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Effects of Perchlorate Exposure on Swimming Performance of Zebrafish (Danio rerio) and Organ Development of Mice (Mus musculus)

Quentin Phillips
Minnesota State University, Mankato

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By

Quentin Phillips

A Thesis Submitted in Partial Fulfillment of the

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This thesis has been examined and approved by the following members of the student's committee.

Dr. Michael Minicozzi
Advisor

Dr. Rachel Cohen
Committee Member

Dr. Frank von Hippel
Committee Member

Table of Contents

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Abstract

Biomimetic properties of endocrine disrupting compounds (EDC) enable these chemicals to induce physiological responses in organisms at the cellular level, affecting fishes, amphibians, reptiles, birds, and mammals. Perchlorate is a ubiquitous EDC to which nearly all humans of industrialized countries are exposed. Perchlorate competitively inhibits the sodium-iodide symporters located within the follicular cells of the thyroid gland, reducing the body's ability to uptake iodide and synthesize thyroid hormone (TH), a crucial hormone in the regulation of many metabolic processes. Threespine stickleback (*Gasterosteus aculeatus*) and zebrafish (*Danio rerio*) are aquatic model organisms used in perchlorate exposure research. Exposure causes many common shared pathologies; however, sex and species-specific results have been observed between the two models, implying the potential for variation in results across other species as well. These models provide much insight into the effects of perchlorate exposure, but mammalian models such as mice (*Mus musculus*) provide a better representation of the effects of perchlorate exposure in humans across all developmental stages. For this project, I aimed to determine the effects of perchlorate exposure on swimming performance in adult wildtype zebrafish and multi-system organ development in sexually mature C57/BL6 mice. Zebrafish embryos were exposed beginning at ~2 hours post-fertilization to either a 0 ppm (control), 10 ppb, 10 ppm, or 100 ppm perchlorate solution until 100-114 days post-fertilization and subject to a standard U_{crit} test to assess swimming performance. Mice were exposed for 49 days after weaning, euthanized, and organs including the thyroid gland, liver, testes, and ovaries were dissected, sectioned, and stained using hematoxylin and eosin. Interestingly, the U_{crit} of perchlorate exposed zebrafish did not differ from that of the control group; however, the ability to acclimate to low flow showed a dose-dependent decline in the number of fish able to complete acclimation as exposure concentration increased. Morphological abnormalities found within the mouse thyroid included a reduction in follicle area, and an increase in lipid deposition and blood vessel frequency. Perchlorate exposure caused an increase in the aggregates of inflammatory cells in exposed livers, suggesting that perchlorate exposure causes insult to hepatic tissue. Within reproductive tissues, perchlorate caused an increase in vacuolation and disorganization in testis and increased the number of follicles in the exposed ovaries. These data support the need for more research on perchlorate exposure as mice display similar and disparate effects to the established aquatic models.

Extended Abstract

Biomimetic properties of endocrine disrupting compounds (EDC) enable these chemicals to induce physiological responses in organisms at the cellular level, affecting fishes, amphibians, reptiles, birds, and mammals. Disruption of normal cellular processes may lead to altered behavior and metabolism, developmental abnormalities, and reduced quality of life, including early death. Perchlorate is a ubiquitous EDC to which nearly all humans of industrialized countries are exposed. Perchlorate competitively inhibits the sodium-iodide symporters located within the follicular cells of the thyroid gland, reducing the body's ability to uptake iodide and synthesize thyroid hormone (TH), a crucial hormone in the regulation of many metabolic processes. Chronically diminished levels of TH may lead to hypothyroidism, a condition characterized by developmental, behavioral, and metabolic abnormalities, affecting fetal development and individuals of all ages. Perchlorate has both natural and anthropogenic sources, largely originating from products of explosive nature due to the widespread use of perchlorate as an oxidizing agent by aerospace and defense companies. Products containing perchlorate typically have a short shelf life and require periodic replacement and disposal, contributing greatly to environmental contamination and organismal exposure.

Threespine stickleback (*Gasterosteus aculeatus*) and zebrafish (*Danio rerio*) are aquatic model organisms used in perchlorate exposure research. Exposure causes many common shared pathologies; however, sex and species-specific results have been observed between the two models, implying the potential for variation in results across other species as well. Alterations to stickleback behavior show a dose-dependent decline in swimming performance and reproductive attempts as well as morphological and developmental abnormalities including lipid deposition in the thyroid and reduction of whole-thyroid area and follicle size. Perchlorate exposed stickleback also develop non-alcoholic fatty liver disease (NAFLD), whereas zebrafish do not. Reduced

overall thyroid area and reduced colloid follicle area have also been observed in a dose-dependent manner in both models. Though aquatic models provide much insight into the effects of perchlorate exposure, mammalian models such as mice (*Mus musculus*) provide a better representation of the effects of perchlorate exposure in humans across all developmental stages. My thesis work continues to elucidate species-specific and generalized effects of perchlorate exposure in vertebrate species.

For this project, I aimed to determine the effects of perchlorate exposure on swimming performance in adult wildtype zebrafish and multi-system organ development in sexually mature C57/BL6 mice. Based on previous work, I predicted swimming performance of perchlorate-exposed zebrafish to decline in a dose-dependent manner. Zebrafish embryos were raised from syngamy and exposed beginning at ~2 hours post-fertilization to either a 0 ppm (control), 10 ppb, 10 ppm, or 100 ppm perchlorate solution until 100-114 days post-fertilization. Swimming performance was assessed using a fish flume and a standard U_{crit} test that began with a 45-minute acclimation period at 4 cm/s. Fish able to successfully resist the acclimation flow were subjected to periodic flow rate increases by 2 cm/s every 180 seconds until the fish could no longer resist flow. Final velocity and elapsed time were recorded and used to determine U_{crit}, the maximum sustained swimming velocity of each fish. Fish unable to resist the flow of the acclimation current were not used in U_{crit} analysis but were documented and used to compare trial success across groups.

Within mice, I predicted NAFLD to be present in exposed livers, average thyroid follicle area to be reduced, and presence of lipid and blood vessel frequency to increase within the mouse thyroid in a dose-dependent manner. Within the mouse testis, I predicted a dose-dependent decline in seminiferous tubule and lumen area but an increase in Sertoli cell and Leydig cell nuclei area as well as a dose-dependent increase in vacuolation and disorganization. Within the

mouse ovary, I predicted a dose-dependent decline in follicle frequency and maturity of follicles. To determine this, mouse pups were weaned at 36 days post-partum, housed by generation and sex, and exposed to either a 0 ppm (control), 10 ppm, or 100 ppm perchlorate solution administered through their drinking water. Mice were exposed for 49 days, euthanized, and organs including the thyroid gland, liver, testes, and ovaries were dissected, paraffin embedded, sectioned, and stained using hematoxylin and eosin. Thyroid tissue was analyzed for average thyroid follicle area and frequency of excess blood vessels and lipid. Within the liver, alterations to gross morphological structure and lipid (steatosis) frequency were recorded. Seminiferous tubule and lumen area, vacuolation and disorganization, and nuclear area of Leydig cells were measured in the testis, and within the ovary, follicle maturity and frequency were recorded.

Although I predicted a dose-dependent decline in swimming performance, the U_{crit} of perchlorate exposed zebrafish did not differ from that of the control group; however, the ability to acclimate to low flow showed a dose-dependent decline in the number of fish able to complete acclimation as exposure concentration increased. Morphological abnormalities found within the mouse thyroid included a reduction in follicle area, and an increase in lipid deposition and blood vessel frequency, in a dose-dependent manner. There was no indication that perchlorate exposure caused lipid accumulation or morphological changes in the mouse liver, suggesting perchlorate may not induce NAFLD as it had in stickleback. Aggregates of inflammatory cells were observed during analysis and their frequency was recorded. Frequency of inflammatory cell aggregates increased in a dose-dependent manner, suggesting that perchlorate exposure causes insult to hepatic tissue, though more work is needed to determine the nature of this phenomenon. In testis, there was no effect on seminiferous tubule or lumen area, though vacuolation and disorganization increased in a dose-dependent manner. Interestingly, there was a non-monotonic reduction in the Leydig cell nuclei area as this occurred only in the 10 ppm group. Within the perchlorate-exposed

mouse ovary, the frequency of primary follicles and overall follicle frequency increased in the 100 ppm group and displayed a dose-dependent increase in the frequency of late antral follicles across treatment groups, contrary to my predictions.

Future work should include swimming performance analysis of zebrafish at key life stages to determine the extent to which perchlorate exposure affects swimming performance throughout life. A series of swimming performance tests may reveal vulnerable periods of development in which the organism is not able to perform to physical demands based on the environment. Histological analysis of these fish may also be done to determine the extent to which gross internal morphology is affected by exposure. Future work involving mammalian models should expose dams 21 days prior to breeding and throughout pregnancy to reduce serum levels of maternal thyroid hormones to expose pups from an embryonic stage and to determine inter-generational effects. Weights of the mother's pre and post-pregnancy, normalized for size of litter, should be recorded and used to determine the effects of perchlorate on birth weight and offspring frequency. Analyzed organs should include those used in this experiment with the addition of the heart and kidneys due to perchlorate being filtered and eliminated by the kidneys and both organs being of the same type of embryonic tissue as bone and skeletal muscle, which are typically affected by hypothyroidism.

