. In some locations in the U.S., perchlorate has been found at part per million concentrations in surface water inhabited by fish. To test the hypothesis that exposure to environmentally relevant concentrations of perchlorate can impact fish thyroid and reproductive function, adult male and female common goldfish (*Carassius auratus*) were exposed in a laboratory flow-through exposure system for 30 days to four concentrations of perchlorate ranging from 14 ppb to 31 ppm, along with controls. Exposure was continued to 60 d for an additional set of controls and 31 ppm treatment groups. Gonadosomatic index (GSI), gonad histology, and plasma sex steroids (17β-estradiol, testosterone, and 11-ketotestosterone) were examined to assess reproductive function. Thyroid activity was assessed by use of a nonparametric rank-order assessment method developed for fish during this study. Significant increases in head kidney thyroid activity occurred at 1200 ppb and 31 ppm in females and at 31 ppm in males. Pharyngeal thyroid activity increased in both male and female goldfish exposed to 31 ppm perchlorate. Exposure to perchlorate concentrations up to 31 ppm for 30 or 60 d had no effect on GSI. Some statistically significant effects on plasma sex steroid concentrations were observed for both sexes, but the differences were small and concentrations remained within normal ranges previously reported for goldfish in this reproductive state.

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Introduction

Perchlorate is a powerful oxidizer used in solid rocket fuels, missiles, fireworks, matches, and munitions (U.S. EPA 2001; Von Burg 1995). The negatively charged perchlorate ion (ClO_4^-) originates as a contaminant in the environment from the solid salts of ammonium, potassium, and sodium (U.S. EPA 2001). These perchlorate salts are highly soluble in water (Urbansky 2000). The low reactivity of the perchlorate ion allows it to remain stable for long periods under normal environmental conditions (Urbansky 1998). High water solubility and resistance to environmental degradation make perchlorate extremely mobile in the environment (U.S. EPA 2001). Areas of greatest contamination generally occur near military and industrial installations where perchlorate is manufactured or handled (Brechner *et al.* 2000; Smith *et al.* 2001; Urbansky and Schock 1999). Perchlorate also is found in fertilizers containing nitrate derived from caliche ores that are rich in sodium nitrate, consequently leading to soil contamination and vascular plant accumulation (Ellington *et al.* 2001).

Perchlorate inhibits normal thyroid function. Thyroid follicular cells accumulate iodide, which is required for synthesis of thyroid hormones. Perchlorate competes with iodide (I^-) for the sodium-iodide transporter, resulting in decreased I^- transport across the plasma membrane of the thyroid epithelial cell; thus, perchlorate inhibits iodide uptake while iodide is discharged from the thyroid gland (Saito *et al.* 1983). Unlike iodide, perchlorate in the thyroid is not incorporated into thyroid hormones (Urbansky 2000). The inhibition of iodide accumulation results in reduced secretion of thyroid hormones. A safe dose or environmental concentration of perchlorate, ensuring no human or wildlife health effects, has not been established (Urbansky 2000).

Recent advances in technology have allowed for enhanced detection of the perchlorate anion in water (Dionex 1998). Use of these new analytical capabilities has produced data suggesting widespread contamination of ground water and surface water in the western United States (Smith *et al.* 2001). Currently, at least 11 states have sites contaminated with perchlorate, and most states have sites where contamination is a possibility (Urbansky and Schock 1999). Perchlorate has been found in surface water inhabited by fish, but little is known about the potential for low-level, chronic perchlorate exposure to produce effects in fish. The Las Vegas Bay of Lake Mead (Nevada-Arizona) is an example of a location where fish receive chronic exposure to small concentrations of perchlorate. Perchlorate enters the Las Vegas Bay via the flow of the Las Vegas Wash, which consists entirely of groundwater discharge that is contaminated with perchlorate, stormwater runoff from the urbanized Las Vegas Valley, and highly treated sewage effluent. The Las Vegas Wash currently contains approximately 500 ppb perchlorate. After mixing and dilution in the lake water, perchlorate occurs at approximately 60 ppb in the inner Las Vegas Bay. Bevans *et al*. (1996) reported evidence of reproductive endocrine disruption in carp (*Cyprinus carpio*) captured from Las Vegas Bay, but a cause has not been identified. This finding has raised concern for the federally endangered razorback sucker (*Xyrauchen texanus*), which inhabits the Las Vegas Bay, and for species important to the Lake Mead fishery.

Previous investigations into potential causes for reproductive endocrine disruption in feral carp from the Las Vegas Bay have focused on synthetic organic compounds (Bevans *et al.* 1996; Goodbred *et al.* 1997) or estrogenic contaminants (Snyder *et al.* 2001). However, to date, the potential role of perchlorate has not been examined.

Thyroid in Fish

Thyroid hormones in fish are the same as those found in other vertebrates (thyroxin or tetraiodothyronine, or T_4, and triiodothyronine, or T_3) (Norris 1997). Thyroid hormone synthesis is primarily under the control of the hypothalamic-pituitary-thyroid axis (HPT-axis). In mammals, environmental cues and factors from the brain cause the hypothalamus to release thyrotropin-releasing hormone (TRH) to the pituitary, where TRH influences the release of thyrotropin (thyroid stimulating hormone, or TSH) from the pituitary thyrotropes located in the pars distalis (Norris 1997). In contrast to what is observed in other vertebrates, hypothalamic control in fish is primarily inhibitory, but excitatory pathways also exist (Eales and Brown 1993; Norris 1997). Furthermore, the specific hypothalamic factors influencing teleost thyrotropes have not been identified, but TRH has been suggested as a candidate inhibitory factor and may be an excitatory factor in salmonids (Eales and Brown 1993). TSH is released from the pituitary and transported in the blood to the thyroid, where it promotes synthesis and release of the thyroid hormones (Cyr and Eales 1996). Under TSH stimulation, fish thyroid tissue takes up iodide and synthesizes T_4, which is released to the blood (Clark 2000; Norris 1997). Peripheral deiodinases convert T_4 to T_3, which is believed to be the active thyroid hormone in fishes (Eales 1984).

To maintain a normal metabolic state, in mammals the hormones TRH, TSH, T_3, and T_4 are regulated by balanced negative feedback (Norris 1997). High concentrations of TRH stimulate production of TSH in the pituitary (Norris 1997). Increased concentrations of TSH stimulate the thyroid to produce T_3 and T_4. In turn, high concentrations of T_3 and T_4 inhibit TRH production in the hypothalamus. Under

perchlorate exposure, thyroid hormone synthesis is inhibited, releasing the negative feedback on the HPT-axis and resulting in increased stimulus of the thyroid. In fish, the HPT-axis responds to an increase or decrease in plasma T_4 level with a compensatory increase or decrease in activity of the axis and resultant modulation of plasma T_4 level (Eales and Brown 1993). T_4 negative feedback appears to occur at the level of the hypothalamus and pituitary (Eales and Brown 1993). T_3 does not appear to influence the HPT-axis in fish as it does in birds and mammals (Eales and Brown 1993).

Thyroid hormones produce a plethora of effects in teleost fishes. The thyroid hormones appear to have a permissive effect on tissues, increasing their responsiveness to other hormones (Norris 1997). Thyroid hormones also are important in somatic growth and development, osmoregulation, gonadal development, and metabolism of lipids, proteins, and carbohydrates (Clark 2000; Leatherland 1982). Of particular interest here are the interactions between thyroid activity and reproductive systems in teleost fishes (Cyr and Eales; Norris 1997).

In fishes, thyroid follicles are scattered throughout the pharyngeal region rather than located in a discrete gland as in mammals (Norris 1997). In addition, although most of the follicles are located between the second and fourth aortic arches, some follicles (termed ‘heterotopic follicles’) may occur outside the pharyngeal region (Norris 1997). In the goldfish, thyroid follicles typically are found in the head kidney as well as in the pharyngeal region, and the activities of follicles in these tissues may differ (Chavin 1956; Peter 1970). Deficient levels of thyroid hormones, as might occur under exposure to a goitrogen, result in increased TSH stimulation of the thyroid tissue in an attempt to compensate (Norris 1997). Resultant hypertrophy and hyperplasia of thyroid follicular

cells may produce goiter (enlarged thyroid). Hyperplasia and hypertrophy of follicular cells are primarily measures of TSH stimulation of the thyroid (Norris 1997). Thyroid follicular cell shape and size also are indicators of thyroid activity. The thyroid follicular cell shape changes from squamous to cuboidal or columnar in response to TSH stimulation (Norris 1997).

Reproductive System in Fish

Goldfish are an oviparous species that spawn in the spring (Aida 1988). Fish undergo seasonal reproductive development, or gonadal recrudescence, in preparation for spawning. During this time, the gonads develop from a regressed state that occurs between annual spawning seasons to the sexually mature state required for spawning. In goldfish, this occurs during the spring and fall (Kobayashi *et al.* 1986; Razani and Hanyu 1986). This development results in an increase in the weight of the gonads relative to the body weight (BW), expressed as the gonadosomatic index (GSI), where GSI = (weight of the gonads / BW) *100. If fish are exposed to chemicals that impact the reproductive system during recrudescence, gonadal growth and development, and thus GSI, might be altered with respect to controls (Jobling *et al.* 1996; Panter *et al.* 1998). Exposure to endocrine disrupting chemicals also can cause alterations in the histology of ovaries (an increase in atretic follicles) and testis (Sertoli cell proliferation and degenerative changes) (Miles-Richardson 1999).

During gonadal recrudescence, gonadal steroids are involved in the growth of the oocytes in the ovary and in spermatogenesis in the testis (Redding and Patiño 1993). Major gonadal steroids in teleost fishes are the estrogen 17β-estradiol (E2) and the

androgen testosterone (T) in females, and the androgens T and 11-ketotestosterone (11-KT) in males (Norris 1997). T is produced in the ovary or testis and can be converted to E2 by the enzyme aromatase. The androgens T and 11-KT are the dominant male sex steroids and are responsible for development of male secondary sex characteristics and sexual behavior (Norris 1997). E2 induces production of the egg yolk precursor vitellogenin (VTG) in the liver or hepatopancreas (Mommsen and Walsh 1988) and thus is important for oocyte development. Alterations in the normal absolute levels of T and E2 (McMaster *et al.* 1992) have been used as indicators of exposure to chemicals that modulate the function of the reproductive endocrine system. It has been reported that the ratio of plasma E2 to plasma 11-KT (E2:11-KT) should be greater than one for female fish, reflecting the dominance of estrogens, and less than one for male fish, reflecting the dominance of androgens (Goodbred *et al.* 1997).

