

PERCHLORATE TOXICITY IN FISH: TROPHIC TRANSFER, DEVELOPMENTAL
WINDOWS, AND HISTOLOGICAL BIOMARKERS

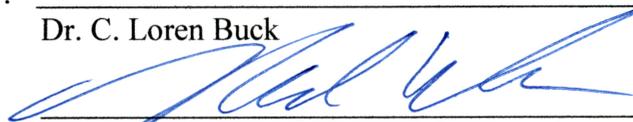
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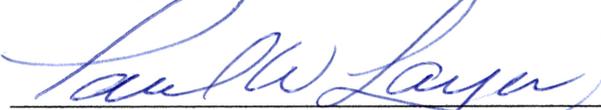


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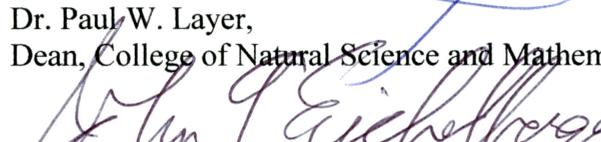


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PERCHLORATE TOXICITY IN FISH: TROPHIC TRANSFER, DEVELOPMENTAL
WINDOWS, AND HISTOLOGICAL BIOMARKERS

A
DISSERTATION

Presented to the Faculty
of the University of Alaska Fairbanks

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By

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Abstract

The perchlorate anion is an oxygenated chlorine compound often used by the military as an oxidizer in solid rocket propellant and by industry in numerous other applications. It is a known endocrine disruptor and competitively inhibits the uptake of iodide into thyroid tissue in a concentration dependent manner, effectively reducing production of thyroid hormones. Perchlorate is highly water soluble, kinetically inert and has low adsorption tendency, making it a persistent and mobile aquatic contaminant. Perchlorate has been detected in drinking water sources throughout the United States and is present in many commercially available food and drink products. The objective of this dissertation was to better understand the bioaccumulation, toxicodynamics and morphological changes caused by perchlorate exposure utilizing northern pike (*Esox lucius*) and the threespine stickleback species (*Gasterosteus aculeatus*) model.

The first research chapter (chapter 2) examines the potential for bioaccumulation and trophic transfer of perchlorate in northern pike exposed to 10 and 100 mg/L perchlorate via ambient water and food. As expected, perchlorate does not biomagnify, but does concentrate in the gastrointestinal tract tissue of pike. At the lower exposure concentration (10 mg/L) for combined water and food exposure, greater than additive tissue concentrations were detected indicating the need for regulatory testing to consider not only contaminated water but the associated contaminated food in the contaminated ecosystem (e.g., some studies provide food that is not contaminated while fish are exposed only to contaminated water).

The following two chapters (chapters 3 and 4) examine the morphologic effects of exposure timing and duration on developing stickleback utilizing a unique upshift/downshift exposure regime to determine if critical developmental windows of perchlorate sensitivity exist for two exposure concentrations (30 and 100 mg/L). In chapter three, gross morphology (body

size and skeletal armor traits) were quantified in sexually mature fish. The results demonstrate that growth can be suppressed with continuous exposure beginning within the first 14 days post fertilization (dpf). Skeletal armor traits responded variably to perchlorate exposure, with some increasing, some decreasing and others developing normally. The traits measured in this study (excluding standard length) were not sensitive to the timing of exposure (i.e., no definitive critical windows), but responses were concentration-dependent.

Chapter four examines thyroid tissue histomorphological endpoints, sex ratio, and gonadal maturity in stickleback exposed to perchlorate (30 and 100 mg/L) for varying times and durations. Thyroid tissue responded with increased follicle hyperplasia, decreased area of colloid, increased angiogenesis, and follicle cell hypertrophy. Within the first 42 dpf, a critical window emerged for follicle hyperplasia and area of colloid. Stickleback rescued (removed from perchlorate contaminated water) anytime up to 305 dpf recovered from follicle hyperplasia and reduced colloid area. Angiogenesis increased in fish exposed to perchlorate and a critical window was detected for fish exposed to 30 mg/L anytime between 7 and 154 dpf. Recovery from angiogenesis did not occur.

The ratio of males to females and gonadal development were altered in stickleback exposed to perchlorate continuously beginning within the first 14 dpf. Sex ratio was skewed toward males in a concentration-dependent manner, which could be due either to a masculinizing effect of perchlorate on sexual development or to differential survival of the sexes. Additionally, gonadal maturation was delayed for both sexes as the proportion of late stage testes and oocytes decreased in perchlorate exposed fish.

Overall, these results demonstrate that the effects of perchlorate on aquatic vertebrates are complex. Movement within and between organisms is complicated due to the iodide

concentrating mechanism of some tissues. Abnormalities of growth and skeletal armor traits are caused by perchlorate exposure and both are important to the survival and reproductive success of stickleback. In addition, as expected, the histomorphology of thyroid tissue is a responsive biomarker of perchlorate exposure in stickleback. Critical windows of sensitivity to perchlorate exist during early development and future research should scrutinize the biochemical mechanisms driving changes in thyroid condition and abnormal development, particularly for reproductive endpoints.

Dedication

I dedicate this dissertation to my wife Alexis Furin and my mom Barbara Lynn Furin. It would not have been possible without their love and support.

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Chapter 1: General Introduction

1.1 Introduction to Perchlorate

Common perchlorate salts consist of the perchlorate anion and several possible cations, with the most common being sodium, potassium, and ammonium; all are highly soluble in water [1]. The polyatomic perchlorate ion is a chlorine atom bound to four oxygen atoms as a tetrahedron (Figure 1.1) [1, 2]. Perchlorate is kinetically stable (i.e., high activation energy), resistant to reduction, and its negative charge resists adsorption to soil minerals [1, 3]. In aqueous media, it is effectively transported with the flow of water and can readily disperse from point sources. This makes perchlorate difficult to contain and remediate, and increases the potential for perchlorate to come into contact with aquatic organisms and impact water sources valuable to humans, including use in agriculture.

1.2 Uses of Perchlorate

The physicochemical properties of perchlorate make it useful in accelerants as an oxidizer such as in explosives, solid rocket propellant, automobile airbags and fireworks. It is also found in many other products including fixers in fabrics and dyes, paint, desiccants, batteries, metal polishers and bleaching powder [1]. The largest user of perchlorate is the U.S. Department of Defense (DoD) for rocket propellant and ammunition [1, 2]. Perchlorate has a manufacturing history in the U.S. beginning in 1895 with millions of pounds produced [1].

1.3 Perchlorate as an Environmental Contaminant

Perchlorate is commonly found in surface and ground waters and a majority of environmental contamination is of anthropogenic origin associated with manufacturing or military sites [1, 2, 4]. It occurs naturally at low levels in arid regions (e.g., Southwestern U.S. [5], Antarctica [6]) and is found in saltpeter (sodium nitrate) deposits in Chile which are mined to produce commercial fertilizers that are sold internationally [1, 2, 5-8]. Fertilizers containing perchlorate are a potential contamination source for agricultural crops and water resources associated with agricultural lands [9]. Most perchlorate occurrence studies target drinking water sources and, as of 2009, perchlorate was detected at greater than 4 µg/L in drinking water sources within 26 U.S. states [10]. Concentrations below 1 mg/L can have detrimental effects on vertebrates [11, 12]. More comprehensive testing would likely detect biologically relevant concentrations of perchlorate in widespread water resources that are utilized by aquatic organisms having economic and ecological value. Like many chlorinated compounds, the physicochemical properties of perchlorate make it an asset to industry and society but also an undesirable environmental contaminant.

1.4 Toxicity of Perchlorate

As an endocrine disruptor, perchlorate decreases iodide (I⁻) uptake into thyroid tissue [3]. As a relatively large anion, perchlorate has a greater affinity than I⁻ for the sodium/iodide symporter (NIS, alias SLC5A5) on the basolateral membrane of thyrocytes and blocks essential I⁻ uptake as a major mechanism of toxicity and past use as a therapeutant (Table 1.1) [13, 14]. To evaluate affinity potential, Wolff [13] used the degree of the anions ability to block uptake of I⁻ in thyroid tissue slices to determine the following potency series: pertechnetate ≥ perchlorate >

perrhenate > thiocyanate > tetrafluoroborate > iodide > nitrate > bromine > chloride. The perchlorate ion competitively inhibits the uptake of I⁻ into the thyroid tissue in a concentration-dependent manner [13-15]. A perchlorate driven functional I⁻ deficiency in thyroid tissue can cause reduced production of thyroid hormones (TH: thyroxine, T4 and triiodothyronine, T3) [13, 16, 17]. Individual vertebrates most at risk to the effects of perchlorate exposure are I⁻ deficient, and/or in early development or metamorphosis when there is a greater demand for TH [16, 17]. Examples for amphibians and fish include: 1) metamorphosis inhibition of Japanese flounder (*Paralichthys olivaceus*) [18] and South African clawed frog (*Xenopus laevis*) [19, 11], 2) skewed sex ratio in zebrafish (*Danio rerio*) [20] and *X. laevis* [21], and 3) abnormal development of skeleton in stickleback (*Gasterosteus aculeatus*) [22] and *X. laevis* [11]. Exposure to perchlorate during developmentally critical time points can have strong effects on fitness related traits and these deserve further scrutiny.

1.5 Regulation of Perchlorate

The U.S. Environmental Protection Agency (EPA) determined perchlorate to be a contaminant of concern due to its presence in drinking water sources and its endocrine disrupting potential. It recommended a reference dose (non-enforceable) of 0.7 µg/Kg/day in 2005 based on the recommendations of the National Resource Council [23]. The EPA also established an interim drinking water health advisory of 15 µg/L [24]. In 2011 the EPA committed to regulating perchlorate under the Clean Water Act [4], but has not provided any such regulations to date. Some U.S. States have set standards for perchlorate in drinking water including California (6 µg/L) and Massachusetts (2 µg/L) [25].

1.6 Bioaccumulation and Biomagnification

The extent of accumulation of contaminants in aquatic organism is a steady state between uptake and elimination/biotransformation. Uptake occurs by way of ambient water (respiratory, dermal surface and drinking) and/or food ingestion. Elimination is through urine, feces and respiratory surfaces. In this thesis, bioconcentration is defined as the net increase of a contaminant from water only (i.e., diffusion or active transport across epithelium in contact with water). Bioaccumulation is the net increase of a contaminant from all sources (e.g., water and ingested food sources) [26]. Biomagnification is the net increase of a contaminant from one trophic level to the next attributable to food [27]. Relative to prey, top predators can increase tissue concentrations of some chemicals (biomagnify; e.g., mercury and dichlorodiphenyltrichloroethane [DDT]) that can also increase over their lifetime (bioaccumulate). The predicted potential for some chemicals to bioconcentrate or bioaccumulate is correlated to its K_{ow} (octanol-water partition coefficient) which is a measure of lipid/water solubility with high values being lipophilic and low values being hydrophilic. Contaminants with low K_{ow} have a greater potential to be eliminated in kidney nephron filtrate [28].