Chapter 1

Effects of Perchlorate Exposure on Swimming Performance in Adult Zebrafish

Introduction

Endocrine disrupting compounds (EDCs) are xenobiotic chemicals capable of eliciting physiological responses in organisms through the disruption of various metabolic processes (Guillette et al. 1995). Acute exposure to EDCs may affect local cells and tissues; however, chronic exposure may lead to widespread downstream effects dependent on the concentration, route, and duration of exposure as well as the individual's sensitivity to that EDC. Perchlorate is a pervasive EDC capable of competitively inhibiting the sodium-iodide symporters (NIS) located within the thyroid gland, binding to the receptor with 30X the affinity of iodide (Wolff 1998 and Tran 2008). Inhibition of the NIS by perchlorate reduces the ability of thyroid follicular cells (TFC) to uptake iodide, a key component in the synthesis of thyroid hormones (TH: thyroxine, T_4 and triiodothyronine, T_3; Clewell et al. 2004). Chronically diminished TH, hypothyroidism, may lead to metabolic syndromes, conditions characterized by altered behavior, abnormal development, and loss of metabolic homeostasis (Chaker et al. 2017).

Synthesis of TH begins with the release of thyrotropin-releasing hormone from the hypothalamus which binds to thyrotrope cells located within the anterior pituitary to signal the release of thyroid stimulating hormone (TSH). TSH binds to receptors on TFCs to initiate the intracellular process of TH synthesis. First, an ion gradient is established across the external membrane of the TFCs to provide the kinematic means necessary for the uptake of iodide into the cell, against its concentration gradient. Sodium-potassium ATPases embedded within the apical cell membrane of TFCs actively transport three sodium ions out of the cell per two potassium

ions into the cell, establishing a negative resting potential within the cell (Darrouzet et al. 2014). The NIS then utilize the charged gradient to transport sodium and iodide into the TFCs via secondary active transport for storage before being moved into the colloid space for further processing. In this process, sodium is moved with its concentration gradient, while iodide is moved against its concentration gradient and stored for TH synthesis. Within the colloid, iodide is bound to the tyrosine residues of thyroglobulin (Tg), the precursor molecule to TH. As Tg precursor molecules are cleaved and modified, molecules of either monoiodotyrosine (MIT, single bound iodide) or diiodotyrosine (DIT, two bound iodides) are produced depending on the number of bound iodide ions to each tyrosine residue. From the colloid space, Tg is endocytosed into the TFCs on the basal side of the cell where MIT and DIT are then cleaved from the parent structure and joined, forming either T_3 or T_4 depending on the MIT/DIT or DIT/DIT combination. One MIT bound to one DIT produces T_3, and two bound DIT molecules produce T_4. Release of TH into circulation is also stimulated by the binding of TSH to its receptor on the TFCs. Within serum, T_4 serves as the inactive form of TH, needing to be converted to T_3, the active form, by deiodinase 2 in order to activate TH receptors. TH expression is maintained by positive and negative feedback loops that respond to fluctuations in TH concentration and activate or inhibit certain processes and mechanisms depending on natural fluctuations and unnatural perturbations. Disturbance of any part of this process will result in various pathologies and the implementation of compensatory mechanisms as the individual attempts to metabolically correct the disruption and reestablish homeostasis.

Perchlorate is commonly used by the aerospace and defense industries as an oxidizing agent for sustained propulsion reactions, such as in military munitions and aerospace vehicles (Fournier and Brady 2005). Products of this nature require periodic replacement to work effectively, subsequently introducing perchlorate salts into the environment, contributing greatly

to soil, water, and organismal contamination and exposure (Fournier and Brady 2005). Common perchlorate-containing products include pyrotechnics such as fireworks, flares, and explosives involved in blasting and fracking, as well as herbicides (Urbansky et al. 2003; Trumpolt et al. 2005). Environmental concentrations of perchlorate vary and depend on the type and amount of products being manufactured and used, and how they are disposed. The production, use, and disposal of perchlorate-containing products introduces remarkable amounts of perchlorate salts into the environment annually, which remain stable in soil and solution for decades (Stetson et al. 2006; Rao et al. 2007). The munitions manufacturing plant in Lubbock, Texas is one such example of this. Perchlorate salts leach from the disposal site and dissociate into their constituent cations and anions (perchlorate, ClO_4^-) within the arid environment, exposing local aquatic and terrestrial ecosystems to this thyrotoxic chemical. A study conducted near this site found tissue concentrations of perchlorate to be higher than that of the local environment in some native fish species, suggesting the potential for bioaccumulation of perchlorate in certain fish species, but not in others (Theodorakis et al. 2005; Furin et al. 2013). Since the early 2000s, numerous studies have been conducted to elucidate the prevalence of perchlorate in the environment, food and drink items, and organisms. In 2006, researchers detected perchlorate in 213 of 285 common food items including bottled beverages, produce, dairy, and meat, consisting of both locally sourced and imported goods (El Aribi et al. 2006). Prevalence among human populations was further elucidated by a study conducted by the Centers for Disease Control (CDC), which found that all 2820 sampled humans from across the United States tested positive for urinary perchlorate (Blount et al. 2007). This study consisted of a wide demographic, including males and females aged six years or older, and detected the highest concentrations of urinary perchlorate in children aged 6-11 (Blount et al. 2007). This raises concern because in 2009, environmental perchlorate had been detected in 45 U.S. states, indicating exposure via external sources (GAO et al. 2010).

Despite the prevalence of perchlorate in ground and surface water, conventional filtration techniques are unable to remove perchlorate from solution and require specialized facilities and equipment in order to remove perchlorate from water destined for human consumption (Brechner et al. 2000; US EPA 2022). These facilities remove thousands of kilograms of perchlorate from solution per year, though environmentally relevant concentrations persist (US EPA 2005).

Chronic perchlorate exposure may lead to hypothyroidism, a condition characterized by diminished TH production accompanied by elevated TSH expression across all tested vertebrate species, including fishes, birds, reptiles, and mammals (Zoeller et al. 2007). Elevated expression of TSH serves as a compensatory mechanism by the individual in response to low concentrations of circulating TH (Brechner et al. 2000). Symptoms of hypothyroidism include fatigue, weight gain, muscle weakness, decreased cognitive abilities, and impaired memory, and if not properly addressed, the individual may experience reduced quality of life and even premature death (Chaker et al. 2017). Animal models provide a clearer view of the effects EDCs have on humans, like perchlorate exposure, due to many developmental processes being conserved across taxa. Fishes are ideal models for testing the effects of EDCs, such as perchlorate, due to ease of exposure and rapid development. Perchlorate may be added to tanks to create a broad but precise range of concentrations, ensuring consistent exposure due to perchlorate being absorbed through the skin, mucus membranes, and by the gills during respiration (Theodorakis et al. 2006). Because fishes are such practical models for exposure experiments, much of our knowledge of the effects and mechanisms of perchlorate comes from research using threespine stickleback (*Gasterosteus aculeatus*) and zebrafish (*Danio rerio*).

Prior experiments to elucidate the effects of perchlorate exposure in stickleback found exposure from an embryonic stage to cause a dose-dependent increase in morphological abnormalities and aberrant behavior (Bernhardt and von Hippel 2006; Bernhardt and von Hippel

2008). Perchlorate exposure in stickleback also caused impaired development of bone and other calcified traits, reduced development and silvering of scales, and induced abnormalities in organ morphology (Bernhardt et al. 2011; Furin et al. 2015). Within the thyroid gland of perchlorate-exposed stickleback, proliferation of TFCs, diminished and irregular follicle morphology, and increased angiogenesis and lipid deposition were observed in a dose-dependent manner (Gardell et al. 2017; Furin et al. 2015; Petersen et al. 2015). Perchlorate-exposed stickleback also developed non-alcoholic fatty liver disease (NAFLD), though zebrafish did not, indicating species-specific effects of perchlorate exposure (Minicozzi et al. 2019; Minicozzi et al. 2021). Slower growth rates, poor survivorship, and male biased sex ratios were also observed in perchlorate-treated stickleback (Bernhardt et al. 2011). Alterations to stickleback behavior included a dose-dependent decline in swimming performance when subjected to environmentally relevant flow within a fish flume, decline in ability to resist acclimation flow, and reduced courtship and mating behaviors (Bernhardt and von Hippel 2008). Courtship and mating abnormalities included a dose-dependent decline in the ability of male fish to build nests, court a female, and guard fry. In higher treatment groups, some genetically female fish displayed male-typical courtship and mating behaviors to other genetic females (Bernhardt and von Hippel 2008).

In zebrafish, perchlorate exposure resulted in many common shared phenotypes when compared to stickleback. Within the zebrafish thyroid, increased TSH expression, diminished follicle volume, and increased frequency of angiogenesis and lipid deposition occurred in a dose-dependent manner (Mukhi and Patiño 2007; Schmidt et al. 2011). Other shared pathologies include diminished development of calcified traits, limited to the jaw and associated structures (Patiño et al. 2002; Mukhi and Patiño 2007). Perchlorate exposure caused a female sex bias in laboratory zebrafish, which unlike stickleback, do not have genotypic sex determination, and did not induce NAFLD at any concentration, even upon replication of the experiment (Sharma and

Patiño 2013; Mukhi et al. 2007; Minicozzi et al. 2021). Less is known about the effects of perchlorate exposure on the behavior of zebrafish, though one study found no alterations in average time spent travelling or distance travelled in perchlorate-exposed zebrafish larvae compared to the controls (Fraser et al. 2017). This is interesting because unpublished data from our lab suggests that perchlorate exposure in zebrafish larvae causes irregular behaviors, indicating further variation in exposure outcomes.