Hypothesis

Perchlorate at environmentally relevant concentrations can interfere with thyroid function and/or reproductive endocrine function in goldfish exposed in a laboratory setting.

Effects on reproductive endpoints in the fish may or may not be mediated through effects on thyroid activity. Assessment endpoints included thyroid follicle activity, plasma sex steroid concentrations, gonad development (GSI and histological assessment of gonad development), and gonad histopathology.

Methods

This study was conducted at Northern Appalachian Research Laboratory, Leetown Science Center, U. S. Geological Survey, Wellsboro, Pennsylvania. Sexually mature male and female goldfish (10-15 cm total length) of the common or comet variety were obtained in early January from Ozark Fisheries Inc. (Stoutland, MO). Fish were acclimated in the laboratory prior to beginning the exposure. Fish of both sexes were held together in polyethylene tanks supplied with degassed, oxygenated well water at 10 exchanges per day. Prior to exposure, goldfish were weighed individually and averaged 100 g for females and 80 g for males. Fish were fed 0.32 cm Hikari Staple koi pellets (Aquatic EcoSystems, Apopka, FL) at 1% body weight per day, with daily ration split into equal portions offered in the morning and afternoon. Food was tested for selected trace elements (including iodide and iodine), organophosphorus and organochlorine pesticides, and polychlorinated biphenyls (PCBs). Exygen Research, State College, Pennsylvania conducted all food analyses, with the exception of iodine and iodide, which were analyzed by Nevada Environmental Laboratories, Las Vegas, Nevada. Complete results of food analyses are provided in Appendix A.

The exposure study encompassed two simultaneous experiments, with one group of fish exposed for 30 d and the second exposed for an additional 30 d, totaling 60 d (Table 1). Nominal perchlorate exposure concentrations for the 30 d exposure were 10 ppb, 100 ppb, 1000 ppb, and 25 ppm, plus a control (Table 1). The 60 d exposure was an extension of the control and 25 ppm treatments. The dilution water for the laboratory exposure was supplied from deep wells. The water was degassed and then oxygenated. The water was analyzed by ion chromatography with conductivity detection and found to

Table 1. Treatment groups for adult male and female goldfish exposed to waterborne perchlorate, including nominal and measured perchlorate exposure concentrations and final sample sizes for each treatment group.

Nominal Concentration (µg/L)	Mean Measured Conc. (µg/L)	Standard Deviation	Exposure Duration (d)	Sample Size Males*	Sample Size Females*
Control	<4	-	30	25	32
10	14	±2	30	26	31
100	130	±7	30	24	36
1000	1198	±159	30	21	37
25,000	30688	±9241	30	33	26
Control (60 d)	<4	-	60	24	29
25,000 (60 d)	31149	±1206	60	19	33

*Sample sizes vary from n= 30 fish per treatment due to mortalities and because some fish were mistaken for fish of the opposite sex and placed into tanks designated for the other sex.

be free of perchlorate (less than the method detection limit of 0.9 µg/L). Calcium chloride ($CaCl_2$, anhydrous food grade, CalTrac brand, Mallinckrodt, St. Louis, MO) was added to the dilution water to achieve a total hardness of 50 mg/L. Concentrated $CaCl_2$ solutions were mixed in deionized water, then filtered through 125-mm diameter glass fiber filter (Whatman 1820125), then filtered again with a 142-mm diameter, 0.45-micron pore size Durapore hydrophilic PVDF membrane filter (Fisher HVLP 14250). Concentrated $CaCl_2$ solution was administered continuously to dilution water with a Manostat E-series peristaltic pump (model 72-410-014, Barnant Company, Parrington, IL). Following addition of $CaCl_2$, dilution water was treated by filtration through (in series) 20 and 10-micron filters and activated carbon, then sterilized with ultra violet light. Concentrated perchlorate stock solutions (sodium perchlorate monohydrate, reagent (ACS) grade, GFS Chemicals, Powell, OH) were mixed in deionized water, then filtered through the Durapore membrane described previously. Perchlorate stock solutions were held in glass carboys and added to dilution water in mixing chambers with

a 7-channel peristaltic pump (Masterflex L/S 7523-50 digital standard pump drive, Masterflex L/S 07519-15 cartridge pump head, and Masterflex L/S 07519-85 small cartridge; Cole-Parmer, Vernon Hills, IL) to achieve the desired exposure concentrations of perchlorate. Diluted and mixed perchlorate solutions were gravity fed to exposure tanks with 0.95-cm teflon-lined tubing (Cole-Parmer, Vernon Hills, IL).

We attempted to expose male and female goldfish in separate tanks, with 3 replicate tanks per sex (10 fish/tank) in each treatment. However, because it is difficult to determine the sex of goldfish through non-invasive methods during the pre-spawning period, some fish were exposed in tanks intended for the opposite sex. Fish were held in 100 L glass tanks in a continuous flow-through diluter system with 10 complete volume exchanges per tank per day. Peristaltic pump flow rates were calibrated 3 times per week. Measurements of dissolved oxygen (DO) (DO meter, YSI model 58 and 30; Yellow Springs, OH) and pH (pH meter, Oakton 35618-Series) were recorded weekly for each exposure tank (Table 2). Total ammonia (ammonia nitrogen test kit, Hach model NI-8, Loveland, CO; or Corning 350 pH/ion analyzer fitted with ion-selective electrode for ammonia, Corning 476130) was measured weekly in one exposure tank per treatment group. Temperature (Digi-Sense ThermoLogR Thermistor, Cole-Parmer, Vernon Hills, IL) was measured in each tank on alternate days (Table 2). Additional water quality analyses to characterize the treated source water and to monitor water quality during the exposure were provided by the Chemistry Laboratory, Southern Nevada Water System, Boulder City, Nevada (Table 3) and by Exygen Research, State College, Pennsylvania (Appendix B). Hardness (calcium and magnesium), alkalinity, and perchlorate concentrations were measured weekly. Perchlorate concentrations in all exposure tanks

Table 2. Basic water quality measurements (conducted in-house). Measurements of pH and dissolved oxygen (DO) were recorded weekly for each exposure tank. Total ammonia (Ammonia) was measured weekly in one exposure tank per treatment group. Temperature (Temp.) was measured in each exposure tank on alternate days. Data are presented as mean ± standard deviation for all observations made for tanks in use within each time period.

Time Period	pH	DO (mg/L)	Ammonia (mg/L)	Temp. (°C)
Initial 15 days	6.80±0.11	9.62±0.46	0.40±0.17	11.71±0.43
Remaining days	7.07±0.23	9.16±0.44	0.30±0.11	13.30±0.42

Table 3. Water quality analyses conducted by the Southern Nevada Water Authority, Boulder City, Nevada. Hardness (calcium and magnesium), alkalinity, and perchlorate concentrations were measured weekly. Perchlorate (ClO_4^-) concentrations in all exposure tanks were measured once per week during weeks 1, 4, and 5. During the other five weeks of the experiment, perchlorate concentrations were measured in one exposure tank per treatment group. Hardness (calcium, or Ca, and magnesium) and alkalinity measurements were taken for three exposure tanks per treatment group during week 1. During the final seven weeks of exposure, hardness and alkalinity measurements were taken for one exposure tank per treatment group. Measurements of chloride (Cl), total organic carbon (TOC), total dissolved solids (TDS), total suspended solids (TSS), sodium, and sulfate were made once for each of three exposure tanks per treatment during weeks 1 and 5. H_20 = dilution water, C-30 d = 30 d exposure control, C-60 d = 60 d exposure control, 25 ppm-30 d = 25 ppm exposure for 30 d, 25 ppm-60 d = 25 ppm exposure for 60 d.

Treatment Group	ClO_4^- µg/L	Cl mg/L	TOC mg/L	TDS mg/L	TSS mg/L	Alkalinity[b] mg/L	Ca mg/L
H_2O	[a]	28.19	0.36	113.17	0.92	35.50	24.28
C-30 d	< 4	29.28	1.70	119.00	0.42	37.14	25.23
10 ppb	13.88	29.26	1.66	117.50	0.58	33.29	25.94
100 ppb	130.12	26.04	3.32	119.00	0.44	34.71	26.10
1000 ppb	1198.41	29.28	0.95	115.50	0.33	35.14	25.94
25 ppm-30 d	30687.85	29.30	1.50	156.50	0.33	33.29	25.84
C-60 d	< 4	30.67	1.99	119.75	0.50	36.38	24.80
25 ppm-60 d	31148.78	30.48	0.83	156.67	0.25	35.85	24.93

Magnesium was less than 3 mg/L and sulfate was less than 10 mg/L for all treatments and dilution water. Sodium was less than 3 mg/L, except in the 25 ppm-60 d (8.845 mg/L) and 25 ppm-30 d (9.153 mg/L) treatment groups. [a] Perchlorate was not detected (< 0.9 µg/L) in laboratory supply water analyzed just prior to the start of the exposure. Perchlorate analysis was conducted by Dr. Kevin Kelly, U.S. Bureau of Reclamation, Denver, CO. [b] Alkalinity= bicarbonate alkalinity; carbonate and hydroxide alkalinity were not detected.

were measured (U.S. EPA Method 314.0, Hautman *et al*. 1999) once per week during weeks 1, 4, and 5. During the other five weeks of the experiment, perchlorate concentrations were measured in one exposure tank per treatment group. Hardness and alkalinity measurements were taken for three exposure tanks per treatment group during week 1. During the final seven weeks of exposure, hardness and alkalinity measurements were taken for one exposure tank per treatment group. Measurements of chloride, total organic carbon (TOC), total dissolved solids (TDS), total suspended solids (TSS), sodium, and sulfate were made once for each of three exposure tanks per treatment during weeks 1 and 5.