1.7 Bioaccumulation and Trophic Transfer of Perchlorate

Sodium perchlorate has a low K_{ow} (-7.18) and for that reason, is unlikely to bioaccumulate as a lipophilic agent. Two properties that may increase its potential to bioaccumulate are its resistance to metabolism (persistence or refractoriness to biotransformation) *in vivo* and affinity for the NIS. Almost all perchlorate is eliminated unchanged relatively quickly (~ 8 hour half-life) via urine in rats and humans [13, 29]. The NIS is very effective at concentrating I^- and will transport perchlorate out of plasma, potentially

reducing its likelihood of being eliminated via the kidneys. Transport of perchlorate by NIS has been demonstrated in rats, both *in vitro* [30, 31] and *in vivo* [32]. Tissues known to express NIS include: thyroid, mammary, gastrointestinal tract (GIT) epithelium, salivary glands, kidney tubular epithelium, and brain [33, 34]. Fish readily take up I⁻ from water via gills and this is likely due to NIS [35].

Perchlorate has a low potential for biomagnification because it is more likely to be eliminated than accumulated in most tissues. A study utilizing largemouth bass (*Micropterus salmoides*) exposed to 1 g/L perchlorate via water and 0.5 g/L contaminated food did not detect bioaccumulation or biomagnification of perchlorate, but did find higher than expected tissue concentrations in some water and food combination treatments [36]. This suggests that perchlorate is differentially retained in some tissues and is further explored in this thesis ([37], Chapter 2).

1.8 Thyroid Endocrine System: General Introduction

Most of our knowledge of hypothalamic-pituitary-thyroid (HPT; Figure 1.2) axis development and the function of TH on the development of organ systems is derived from mammalian studies, mainly in rats, sheep (*Ovis aries*) and humans [38]. TH is crucial to normal development and maturation processes and organizational effects include coordinating the onset of brain, bone, retina, cardiac, liver, lung, gonadal and heart tissue among others [33, 38]. In particular, nearly every aspect of central nervous system development is dependent on TH [38, 39, 40]. Deficiency of TH during the developmental (organizational) period often results in abnormal development of the affected systems. Examples of activational effects of TH include basal metabolic regulation [33, 41], metamorphosis [16, 18] and immune response [42]. In

humans, hypothyroidism in infants leads to irreversible and major neurological deficits (organizational effects) while symptoms of adult onset hypothyroidism (activational effects) are generally reversible [43].

The thyroid follicle is the functional unit of TH production and consists of a single layer of follicle cells surrounding an intercellular lumen which contains thyroglobulin, a glycoprotein precursor of TH, and stores of TH. The hormone is synthesized as follows: 1) I⁻ is concentrated by follicle cells via the NIS into the lumen, 2) I⁻ is oxidized by thyroperoxidase (TPO), 3) it is added to tyrosine residues on thyroglobulin to form monoiodotyrosine (MIT) and diiodotyrosine (DIT), and 4) TH is liberated by proteolysis of thyroglobulin via lysosomes and exocytosed from the follicle cell into circulation [17, 44]. This process, as well as I⁻ uptake and thyroglobulin production, is stimulated by thyroid stimulating hormone (TSH).

T4 is the primary product released from thyroid follicles and is generally present in plasma at higher concentrations than T3, but varies by species. Plasma carrier proteins bind most TH and include thyroxine binding globulin (TBG), transthyretin (TTR) and plasma albumin [17]. In humans, TBG binds most T4, while in fish; TTR is thought to be the main carrier protein for TH [33, 45, 46]. In peripheral tissues near the site of action, iodothyronine deiodinases (D1 and D2) remove one of the iodides from the outer ring resulting in the formation of T3, which is the more biologically active of the two hormones. An additional deiodinase (D3) removes an iodide from the inner ring forming reverse-T3, which is not biologically active and is important in the clearance of TH from serum.

The action of TH is mediated by: 1) transport into target cells, 2) binding to nuclear receptors on thyroid response elements, 3) replacement of co-repressor complex with co-activator complex, 4) and regulation of gene expression. Thyroid receptors (TR) belong to a

superfamily of ligand-dependent transcription factors [17]. Two major TR have been recognized in vertebrates, TR α and TR β [17]. Ligand (T3) binding to TR generally induces increased gene transcription, but can suppress transcription as well and is specific to cell, tissue, developmental stage and species [17].

TH is eliminated after conjugation or glucuronidation, mainly taking place in the liver, which renders it more water soluble and it is then eliminated via bile (hepatic route). Conjugation involves sulfation or sulfonation by the action of a sulfotransferase [17]. Glucuronidation is facilitated by UDP-glucuronosyl transferases [17]. To a lesser extent, oxidative deamination and ether-linked cleavage pathways also metabolize plasma THs.

The HPT-axis is responsible for maintaining homeostasis of TH and the functional units are highly conserved among vertebrate taxa [17]. The paraventricular nucleus of the hypothalamus synthesizes and secretes thyrotropin-releasing hormone (TRH) which stimulates the release of TSH from the adenohypophysis (Figure 1.2). TSH then stimulates thyroid follicles to produce and secrete TH into circulation. There is a negative feedback loop per TH action upon the hypothalamus and pituitary (Figure 1.2).

1.9 Thyroid Endocrine System of Teleost Fishes

Many aspects of the HPT-axis are conserved across taxa, but there are some important differences between mammals and fish [47, 48]. A majority of fish species have thyroid follicles that are not contained within a confined gland (as in mammals) and are found dispersed in the pharyngeal region in the proximity of the brachial arches [44, 49]. A difference from mammals in the neurological control of the HPT-axis is a lack of a hypothalamic-hypophyseal portal system in fish. Instead, the anterior pituitary is stimulated by direct neurosecretory fibers from

the paraventricular nucleus in the hypothalamus [47]. T3 is regulated (deiodination and/or degradation) in peripheral tissues in fish and is dependent on the presence of appropriate enzymes [50, 51]. Changes in T4 levels do not always correspond with changes in T3 levels in mammals and fish, but such decoupling may be more important in some species of fish due to a greater separation of feedback between the HPT-axis and peripheral deiodination; further research is needed to fully characterize these interactions [47, 52].

TH levels can vary with season, photoperiod, temperature and the age and physiological state of the fish, among other factors [53]. Examples of known developmental events under control of TH in fish include metamorphosis (larval to juvenile transformation) and smolting (preparation of juveniles for migration from freshwater to saltwater). For example, eye and fin migration of the Japanese flounder (*Paralichthys olivaceus*) and transition from larval to juvenile form in lamprey (*Petromyzon marinus*) are dependent on plasma TH levels [18, 54]. Smolting in salmonids is associated with a spike in TH [47, 55]. Bone development in fish has also been shown to be under the influence of TH [18, 56-58]. TH has a wide influence over many physiological systems and development during all life stages and its disruption can have profound direct and indirect effects.

1.10 Teleost Gonadal Sex Determination and Development

Sex determination in teleosts is highly variable and includes one or a combination of: gonochoristic, parthenogenic or hermaphroditic modes (for review see [59, 60]). There are two classes of gonochorism: differentiated and undifferentiated. In undifferentiated gonochorists all germ cells initially develop as ovaries and later a percentage transform to testes (e.g., Cyprinids including zebrafish) [61]. In differentiated gonochorists gonads develop into ovaries or

testes directly from germ cells. Parthenogenic individuals reproduce asexually. There are variations of hermaphroditism, but general categories include: 1) protandry; develop as males and change to females, 2) protogyny; develop as females and change to males, and 3) simultaneous hermaphrodites (both testicular and ovarian tissues present). The labile nature of sex determination in teleosts can lead to different outcomes depending on the influence of genetic, environmental or other factors [62]. Genetic sex can be overridden by environmental factors or sex steroids [62, 63]. Intersex is another condition some gonochorists express in which the individual functions as one sex, but has both testicular and ovarian tissues. Individuals with intersex gonads are found at low frequency in some species [63].

Some time after sex determination, sexual differentiation including gametogenesis of ovaries and testes which progress through several developmental stages including proliferation of undifferentiated cells, meiotic divisions, and maturation (vitellogenesis and oocyte maturation in females and spermatogenesis and spermiogenesis in males) [64, 65]. These processes are generally under the control of sex steroids (androgens and estrogens) which are produced in gonads. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary regulate sex steroid production [64-66]. The mechanisms of TH influence on teleost gonadal development is largely unknown, but it is generally thought to inhibit reproductive processes in favor of somatic growth by interacting with multiple targets of the HPG-axis and possibly reducing LH and FSH synthesis [53, 67].

Three studies utilizing fish that were exposed to a goitrogen known to induce a hypothyroid state found skewed sex ratios; specifically, a female bias in zebrafish [20, 68] and a male bias in threespine stickleback (*Gasterosteus aculeatus*) [69]. A balanced sex ratio is thought to maximize fitness of both sexes in genotypic determinant species and are usually 1:1 in

randomly mating populations [62]. A shift to bias one sex can cause fewer fertilization events (lower recruitment) and affect population size and genetic variation; and indicates a significant biological response that could have other underlying adverse effects.

1.11 Threespine Stickleback as a Biomonitoring Tool

Despite being a small, non-commercially harvested fish, the threespine stickleback has received significant attention by the research community. Indeed, stickleback have been studied for well over a century and there is a large body of literature describing their biology and ecology [70]. This teleost in the family *Gasterostidae* is widespread in the Holarctic and inhabits diverse habitats including marine habitats, estuaries, low gradient streams and rivers, and lakes. The marine ecotype is ancestral and colonizes freshwater systems where fish adapt to local conditions, a process that has occurred in parallel in many thousands of locations [71, 72]. Their propensity to colonize new habitat, wide distribution and phenotypic diversity make them ideal for ecotoxicology studies because populations within contaminated site(s) can be compared to reference site(s), often with replicates of phenotypically convergent populations [70]. They are a hardy species that tolerate a range of temperatures, dissolved oxygen and salinities [73, 74]. Their tolerance for contaminant exposure often allows them to survive in environments where sensitive species perish, permitting studies to determine mechanisms of action. Their usefulness as a biomonitoring tool has great potential due to the availability of sophisticated molecular tools, and a diverse set of biomarkers including for both estrogen-modulating and androgen-modulated xenobiotics [75, 76].

1.12 Threespine Stickleback as a Model in the Laboratory

In laboratory settings, stickleback are easily propagated and cared for. A complete lifecycle can be completed in a year or less. More recently, their whole genome was sequenced and they have become a model for evolution-development studies (evo-devo) [77] and endocrine disruption [75, 78]. Many tools exist to evaluate morphological, genetic and endocrine endpoints [63, 70, 77-79]. Stickleback are gonochoristic with male heterogametic sex determination thus are useful for evaluating sex change and intersex under a variety of experimental conditions [63, 80]. Species in the stickleback family are the only fish known to produce a protein in response to androgens (spiggin). Spiggin is a glue-like protein produced by males in modified caudal kidney tissue that is used in nest building [79]. It can be used to quantify effects of and/or detect androgenic and anti-androgenic compounds and in combination with vitellogenin, estrogen modulating compounds. Thus, the stickleback is a useful laboratory and sentinel species for the study of hypothalamus-pituitary-gonad axis disruption [75, 76].