Due to the nature and extent of the disparate sex and species-specific effects found previously, my project aims to expand upon our current knowledge of the effects of perchlorate exposure on behavior by comparing the average U_{crit} of adult wildtype zebrafish exposed to a range of environmentally relevant perchlorate concentrations beginning at an embryonic stage. These data will further be compared to previous experiments which have used stickleback as the model organism to continue to elucidate species-specific and generalized effects of exposure. I predict a dose-dependent decline in average U_{crit} across groups as concentration increases. I also predict there to be a dose-dependent decline in the ability of fish to successfully complete the low flow acclimation period prior to the experimental trial.

Materials and Methods

Fish Husbandry

Housing and Feeding

All animal husbandry and protocols were approved by Minnesota State University's Institutional Animal Care and Use Committee (Protocol # 21-01). Ten adult male and ten adult female wildtype zebrafish were purchased from Aquatic Research Organisms, INC and housed separately by sex in 37.85 L tanks consisting of DI water mixed with 1.0 g/L Instant Ocean©

(Spectrum Brands Blacksburg, VA) at a volume of 20 L/ tank or 2 L/ individual. For non-breeding conditions, adult fish were fed 175 mg of 300-micron GEMMA MICRO zebrafish food per tank once a day, photoperiod was maintained at 10:14 light:dark, and water temperature was maintained at 24℃. Tanks were equipped with a sponge filter to aerate the water as well as activated carbon filters which were cleaned weekly, and carbon replaced every two weeks. Water changes were conducted every week by removing and replacing approximately 90% of the water volume, during which time tanks were scrubbed using a firm bristle brush to remove any hard stuck algae or food debris.

Breeding Conditions

In preparation for breeding, adult fish were fed 175 mg of 300-micron GEMMA MICRO zebrafish food per tank twice a day approximately 12 hours apart with supplemental 3 mL brine shrimp feedings twice a day for female fish beginning one week prior to breeding. Photoperiod was shifted to 14:10 light:dark and water temperature was raised to 28℃ to replicate normal seasonal breeding conditions. For breeding, adult fish were removed from their respective 37.85 L housing tanks and placed into 9.46 L breeding tanks at a ratio of three male to four female fish, consisting of the same water preparation standards and seeded with 100 mL of water from each housing tank. Bottoms of the breeding tanks were lined with sterile 1 cm marbles to prevent adult fish from consuming eggs and embryos. Breeding tanks were scrubbed and sterilized with 5% bleach solution and marbles were autoclaved. A total of five tanks were prepared and fish were placed into their respective tanks four hours before lights-out to acclimate to their new environment. Fish were left undisturbed overnight and for three hours after lights-on to provide adequate time for breeding, and not fed during this time. Adult fish were collected and returned to

a same-sex tank at a frequency of ten individuals per 37.85 L tank, water was changed, and fish were fed.

Embryo, Larval, Juvenile, and Adult Care

Marbles from the breeding tanks were removed and viable embryos were collected using a 3 ml transfer pipette and placed into a 250 mL beaker filled to 150 mL with an antifungal solution composed of 2 mL 0.1% methylene blue added to 1 L E2 media (Westerfield 2007). Embryos were randomized and rinsed with fresh antifungal solution four times, then placed into sterile glass Petri dishes at a density of 40 embryos per dish filled with 50 mL of fresh antifungal solution mixed with perchlorate stock to create a 10 ppb, 10 ppm, or 100 ppm concentration, in addition to the control treatment (0 ppm perchlorate). Embryos were kept in the antifungal solution until all embryos hatched (~ 3 dpf), at which point methylene blue was no longer added to the E2-perchlorate media. E2 media and perchlorate concentrations were prepared to the same standards throughout the experiment. Water changes were conducted four times per day while in Petri dishes, spaced approximately three hours apart. Larval fish were allowed to rely on their yolk sac for nutrition until 6 dpf and then started on a diet of live rotifers given four times per day approximately one hour before each water change. Larval fish were allowed to remain in the Petri dishes until 15 dpf when they were transferred into 450 mL glass jars filled with 250 mL of E2-perchlorate media. The jar volume was increased to 400 mL at 21 dpf and fish were kept on the same rotifer feeding schedule with the addition of 1mL of brine shrimp per day. Water changes were reduced to two per day approximately 12 hours apart and the density was maintained at 40 fish per jar. Embryos and larval fish were kept in an incubator at 28℃ with a 14:10 light:dark photoperiod. At 30 dpf, fish were transferred from their respective jar into 9.46 L tanks filled to a volume of 120 mL per fish and fed 3 mL of brine shrimp five times per day per tank.

Temperature was reduced to the ambient 24℃ (room temperature) and photoperiod was maintained at 14:10 light:dark, and fish were housed at a density of 75 fish per 9.46 L tank. At 60 dpf, fish were transferred into their permanent 37.85 L housing tanks and water volume was increased to 200 mL per fish. Fish were euthanized immediately after acclimation failure or trial completion via Tricaine (MS-222) overdose and fixed in 10% buffered formalin.

Experimental Design

E2 media was comprised of three separate solutions, E2A, E2B, and E2C, mixed in 10 L of nanopure water to create the final E2 media used to house fish. E2 media was prepared based on the methods of Nusslein-Volhard et al. (2002). Perchlorate stock was prepared by dissolving 2.0 g of sodium perchlorate into 200 mL of DI water, resulting in a 10,000 ppm solution. Methylene blue stock was prepared by adding 0.20 g of methylene blue to 200 mL of DI water. The E2 methylene blue solution was made by adding 2 mL of the methylene blue stock to 998 mL of E2 media (Nusslein-Volhard et al. 2002). Perchlorate stock was added to master mix solutions dependent on the desired concentration and for juvenile and adult tanks, perchlorate stock was added directly to tank water. Master mix solutions were incubated at 28℃ for the initial 30 days to reduce temperature shock when water changing.

Critical Swimming Velocity Trials

Flume Design

Flume design utilized two spherical-impeller pumps connected in series, capable of independent or dual control. Pumps can achieve up to 50 cm/s and be restricted to flow rates as low as 1.50 cm/s with the use of an electronic modulating control valve. An Omega flow meter capable of high-resolution control was used to assess flow velocity. Flow rate was controlled

through a closed-loop PI (proportional-integral) control scheme implemented using myRIO (National Instruments) capable of recording flow velocity data every 100 ms to minimize deviation in desired flow rate and overshoot when changing velocity.

U_{crit} Parameters

Assessment of adult fish (100-114 dpf) critical swimming velocity (U_{crit}) began with a 45-minute acclimation period at 4 cm/s (Napolitano et al. 2018). Because each trial took several hours, concentration was randomized to spread the variation of age across all treatment groups. Three fish from each concentration were run through the flume at a time and a top-down camera recorded the trials to keep track of individuals. Fish able to successfully complete acclimation were immediately subjected to periodic flow rate increases of 2 cm/s every 180 s. Failure of the trial occurred when the fish could no longer resist flow or if the tail touched the mesh three times at the back of the conduction portion of the flume. Upon failure, current velocity and elapsed time for that interval were recorded for U_{crit} assessment. Fish unable to complete acclimation were not used for determining average U_{crit} but frequency of success was recorded for comparison across experimental groups.

Statistical Analysis

All statistical analyses were conducted with IBM SPSS statistics 27 (IBM Corp. 2020). A Shapiro-Wilk test was used to determine normality and a Levene's test used to test for homogeneity of variance. An analysis of variance (ANOVA) was used to compare average U_{crit} between groups. A Tukey's post hoc was used to determine which groups differed if a significant ANOVA was detected. U_{crit} values were divided by the standard length (SL) of tested fish, the distance between the tip of the snout and base of the caudal fin, to normalize for size variation.

Results

Perchlorate exposure caused a dose-dependent decrease in the ability of fish to complete acclimation despite no differences being detected in the standard length of fish across all groups (ANOVA, F= 0.293, df= 3, p= 0.831, Fig. 1). An ANOVA revealed differences in average U_{crit} (ANOVA, F= 3.561, df= 3, p= 0.016, Fig. 1) but a Tukey's post hoc test failed to detect any differences in U_{crit} across treatment groups (all p-values above 0.05, Table 1). Notably, some of the 100 ppm fish developed a large red protrusion that distended the abdomen, a phenotype that to our knowledge has not previously been observed (Fig. 2). A small population of the 100 ppm fish, including individuals with visibly red and non-red abdomens, were sequestered and allowed to persist in a separate tank. The phenotype ceased to express after approximately 180 dpf, and no mortalities occurred during this time.

Discussion

Behavioral analysis of perchlorate-treated zebrafish revealed no significant difference in average U_{crit} or SL across groups. There was, however, a dose-dependent decline in ability to complete acclimation to low flow, as seen in perchlorate treated stickleback when subjected to low flow conditions (Bernhardt et al. 2011). Fish able to complete acclimation were able to swim statistically similar to untreated fish, suggesting perchlorate may be affecting multiple pathways associated with swimming performance and fish that completed the acclimation may have ameliorated the effects that caused the decline by the time trials commenced. Tests of swimming performance across developmental life stages could be used to test for an interaction between

perchlorate exposure and development in regard to swimming performance. If performance is dependent on developmental stage, histological analysis of this developmental series may reveal disruptions to musculoskeletal or nervous system morphology that may cause the dose-dependent decline in acclimation success, despite all groups being able to achieve a statistically similar U_{crit}. Histological analysis of the 100 ppm fish should be compared to determine the origins of the protrusions. The protrusion may be a combination of thyroid goiter and hypertrophied liver, likely due to lipid accumulation because of oxidative stress in response to exposure (Garcia-Ruiz et al. 2007; Chen et al. 2014; da Cunha et al. 2017). A study conducted by Schmidt and Braunbeck 2011 exposed zebrafish to various thyrotoxic chemicals and found some to develop an increase in vasculature in the opercular region, however, these protrusions did not extend beyond this region, suggesting disruption to multiple organ systems in fish with visibly red abdomens.