For the 30 d exposure, photoperiod and temperature were maintained at 10L: 14D and 11.6 ^{o}C ±0.4 (mean ± standard deviation) for 15 days, then increased to 12L: 12D and 13.5 ^{o}C ±0.3 for the remaining 15 d. This regimen was intended to mimic spring (pre-spawning) conditions for goldfish. The 60 d exposure was an extension of the control and 25 ppm treatments. The goldfish were kept under a 10 L: 14 D photoperiod for 45 d. The photoperiod was then increased to 12 L: 12 D for the remaining 15 d of the exposure period. The temperature was maintained at 11.6 ^{o}C ±0.4 for the initial 15 d of exposure and 13.1 ^{o}C ±0.4 for the final 45 d of exposure. Both 30 d and 60 d exposures were initiated with a staggered start, enabling post-exposure sampling of fish from a single treatment over a period of 5 d or 2 d for the 30 d exposure and 60 d exposure treatments, respectively. All diluter effluent water was treated by filtration with strong base anion exchange resin (US Filter, Roseville, MN) to remove perchlorate before release, and treated effluent was monitored for breakthrough.

Post-exposure, all fish of both sexes from a single treatment were sampled on one day. Following overdose of fish with MS-222 mixed with exposure water, blood was sampled from the caudal vasculature with a heparinized 25G × 1.59-cm needle and syringe (1 cc, Becton-Dickinson). Blood was sampled within a consistent 1.5 hr period each morning. Blood samples were immediately centrifuged at 3,000 × g for 10 min. at 4 ^{o}C, and plasma was frozen and stored at –80 ^{o}C. Individual fish were weighed to the nearest 0.1 g and measured (standard length) to the nearest millimeter. Gonads were removed and weighed to the nearest 0.001 g. Head kidney and pharyngeal thyroid tissue and one gonad from each fish were preserved in 10% neutral-buffered formalin for histological examination. After fixation for a period of 7 d, tissue samples were rinsed and placed in 70% ethanol. Pharyngeal thyroid tissue was isolated further by taking a transverse section through the pharyngeal connective tissue and the bases of the first (rostral) set of gill arches. Tissues were sectioned, embedded, and stained by the Michigan State University Clinical Histology Laboratory, East Lansing, Michigan. Both pharyngeal thyroid tissues and head kidneys were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. If thyroid follicles were not found in the initial sections, deeper sections were taken until at least 10 total follicles were found in head kidneys and in the pharyngeal region for each fish.

Measurements of the thickness of epithelial cells surrounding the follicle (epithelial cell height, ECH) commonly are used to assess thyroid follicle activity in fish. ECH increases with increasing TSH stimulation. However, measuring ECH can be laborious for large numbers of samples, and it is most easily accomplished by using a digital imaging system, a piece of equipment that is not available in many laboratories.

To facilitate faster and less expensive assessment, we developed a subjective nonparametric rank-order assessment method to assign thyroid follicle activity scores to fish thyroid tissue and compared results obtained with this method to results based on the more traditional ECH measurements. Although Raine *et al*. (2001) previously used an arbitrary scale (1 – 8) to assess colloid vacuolation, the method proposed here integrates assessment of epithelium thickness, colloid depletion, and proportion of follicles in each stage within one score. Tissue was given a score of 1 if the majority of the follicles had squamous epithelial cells with no colloid depletion (photos in Appendix C). A score of 2 was given if the majority of the follicles consisted of squamous epithelial cells with a few cuboidal or columnar epithelial cells located adjacent to isolated vacuoles in the otherwise full colloid or if the majority of follicles showed evidence of uniform, slight elevation in epithelial cell height (short of cuboidal in shape) and no colloid depletion. Tissue scored 3 contained a majority of follicles with cuboidal to columnar epithelial cells and little or no apparent colloid depletion. A score of 4 was assigned when the majority of follicles had columnar epithelial cells and evident colloid depletion, while a score of 5 was given when the majority of follicles had columnar epithelial cells and complete or nearly complete colloid depletion. Thyroid activity for head kidney and pharyngeal region also was assessed by measuring the mean ECH for 10 follicles per tissue for each fish, consistent with the methodology described previously by Hurlburt (1977). This was accomplished by measuring the minimum and maximum epithelial cell height for ten representative follicles. The grand mean of the ten follicle means then was used as the measurement of follicle activity for each tissue. Follicle epithelial cell height

was measured with a Nikon DN100 digital camera and Eclipse Net version 1.16.2 digital imaging software developed by Laboratory Imaging for Nikon Instruments.

Gonad tissue samples were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for histological evaluation. Testes were sectioned longitudinally, while ovaries were sectioned transversely. Testes or ovaries were classified according to four stages of sexual maturation described previously (Lee *et al.* 1999) and similar to methods described by Goodbred *et al*. (1997).

Plasma sex steroid (E2, T, and 11-KT) analyses were conducted in the laboratory of Dr. Glen Van Der Kraak, University of Guelph, Guelph, Ontario, using a radioimmunoassay method previously developed and validated for goldfish plasma (McMaster *et al.* 1992). The method detection limit (MDL) for each hormone was 0.08 ng/mL.

Statistical analyses were conducted with MINITAB 13 (State College, PA). For normally distributed data and for thyroid activity scores, differences among treatment groups were analyzed by analysis of variance (ANOVA), followed by Tukey pairwise comparison test. When data were not normally distributed, differences among treatment groups were explored by Kruskal-Wallis test followed by Tukey test on ranked data. When sex steroid concentrations were below the MDL, ½ MDL was substituted for the non-detects in the statistical analysis. Time effects were analyzed with a nested ANOVA, with treatment nested in exposure duration. Data used in analysis of time effect included only controls and 31 ppm treatment groups. Where appropriate, data are depicted in box and whisker plots. Each box represents the inter-quartile range, i.e., values falling within the first quartile (Q_1) and third quartile (Q_3). The horizontal line

within each box represents the median. If the inter-quartile range encompasses only two discrete variables, there will be no median line within the box. In this case, the median is either the Q_1 or Q_3. If the inter-quartile range encompasses only one discrete variable, there will only be a horizontal line. Whiskers represent the upper and lower limits (approximately 95%), with asterisks denoting outliers (points outside the upper and lower limits). Lower limit: $Q_1 - 1.5(Q_3 - Q_1)$. Upper limit: $Q_3 + 1.5(Q_3 - Q_1)$. In the Discussion, 'significant difference' refers to a significant difference of the 30 d treatments from the 30 d control or a significant difference of the 31 ppm-60 d treatment from the 60 d controls, unless otherwise noted. However, all significant differences are represented in the figures by letters indicating Tukey groupings.

Results

Thyroid Endpoints

In females, a significant difference in pharyngeal thyroid activity score from the controls occurred at 31 ppm following both 30 d and 60 d exposure durations (Figure 1). Duration of exposure significantly affected pharyngeal thyroid activity score (P=0.003). Thyroid activity was significantly greater in the 31 ppm-60 d treatment group than in the 31 ppm-30 d treatment group. Pharyngeal thyroid ECH in females differed significantly from the controls at 31 ppm at both 30 d and 60 d exposure durations (Figure 2), and exposure duration had no significant effect on this endpoint.

For males, the only significant difference in pharyngeal thyroid activity score from the controls occurred at 31 ppm after 60 d exposure (Figure 3). There were no significant effects of exposure duration on pharyngeal thyroid activity score. No significant differences in pharyngeal thyroid ECH occurred among treatment groups (Figure 4), and exposure duration had no significant effect on this endpoint.

For females, a significant difference in head kidney thyroid activity score from the controls occurred at both 1200 ppb and 31 ppm after 30 d exposure and at 31 ppm after 60 d (Figure 5). Exposure duration had no significant effect on head kidney activity score for females. In males, a significant difference in head kidney thyroid activity score from the controls occurred only at 31 ppm after 60 d exposure (Figure 6). Head kidney thyroid activity score was significantly affected by exposure duration (P=0.000) in males. For both males and females, head kidney ECH differed significantly from that in controls only in the 31 ppm-60 d treatment group (Figures 7, 8). Head kidney ECH was

significantly affected by exposure duration in both males and females (P=0.000 and P=0.006, respectively).

Reproductive Endpoints

For both females (Figure 9) and males (Figure 10), there were no significant differences in GSI among fish that received different treatments. At the end of the study, all male goldfish were in the same stage of reproductive development (stage 3, late spermatogenic), as assessed histologically. The only occurrences of Sertoli cell proliferation were seen in three fish exposed to 31 ppm perchlorate. Female fish had gonads in stages of maturation ranging from 1-3 (previtellogenic to late vitellogenic). There were no significant differences in stages of reproductive development among females in different treatment groups. No significant ceroid lipofuscin accumulation was observed in gonads of males or females.

For females, a small but statistically significant decrease in plasma E2 concentration relative to the control group occurred only at 14 ppb for fish exposed to perchlorate for 30 d (Figure 11). For males, a small but significant increase in plasma E2 relative to the controls occurred at 31 ppm for both 30 d and 60 d exposure durations (Figure 12). No significant differences in plasma T concentrations were detected among females in the different treatment groups (Figure 13). In males, significant decreases in plasma T concentrations relative to controls occurred at both 14 ppb and 1200 ppb, but not in other treatment groups (Figure 14). No significant differences in plasma 11-KT concentrations were detected among females in the different treatment groups (Figure 15). In males, a significant decrease in plasma 11-KT concentrations relative to the

controls occurred at only at 14 ppb and 1200 ppb (Figure 16). Exposure duration did not affect sex steroid concentrations in females. In males, plasma androgen concentrations were not affected by exposure duration; however, there was a statistically significant effect of exposure duration on plasma E2 concentrations in males (P=0.000).