1.13 Teleost Toxicity Testing of Perchlorate

The exposure studies to date involving fish and perchlorate mainly involve zebrafish, while fathead minnows, mosquitofish and stickleback have been used to a lesser extent. Exposure periods ranged from 30 days to one year. Six chronic exposure studies, four using zebrafish [19, 68, 72, 81], one using fathead minnows [82], and one using stickleback [69] began exposures during development (<6dpf) and had exposure periods of 35 days or less with the exception of the stickleback study in which fish were exposed for a year. Zebrafish reach sexual maturity at approximately 90 dpf and fathead minnows at approximately 120-150 dpf [83, 84]. These six studies (with the exception of stickleback) provide good information on the acute

responses of perchlorate including thyroid tissue and gonadal perturbations but do not cover the entire lifecycle and give no information on the mechanisms involved or differential temporal effects. Potential recovery from developmental period exposures are possible and results can be quite different, depending on the time of sample collection. Fitness consequences are not adequately evaluated with acute exposures.

1.14 Standard Endocrine Disruptor Testing Procedures

The U.S. Environmental Protection Agency (EPA) has established standard testing procedures for screening potential endocrine disrupting compounds (EDC) under the Endocrine Disruption Screening Program (EDSP) [85]. It is a two tier system intended to minimize the usage of experimental animals and false negative outcomes [86]. The first tier is a battery of tests designed to detect endocrine perturbations. The second tier tests determine adverse effects and establish risk assessments. Currently only tier one tests have been carried out for the EPA's initial list of chemicals for screening [86]. Current EDC testing procedures utilizing aquatic vertebrates mainly involve acute toxicity assays in which fish are exposed for a relatively short period followed by evaluation of endocrine related endpoints to determine the presence or absence of abnormal endocrine function (HPT and HPG being the main focus). Tier two assays involve multigenerational studies but are still under development.

1.15 Thesis Goals

The intent of this dissertation is to clarify bioaccumulation potential and investigate the importance of timing of perchlorate exposure and its impacts on stickleback thyroid, gross and microscopic morphology and sexual development and outcomes. The validity of current

endocrine disruptor testing procedures will be addressed in light of experimental designs used in this dissertation. The objectives of this dissertation are listed below and the grounds for each are described in the respective chapter:

Chapter 2: Presents a study designed to determine the potential for perchlorate to bioaccumulate and biomagnify in a fish predator-prey model, and evaluate the importance of the gastrointestinal route of exposure with respect to waterborne exposure.

Chapter 3: Characterizes the response of stickleback gross morphology, specifically dermal skeletal traits and growth, to differential timing and duration of exposure to perchlorate during developmentally critical windows.

Chapter 4: Evaluates the response of stickleback thyroid tissue histomorphology to differential timing and duration of exposure to perchlorate during developmentally critical windows and determines if thyroid tissue will recover with rescue. Additionally, the effects of perchlorate on sexual development and sex determination in stickleback was evaluated in chronically exposure fish.

This work will contribute to teleost thyroid histomorphology, developmental biology and ecotoxicology and has important implications for aquatic toxicant testing procedures and natural resource management related to an important contaminant of water.

1.16 References

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1.17 Tables

Table 1.1: Physicochemical properties of perchlorate and iodide

	Perchlorate	Iodide
Molar mass (g/mol)	99.45	126.90
Water solubility (g/L @ 25°C)	2,096*	1,840*
Ionic radii (Angstrom)	2.40	2.22

*Sodium cation

1.18 Figures

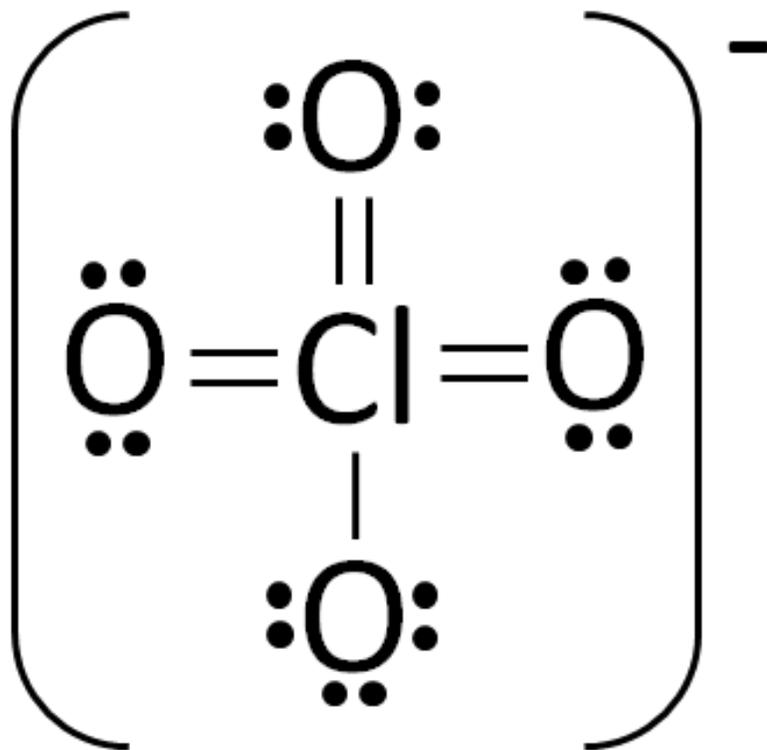


Figure 1.1: Lewis structure of the perchlorate ion

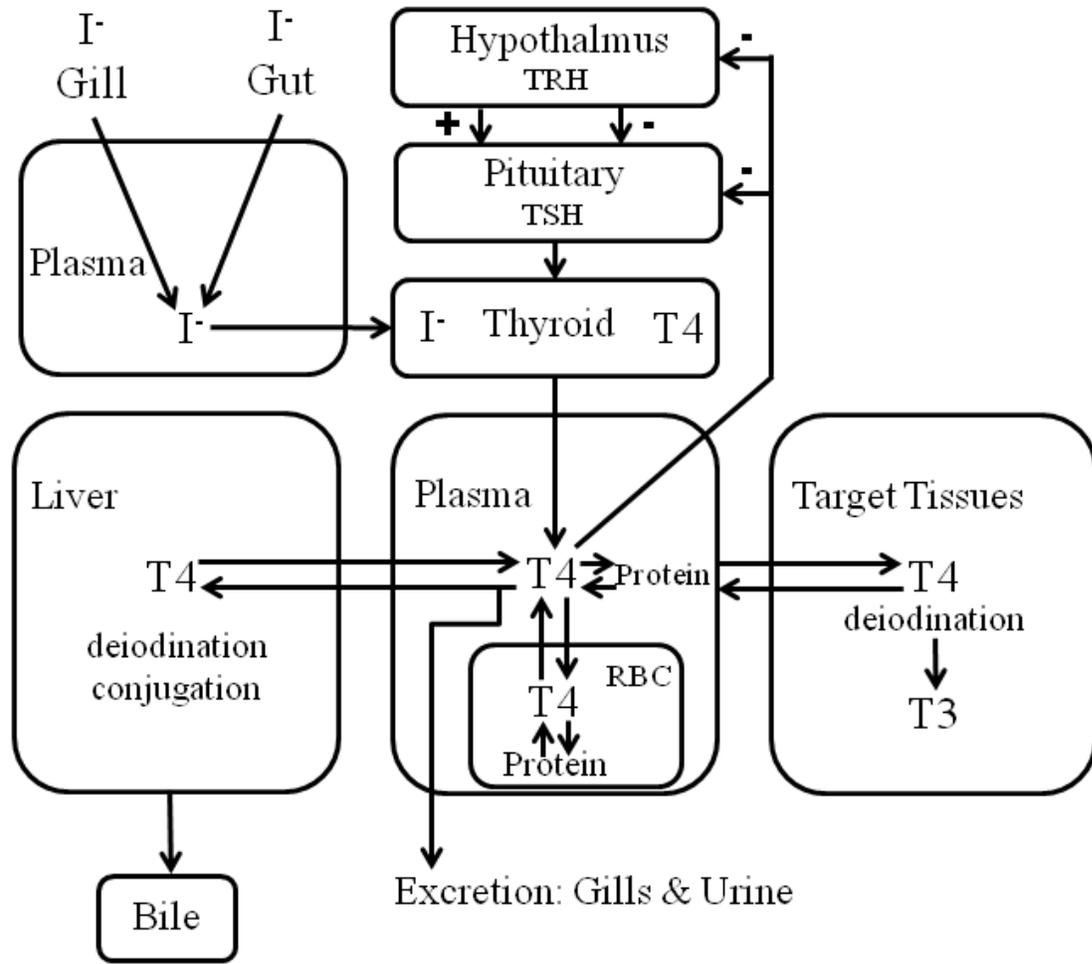


Figure 1.2: Teleost thyroid cascade: Thyroxine (T4) synthesis, secretion and metabolism. TRH = thyrotropin-releasing hormone; TSH = thyroid stimulating hormone; T3 = triiodothyronine; RBC = red blood cell. Adapted from Brown et al. 2004 [44]

Chapter 2: Perchlorate trophic transfer increases tissue concentrations above ambient water exposure alone in a predatory fish¹

2.1 Abstract

This study examined effects of varying concentrations of the environmental contaminant perchlorate in northern pike (*Esox lucius*) based on exposure in water and/or from prey (threespine stickleback, *Gasterosteus aculeatus*). Routes of exposure to pike were through contaminated water at 0, 10 or 100 mg/L perchlorate for 49 days and/or through feeding one stickleback per day over 14 days that were previously maintained in water at 0, 10 or 100 mg/L perchlorate. Both water and food significantly contributed to pike tissue concentrations of perchlorate as compared to controls, but, as expected for a water-soluble contaminant, perchlorate did not biomagnify from prey to predatory fish. Pike gastrointestinal tissue retained significantly more perchlorate than other tissues combined. Route of exposure and concentration of perchlorate in various media are important to consider in risk assessment when evaluating uptake and tissue concentration of perchlorate because significantly higher tissue concentrations may result from combined prey and water exposures than from prey or water exposures alone in a concentration dependent manner.

2.2 Key Words

Bioconcentration, *Esox lucius*, *Gasterosteus aculeatus*, Risk Assessment, Tissue Residue.

¹Furin, C. G., F. A. von Hippel, B. Hagedorn, and T. M. O'Hara. 2013. Perchlorate trophic transfer increases tissue concentrations above ambient water exposure alone in a predatory fish. *J. Toxicol. Environ. Health, A* 76: 1072-1084.

2.3 Introduction

Aquatic ecosystems contain xenobiotics that are persistent and/or accumulate in organisms (Lapworth et al. 2012; van der Oost et al. 2003). A fundamental understanding of the uptake, elimination, biotransformation and retention (disposition) of contaminants in tissues is essential when evaluating the impact of contaminants and their ability to concentrate between trophic levels. Bioaccumulation is the net increase of a contaminant concentration in an organism from all sources, including food, during its lifetime, while biomagnification is the increase of contaminant concentration from one trophic level (prey) to the next trophic level (predator) attributable to accumulation from food (Newman 2010). Some contaminants are well known to both bioaccumulate and biomagnify (e.g., methyl-mercury), but the behavior of many contaminants is unknown, particularly for hydrophilic inorganic compounds.