Future work should also include histological analysis of musculoskeletal structures associated with locomotion and respiration, including metrics of the gills, vascular system, pectoral and caudal fins, and caudal peduncle. Analysis may reveal morphological abnormalities responsible for the dose-dependent decline in acclimation success. Considering that there was no difference in SL, alterations to musculoskeletal morphology may not be significantly affected, and the dose-dependent decline may be related to metabolism or control of these systems. Alterations to thyroid hormones are linked to disorders of the nervous system related to regulation of cognition, visual processing, and motor control, which may be brought on by perchlorate exposure and affect some individuals more than others (Baksi and Pradhan 2021). It is possible that exposure to perchlorate may not affect the development of the musculoskeletal system but may alter neural control of these systems, allowing for the dose-dependent decline in acclimation success, but also the ability to perform similarly to untreated fish if these effects are ameliorated. Histological analysis of 100 ppm fish should be used as a guide for future investigation of these

structures. Internal anatomy of the 100 ppm fish visually expressing the red abdomen phenotype should be compared to visibly unaffected 100 ppm fish and to other 100 ppm fish unable to complete acclimation. If alterations to skeletal, muscular, or organ development are detected, results should be compared across groups to determine the frequency and severity to which associated pathologies present. It is also possible that perchlorate may affect metrics of metabolism that some individuals are able to ameliorate. Molecular work analyzing expression of thyroid hormones, hemoglobin concentration and oxygen binding, blood volume, and stress markers could explain these differences and should be used in conjunction with histological findings (Dorgalaleh et al. 2013).

Conclusion

Zebrafish exposed to environmentally relevant concentrations of perchlorate did not perform significantly different compared to the controls, though exposure did cause a dose-dependent decline in ability to complete the acclimation period (Fig. 1). SL did not differ across groups and aside from some of the 100 ppm fish presenting with protrusive red abdomens (Fig. 2), no further gross morphological abnormalities were observed. Histological analysis will need to be done to determine the nature of the red protrusion and its effects on surrounding tissue.

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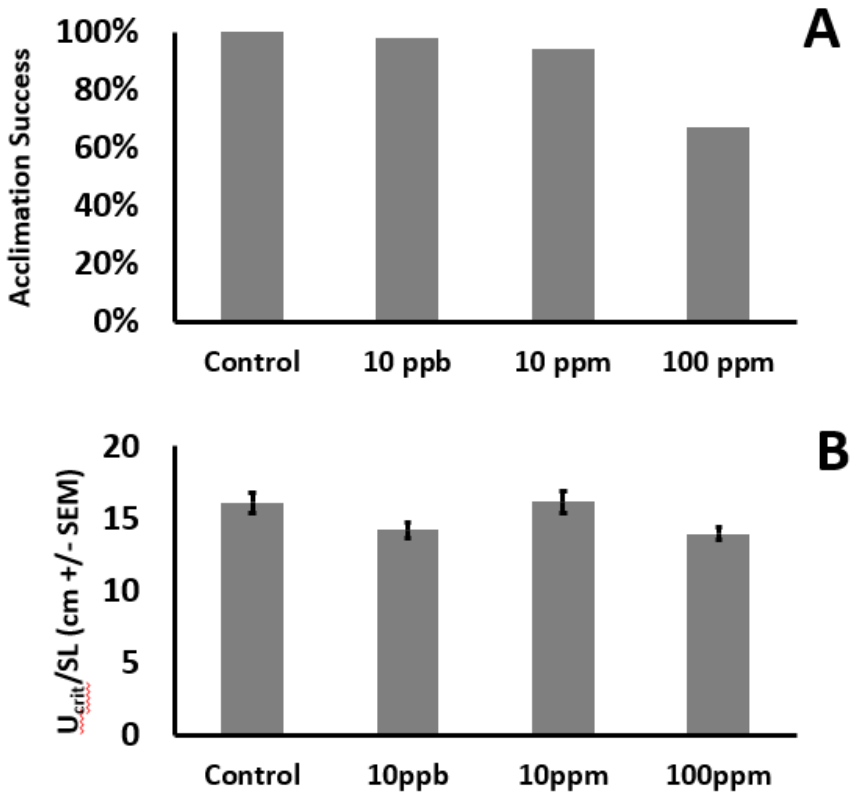

Figure 1: Perchlorate caused a dose-dependent decline in acclimation success but fish able to complete acclimation had similar U_{crit} across treatment groups. (A) All control fish were able to complete acclimation. Only fish that completed acclimation were analyzed for U_{crit}. (B) There were no differences in U_{crit} across treatment groups (ANOVA, all post hoc, $p > 0.05$). Sample size varied across treatment based on larval survival and acclimation success, control n= 31, 10 ppb n= 45, 10 ppm n= 36, and 100 ppm n= 46.

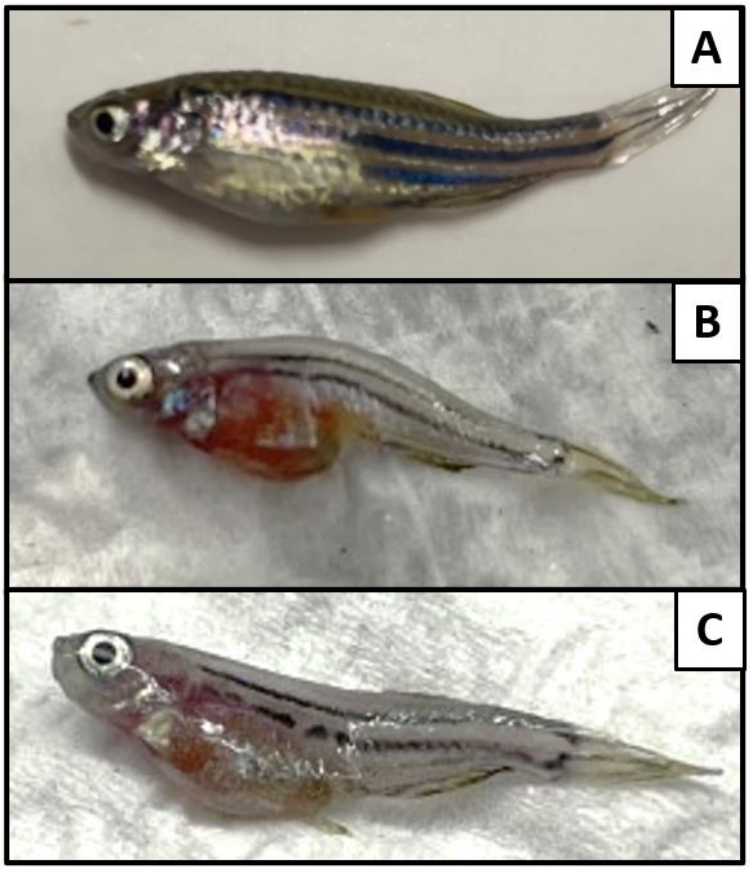

Figure 2: Protrusive red abdomen phenotype. Image (A) is of a control fish displaying no external morphological abnormalities. Images (B) and (C) are of fish in the 100 ppm group that developed visibly red and protrusive abdomens. Fish in these images were fasted for 24 hours.

Table 1: Results of Tukey's post hoc tests for the significant ANOVA for U_{crit}. Despite the significant ANOVA, no significant differences were apparent among the treatment groups.

Comparison	Mean Difference	Standard Error	P-value
0 ppm and 10 ppb	1.92	0.86	0.121
0 ppm and 10 ppm	-0.01	0.92	1
0 ppm and 100 ppm	2.18	0.94	0.098
10 ppb and 10 ppm	-1.92	0.10	0.103
10 ppb and 100 ppm	-1.94	0.99	0.991
10 ppm and 100 ppm	2.19	0.84	0.084

Chapter 2

Effects of Perchlorate Exposure on Multi-System Organ Development in Mice

Introduction

Endocrine disrupting compounds (EDC) are xenobiotic chemicals capable of altering various metabolic processes in organisms, eliciting non-natural physiological responses which may lead to acute and chronic pathologies (Guillette et al. 1995). Sub-chronic and chronic exposure to EDCs may affect local and global tissues and cell types depending on the concentration, route, and duration of exposure as well as the individual's sensitivity to that EDC. Perchlorate is a pervasive EDC capable of competitively inhibiting the sodium-iodide symporters (NIS) located within the thyroid gland, binding at 30X the affinity of iodide to the receptor (Wolff 1998 and Tran 2008). Inhibition of the NIS by perchlorate reduces the ability of thyroid follicular cells (TFC) to uptake iodide, a key component in the synthesis of thyroid hormones (TH: thyroxine, T_4 and triiodothyronine, T_3; Clewell et al. 2004). Chronically diminished TH may lead to metabolic syndromes, like hypothyroidism, conditions characterized by aberrant behavior, abnormal development, and loss of metabolic homeostasis (Chaker et al. 2017).