In females, a significant decrease in the ratio of plasma E2:T relative to that in controls occurred only for fish exposed at 14 ppb (Figure 17). For males, a significant increase in plasma E2:T relative to that in controls occurred only for the group exposed to 31 ppm for 30 d (Figure 18). For females, a significant increase in plasma E2:11-KT relative to that in the controls occurred only for the 31 ppm-60 d treatment group (Figure 19). In males, a significant increase in plasma E2:11-KT relative to that observed in the controls occurred only for the 31 ppm-30 d treatment group (Figure 20).

Discussion

Significant increases in pharyngeal thyroid activity in females occurred at 31 ppm, with a significant effect of exposure duration. In males, a significant increase in pharyngeal thyroid activity occurred at 31 ppm, with no significant effect of exposure duration. Significant increases in head kidney thyroid activity in females occurred at 1200 ppb and 31 ppm, with a significant effect of exposure duration on ECH. It has been reported previously that goldfish thyroid activity parallels temperature change (Gorbman 1969). The increase in ECH for control females over time (control-60 d relative to control-30 d) may be attributed to the extended exposure duration at greater temperature for the control-60 d treatment group. A significant increase in head kidney thyroid activity in males occurred at 31 ppm, with a significant effect of exposure duration on both activity score and ECH. Goldfish strongly retain iodine and recycle it slowly from the tissues to the thyroid (Gorbman 1969). The significant effect of exposure duration on male and female goldfish might be attributed to the slow turnover rate of iodide in the goldfish thyroid. Because exposure duration had a significant effect on some thyroid endpoints, impacts at concentrations less than or equal to 1200 ppb might become evident if the fish were exposed to perchlorate for at least 60 d. Consistent with the findings of Chavin (1956), head kidney thyroid activity was greater than pharyngeal thyroid activity in both males and females.

Thyroid tissue in female goldfish appears to be more sensitive than that in males to the goitrogenic effects of perchlorate. This finding conflicts with results of three previous studies with goldfish that indicate that there is no relationship between sex and thyroid ECH or magnitude of the increase in ECH in response to experimental

manipulations (Chavin 1956; Hoar and Eales 1963; Ortman and Billig 1966). Female fish sequester iodide into eggs during recrudescence, securing a source of iodide for the developing embryo (Norris 1997). The additional iodide demand in females undergoing recrudescence might increase sensitivity to goitrogens like perchlorate.

The difference between results of the thyroid activity assessment method and measurement of ECH illustrates that the activity score method is the more sensitive method for assessing thyroid activity. The staging method takes into account criteria in addition to epithelial cell height, including percent follicles affected and amount of colloid.

A significant decrease in E2 and E2:T occurred in females at 14 ppb, and a significant increase in E2:11-KT occurred in the 31 ppm-60 d treatment group. However, female E2:11-KT values were greater than 1 in all treatment groups, indicating that the normal dominance of estrogens over androgens has been maintained. In males, a significant increase in E2 occurred in the 31 ppm-30 d treatment group and in the 31 ppm-60 d treatment group, and an increase in E2:T and E2:11-KT occurred in the 31 ppm-30 d treatment group. A significant decrease in T and 11-KT occurred for males at 14 ppb and 1200 ppb. The majority of male E2:T and E2:11-KT values were less than 1, indicating that the normal dominance of androgens over estrogens in males was maintained. A small number of E2:11-KT outliers greater than 1 was observed in males; however, there appears to be no relationship between occurrence of these outliers and concentration of perchlorate to which the fish were exposed. The plasma E2 increase at 31 ppm in males coincides with the increase in thyroid activity at 31 ppm. Exposure duration had no significant effect on female plasma sex steroid concentrations; however,

exposure duration significantly affected E2 concentrations in males. Because exposure duration significantly affected male plasma E2 concentrations, impacts at lesser perchlorate exposure concentrations might occur if exposure was continued to 60 d.

Although some significant differences in plasma sex steroid concentrations among treatment groups were observed, all mean and median sex steroid concentrations and ratios were within normal ranges for goldfish in this part of the reproductive cycle and at this temperature and photoperiod (Rosenblum *et al.*1985). Nonmonotonic dose-response curves were observed for female plasma E2 and male plasma T and 11-KT. Typically, a toxic response increases in accordance with increasing dose or exposure concentration. 'Nonmonotonic dose-response' refers to cases in which effects observed at lesser exposure concentrations or doses are not observed at greater doses, or when lesser doses produce a more substantial effect than do greater doses. Endocrine disrupting chemicals are known for their ability to produce these atypical dose-response curves. Others have reported nonmonotonic dose-response curves for fish exposed to endocrine disrupting chemicals. Fathead minnows exposed to 4-nonylphenol demonstrated 'inverted U-type' dose-response relationships for egg production and for plasma concentrations of vitellogenin and E2 (Giesy *et al.* 2000).

The observed GSI for both males and females are consistent with those previously reported for goldfish in this stage of gonadal development (Kagawa *et al.* 1983; Razani *et al.* 1988). The stages of gonad maturation assessed histologically agree with the stages of gonadal maturation suggested by GSI. Impacts on GSI are relatively serious, since they are linked more directly to decreased reproductive performance than are changes in circulating sex steroids; however, no impacts on GSI were seen in the exposed male and

female goldfish. Because perchlorate exposure did not affect GSI and did not shift circulating sex steroid concentrations outside the normal range for goldfish, perchlorate does not appear to produce serious impacts on reproductive system function in adult goldfish, at least under these exposure conditions. However, exposure to perchlorate at > 130 ppb to ≤ 1200 ppb can increase activity of thyroid follicles in head kidney of female goldfish. With perchlorate concentrations in surface water ranging up to 31 ppm (Smith 2001), it is possible that thyroid function in fish in these areas is affected.

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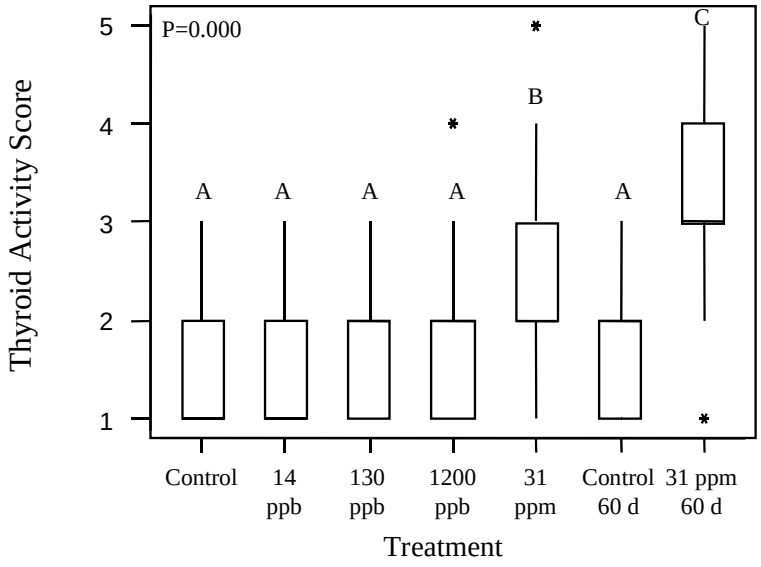

Figure 1. Thyroid activity score for pharyngeal thyroid tissue in female goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=25, 14 ppb=32, 130 ppb=30, 1200 ppb=30, 31 ppm=18, Control 60d=22, and 31 ppm 60d=23. Differences among treatment groups were explored by analysis of variance. Treatment groups marked by common letters were not significantly different by Tukey test.

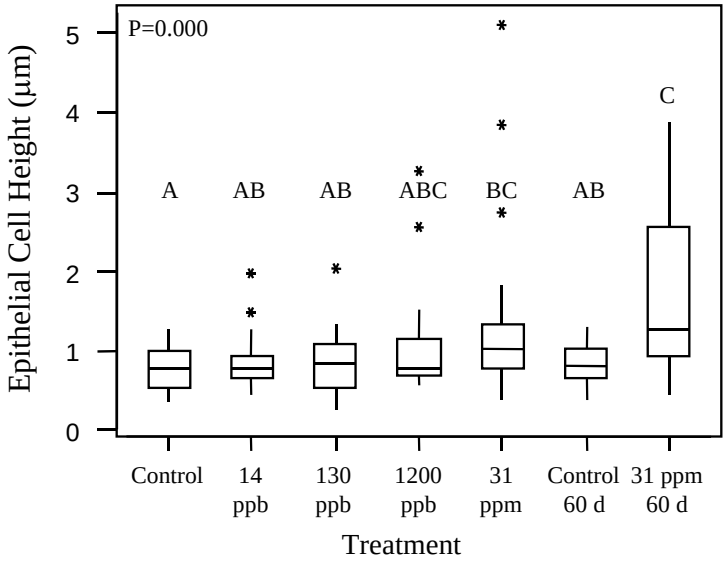

Figure 2. Measurements of epithelial cell height for pharyngeal thyroid follicles in female goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=25, 14 ppb=32, 130 ppb=29, 1200 ppb=29, 31 ppm=17, Control 60d=22, and 31 ppm 60d=23. Differences among treatment groups were explored by Kruskal-Wallis test. Treatment groups marked by common letters were not significantly different by Tukey test on ranked data.

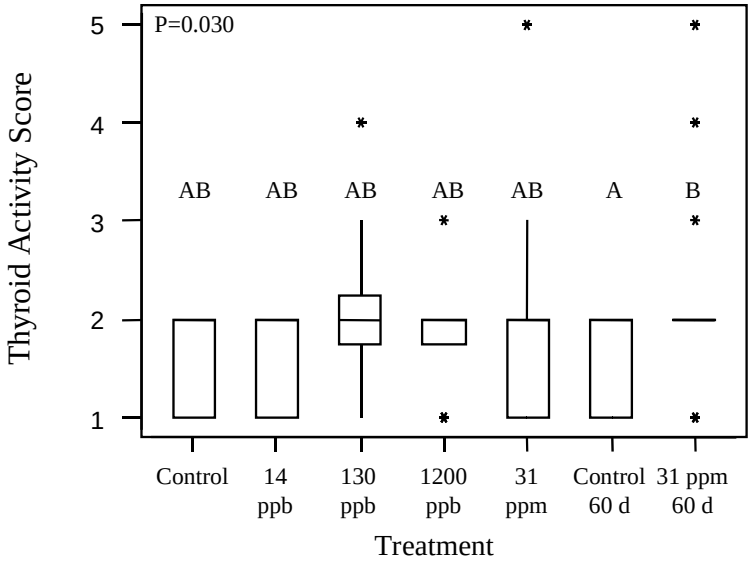

Figure 3. Thyroid activity score for pharyngeal thyroid tissue in male goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=19, 14 ppb=21, 130 ppb=18, 1200 ppb=14, 31 ppm=17, Control 60d=23, and 31 ppm 60d=16. Differences among treatment groups were explored by analysis of variance. Treatment groups marked by common letters were not significantly different by Tukey test.