The perchlorate anion is a persistent environmental contaminant found in many water sources throughout the United States (Brandhuber et al. 2009; EPA 2011). Perchlorate salts are both naturally occurring and synthesized for industrial uses (e.g., ammonium perchlorate for solid rocket fuel) (Trumpolt et al. 2005; Morrison et al. 2006). Anthropogenic sources are the main cause of contamination of most affected aquatic systems (Trumpolt et al. 2005; Morrison et al. 2006). Perchlorate salts are highly soluble and the perchlorate ion is stable once in solution, where it can reside for decades (Urbansky 2002,1998; Morrison et al. 2006). Perchlorate is not expected to biomagnify in aquatic trophic webs because it is water soluble and likely easily eliminated from most organisms. The exception being plants, including important agricultural species such as spinach (*Spinacia oleracea*), which can accumulate high concentrations of perchlorate and may be significant sources of dietary exposure (Ha et al. 2011; Jackson et al. 2005; Tan et al. 2004; Sanchez et al. 2005a; Voogt and Jackson 2010).

The known mechanism of toxic effects of perchlorate is competitive binding to the sodium/iodide symporter (NIS) in thyroid tissue (Wolff 1998). This effectively blocks the uptake of iodide and thereby disrupts production of thyroid hormone (TH) which is critical for the development and maintenance of many body systems (Wolff 1998). Additionally, McDougal et al. (2011) showed that perchlorate has effects on thyroid unrelated to iodide deficiency.

Perchlorate has been shown to interfere with development and reproduction in African clawed frogs (*Xenopus Laevis*) (Goleman et al. 2002b; Goleman et al. 2002a), rats (*Rattus norvegicus*) (Baldrige et al. 2004; Gilbert and Sui 2008), mosquitofish (*Gambusia holbrooki*) (Park et al. 2006), zebrafish (*Danio rerio*) (Patiño et al. 2004; Mukhi et al. 2005; Patiño and Mukhi 2007; Mukhi and Patiño 2007), and threespine stickleback (*Gasterosteus aculeatus*) (Bernhardt et al. 2006; Bernhardt et al. 2011; Bernhardt and von Hippel 2008). Exposure occurs through contaminated water and/or food (Murray et al. 2008; Huber et al. 2011).

Organisms in aquatic environments are susceptible to perchlorate because of its solubility and stability. They are exposed to perchlorate by way of ambient water (gills and integument) and/or gastrointestinal tract (GIT) (Theodorakis et al. 2006a). Exposure via water may be continuous or sporadic depending on the source of the contaminant, hydrological setting, and route of exposure. Exposure through diet might be important and has been implicated in higher than expected tissue residue concentrations in fish and other vertebrates at perchlorate contaminated sites (Smith et al. 2001; Theodorakis et al. 2006b). The importance of total diet intake for humans has been previously investigated (Murray et al. 2008; Sanchez et al. 2009; Wang et al. 2009; Trumbo 2010; Valentin-Blasini et al. 2011), but few studies address routes of exposure for aquatic organisms, but see: (Park et al. 2005). Routes of perchlorate exposure may be more important for aquatic organisms because of presence in ambient water and physiological

differences in exposed tissues such as gills, GIT and integument, species specific variation and the presence or absence of NIS. This drives concern for a better understanding of the dynamics of perchlorate within aquatic food webs and of the relative merits of assessment techniques such as free ranging fish vs. aquatic animal models in lab settings.

Toxicokinetic studies of perchlorate in fish have been conducted using zebrafish (Liu et al. 2006; Patiño et al. 2003), Bluegill sunfish (*Lepomis macrochirus*, (Dean et al. 2004)), mosquitofish (Bradford et al. 2006) and channel catfish (*Ictalurus punctatus*, (Park et al. 2007)). General conclusions based on these studies include: 1) perchlorate is rapidly taken up from water, 2) tissue concentrations are well below the ambient water concentrations once a steady state is reached, 3) there are species differences in the concentrations of tissue residue and the tissue in which it is found (head, fillet, GIT, etc.). Bioconcentration factors (BCF) ranged between 0.02 and 0.70 and were concentration dependent. Two field studies of highly contaminated military sites found perchlorate was sporadically present in fish but were generally correlated with proximity to the point source (Smith et al. 2001; Theodorakis et al. 2006b). In contrast to the lab studies, tissue concentrations in the field caught fish were often above the ambient water concentrations. A study by Park et al. (2005) examined water and food routes of exposure in largemouth bass (*Micropterus salmonides*) and determined that perchlorate did not bioconcentrate but food exposure was more than additive to body burden when fish were exposed via water and food.

The purpose of this study was to determine if varying concentrations of sodium perchlorate provided in prey only or prey plus water resulted in additive or greater than additive differences in predator tissue concentrations as compared to water only exposure. Monitoring water concentrations alone are not sufficient to evaluate fish exposure if it is not the sole route by

which they are exposed. A 3X3 factorial study is used to evaluate predatory fish tissue concentrations and interactions between water and food routes of exposure. It is hypothesized that water and food routes of exposure will be additive to tissue concentrations. This study is expected to provide insights into the most appropriate aquatic animal models and experimental procedures for testing the food web dynamics of perchlorate over a range of concentrations.

2.4 Methods

2.4.1 Collection and husbandry

Threespine stickleback with a mean mass \pm SD of 0.39 ± 0.2 g were collected with minnow traps from Bear Paw Lake ($61^{\circ} 36.830'$ N, $149^{\circ} 45.413'$ W) in the Matanuska-Susitna Valley, Alaska, on 31 July, 2007. Juvenile northern pike (*Esox lucius*) with a mean mass \pm SD of 14.5 ± 5.7 g and mean standard length \pm SD (distance from the tip of the upper jaw to the posterior end of the hypural plate) of 139 ± 12.3 mm; (n = 43) were collected with a hand seine from Fire Lake ($61^{\circ} 20.978'$ N, $149^{\circ} 32.837'$ W) in Eagle River, Alaska between 19 July and 7 August, 2007. All fish were transported in coolers with aerated lake water to the lab at the University of Alaska Anchorage, where they were housed in outdoor 1400 L polyethylene pools (diameter = 152 cm, height = 86 cm) filled with 1000 L of de-chlorinated city water. Instant Ocean[®] was added to maintain salinity at 1 g/L, which was previously determined optimal for the juvenile pike. Mean water temperature was 12°C during the experiment. Stickleback were fed frozen brine shrimp (*Artemia* spp.) twice a day to satiation. Pike were fed unexposed stickleback from Cheney Lake ($61^{\circ} 12.045'$ N, $149^{\circ} 45.660'$ W) *ad lib* until the experiment began.

2.4.2 Experimental design

A 3 X 3 static renewal experiment with three replicates was used for pike exposures. There were 4 pools each with no perchlorate added (control), 10 or 100 mg/L perchlorate. These concentrations were chosen for this study to ensure detectable tissue concentrations in exposed fish. Environmental water concentrations > 10 mg/L have been found (Smith et al. 2001) but most water sources have < 4 µg/L (Brandhuber et al. 2009). The four pike in each pool were individually marked by fin clipping. Within every pool, each pike was randomly assigned a feeding treatment and fed individually in a large dip net. Stickleback used as prey for the feeding treatments were exposed to 10 or 100 mg/L perchlorate in two separate pools, and one control pool with no perchlorate. From day one of exposure, 5 stickleback from each treatment were collected every two days to generate a perchlorate uptake curve and determine steady state tissue concentrations.

2.4.3 Experimental procedures

Sodium perchlorate (> 98% purity, Sigma-Aldrich, St. Louis, MO, USA) was added to each tank by drying it in an oven at 90° C before weighing out 12.3 g or 123 g for 10 and 100 mg/L concentrations respectively. The salt was thoroughly dissolved in water and added to each tank. Perchlorate concentrations were verified by ion chromatography (IC) over the exposure period and were within 1 to 10% of the target concentrations throughout (Figure S2.1). Slight contamination was detected in the control tanks due to the feeding treatments (Figure S2.1).

Stickleback and pike were exposed to perchlorate on the same day (August 1st) and allowed to reach a steady state (tissue concentration reached a plateau) prior to the feeding treatment. This was done to ensure perchlorate contributed from food was detectable in the

interaction treatments. Following this period of water exposure, pike were fed according to their assigned feeding treatment for 14 days, beginning on September 1st. A single stickleback was fed to each pike per day. The mass of each stickleback was recorded before it was fed to the pike. Mean \pm SD daily ration (wtt/wtt) over the duration of the feeding treatment for all pike was 3.07 ± 2.13 . Feeding treatments are referred to as the 10 and 100 mg/L exposure of the prey throughout the manuscript. At the end of the two week feeding period and approximately 24 hr after the last feeding, pike were killed instantly in liquid nitrogen and stored in a -80°C freezer until analysis. We assumed steady state was reached for the feeding treatments after two weeks. Pike mass and standard length were recorded at the beginning of the water exposure, at the beginning and after the two week feeding period. Throughout the manuscript, pike treatments are expressed as water:feeding (e.g., 0:100 = control water and prey exposed to 100 mg/L water).

2.4.4 Perchlorate analysis of tissue and water

Fish tissue concentrations of perchlorate were determined using a modified method of Dodds et al. (2004). Stickleback were thawed and homogenized to produce a single homogenate for each sampling date for each treatment condition. The 5 fish collected from each pool every two days were treated as one sample to produce enough tissue to analyze. Pike were measured individually. Fish were thawed and the GIT from esophagus to vent was removed. The GIT contents, GIT and fish body were homogenized separately. Mean fractional weight of the GIT contents and GIT were: 5.65% and 3.04% respectfully.

Perchlorate was extracted from homogenates using an Accelerated Solvent Extraction system (ASE 200; Dionex, Sunnyvale, CA, USA) with ultra-purified water (>18 mega ohm) as solvent. A 2 g sample was mixed with hydromatrix (diatomaceous earth) and placed in a 10 ml

stainless steel ASE cell and extracted at a pressure of 689.5 kilopascal and 100°C. Two blanks and two spikes (3 ml of 1 µg/L perchlorate) were extracted and analyzed for quality control. After the extraction, 1 mL of hydrogen peroxide was added to the extract, which was heated to 90°C for approximately one hour to break down organic material. After organic material was removed, samples were reconstituted to 25 ml with ultra-purified water. For measurement, 2 ml of each sample was filtered at 0.45 µm and analyzed with a Dionex DX-500 ion chromatograph using a 4mm Dionex IonPac As16 column, 38mM KOH eluent and the suppresser method. Quantification was reached using linear regression of peak area (µS/cm * min) with 4 level calibration (10 to 500 ppb) prepared in 1:1 diluted Instant Ocean®. The perchlorate peak was readily observed at 9.22 ± 0.1 min. All reported measurements were within the range of the calibration curve. Water samples were diluted to match calibration concentration, filtered, and analyzed using the same IC method.

The detection limits for perchlorate (LOD) were 10 ng/ml in water and 30 ng/ml in tissue extracts. LOD was calculated from the regression line of the calibration curve as:

$$\text{LOD} = t * \sigma_{\text{intercept}}/m$$

with t being the student t distribution, $\sigma_{\text{intercept}}$ the standard variation of the intercept and m the slope. Recovery was determined on 5 spiked pike tissue samples and was between 65 and 80%. Uncorrected, measured values are reported below. All analyses were performed in the Applied Science Engineering and Technology (ASET) Laboratory at the University of Alaska Anchorage.