Perchlorate is commonly used by aerospace and defense companies as an oxidizing agent for sustained propulsion reactions, like military munitions and aerospace vehicles (Fournier and Brady 2005). Products of this nature require periodic replacement to work effectively, subsequently introducing perchlorate salts into the environment when disposed, contributing greatly to soil, water, and organismal contamination and exposure (Fournier and Brady 2005). Common perchlorate-containing products include pyrotechnics such as fireworks, flares, and techniques involved in blasting and fracking, as well as herbicides (Urbansky et al. 2003;

Trumpolt et al. 2005). Environmental concentrations of perchlorate vary and depend on the type and amount of product being manufactured and used, and how they are disposed. Combined, production, use, and disposal of perchlorate-containing products introduces remarkable amounts of perchlorate salts into the environment annually, remaining stable in soil and solution for decades (Stetson et al. 2006; Rao et al. 2007). Environmental concentrations of perchlorate depend on multiple factors, including the type and amount of perchlorate-containing product manufactured and used, as well as the methods in which those products were disposed. Examples of this can be seen near the munitions manufacturing plant located in Henderson, Nevada. Perchlorate salts within this arid environment readily dissociate into their constituent cation and anion (perchlorate, ClO_4^-) and are capable of leaching into the Las Vegas Wash which drains into Lake Mead and some parts of the Colorado River, contaminating the surface and groundwater received by over 20 million people throughout the Southwest United States (Brechner et al. 2000). Water from these sources is also used to irrigate crops and provide water for livestock throughout this region, contributing greatly to the spread of anthropogenic perchlorate through the distribution of contaminated crops, meat, dairy, and water (Rao et al. 2007; Urbansky et al. 2003). In 2006, researchers detected perchlorate in 213 of 285 common food items including bottled beverages, produce, dairy, and meat, consisting of both locally sourced and imported goods (El Aribi et al. 2006). Prevalence among human populations was further elucidated by a study conducted by the Centers for Disease Control (CDC), finding that all 2820 sampled humans from across the United States tested positive for urinary perchlorate (Blount et al. 2007). This study consisted of a wide demographic, including males and females aged six years or older, and detected the highest concentrations of urinary perchlorate in children aged 6-11 (Blount et al. 2007). This raises concern because in 2009, environmental perchlorate had only been detected in 45 U.S. states, indicating that exposure to perchlorate had been received via external sources

(GAO et al. 2010). Despite the prevalence of perchlorate in ground and surface water, conventional filtration techniques are unable to remove perchlorate from solution and require specialized facilities and equipment to remove perchlorate from water destined to be drinking water (Brechner et al. 2000 and US EPA 2022). These facilities do exist and remove thousands of pounds of perchlorate from solution per year, though environmentally relevant concentrations persist (US EPA 2005).

Chronic perchlorate exposure may lead to hypothyroidism, a condition characterized by diminished TH production accompanied by elevated TSH release across all tested vertebrate species, including fish, birds, reptiles, and mammals (Zoeller et al. 2007). Elevated expression of TSH serves as a compensatory mechanism by the individual in response to low circulating TH concentrations (Brechner et al. 2000). Symptoms of hypothyroidism include fatigue, weight gain, muscle weakness, decreased cognitive abilities, and impaired memory, and if not properly addressed, the individual may experience reduced quality of life and even premature death (Chaker et al. 2017). Because the thyroid has such an important role in early development, young children are particularly susceptible to the detrimental effects of hypothyroidism. This includes developing fetuses due to their dependence on maternal TH to initiate and maintain processes such as brain and skeletal development. Perchlorate crosses the blood-placenta barrier, indicating additional risk of exposure during early crucial developmental stages (Blount and Valentin-Blasini 2006 and Zhang et al. 2016). The compounding effects on the fetus from direct perchlorate exposure combined with receiving sub-adequate TH from the mother may contribute greatly to the frequency of developmental abnormalities in industrialized countries. A study conducted in Arizona sampled TSH levels in two separate populations of neonates located in either Yuma (southern AZ) or Flagstaff (northern AZ). Yuma drinking water is sourced from the Colorado River, known to carry perchlorate-contaminated water, while drinking water in

Flagstaff is sourced from local aquifers which do not contain perchlorate. Researchers found that neonates in Yuma expressed greater amounts of TSH compared to neonates in Flagstaff who had not been exposed to perchlorate-containing water (Brechner et al. 2000). Another study conducted in Southern California found an increase in frequency of neonates born with hypothyroidism whose mothers had consumed water from known perchlorate-containing sources during pregnancy (Buffler et al. 2006). These findings are concerning due to the immense health impacts perchlorate may have on individuals of all ages and the frequency in which one is likely to be exposed. Many factors contribute to the degree to which a person is affected by an EDC, including concentration of exposure, route of exposure, and the individual's sensitivity to that chemical (Scinicariello et al. 2017). Animal models provide a clearer view of the effects EDCs have on humans due to many developmental processes being conserved across taxa. Mice serve as a great model organism in experiments related to human health outcomes due to the genetic and developmental similarities shared within the class Mammalia.

Prior experiments to elucidate the effects of perchlorate exposure in vertebrates has primarily used the aquatic models threespine stickleback (*Gasterosteus aculeatus*) and zebrafish (*Danio rerio*). Between the two species, perchlorate exposure causes many common shared pathologies, though sex and species-specific effects of exposure have been observed as well. From a morphological perspective, perchlorate exposure caused impaired development of bone and other calcified traits, leading to anatomical and physiological impairments; however, the affected components differed between species. In stickleback, dermal bone and scale development was hindered, but in zebrafish the lower jaw and other associated components were. (Bernhardt et al. 2011; Mukhi and Patiño 2007). Morphological abnormalities within the thyroid gland of each species included a dose-dependent reduction in overall thyroid and follicle area, proliferation of TFCs, and increased presence of angiogenesis and lipid deposition (Furin et al.

2015; Gardell et al. 2017; Patiño et al. 2002). Alterations to hormone expression were also consistent and presented as a dose-dependent increase in TSH and decrease in TH (Gardell et al. 2017; Furin et al. 2015; Schmidt et al. 2012). Sex biases were also observed, however, in stickleback sex was skewed toward male, whereas in zebrafish sex was skewed toward female (Furin et al. 2015; Mukhi et al. 2007). Stickleback also developed non-alcoholic fatty liver disease (NAFLD), but exposed zebrafish did not (Minicozzi et al. 2019; Minicozzi et al. 2021). Interestingly, perchlorate-exposed molly fish (*Poecilia sphenops*) also developed hepatic steatosis (NAFLD), but within this species pathogenesis extended to fibrosis and necrosis of hepatic tissue in some individuals (Ahmad et al. 2009).

Disparate results within the exposed stickleback gonad have also been documented. For example, one study determined that perchlorate delayed gonad maturity, while another study determined that perchlorate exacerbated gonad development (Furin et al. 2015; Petersen et al. 2015). Within the stickleback gonad, there is a female-specific premeiotic increase in the number of primordial germ cells (PGC) that occurs before sexual differentiation, followed by an increase in apoptosis of these cells, which male stickleback do not undergo (Lewis et al. 2008). Perchlorate exposure to the stickleback gonad reduces the number of primordial germ cells present in exposed fish, suggesting that perchlorate disrupts processes associated with sexual differentiation and gonadal development (Petersen et al. 2016). Perchlorate also had a masculinizing effect on both male and female stickleback. Enlarged and developmentally mature testes were observed in genetically male stickleback while intersex gonads and functional hermaphroditism were observed in genetically female stickleback (Bernhardt et al. 2006; Bernhardt and von Hippel 2008). Despite the effects on sexual development, the F_1 generation of perchlorate-treated fish were not affected when reared in perchlorate-free water (Bernhardt et al. 2011). These findings present an interesting anomaly due to stickleback sex being genetically

regulated while laboratory zebrafish sex is epigenetically regulated, allowing for exogenous factors to influence sex determination (King et al. 2020).

Use of aquatic models offers many benefits in exposure experiments; however, the use of mammalian models provides more accurate insight into human health outcomes. Because of this, mice were selected as the model for this experiment due to the many genetic and developmental similarities shared between mice and humans. Previous experiments assessing the effects of perchlorate exposure in mammals has primarily used laboratory rats (*Rattus rattus*) and found many pathologies to be shared between this species and those found in aquatic models. Within the rat thyroid, dose-dependent alterations to thyroid morphology and hormone expression have been observed. Effects to hormones include a dose-dependent increase in TSH expression and a dose-dependent decrease in TH expression (Serrano-Nascimento et al. 2017). Developmental morphological abnormalities included a dose-dependent reduction in overall thyroid and follicle area and a dose-dependent increase in angiogenesis and lipid deposition (Khan et al. 2005; Serrano-Nascimento et al. 2017). A case study analyzed TSH and TH expression in various mammalian species, including humans, and found rats to be much more sensitive to the hormonal perturbations induced by perchlorate exposure, indicating species-specific effects on thyroid-related hormone expression in mammals (Lewandowski et al. 2004). Within the mouse liver, perchlorate exposure caused deiodinase-2 to be upregulated, suggesting a necessity for TH (T_3) or free iodide in hepatic cells which also express NIS (Chen et al. 2009; Wu et al. 2010). Though perchlorate and some EDCs may induce enough oxidative stress to cause NAFLD in the livers of multiple species, it has yet to be determined if perchlorate exposure causes NAFLD in mice (Garcia-Ruiz et al. 2007; da Cunha et al. 2017). Catalase activity within the rat thyroid decreased in a dose-dependent manner, allowing for the potential of increased oxidative stress and the associated symptoms; however, there was no difference in catalase

activity in the rat liver, suggesting minimal oxidative stress to the rat liver when exposed to perchlorate (Chen et al. 2014). To our knowledge, no literature exists pertaining to the effects of perchlorate exposure on the mouse gonad. Because so many disparate sex and species-specific results exist between aquatic and mammalian models, I aim to determine the effects of perchlorate exposure on thyroid, liver, testis, and ovary morphology in mice exposed to perchlorate as juveniles until sexual maturity.