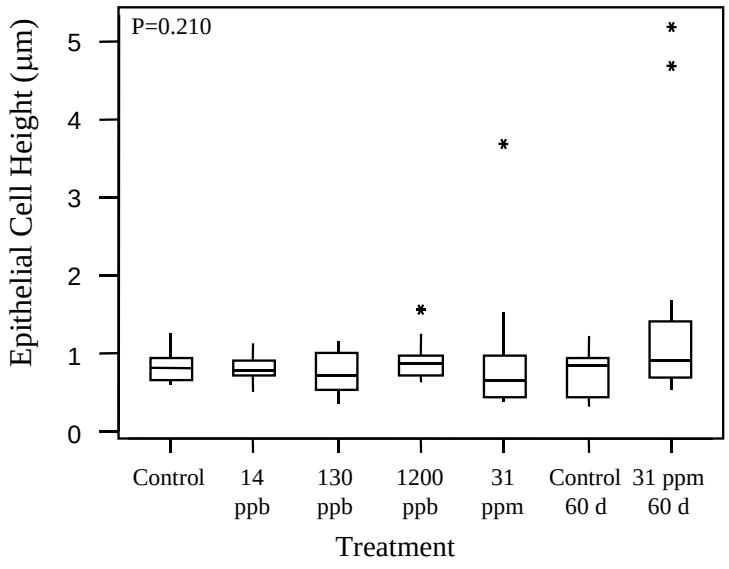

Figure 4. Measurements of epithelial cell height for pharyngeal thyroid follicles in male goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=19, 14 ppb=21, 130 ppb=18, 1200 ppb=14, 31 ppm=17, Control 60d=23, and 31 ppm 60d=16. Differences among treatment groups were explored by Kruskal-Wallis test. Treatment groups marked by common letters were not significantly different by Tukey test on ranked data.

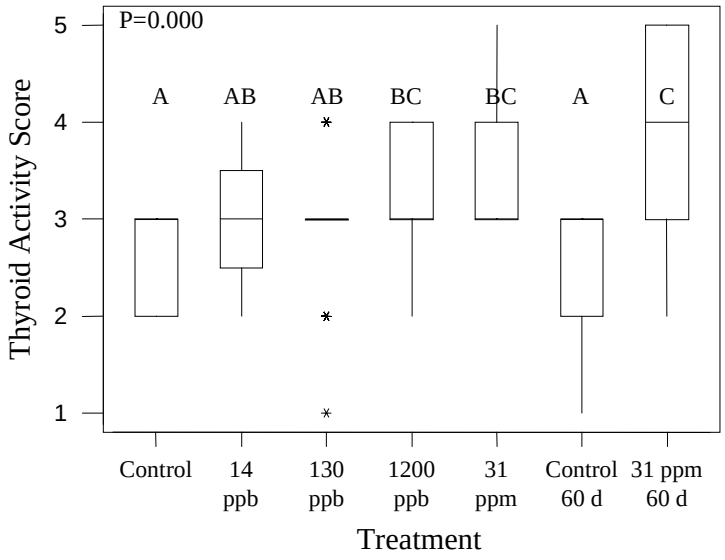

Figure 5. Thyroid activity score for thyroid follicles in the head kidney of female goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=25, 14 ppb=29, 130 ppb=27, 1200 ppb=28, 31 ppm=16, Control 60d=21, and 31 ppm 60d=22. Differences among treatment groups were explored by analysis of variance. Treatment groups marked by common letters were not significantly different by Tukey test.

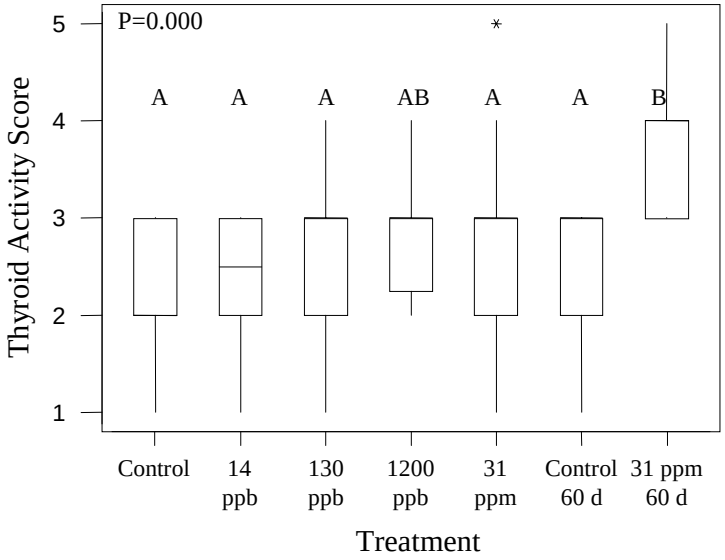

Figure 6. Thyroid activity score for thyroid follicles in the head kidney of male goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=20, 14 ppb=20, 130 ppb=18, 1200 ppb=16, 31 ppm=19, Control 60d=22, and 31 ppm 60d=19. Differences among treatment groups were explored by analysis of variance. Treatment groups marked by common letters were not significantly different by Tukey test.

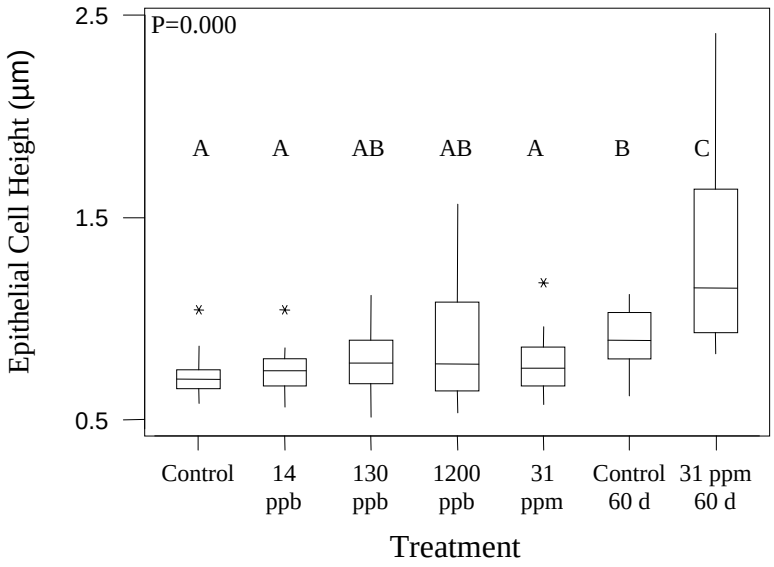

Figure 7. Measurements of epithelial cell height for thyroid follicles in the head kidney of female goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=25, 14 ppb=25, 130 ppb=27, 1200 ppb=26, 31 ppm=15, Control 60d=21, and 31 ppm 60d=22. Differences among treatment groups were explored by Kruskal-Wallis test. Treatment groups marked by common letters were not significantly different by Tukey test on ranked data.

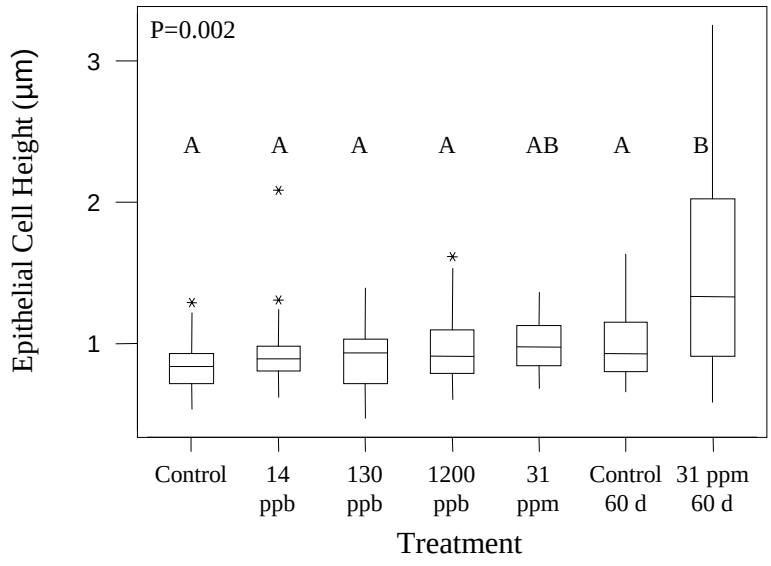

Figure 8. Measurements of epithelial cell height for thyroid follicles in the head kidney of male goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=19, 14 ppb=21, 130 ppb=18, 1200 ppb=14, 31 ppm=17, Control 60d=23, and 31 ppm 60d=16. Differences among treatment groups were explored by Kruskal-Wallis test. Treatment groups marked by common letters were not significantly different by Tukey test on ranked data.