2.4.5 Statistical analysis

All statistical analyses were performed using R (version 3.0.1). Data were tested for normality using the Shapiro-Wilk test and homogeneity of residuals with the Modified Levenes

test. Differences in tissue residue were analyzed using ANOVA. For multiple comparisons, a TukeyHSD contrast was used. Nonparametric tests (Kruskal-Wallis and Dunnett's Modified Tukey-Kramer Pairwise Multiple Comparison Test) were used as well and were qualitatively equal to the parametric tests. A paired Wilcoxon rank sum test was used to determine if stomach contents and GIT perchlorate concentrations differed significantly. In addition, Response surface method (RSM) was used to evaluate interactions of effects between routes of exposure. A second order polynomial response function was used to estimate $f(x)$:

$$E[\gamma_i] = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2,$$

where $X_1 = [\text{ClO}_4^-]$ in water, $X_2 = [\text{ClO}_4^-]$ in food, β_1, β_{11} = unknown effects associated with $[\text{ClO}_4^-]$ in water, β_2, β_{22} = unknown effects associated with $[\text{ClO}_4^-]$ in food, and β_{12} = unknown effect from interaction of $[\text{ClO}_4^-]$ in water and $[\text{ClO}_4^-]$ in food. To equilibrate scaling factors, factorial data were transformed using the formula: $X_j = (\text{Actual level} - (\text{max level} + \text{min level})/2) / ((\text{max level} - \text{min level})/2)$, where the level X_j is the j th factor (Kutner et al. 2005; Lenth 2009).

To determine if there was a significant effect of route of exposure, an expected value for each treatment (10:10, 10:100, 100:10 and 100:100) was calculated as: Expected value = water $[\text{ClO}_4^-]$ from tanks with food+water treatments * BCF from water only treatments + tissue $[\text{ClO}_4^-]$ from food only treatments. The expected value was then subtracted from actual tissue concentrations and a one sample t-test performed to detect differences from zero.

Pike body condition was calculated using a regression index to control for body size differences (Jakob et al. 1996). Body mass and length were transformed using the natural log before generating residuals by regression. The differences in residuals were then used to compare between treatments of three different time points during the perchlorate exposure: 1) 14 days

after water exposure began, 2) 4 days into the feeding trial, and 3) the day of fish collection after the feeding trial.

2.5 Results

2.5.1 Perchlorate exposure from food

The dose of total perchlorate from contaminated stickleback over the 14 day feeding period was 0.16 – 0.41 µg/day for 10 mg/L exposed stickleback and 2.07 - 3.47 µg/day for 100 mg/L exposed stickleback. The mean ± SE total doses to pike for the whole two week exposure were 3.37 ± 0.3 and 37.1 ± 1.6 µg, respectively and the mean body mass specific total dose was 0.21 mg/kg for pike eating 10 mg/L exposed prey and 2.2 mg/kg for pike eating 100 mg/L exposed prey.

2.5.2 Pike body condition

Pike sample sizes for each treatment were: 0:0 - five, 0:10- three, 0:100 - six, 10:0 - five, 10:10 - five, 10:100 - five, 100:0 - five, 100:10 - five, 100:100 - four. Pike body condition did not change significantly among treatments during the exposure period (K-W $\chi^2 = 10.03$; df = 8; p = 0.26) or during the feeding trial alone (K-W $\chi^2 = 3.75$; df = 8; p = 0.88). Mean body condition increased over the whole exposure period.

2.5.3 Tissue

Stickleback tissue concentrations reached a steady state with contaminated water in 50-100 hr (Figure 2.1). Mean ± SE bioconcentration factors (BCF) were 0.047 ± 0.001 for 10 mg/L and 0.051 ± 0.003 for 100 mg/L perchlorate contaminated water. In contrast, the BCF of total

tissue (body + GI) of pike in 10 mg/L and 100 mg/L contaminated water were 0.132 ± 0.024 and 0.05 ± 0.004 , respectively. Tissue residue increased in concentration in a dose dependent relationship. Water-only exposure resulted in greater tissue residues than food-only exposures at 10 mg/L (0:10, 10:0) while there was no difference at the highest exposure level (0:100, 100:0; Figure 2.2). Food-borne exposure at the 100 mg/L level resulted in significant differences from water-borne only exposure (0:100, 10:100), with the exception 100 mg/L water in which food-borne exposure did not contribute to tissue residue (Figure 2.2). The GIT retained more perchlorate than the other tissues combined (Figure 2.2).

Total tissue concentrations of perchlorate in pike were significantly different between some of the food only, water only and food+water treatments and were concentration dependent (ANOVA: $F = 55.1$, $df = 8$, $p < 0.0001$, Figure 2.2). Pike exposed to contaminated water only (10:0 and 100:0) had total tissue concentrations significantly different from controls and each other (Tukey HSD, $p < 0.05$). Pike from 0:100 had significantly higher perchlorate concentration in their tissues than the two other food only treatments 0:0, 0:10 and the 10:10 treatment. In the 0:100 and 10:100 treatments, diet-borne perchlorate from the 100 mg/L exposed prey resulted in significantly more tissue residue than controls (Figure 2.2). The pike in 100:0, 100:10 and 100:100 showed no significant effect of diet-borne perchlorate on tissue levels (Figure 2.2). The interaction term between water and food routes was significant in the ANOVA ($F = 10.17$, $df = 4$, $p < 0.0001$). Smaller differences were detected in the 100 mg/L treatments compared to control and 10 mg/L (Figure S2.2). Tissue concentrations clearly increase with increasing exposure concentrations but there is a change in response with the highest concentrations. Gastrointestinal tract tissue did not contain significantly different perchlorate than associated stomach contents for pike in any of the treatments (Mann-Whitney: $V = 0$, $p > 0.05$; Figure 2.2),

although the 10:0 and 10:10 treatments had notable differences between these two tissues (Figure 2.2).

The response surface analysis resulted in a significant second order polynomial function ($F_2 = 7.20$, $p = 0.002$; lack of fit: $F_3 = 2.40$, $p = 0.088$; Figure 2.3). The regression coefficient and parameters showed that increasing concentrations of both water and food exposure resulted in rising pike tissue residues (Table 2.1). The negative interaction parameter indicates that there is not a greater than additive relationship between routes of exposure for all concentrations (Carter Jr et al. 1988). The stationary points (maximum estimated response) for food and water perchlorate treatments were 65.1 and 66.2 mg/L, respectively (Figure 2.3).

Actual pike tissue concentrations differed significantly from calculated expected values in two of the four combination treatments. The 10:10 treatment resulted in greater than expected tissue concentrations while the 100:100 treatment resulted in less than expected (Figure 2.4). The 10:100 and 100:10 treatments trended toward more than expected, but were not significantly different.

2.6 Discussion

The perchlorate ion was retained in the tissue of both fish species at varying concentrations and was related to water and prey contamination. Stickleback tissue reached steady state with contaminated water after approximately two days of exposure (Figure 2.1), which is similar to other studies for uptake of perchlorate in small fish (Patiño et al. 2003).

The water and food routes of exposure produced apparent concentration-dependent increases in pike tissue concentration of perchlorate. The 10:10 treatment resulted in significantly greater tissue residues than would be expected from simple addition (additivity) of

the results from food only and water only treatments. This suggests an interaction is occurring between these two routes at the lower concentration used in this study (Figure 2.3 and 2.4). This demonstrates that perchlorate does not act as a strict water soluble compound in some fish and that food can be a significant source of perchlorate for aquatic vertebrates. The RSM model resulted in a negative interaction coefficient, indicating an overall non additive relationship between routes, which is most likely due to the 100 mg/L water treatments where food contamination had no effect (Figure 2.2). Further, comparing the 100:0 and the 0:100 treatments, there was no marked difference in perchlorate levels in the GIT tissue concentration, but the body tissue concentration in the water only treatment was significantly greater (Figure 2.2) suggesting dispositional differences of the tissues involved. These results are consistent with a similar study by Park et al. (2005) who found higher than expected tissue concentrations in largemouth bass (*Micropterus salmoides*) that were exposed to both contaminated water and contaminated prey. Smith et al. (2001) and Theodorakis et al. (2006c) detected greater concentrations of perchlorate in some fish than in ambient water at highly contaminated sites in Texas, and consuming contaminated food may have contributed to the high tissue concentrations among other possible factors.

The higher than expected concentration of perchlorate in pike was in large part a result of the higher concentrations in the GIT (Figure 2.2). Some limitations of this study include the two week food exposure period and a small ration. Two weeks may not have been long enough to reach a steady state for this route. Further research is required to determine accumulation curves via exposure from food. Pike eating more contaminated prey would potentially accumulate more perchlorate. The probable explanation for greater concentrations in the GIT is that GIT epithelium expresses NIS (Wolff 1998; Yu et al. 2002) and/or because this is the primary tissue

exposed to ingested perchlorate. Perchlorate has a higher affinity than iodide for the NIS binding site and is 30 fold more potent than iodide at blocking $^{125}\text{I}^-$ transport across the cell membrane (De Groef et al. 2006; Tonacchera et al. 2004). Perchlorate may also be transported across plasma membranes (Tran et al. 2008; Attanasio et al. 2011; Cianchetta et al. 2010; Dohan et al. 2007). In pike, this would result in concentrating perchlorate in the gastric mucosa, which might account for the higher concentrations found in the GIT tissue. Indeed, three treatment combinations had higher concentrations of perchlorate in the GIT than in the associated stomach contents (Figure 2.2). Feeding on contaminated food and/or drinking contaminated water results in greater GIT tissue residue of perchlorate. Iodide, which perchlorate is chemically similar, may be a rare anion in fresh water (Watanabe et al. 1997) and a mechanism to concentrate it from diet would likely increase fitness. Geven et al. (2007) found that Mozambique tilapia (*Oreochromis mossambicus*) accumulate ^{125}I in their intestinal tract and may therefore act as a reservoir for iodide, and therefore perchlorate, in fish.

There was no significant change in tissue residue for pike eating contaminated food in the 100 mg/L water treatment (i.e., exposure via food did not alter tissue concentrations, Figure 2.2) indicating there was a change in uptake and/or elimination at this water concentration. This is in agreement with the RSM analysis that resulted in the maximized perchlorate exposure being approximately 65 mg/L water concentration. Uptake in the gut may have been limited by the density of NIS, or perhaps pike in the 100 mg/L water treatment reduced perchlorate and/or enhanced elimination of the ion. Perchlorate is known to be eliminated through urine (Wolff 1998). In fish, an additional site of ion exchange occurs at the gills and may play a role in the observed tissue concentrations

The ubiquitous presence of perchlorate in plants and animals at contaminated sites opens up the possibility that food webs contribute to the biotic distribution of this contaminant (Smith et al. 2001). Some plants bioaccumulate perchlorate to relatively high levels (Jackson et al. 2006). Herbivores may be exposed to high concentrations from contaminated plants and then become food for carnivores, effectively bridging the gap between bioaccumulating plants and non-bioaccumulating animals. Chronic exposure to high concentrations of perchlorate through diet in combination with exposure through water might result in body concentrations or burdens of perchlorate that exert detrimental health effects and would go undetected if models relied solely on water-borne exposure studies. Humans are exposed to perchlorate from green leafy vegetables grown with contaminated irrigation water (Sanchez et al. 2005b; Murray et al. 2008), drinks such as milk (Kirk et al. 2005) and water (Murray et al. 2008; Huber et al. 2011) and potentially through contaminated animals. Based on the outcome of the present study these multiple matrices and associated routes of exposure need to be considered in human as well as ecological risk assessments.