Due to the sex and species-specific variation in exposure outcomes, my project aims to analyze the effects of perchlorate exposure on mouse thyroid, liver, testis, and ovaries to compare to findings in previous studies using other species and to define pathologies not previously tested for. Within the thyroid, I predict a dose-dependent decline in average follicle area, and an increase in the presence of blood vessels and lipid. Within the mouse liver, I predict a dose-dependent increase in the presence of NAFLD. Within the mouse gonads, I predict a dose-dependent decline in testicular and ovarian development. Within the testis, I predict a dose dependent decrease in seminiferous tubule and lumen area, and an increase in average nuclei area of Sertoli and Leydig cells. Within the ovary, I predict a dose-dependent decline in follicle maturity and frequency.

Materials and Methods

Mouse Husbandry

All animal husbandry and protocols were approved by Minnesota State University's Institutional Animal Care and Use Committee (Protocol # 21-02). Adult C57/B16 strain laboratory mice were bred, and offspring were allowed to remain with their respective parents until being weaned at 36 days post-partum. Mouse juveniles were transferred into new cages by

sex and generation and administered either a 0 ppm (control), 10 ppm, or 100 ppm perchlorate solution orally via water supply for 49 days. Solutions were changed every four days and volume recorded for consumption analysis. Perchlorate solutions were prepared by mixing a 10,000 ppm stock solution into the respective amount of tap water to achieve either 10 ppm or 100 ppm concentration. Stock solution was created by dissolving 2.0 g of sodium perchlorate into 200 mL of DI water and stored at 4℃ in solution. Bedding changes and feeding schedule were maintained by the Minnesota State University, Mankato's animal care staff.

Histology

At 85 dpf, mice were euthanized via CO_2 asphyxiation, weighed and organs including the thyroid, liver, testis, and ovaries were dissected and fixed in 10% buffered formalin for a minimum of 7 days. Organs were processed, paraffin embedded, sectioned at 10 microns, mounted on positively charged microscope slides, and stained using hematoxylin and eosin. Liver and testis were weighed before being transferred to formalin. Kidneys were also weighed but not used for histological analysis. The hepatosomatic, renosomatic and gonadosomatic (male only) indices were determined by dividing the organ weight by the mouse's body mass. Thyroid tissue was positioned to be sectioned superiorly to inferior, along the trachea and liver samples were taken from the periphery of the organ. Testis and ovaries were sectioned serially, and core sections (largest) used for analysis.

The largest section of thyroid was used for data collection and peripheral thyroid follicles were excluded due to zonal variation (Lee et al. 2016). The three largest non-peripheral follicles were measured for total follicle area and the median value was used for statistical analysis. All follicles were counted in this section of thyroid for the follicle count. Also, within the thyroid, increased blood vessel presence was determined by the presence of vessels running through the

thyroid parenchyma. Blood vessel frequency analysis excluded the superior and inferior branches of the thyroidal arteries due to these vessels being part of normal mouse anatomy. Thyroid tissue was considered to have increased vasculature if two or more vessels were present within the thyroid parenchyma. Lipids are not normally found in thyroid tissue and presence of lipids was considered if clusters of cells containing round, unstained vacuoles were in the thyroid tissue. Liver sections were generally uniform in morphology and were investigated at 40x total magnification for inflammatory cell (lymphocyte) clusters. Lymphocytes can be normally found in the rodent liver (uniformly distributed) but generally do not aggregate to form clusters. Presence of these clusters were considered if the lymphocytes aggregated locally to areas of the liver.

For testis, the three largest non-peripheral seminiferous tubules were selected for area measurements and the median value from each individual was used in statistical analysis. The seminiferous tubule area was measured by drawing a polygon around the basement membrane of the tubule. The lumen area was measured by drawing a polygon where the cell bodies (spermatids) clustered. Flagella were not included as part of the cell bodies. Leydig cells were also measured for the area of the nuclei. The three largest nuclei were selected and the median value for each individual was used for statistical analysis. Testis were also analyzed for the presence or absence of vacuolization and disorganization of the seminiferous tubule. Vacuolization generally occurs in the cytoplasm of Sertoli cells and is round in morphology and displaces the Sertoli cell nuclei (Creasy, 2012). Presence of vacuolization was considered if three or more Sertoli cells displayed this phenotype in a 100x field of view. Disorganization of seminiferous tubules occurs when spermatogonia or Sertoli cells are displaced from the basal layer of the tubule toward the lumen (Creasy et.al. 2012). Presence of disorganization was

considered if three or more large nuclei were found towards the lumen of seminiferous tubules in a 100x field of view. Image J software was used to measure all histological metrics.

Statistical Analysis

All statistical analyses were conducted with IBM SPSS statistics 27 (IBM Corp. 2020). A Shapiro-Wilk test was used to determine normality and a Levene's test used to test for homogeneity of variance. For continuous variables, an analysis of variance (ANOVA) was used to compare effects between experimental groups. A Tukey's post hoc was used to determine which groups differed if a significant ANOVA was detected. For presence/absence variables, a non-parametric binomial test was used. Each perchlorate treatment was analyzed separately and compared to a test proportion (TP) that was the proportion observed in the controls. A binomial test was not used when the control proportion was 0 or 1 as this violates the assumption of the test (IBM Corp. 2020).

Results

Thyroid

Follicle area declined (ANOVA, $F= 12.036$, $df= 2$, $p <0.001$, Fig. 1) in the 100 ppm perchlorate-exposed group (Tukey post hoc, $p <0.001$) but not in the 10 ppm exposed group (Tukey post hoc, $p = 0.45$) when compared to the controls. Presence of blood vessels increased within the mouse thyroid in the 100 ppm group (binomial test, OP= 0.78, TP= 0.048, $p <0.001$) but not in the 10 ppm exposed mice (binomial test, OP= 0.25, TP= 0.048 $p= 0.001$, Fig. 2). Lipid also increased in both the 10 ppm and 100 ppm exposed thyroids in a dose-dependent manner. Thyroid follicles in the 100 ppm group lost their typical round morphology (Fig. 3), presenting

with irregular shapes in addition to their diminished size, though density of thyroid follicles did not change across treatment groups (ANOVA, F= 0.104, df= 2, p = 0.902, Fig. 1).

Liver

Perchlorate exposure did not induce NAFLD or cause any observable alterations to gross liver morphology. 10 ppm and 100 ppm perchlorate exposure caused a dose-dependent increase in the frequency of inflammatory cell aggregates (Fig. 4) when compared to the control group (binomial test, OP= 0.3, TP= 0.1, p= 0.001 and binomial test, OP= 0.6, TP= 0.1, p <0.001, Fig. 5).

Testis

There was no difference in the total area of seminiferous tubules or lumen area (ANOVA, F= 2.790, df= 2, p= 0.067; F= 2.592, df= 2, p= 0.081, respectively; Fig. 6). Average Leydig cell nuclei area was consistent in the control and 100 ppm group but demonstrated a non-monotonic reduction in the 10 ppm group (ANOVA, F= 5.022, df= 2, p= 0.009, Fig. 6). Presence of seminiferous tubule disorganization increased in both 10 ppm and 100 ppm exposed mice (binomial test, OP= 0.571, TP= 0.286, p >0.001 and binomial test, OP= 0.679, TP= 0.286, p >0.001, Fig. 6). Presence of vacuolation also increased in both 10 ppm and 100 ppm mice when compared to the controls (binomial test, OP= 0.486, TP= 0.286, p= 0.010 and binomial test, OP= 0.536, TP= 0.286, p= 0.005, Fig. 6).

Ovary

Perchlorate exposure caused an increase in the frequency of primary follicles and total number of follicles present within the 100 ppm mouse ovary (ANOVA, F= 5.751, df= 2, p=

0.006, Fig. 7) (ANOVA, F= 6.899, df= 2, p= 0.002, Figure 5). Exposure also caused a dose-dependent increase in the frequency of late antral follicles (ANOVA, F= 12.120, df= 2, p >0.001, Fig. 7). There were no differences in the frequency of other follicle types across groups.

Water consumption and Organ Indices

Statistical analysis revealed no difference in the gonadosomatic, hepatosomatic, or renosomatic indices in mice treated with perchlorate compared to that of the control group (ANOVA, F= 0.986, df= 2, p= 0.381; F= 0.487, df= 2, p= 0.616; F= 0.136, df= 2, p= 0.873, respectively; Table 1). There was no difference between the mass of the mice before euthanasia or the total amount of water consumed between each group (ANOVA, F= 2.305, df= 2, p=0.106; F= 0.803, df= 2, p= 0.458, respectively; Table 1).

Discussion

Exposure to perchlorate had profound effects on the mouse thyroid, consistent with findings in experiments using aquatic models when exposed to similar concentrations (Furin et al. 2015; Schmidt et al. 2011). Thyroid follicle area declined, and presence of blood vessels and lipid within the thyroid increased in a dose-dependent manner. Reduced follicle area is often indicative of insufficient iodide uptake, and if prolonged, may result in reduced expression of T_3 and T_4 (Lewandowski et al. 2004). Presence of increased blood vessels may serve as a compensatory mechanism by the body to increase hormone motility in response to reduced synthesis and serum concentrations of the bioavailable hormone (Fitzgerald et al. 2018). Presence of lipid within the thyroid may be a result of oxidative stress on thyroidal cells by perchlorate (Garcia-Ruiz et al. 2007; Chen et al. 2014; da Cunha et al. 2017). Oxidative stress may reduce the individual's

ability to heal, resulting in the deposition of lipid in place of what was previously functional tissue that is now too damaged to be healed. It is possible that some exposed individuals were able to ameliorate the expression of one, both, or neither of these phenotypes; therefore, molecular work should be done to determine the genes affected in thyroidal tissue to better understand the reason for variation in expression and severity of phenotypes. Within the 100 ppm group, follicle morphology was highly irregular, which may be due to the loss of the ability to maintain structure and organization of cytoskeletal elements, a phenotype that has been documented in perchlorate-exposed aquatic models, but not in mammals (Schmidt et al. 2011). It is possible that follicle area reduction accompanied by loss of regular morphology is circumstantial in the presence of extensive TFC proliferation due to cells rapidly dividing in this region accompanied by reduced follicle contents, however, this phenotype has not been documented in other perchlorate-exposed mammalian thyroids.