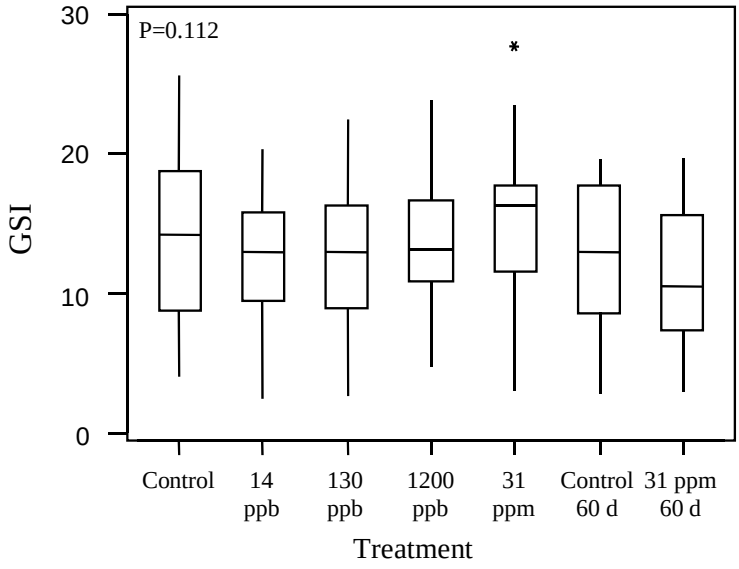

Figure 9. Female gonadosomatic index (GSI), where GSI= (gonad weight / body weight)*100. Sample sizes are: Control=26, 14 ppb=33, 130 ppb=30, 1200 ppb=31, 31 ppm=19, Control 60d=23, and 31 ppm 60d=27. Differences among treatment groups were explored by analysis of variance. No significant differences among treatment groups were detected by Tukey test.

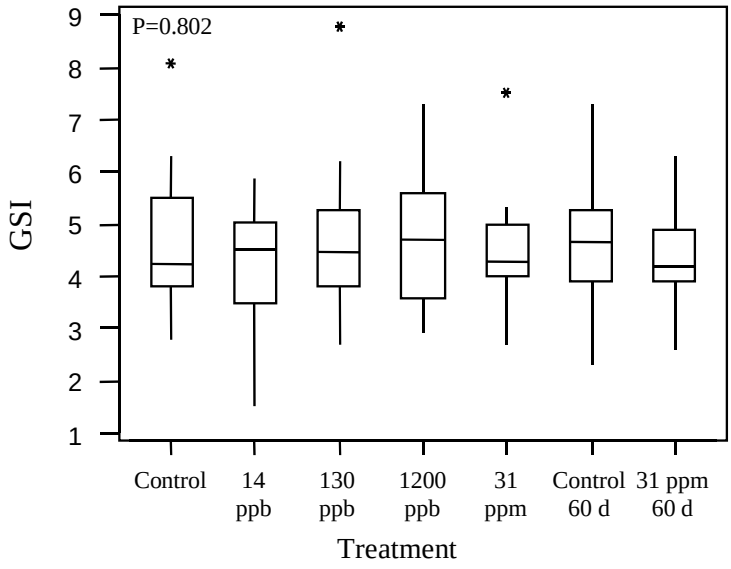

Figure 10. Male gonadosomatic index (GSI), where GSI= (gonad weight / body weight)*100. Sample sizes are: Control=20, 14 ppb=21, 130 ppb=18, 1200 ppb=15, 31 ppm=19, Control 60d=24, and 31 ppm 60d=19. Differences among treatment groups were explored by analysis of variance. No significant differences among treatment groups were detected by Tukey test.

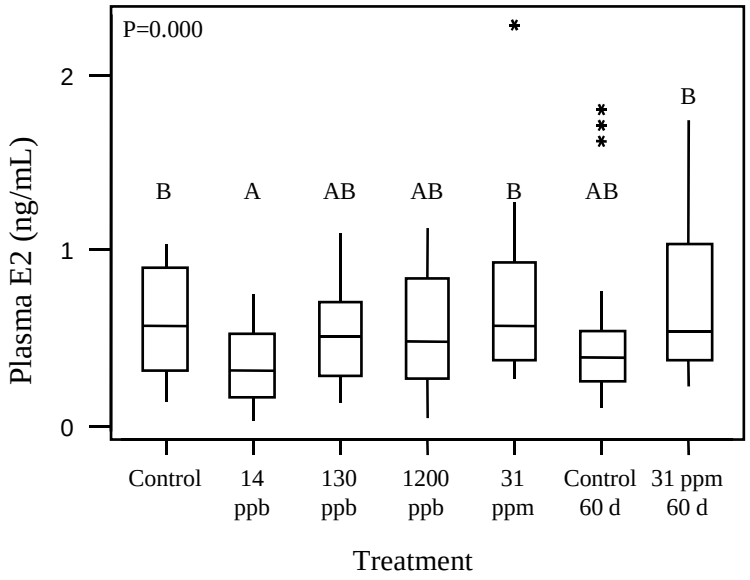

Figure 11. Plasma estradiol (E2) concentrations in female goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=32, 14 ppb=41, 130 ppb=36, 1200 ppb=37, 31 ppm=23, Control 60d=29, and 31 ppm 60d=32. Differences among treatment groups were explored by Kruskal-Wallis test. Treatment groups marked by common letters were not significantly different by Tukey test on ranked data.

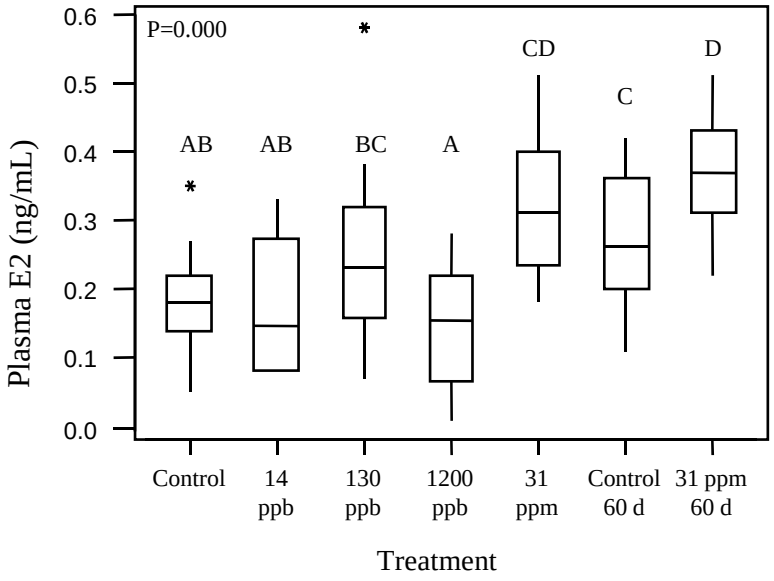

Figure 12. Plasma estradiol (E2) concentrations in male goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=28, 14 ppb=27, 130 ppb=24, 1200 ppb=21, 31 ppm=25, Control 60d=24, and 31 ppm 60d=19. Differences among treatment groups were explored by Kruskal-Wallis test. Treatment groups marked by common letters were not significantly different by Tukey test on ranked data.

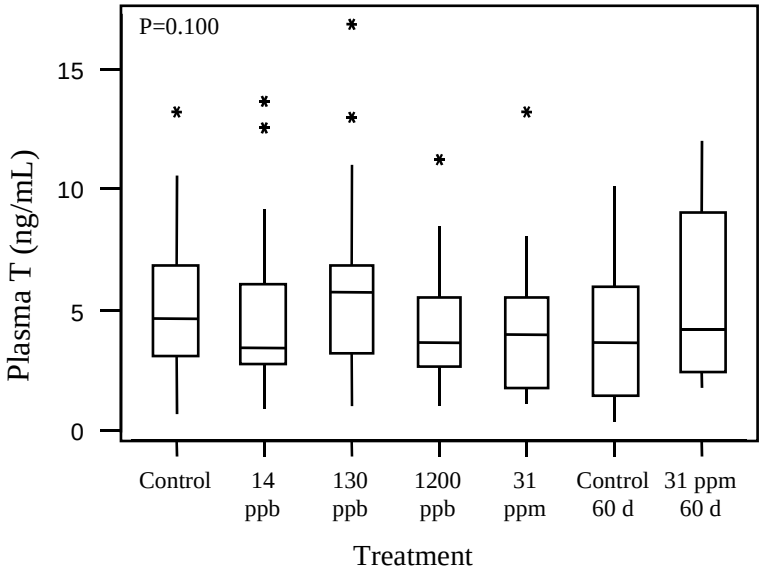

Figure 13. Plasma testosterone (T) concentrations in female goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=32, 14 ppb=40, 130 ppb=36, 1200 ppb=37, 31 ppm=23, Control 60d=29, and 31 ppm 60d=32. Differences among treatment groups were explored by Kruskal-Wallis test. No significant differences among treatment groups were detected by Tukey test on ranked data.

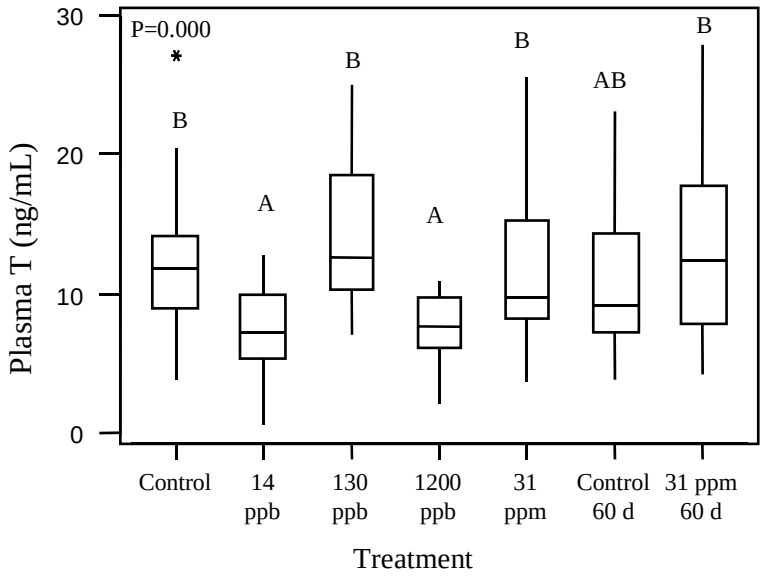

Figure 14. Plasma testosterone (T) concentrations in male goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=27, 14 ppb=27, 130 ppb=24, 1200 ppb=21, 31 ppm=25, Control 60d=24, and 31 ppm 60d=19. Differences among treatment groups were explored by Kruskal-Wallis test. Treatment groups marked by common letters were not significantly different by Tukey test on ranked data.