2.6.1 Conclusions

This study determined that perchlorate did not bioconcentrate (uptake from water) or bioaccumulate (food and water) in fish; which confirm the expectation for a hydrophilic anion. Perchlorate did not biomagnify from fish prey to fish predator with the possible exception of the 10:10 treatment in which tissue concentration was greater than expected. Perchlorate tissue residue was correlated with exposure concentrations and varied by tissue compartment. These results demonstrate that risk assessment of perchlorate in aquatic organisms needs to take multiple routes of exposure into consideration, especially the contributions due to exposure to

water and ingestion of food. The difference in ambient iodide concentrations in fresh water versus salt water, and the adaptations of animals for efficient ion uptake or elimination, provide additional complications that need to be investigated to better understand health risks and selection of model organisms.

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2.9 Tables

Table 2.1: Regression coefficients for pike tissue concentrations of perchlorate.

	Regression coefficient	Parameter estimate	p value
Intercept	β_0	9127.5	<0.001
[Water]	β_1	2255.4	<0.001
[Food]	β_2	1416.6	<0.001
[Water]^2	β_{11}	-3330.3	0.003
[Food]^2	β_{22}	-1843.4	0.088
Interaction	β_{12}	-742.6	<0.001

2.10 Figures

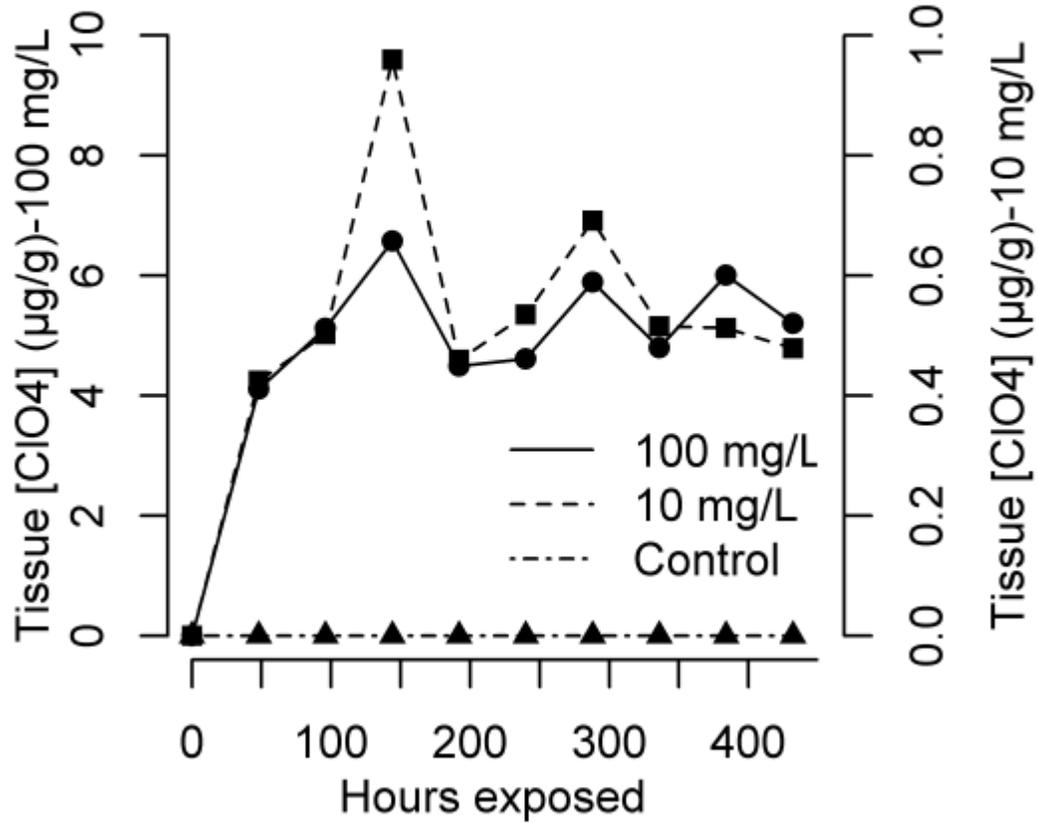


Figure 2.1: Time series of perchlorate concentrations in tissue of stickleback: Concentration ($\mu\text{g/g}$ wet weight) of perchlorate in stickleback tissue in control, 10 and 100 mg/L contaminated water. Measurements are from the homogenate of 5 stickleback collected at each time point.

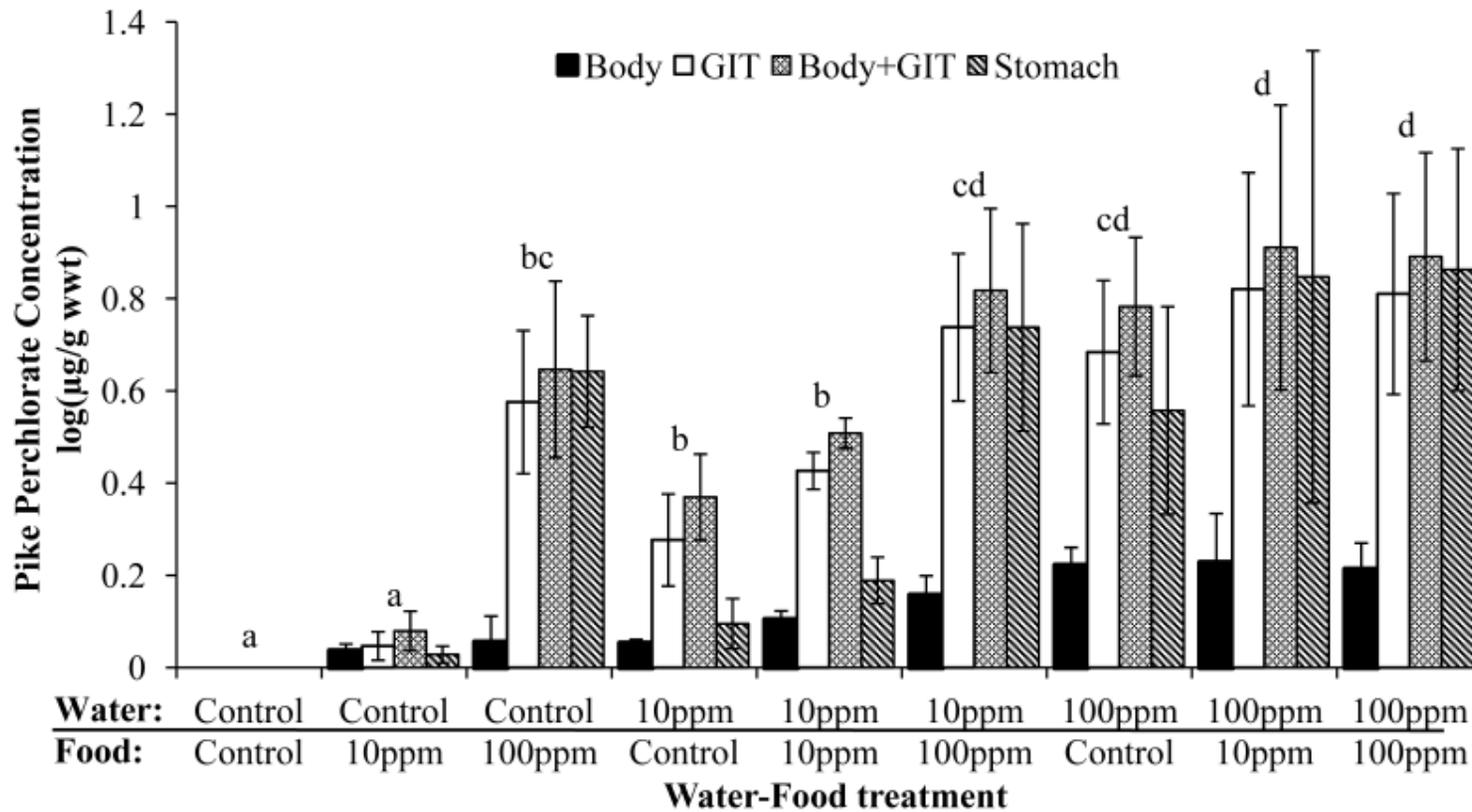


Figure 2.2: Mean concentration ($\mu\text{g/g}$ wet weight) of perchlorate in pike tissue.

Black bar = whole body – gastrointestinal tract and GIT contents; White bar = only GIT; Diagonal cross bar = whole body + GIT; Diagonal bar = stomach contents only. C = control, 10 = 10 mg/L perchlorate, 100 = 100 mg/L perchlorate. Food concentrations on the X-axis are the levels of perchlorate stickleback were exposed to prior to feeding to the pike. Different letters indicate significant differences between treatments by tissue type; Body, GIT and Body+GIT followed the same significance trend ($\alpha = 0.05$; TukeyHSD contrast).

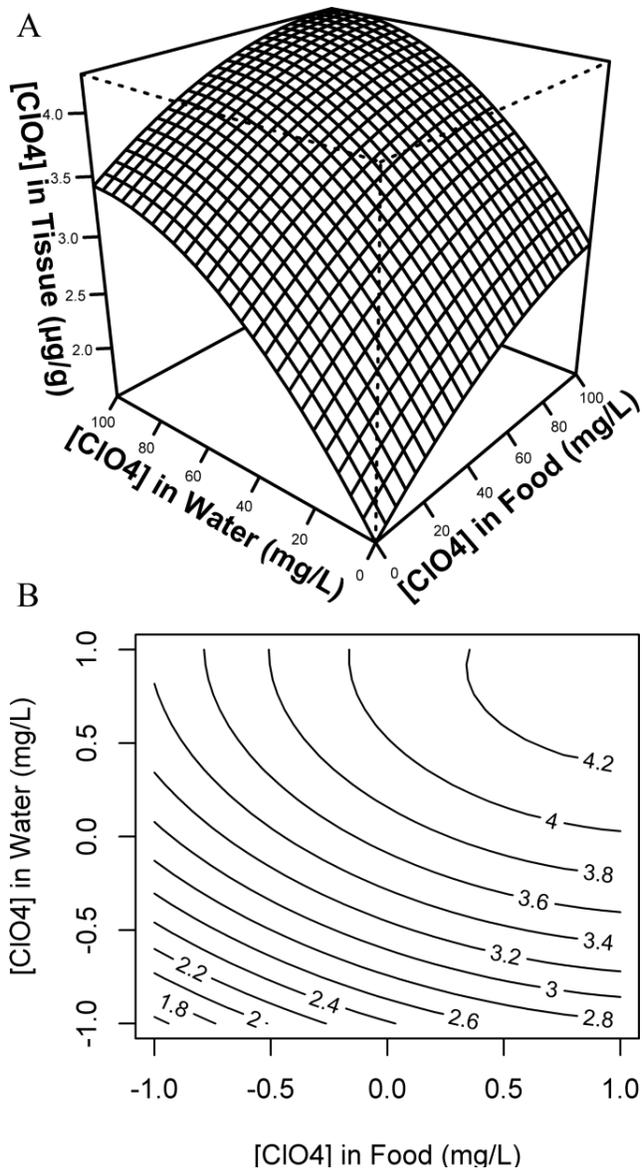


Figure 2.3: Response surface plots of perchlorate in pike tissue: (A) Perspective plot of response surface of perchlorate tissue concentration in pike (body + gastrointestinal tract) for all water and food treatments. The stationary point (peak) for food is 65.1 mg/L and for water is 66.2 mg/L. Dose response curves are on the edge of the food and water axes. (B) Contour plot of response surface of perchlorate tissue concentration (centered on 0) in pike (body + gastrointestinal tract) for all water treatments. Concentration in food is given in mg/L because this is the concentration of perchlorate that stickleback were exposed to prior to feeding to pike.