There were no observable alterations to liver morphology due to perchlorate exposure, however, there was a dose-dependent increase in the presence of inflammatory cell aggregates. Aggregates are not uncommon within normal hepatic tissue but can be indicative of and pronounced in livers chronically exposed to xenobiotic chemicals (NTP 2014). Aggregates of inflammatory cells are found in regions of hepatic tissue suffering from insult, which if unable to improve, may lead to the development of steatosis and necrosis (NTP 2014). The dramatic dose-dependent increase in the presence of inflammatory cell aggregates suggests that perchlorate exposure causes insult to hepatic tissue. I speculate that the presence of the aggregates signifies increased and more severe signs of damage to the liver. Future work should expose mice for longer periods of time to determine if the exposed mouse livers express NAFLD toward middle and later life. Although different phenotypes exist between aquatic and mammalian models,

perchlorate has clear effects on the liver and future work should elucidate why differences exist between taxa despite liver function being highly conserved in vertebrate animals.

Perchlorate exposure yielded a nonmonotonic reduction in Leydig cell nuclei area in the 10 ppm group, as well as a dose-dependent increase in the presence of disorganization and vacuolation, signs of Sertoli cell damage in the mouse testis (Creasy et al. 2012). It is possible that male individuals in the 100 ppm group are engaging compensatory mechanisms brought about by the increase in exposure concentration. Molecular work to measure the effects of perchlorate exposure on testosterone expression may provide insight on how exposure affects the rate of male hormone transcription. Damage to Sertoli cells may result in alterations to sperm morphology over time and have profound effects on the individual related to infertility (Creasy et al. 2012). In replication of this experiment, Sertoli cell nuclei and morphology, and metrics of the developing sperm, should be examined to determine if insult to somatic cells translates to gametes. There was no effect on the area or gross presentation of seminiferous tubules or their lumen; however, if mice were exposed in utero and allowed to develop through sexual maturity, it is possible these structures may be underdeveloped, contributing to the reduced transduction of sperm. In contrast to exposed males, exposure to the mouse ovary caused an increase in the development, maturity, and frequency of ovarian follicles. Primary follicle and total follicle frequency increased in the 100 ppm group and frequency of late antral follicles increased in a dose-dependent fashion.

The differences observed between male and female mice in response to perchlorate are likely due to the role thyroid hormone plays in sexual maturity in mammals. Thyroid hormone is linked to maturity in mammalian testes (Wagner et al. 2008) but inhibits follicular development in mammalian ovaries (Cecconi et al. 2004). The trends observed from perchlorate are consistent if thyroid hormone was decreased in exposed mice. Reduced levels of thyroid hormone inhibit

testis development and Sertoli cell maturation (Wagner et al. 2008) and many phenotypes observed in the perchlorate-exposed testis (disorganization and vacuolization) were likely due to insult to Sertoli cells. The increased maturity of the perchlorate-exposed ovaries could also be attributed to reduced levels of thyroid hormone from perchlorate exposure. Thyroid hormone acts directly on follicle cells and inhibits follicle maturity and proliferation, even in the presence of follicle stimulating hormone (Cecconi et al. 2004).

Conclusions

Perchlorate exposure causes morphological abnormalities to developing thyroid glands, liver, testes, and ovaries. Effects to the thyroid and ovaries were consistent with those found in aquatic and other mammalian models, presenting as a reduction in thyroid follicle size and increase in the presence of blood vessels and lipid in the thyroid. Other consistencies include a loss of regular thyroid follicle morphology as seen in perchlorate-exposed aquatic models, reduction in core follicular area in low treatment groups without peripheral follicles being affected, and no alterations to follicle frequency between control and exposed organisms. Presence of inflammatory aggregates increased in a dose-dependent manner within the mouse liver with no effect on the presence of lipid. Effects to testes suggest insult to the Leydig and Sertoli cells, but not to the overall architecture of the organ. Mouse ovaries had on average a higher number of primary follicles and total follicle number in higher treatment groups, and a dose-dependent increase in late antral stage follicles.

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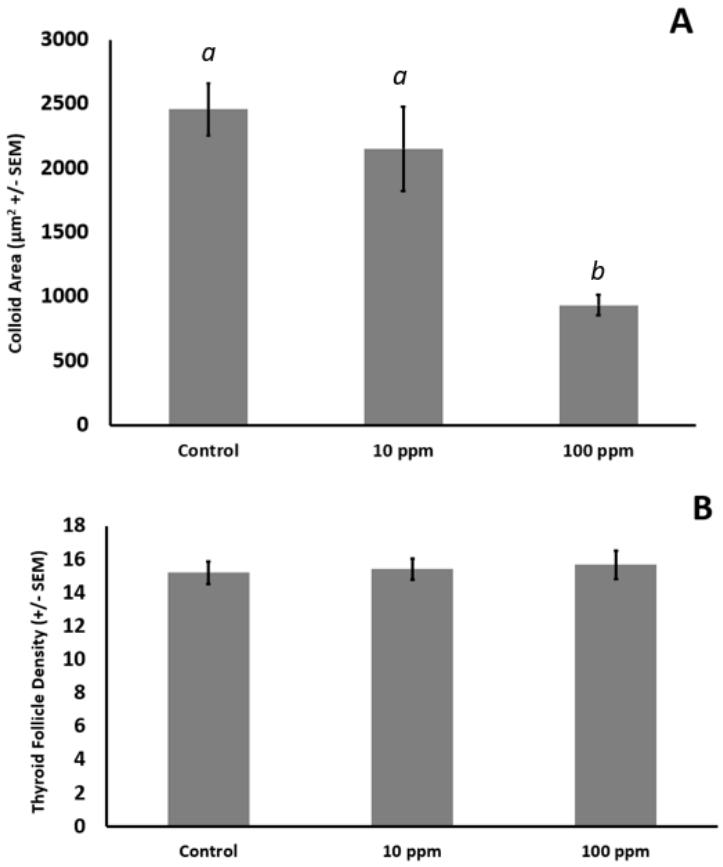

Figure 1: Perchlorate caused a decrease in follicle area but not the number of follicles present in the mouse thyroid. (A) Follicle area declined in a dose-dependent manner (ANOVA, $p<0.001$). (B) Perchlorate exposure did not affect the number of follicles in the mouse thyroid (ANOVA, $p=0.902$). Letters denote significant differences across treatment groups. Control n=21, 10 ppm n=24, and 100 ppm n=18.

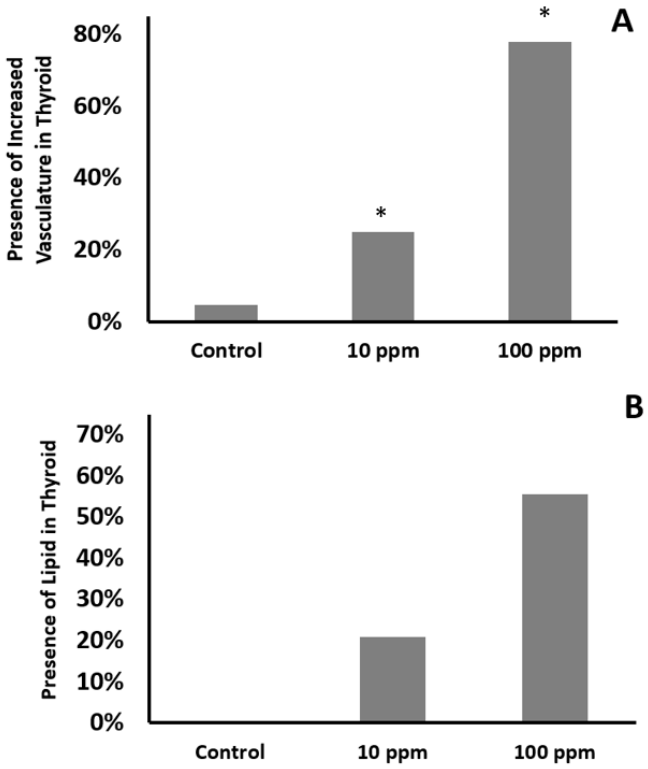

Figure 2: Presence of vasculature and lipid increased in perchlorate-exposed thyroid in a dose-dependent manner. (A) Perchlorate exposure caused a dose-dependent increase in the presence of blood vessels within the thyroid parenchyma. (B) Perchlorate exposure caused a dose-dependent increase in lipid deposition within the mouse thyroid. Asterisk denotes significant difference of a binomial test when compared to the frequency of the control group. No statistics were conducted on presence of lipid in thyroid because the frequency of the control was 0. Control n= 21, 10 ppm n= 24, and 100 ppm n= 18.