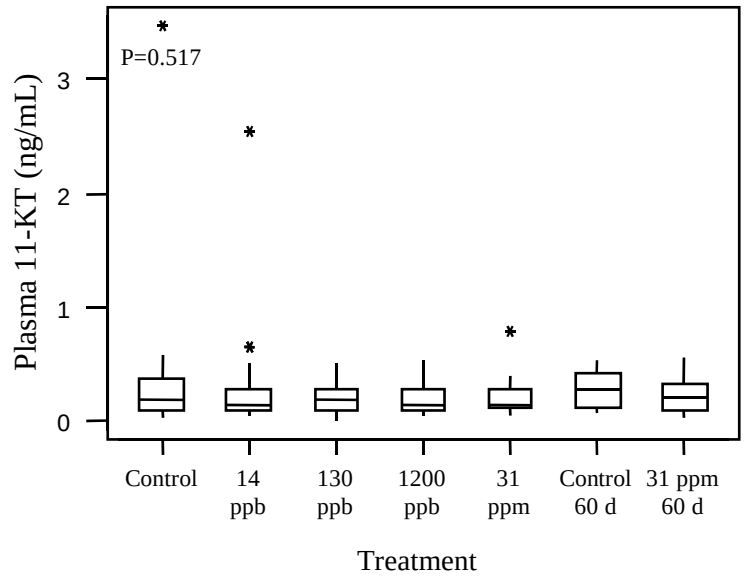

Figure 15. Plasma 11-ketotestosterone (11-KT) concentrations in female goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=32, 14 ppb=41, 130 ppb=36, 1200 ppb=37, 31 ppm=23, Control 60d=29, and 31 ppm 60d=32. Differences among treatment groups were explored by Kruskal-Wallis test. No significant differences among treatment groups were detected by Tukey test on ranked data.

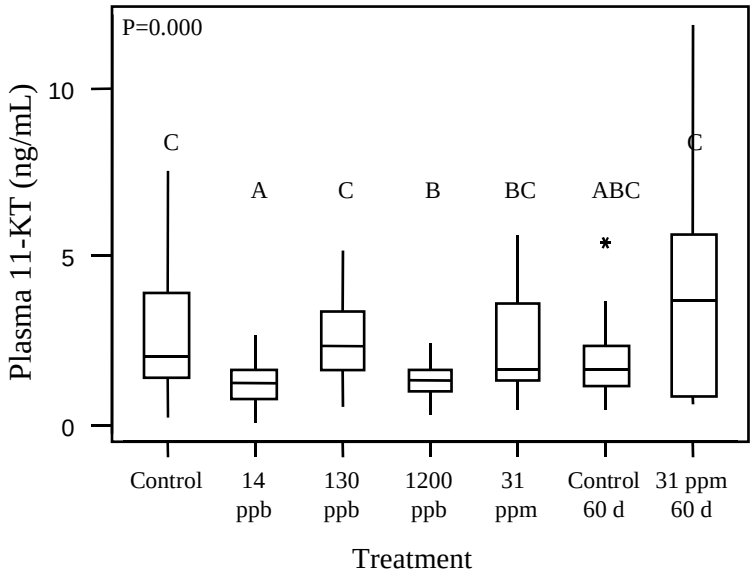

Figure 16. Plasma 11-ketotestosterone (11-KT) concentrations in male goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=27, 14 ppb=27, 130 ppb=24, 1200 ppb=21, 31 ppm=25, Control 60d=24, and 31 ppm 60d=19. Differences among treatment groups were explored by Kruskal-Wallis test. Treatment groups marked by common letters were not significantly different by Tukey test on ranked data.

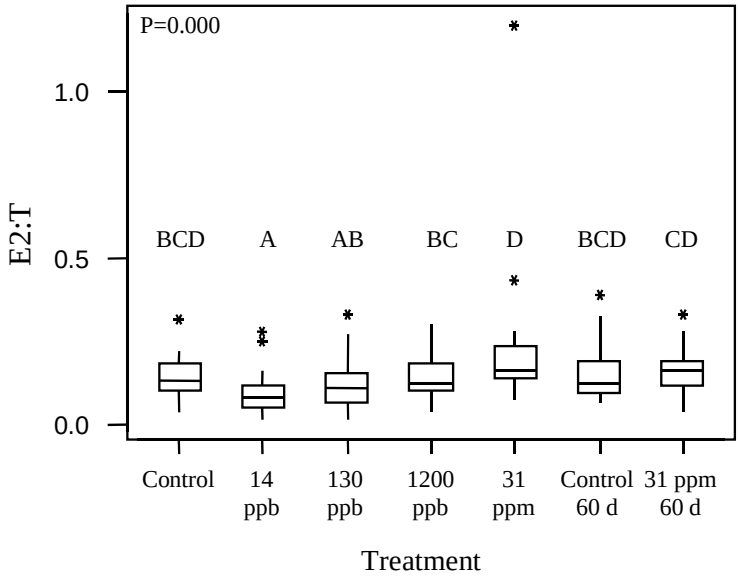

Figure 17. Ratio of plasma estradiol to plasma testosterone (E2:T) in female goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=32, 14 ppb=40, 130 ppb=36, 1200 ppb=37, 31 ppm=23, Control 60d=29, and 31 ppm 60d=32. Differences among treatment groups were explored by Kruskal-Wallis test. Treatment groups marked by common letters were not significantly different by Tukey test on ranked data.

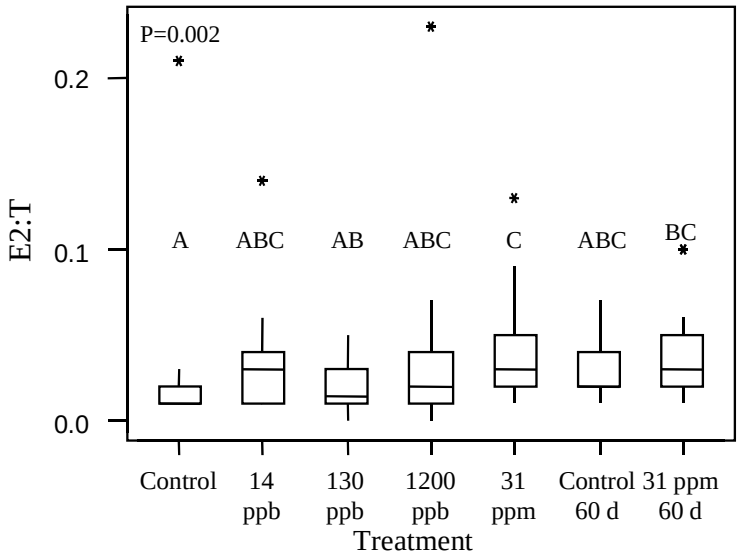

Figure 18. Ratio of plasma estradiol to plasma testosterone (E2:T) in male goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=27, 14 ppb=27, 130 ppb=24, 1200 ppb=21,31 ppm=25, Control 60d=24, and 31 ppm 60d=19. Differences among treatment groups were explored by Kruskal-Wallis test. Treatment groups marked by common letters were not significantly different by Tukey test on ranked data.

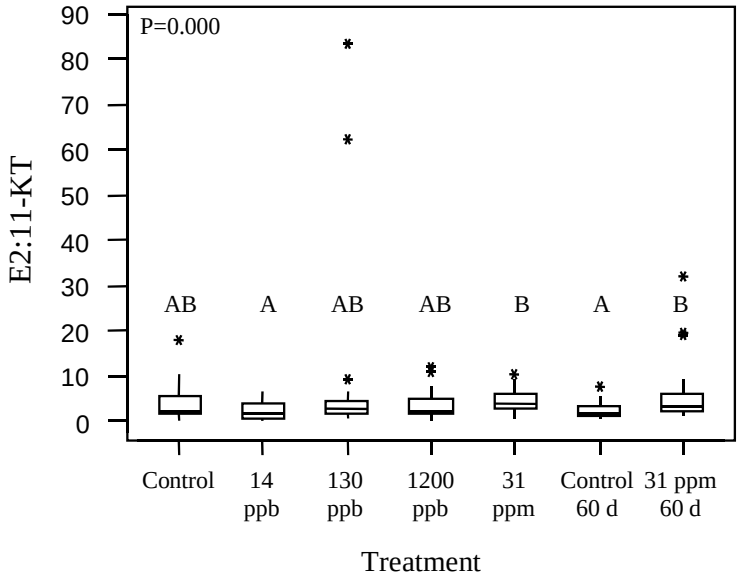

Figure 19. Ratio of plasma estradiol to plasma 11-ketotestosterone (E2:11-KT) in female goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=32, 14 ppb=41, 130 ppb=36, 1200 ppb=37, 31 ppm=23, Control 60d=29, and 31 ppm 60d=32. Differences among treatment groups were explored by Kruskal-Wallis test. Treatment groups marked by common letters were not significantly different by Tukey test on ranked data.