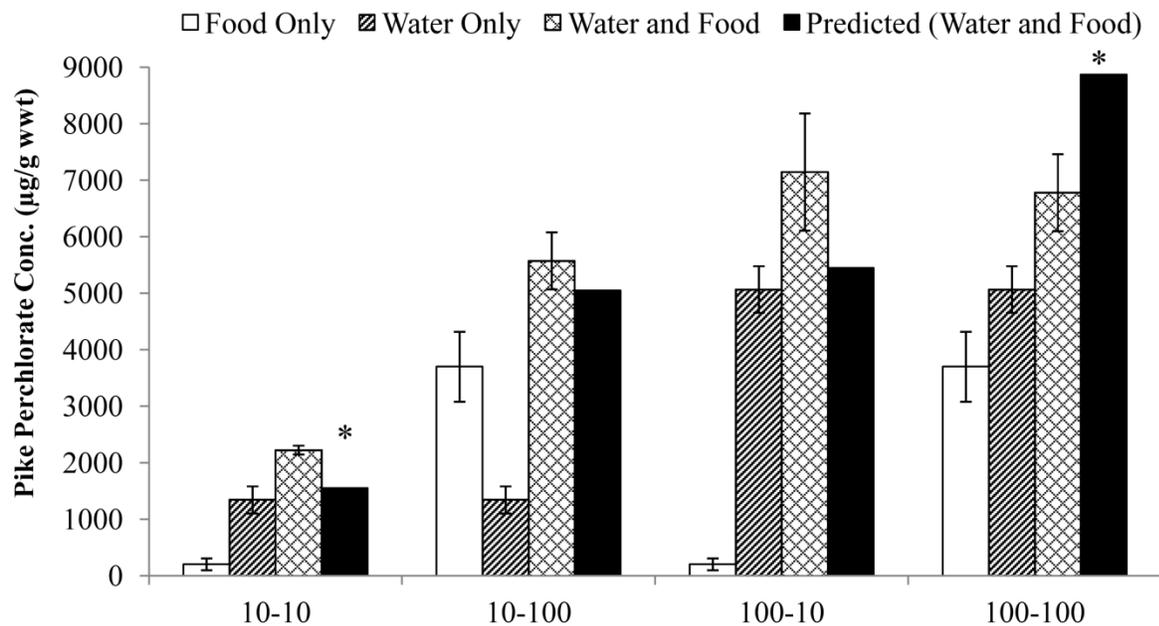


Figure 2.4: Pike tissue concentrations of perchlorate for the four combination (water and food) perchlorate exposure treatments: Black bars represent the predicted values of tissue residue calculated as: Expected $[ClO_4] = [ClO_4]$ in water from combination tanks * BCF from water only treatments + tissue $[ClO_4]$ from food only treatments. Asterisk indicates a significant ($\alpha = 0.05$) difference between expected and water and food treatments.

2.11 Supplemental Material

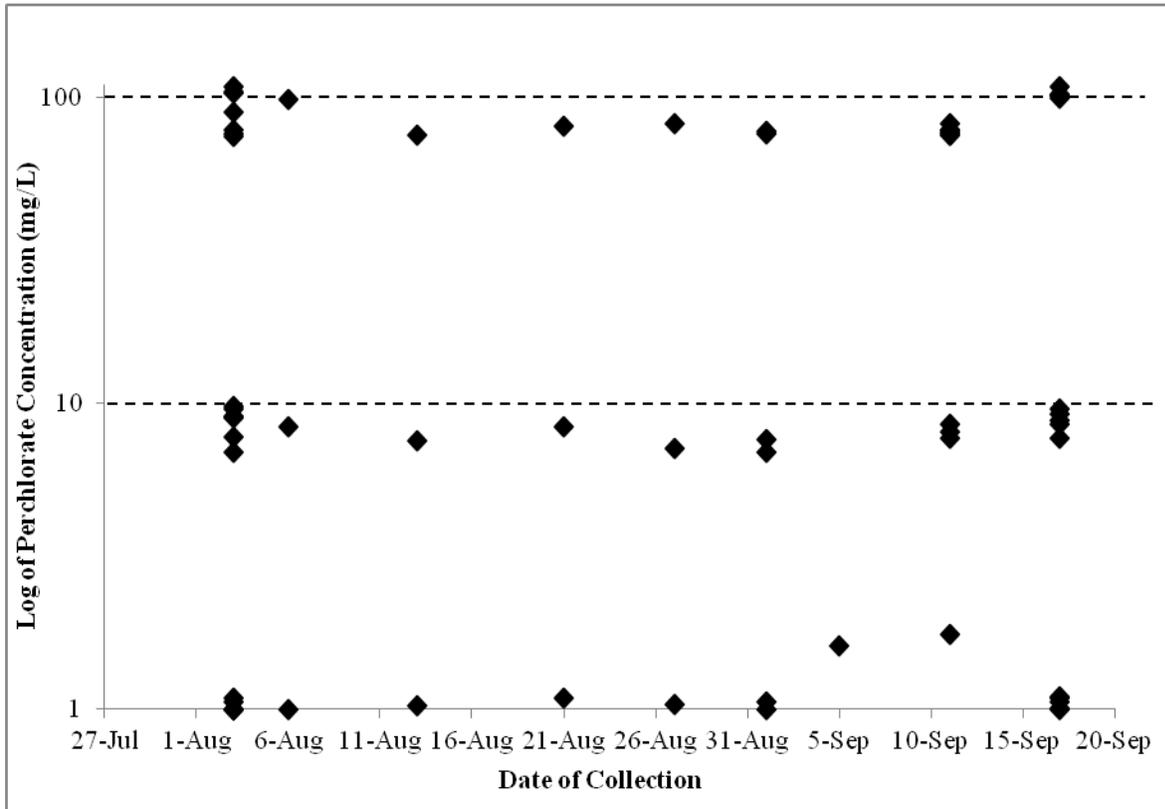


Figure S 2.1: Perchlorate concentration in experimental tanks measured over the experimental period. Measurements were generated using ion chromatography

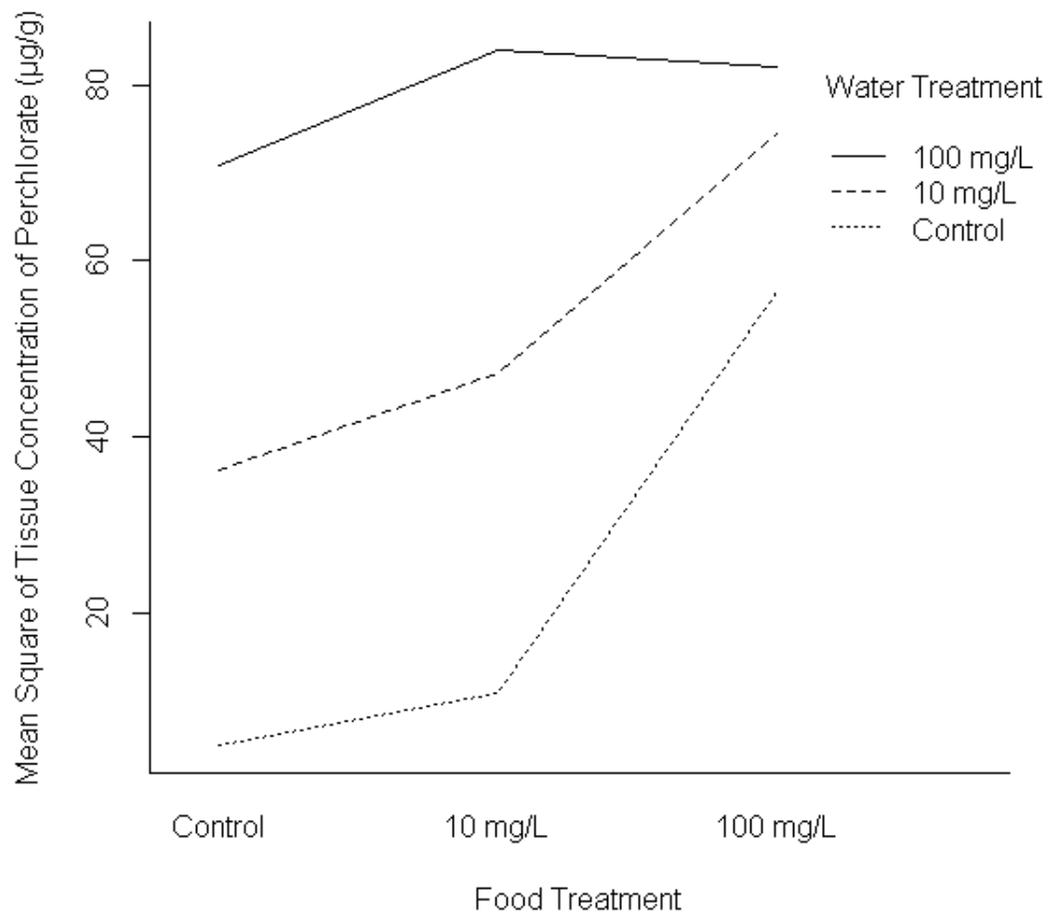


Figure S 2.2: Interaction plot of pike tissue concentration of perchlorate for the food and water routes of perchlorate exposure.

Chapter 3: Timing of perchlorate exposure during development differentially alters threespine stickleback dermal bone¹

3.1 Abstract

Thyroid hormone is critical during development and metamorphosis and for maintaining metabolic homeostasis. Perchlorate, a common water contaminant, is known to inhibit thyroid function in vertebrates. We utilized threespine stickleback (*Gasterosteus aculeatus*) to determine if timing of exposure to perchlorate during development has an impact on adult dermal skeletal phenotype. Fish were chronically exposed to water contaminated with perchlorate (30 mg/L and 100 mg/L) beginning at either 0, 3, 7, 14, 21, 42, 154 or 305 days post fertilization until sexual maturity at one year of age. A reciprocal treatment (contaminated water to clean water) was carried out on the same schedule providing for different stages of initial exposure as well as durations. Perchlorate exposure caused abnormal growth in some bony traits and the effects were concentration-dependent. Continuous exposure initiated in the first 14 days post fertilization had the greatest effects on skeletal traits.

¹ Furin CG, von Hippel FA, Cresko WA, Buck CL, Postlewait J and O'Hara TM. Timing of perchlorate exposure during development differentially alters threespine stickleback dermal bone. Prepared for submission to *Environ Toxicol and Chem*.

3.2 Key Words

Gasterosteus aculeatus; hypothyroidism; thyroid hormone; skeletal abnormality; endocrine disruption

3.3 Introduction

Aquatic environments are susceptible to contamination by water soluble contaminants and aquatic organisms exposed to those contaminants can have decreased fitness if effects are harmful, as is often the case when homeostasis is interrupted [1, 2]. Xenobiotics are introduced foreign compounds found in organisms that make their way into water resources through waste, runoff or spills [1, 3, 4]. Some examples include pharmaceuticals, personal care products and pesticides. Many xenobiotics are endocrine disruptors and even low concentrations can have deleterious effects [2]. Understanding the effects of these compounds during critical developmental stages is necessary for risk assessment, establishing regulations, and directing remediation efforts.