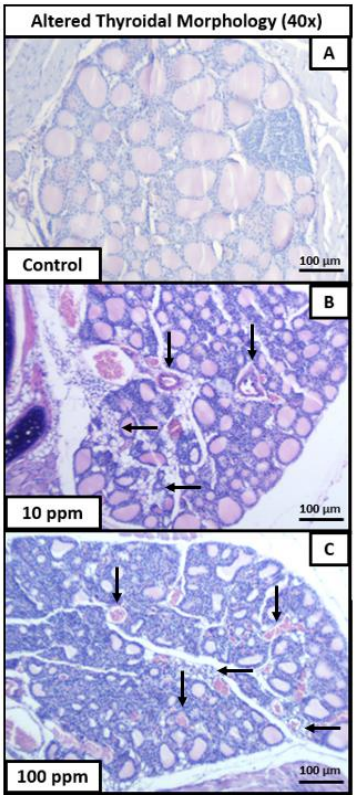

Figure 3: Perchlorate exposure altered thyroid gland morphology. Control mouse thyroid displaying normal thyroid morphology (A). Reduced follicle area and increased presence of blood vessels (vertical arrows) and lipid (horizontal arrows) in the 10 ppm group (B). Severely reduced follicle area, loss of round morphology, and increase in the presence of blood vessels (vertical arrows) and lipid (horizontal arrows) in the 100 ppm group (C).

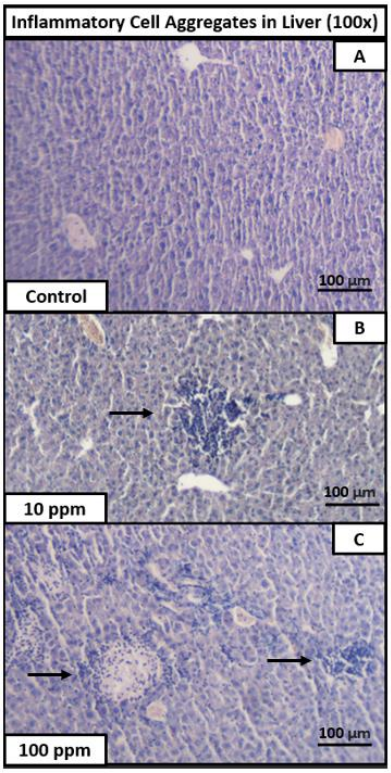

Figure 4: Perchlorate exposure caused an increase in the presence of inflammatory cell aggregates in the mouse liver. Control mouse liver showing normal liver morphology (A). An inflammatory cell aggregate (horizontal arrow) in the parenchyma of a mouse liver exposed to 10 ppm perchlorate (B). Inflammatory cell aggregates surrounding a vessel and within the parenchyma in the liver of a 100 ppm exposed mouse (C).

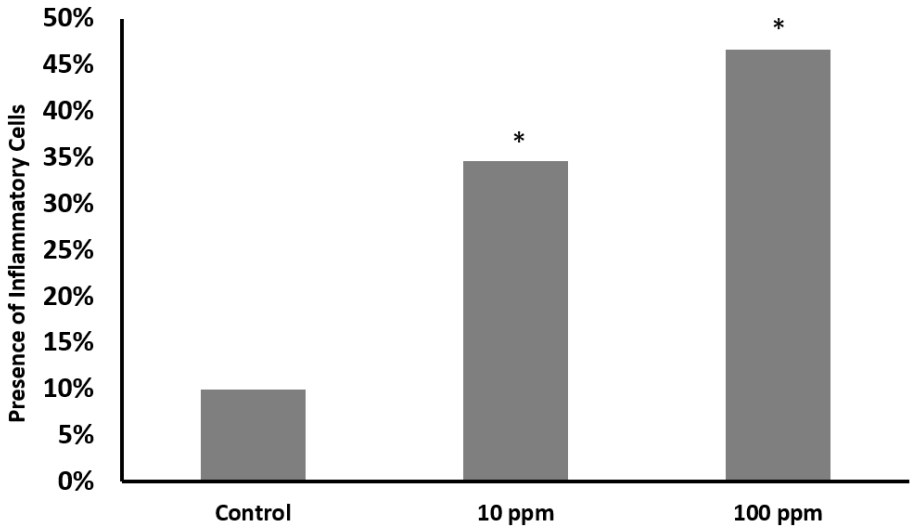

Figure 5: Presence of liver inflammatory cell aggregates increased in a dose-dependent manner. Asterisk denotes significant difference of a binomial test when compared to the frequency of the control group. Control n= 20, 10 ppm n= 26, and 100 ppm n= 19.

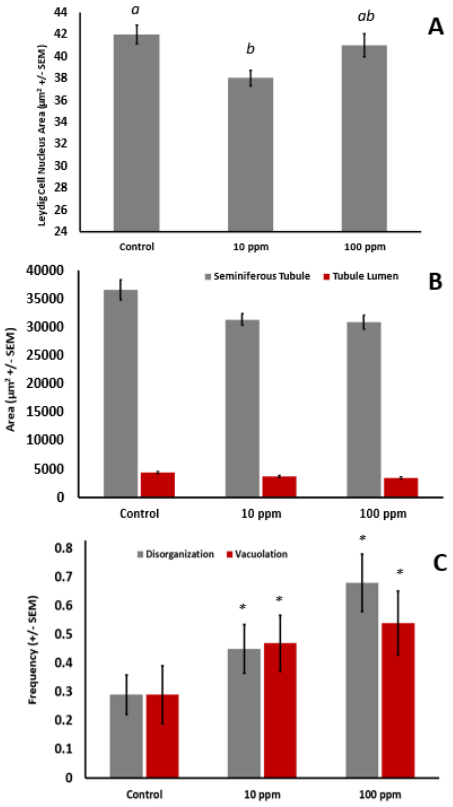

Figure 6: Perchlorate exposure caused a non-monotonic reduction of 10 ppm Leydig cell nuclei area and an increase in the presence of disorganization and vacuolation without affecting the area of seminiferous tubules or their lumen. (A) Perchlorate exposure caused a non-monotonic reduction in the area of Leydig cell nuclei in the 10 ppm group with no significant difference being detected between the control and 100 ppm treatment group. Control n= 28, 10 ppm n= 33, and 100 ppm n= 24. (B) Perchlorate exposure did not alter mouse seminiferous tubules (control n= 25, 10 ppm n= 32, and 100 ppm n= 25) or tubule lumen area (control n= 22, 10 ppm n= 32, and 100 ppm n= 25). (C) Perchlorate exposure caused a dose-dependent increase in disorganization and vacuolation. Control n= 28, 10 ppm= 35, and 100 ppm n= 28. Letters denote significant difference in A. Asterisk denotes significant difference of a binomial test when compared to the frequency of the control group in C.

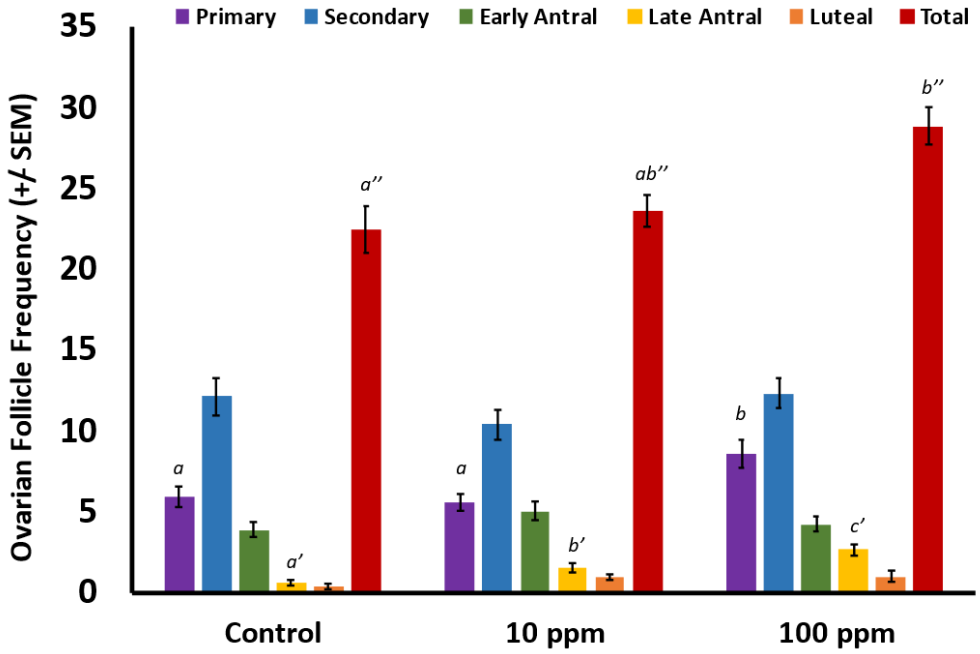

Figure 7: Perchlorate exposure caused an increase in the frequency of ovarian follicle number and maturity. Perchlorate exposure caused a dose-dependent increase in the frequency of late antral follicles, as well as an increase in primary follicle frequency and total follicle count in the 100 ppm treatment group. Letters denote significant difference across treatment groups and only significant effects are displayed to reduce redundancy in the figure. Control n=18, 10 ppm n=21, and 100 ppm n=15.

Table 1: Summary of water consumption and organ indices.

Variable	Concentration	Average	Standard Error	ANOVA p-value
Water Consumption (mL per individual)	0 ppm	263.8	19.58	
	10 ppm	288.8	14.48	
	100 ppm	252.6	9.797	0.458
Renosomatic Index	0 ppm	0.0163	0.00030	
	10 ppm	0.0166	0.00039	
	100 ppm	0.0165	0.00023	0.873
Gonadosomatic Index (male only)	0 ppm	0.0078	0.00010	
	10 ppm	0.0079	0.00014	
	100 ppm	0.0082	0.00015	0.381
Hepatosomatic Index	0 ppm	0.0577	0.00120	
	10 ppm	0.0600	0.00093	
	100 ppm	0.0591	0.00246	0.616