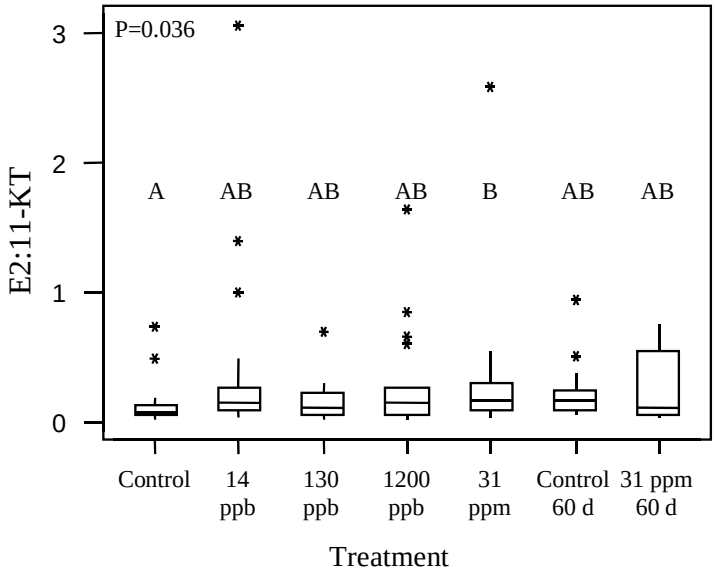

Figure 20. Ratio of plasma estradiol to plasma 11-ketotestosterone (E2:11-KT) in male goldfish exposed to waterborne perchlorate. Treatments consist of combinations of perchlorate exposure concentration and exposure duration. Sample sizes are: Control=27, 14 ppb=27, 130 ppb=24, 1200 ppb=21, 31 ppm=25, Control 60d=24, and 31 ppm 60d=19. Differences among treatment groups were explored by Kruskal-Wallis test. Treatment groups marked by common letters were not significantly different by Tukey test on ranked data.

Appendix A. Analysis of Hikari koi food (Aquatic Ecosystems, Inc., Apopka, Florida). All analyses were conducted by Exygen Research, State College, Pennsylvania, with the exception of iodide and iodine analyses, which were conducted by Nevada Environmental Laboratories, Las Vegas, Nevada.

PARAMETER	RESULT	DETECTION LIMIT
Trace Elements	**mg/kg (dry)**	**mg/kg (dry)**
PERCENT SOLID	91.85%	0.01
MERCURY	<0.0002	0.051
PERCENT SOLID	91.87%	0.01
ALUMINUM	147	0.595
ARSENIC	0.439	0.0894
CADMIUM	0.322	0.0894
CHROMIUM	2.32	0.714
COPPER	16	1.19
NICKEL	1.9	0.179
LEAD	<0.358	0.358
ZINC	104	0.595
IODIDE	29	1
IODINE	<2.5	2.5
Organochlorine Pesticides	**mg/kg (dry)**	**mg/kg (dry)**
PERCENT SOLIDS	91.83%	0.01
ALPHA-BHC	<0.0003	0.0003
GAMMA-BHC	<0.0003	0.0003
BETA-BHC	<0.0003	0.0003
HEPTACHLOR	<0.0003	0.0003
DELTA-BHC	<0.0003	0.0003
ALDRIN	<0.0003	0.0003
HEPTACHLOR EPOXIDE	<0.0003	0.0003
ENDOSULFAN I	<0.0003	0.0003
DIELDRIN	<0.0003	0.0003
4,4'-DDE	<0.0003	0.0003
ENDRIN ENDOSULFAN II	<0.0003	0.0003
4,4'-DDD	<0.0003	0.0003
ENDRIN ALDEHYDE	<0.0003	0.0003
4,4'-DDT	<0.0003	0.0003
ENDOSULFAN SULFATE	<0.0003	0.0003
ENDRIN KETONE	<0.0003	0.0003
METHOXYCHLOR	<0.0003	0.0003
AROCLOR-1016/1242	<0.017	0.017
TOXAPHENE	<0.017	0.017
CHLORDANE	<0.017	0.017
AROCLOR-1221	<0.017	0.017
AROCLOR-1232	<0.017	0.017
AROCLOR-1248	<0.017	0.017
AROCLOR-1254	<0.017	0.017
AROCLOR-1260	<0.017	0.017

Appendix A (cont.)

PARAMETER	RESULT	DETECTION LIMIT
Organophosphorus Pesticides	**μg/kg (dry)**	**μg/kg (dry)**
PERCENT SOLIDS	91.78%	0.01
THIONAZIN	<1.6	1.6
PHORATE	<1.6	1.6
SULFOTEPP	<1.6	1.6
DISULFOTON	<1.6	1.6
METHYL PARATHION	<1.6	1.6
PARATHION	<1.6	1.6
FAMPHUR	<1.6	1.6

Appendix B. Water analyses to characterize dilution water and to test for effects of perchlorate addition on water quality. Analyses were conducted by Exygen Research, State College, PA.

SAMPLE TYPE	PARAMETER	RESULT (mg/L)	DETECTION LIMIT (mg/L)
Adj. Dil.	AMMONIA	<0.2	0.2
25 ppm	AMMONIA	<0.2	0.2
tank 9	AMMONIA	0.334	0.2
tank 1	AMMONIA	0.269	0.2
Adj. Dil.	NITRITE	<0.1	0.1
Adj. Dil.	NITRATE	<0.1	0.1
Adj. Dil.	SULFATE	9.44	1
25 ppm	NITRITE	<0.1	0.1
25 ppm	NITRATE	<0.1	0.1
25 ppm	SULFATE	9.48	1
tank 9	NITRITE	<0.1	0.1
tank 9	NITRATE	<0.1	0.1
tank 9	SULFATE	9.76	1
tank 1	NITRITE	<0.1	0.1
tank 1	NITRATE	<0.1	0.1
tank 1	SULFATE	9.58	1
Adj. Dil.	IODIDE	<0.1	0.1
25 ppm	IODIDE	<0.1	0.1
Adj. Dil.	PHOSPHATE	<0.3	0.3
25 ppm	PHOSPHATE	<0.3	0.3
Adj. Dil.	MERCURY	<0.0002	0.0002
Adj. Dil.	ALUMINUM	0.0136	0.0005
Adj. Dil.	ARSENIC	<0.0003	0.0003
Adj. Dil.	CADMIUM	<0.0003	0.0003
Adj. Dil.	CHROMIUM	0.00121	0.0006
Adj. Dil.	COPPER	<0.001	0.001
Adj. Dil.	NICKEL	0.000646	0.0006
Adj. Dil.	LEAD	0.000449	0.0003
Adj. Dil.	ZINC	0.00395	0.0005
	Organochlorine Pesticides	**(μg/L)**	**(μg/L)**
Adj. Dil.	ALPHA-BHC	<0.02	0.02
Adj. Dil.	GAMMA-BHC	<0.02	0.02
Adj. Dil.	BETA-BHC	<0.02	0.02
Adj. Dil.	HEPTACHLOR	<0.02	0.02
Adj. Dil.	DELTA-BHC	<0.02	0.02
Adj. Dil.	ALDRIN	<0.02	0.02
Adj. Dil.	HEPTACHLOR EPOXIDE	<0.02	0.02
Adj. Dil.	ENDOSULFAN I	<0.02	0.02
Adj. Dil.	DIELDRIN	<0.02	0.02
Adj. Dil.	4,4'-DDE	<0.02	0.02
Adj. Dil.	ENDRIN ENDOSULFAN II	<0.02	0.02
Adj. Dil.	4,4'-DDD	<0.02	0.02

Appendix B (cont.)

SAMPLE TYPE	PARAMETER	RESULT (mg/L)	DETECTION LIMIT (mg/L)
Adj. Dil.	ENDRIN ALDEHYDE	<0.02	0.02
Adj. Dil.	4,4'-DDT	<0.02	0.02
Adj. Dil.	ENDOSULFAN SULFATE	<0.02	0.02
Adj. Dil.	ENDRIN KETONE	<0.02	0.02
Adj. Dil.	METHOXYCHLOR	<0.02	0.02
Adj. Dil.	AROCLOR-1016/1242	<1.01	1.01
Adj. Dil.	TOXAPHENE	<1.01	1.01
Adj. Dil.	CHLORDANE	<1.01	1.01
Adj. Dil.	AROCLOR-1221	<1.01	1.01
Adj. Dil.	AROCLOR-1232	<1.01	1.01
Adj. Dil.	AROCLOR-1248	<1.01	1.01
Adj. Dil.	AROCLOR-1254	<1.01	1.01
Adj. Dil.	AROCLOR-1260	<1.01	1.01
	Organophosphorus Pesticides	**(μg/L)**	**(μg/L)**
Adj. Dil.	THIONAZIN	<0.1	0.1
Adj. Dil.	PHORATE	<0.1	0.1
Adj. Dil.	SULFOTEPP	<0.1	0.1
Adj. Dil.	DISULFOTON	<0.1	0.1
Adj. Dil.	METHYL PARATHION	<0.1	0.1
Adj. Dil.	PARATHION	<0.1	0.1
Adj. Dil.	FAMPHUR	<0.1	0.1

Adj. Dil.= adjusted dilution water, Ammonia= total ammonia. Samples of adjusted dilution water and water containing 25 ppm perchlorate were collected from tubing entering exposure tanks, not from within the exposure tanks containing fish. The purpose of the ammonia, nitrite, nitrate, sulfate, iodide, and phosphate measurements was to determine whether the addition of perchlorate introduced other pollutants into the water. Comparisons indicate that dosing the water with perchlorate did not alter these parameters. Water samples also were collected from within Tank 1 (31 ppm perchlorate) and Tank 9 (1200 ppb perchlorate) for analysis of ammonia, nitrite, nitrate, and sulfate to determine whether the presence of fish or their uneaten food or waste altered these water quality parameters. With the exception of ammonia, none of these parameters appears to have been affected by the presence of the fish.

Appendix C. Scores assigned to individual thyroid follicles in a subjective nonparametric rank-order assessment method used to assess activity of fish thyroid tissue. The follicle marked with a black arrow in each photograph received a score corresponding to the number on the photograph. Tissue was given a score of 1 if the majority of the follicles had squamous epithelial cells with no colloid depletion, i.e., if the majority of follicles received a score of 1. A score of 2 was given if the majority of the follicles consisted of squamous epithelial cells with a few cuboidal or columnar epithelial cells located adjacent to isolated vacuoles in the otherwise full colloid or if the majority of follicles showed evidence of uniform, slight elevation in epithelial cell height (short of cuboidal in shape) and no colloid depletion. Tissue scored 3 contained a majority of follicles with cuboidal to columnar epithelial cells and little or no apparent colloid depletion. A score of 4 was assigned when the majority of follicles had columnar epithelial cells and evident colloid depletion, while a score of 5 was given when the majority of follicles had columnar epithelial cells and complete or nearly complete colloid depletion.