Perchlorate is a widespread inorganic anion found in ground and surface waters throughout the United States [5, 6]. Perchlorate salts occur naturally at low levels in arid regions such as the Southwestern U.S., Antarctica and the Atacama Desert in Chile [7-10]. Perchlorate is highly soluble, persistent and stable in aqueous environments [11]. These properties make it mobile and available to interact with biota in surface waters. As a strong oxidizer, perchlorate has been manufactured for use in solid rocket propellant, munitions and many other industrial products [12]. Anthropogenic sources (military and manufacturing sites) are responsible for most of the environmental contamination in the U.S. [12, 13].

Perchlorate competitively inhibits the uptake of iodide from the bloodstream into thyroid tissue. As a large anion similar to iodide, it has a greater affinity for the sodium/iodide symporter (NIS, alias SLC5A5) located in the basolateral membrane of thyrocytes [11, 14]. Interruption of iodide concentration into thyroid tissue can impair synthesis of thyroid hormone (TH, which includes both thyroxine [T4] and triiodothyronine [T3]), as has been demonstrated in several vertebrate animal models [15-22]. If the supply of iodide is sufficiently decreased for an extended length of time (i.e., TH stores exhausted) hypothyroidism can develop [14]. TH is critical to normal development, growth and metabolism in vertebrates [23, 24]. Individuals most at risk to the effects of perchlorate exposure are iodide deficient, and/or in early development or metamorphosis [2, 25-29]. In addition, perchlorate may have effects independent of iodide deficiency [30, 31]. Exposure in fish occurs via the gills, integument and sometimes ingested food [32]. Oviparous fish embryos are exposed to contaminants in the ambient water and/or pore water of sediments that can pass through the chorion.

Disruption of TH can profoundly impact teleost development and metamorphosis [2, 23, 33, 34]. The role of TH in skeletal development has been studied in some fish species including zebrafish (*Danio rerio*) [35, 36], African barb (*Labeobarbus intermedius*) [36], Medaka (*Oryzias latipes*) [37], threespine stickleback (*Gasterosteus aculeatus*) [38], and Japanese flounder (*Paralichthys olivaceus*) [39, 40]). Bony structures differed in their response to hypo- and hyperthyroid conditions; some fail to develop entirely, some overdevelop and others are relatively unchanged [23, 36]. Effects depend on species and character with many being permanent. Evidence suggests that variation in timing of appearance (heterochrony) and rate of growth (allometry) drive these adverse effects under different TH levels [36].

Bernhardt et al. [38] determined that chronic exposure to perchlorate reduces development of bony structures in young threespine stickleback in a dose dependent manner. They found that stickleback exposed to greater than 12 mg/L perchlorate exhibited phenotypic abnormalities. Of the 25 measured bony characters, 24 were significantly modified (after size correction), and gross abnormalities occurred (e.g., missing lateral plates, lack of skin pigments, extra dorsal spines). The exposed fish had reduced fitness with abnormal locomotion, calcification, pigmentation and reproduction [38, 41, 42].

Our current study delves further into the effects of perchlorate exposure on threespine stickleback by examining developmental effects with controlled timing of exposure and varying durations of exposure using two concentrations known to alter skeletal development. Concentration and critical developmental windows are considered in light of expression of some dermal skeleton features, with a focus on defensive traits, already determined to be affected by perchlorate [38]. Based on the conclusions of Bernhardt et al. [38], we hypothesis that overall growth and skeletal characters will be reduced with exposure to perchlorate. Critical windows are expected during early development between 0 and 21 dpf.

3.4 Materials and Methods

3.4.1 Experimental design

Threespine stickleback were exposed to one of two different concentrations of sodium perchlorate (30 and 100 mg/L) at different time points for varying durations across development in a static renewal experiment. Some exposures began at syngamy and embryos started in either perchlorate treated or control water. At 0, 3, 7, 14, 21, 154 (5 months) and 305 (10 months) days post fertilization (dpf), fish were translocated into or out of contaminated water. Fish that began

in contaminated water and subsequently moved into clean water were in the downshift (rescue) exposure regime and those moved from clean water to contaminated water were in the upshift exposure regime (Figure 3.1). In combination, this exposure regime should pinpoint the stage(s) of development when perchlorate has its greatest effect; if such a window occurs at these concentrations. Fish were raised in their respective treatments until approximately one year of age when they were collected and processed for morphological analysis. Due to differential survivorship and use for other experimental endpoints, sample sizes varied (Table 3.1).

Fish collection and experimental procedures

Threespine stickleback were collected from Rabbit Slough (61.534° N, 149.266° W) with un-baited wire-mesh (0.64 cm) minnow traps on 4 June, 2008. Anadromous Rabbit Slough fish were chosen to represent the ancestral marine ecotype and anadromous *G. aculeatus* exhibit little genetic divergence locally and regionally [43, 44]. They were transported to the University of Alaska Anchorage in aerated coolers where they were kept in outdoor 1600-L pools. De-chlorinated city water with Instant Ocean[®] added to 3 g/L was used in all pools.

On 10 June, 2008, a mass cross was performed to generate a representative study population with genetic variation randomly distributed throughout the treatments. Testes were collected from 40 males and eggs from 40 females. Egg clutches from all females and testes from all males were combined to randomize the genetic pool before fertilizing batches in Petri dishes (100X20mm) for all treatments. Sterilized reverse osmosis (RO) purified water with Instant Ocean[®] added to 4 g/L was used as the embryo medium. Sodium perchlorate (> 98% purity, Sigma-Aldrich, St. Louis, MO, USA) dried in an oven at 90° C before weighing, was added to produce embryo medium at 30 and 100 mg/L. Water was changed daily and dead embryos were removed. Day 0 downshift fish were fertilized in perchlorate treated water and

then moved into uncontaminated water after 15 minutes. Embryos in the initial Petri dishes were divided into three replicates within 3 dpf.

Embryos were kept in an incubator held at $20 \pm 0.5^\circ \text{C}$ for the first ten days and then transferred to aerated 56-L aquaria (60cm×31cm×32cm) with an AZOO[®] multi sponge filter (65mm diameter). Water level was adjusted in aquaria to maintain a ratio of at least 1-L water per 1 cm of fish and Bacta-pur[®] N3000 (IET-Aquaresearch Ltd., Quebec, Canada) was added to control nitrogenous waste. Water was changed every two weeks or as needed and dead fish were removed daily. RO water was added weekly to replace evaporative loss and maintain desired perchlorate concentrations. Lighting cycle mimicked the natural diurnal cycle for Anchorage, Alaska and the average water temperature during the experimental period was $13.5 \pm 0.5^\circ \text{C}$. Salinity (4-5 g/L), pH (7.0-8.0) and Ammonia (<1.9 mg/L Total Nitrogen [45]) were measured with a YSI photometer model 9100 (Yellow Springs Instrument Co., Yellow Springs, OH, USA) periodically to check for abnormal levels; none were detected. Perchlorate concentrations were measured using an Acorn Ion 6 meter (Oakton Instruments, Vernon Hills, USA) with a perchlorate ISE electrode (Cole-Parmer, Vernon Hills, IL, USA). Fry (<2 months old) were fed live brine shrimp and a mixture of Golden Pearls 100 (a commercial larval food), Artemia food (both from Aquatic Ecosystems, Apopka, FL, USA), and frozen ground brine shrimp (Brine Shrimp Direct, Ogden, UT, USA). Once large enough (at approx. 50 dpf), fish were fed frozen brine shrimp daily. Perchlorate intake from food sources was assumed to be negligible.

At the age of 12 months, fish were euthanized with an overdose of tricane methanesulfonate (MS-222; Argent Chemical Laboratories, Richmond, WA, USA) and fixed in 10% neutral buffered formalin. After 14-20 days, fish were thoroughly rinsed and transferred to 70% un-denatured ethanol for storage. Mineralized tissue was stained using alizarin red S.

Digital calipers (Fowler High precision, Newton, Massachusetts, USA) were used to measure: standard length (SL), body depth (BD), length of 2nd dorsal spine (DS), length of left and right pelvic spines (PS), height of plate anterior to the plate aligning with the ascending branch of the pelvic girdle (AP), and height of the plate dorsal to the anal spine (PP; Figure 3.2). The number of lateral plates (LP) was also recorded. Both left and right sides of bilateral characters were measured and added together. One individual researcher measured all fish in this study to maintain consistency.

3.4.2 Statistical analysis

Morphometric (continuous) characters were size corrected to control for covariation of character size and body size of fish, which have indeterminate growth [38, 46]. For each treatment, all bony characters were regressed against SL and the residuals, slope and Y intercept were calculated. The y value for each character for each treatment was then calculated using the regression equation with x set as the global mean of SL. This y value was added to the residuals to standardize all characters to a fish of the same SL. When the regression for a given treatment was non- significant, it was discarded (~5% of measurements) and size correction was calculated using a larger pool (within concentrations) of individuals.

Concordance was determined from 30 (10 each from control, 30 and 100 mg/L) randomly chosen individuals that were measured blindly a second time at least two days after the initial measurement [47]. A concordance coefficient was calculated for morphometric characters by dividing the smaller value by the larger value and averaging for all traits to determine the strength of correlation between repeated measures [48]. In addition, for the three bilateral morphometric traits, a two-way mixed model ANOVA with side (fixed) and individual (random)

as factors and trait as the dependent variable was utilized to test for directional asymmetry (DA) and to determine if measurement error (ME) contributed significantly to between-side variance [47].

Normality of dependent variables was determined by visually inspecting plotted data and via the Shapiro-Wilk test. In most cases, due to small or uneven sample sizes, non-parametric tests were used. Parametric tests were used only when all assumptions were met. Density effects were evaluated for control fish by regressing fish density in aquaria against trait values. Measurements of bony characters were done on preserved fish, but ~ 50% of the SL, pelvic spine and body depth measurements were conducted on digital images using ImageJ [49]. A t-test was used to determine if the methods resulted in significantly different measurements. Mortality during the first 15 dpf and survivorship at 374 dpf was compared between perchlorate exposures and controls using ANOVA.

After size adjusting, treatments were compared using a Kruskal-Wallis test and non-parametric Tukey multiple comparisons. Upshift and downshift exposure regimes were analyzed separately and compared to controls. A Hotellings T^2 test was used to test for differences between upshift and downshift exposure regimes at each dpf across all measured traits. Mann-Whitney U tests (with Bonferroni correction) were then used to test for differences between upshift and downshift for each character at each dpf. Directional asymmetry of bilateral morphometric traits was assessed using plots and a t-test (with Bonferroni correction) to determine if R-L was different from zero. Fluctuating asymmetry was determined using $|R-L|$ for each trait value and differences between treatments determined using a non-parametric Levene's test [47].

3.5 Results

3.5.1 Density, validation, mortality and asymmetry

Mean perchlorate concentrations in aquaria were maintained close to the target concentration for the duration of the experimental period (Figure 3.3). Fish density in aquaria was statistically associated with two of the five traits measured. After size correction, BD and PS were negatively correlated with the number of fish per tank (slope = -0.029, $t = -3.443$, $p < 0.001$; slope = -0.047, $t = -2.514$, $p = 0.013$, respectively). These effects were primarily driven by two aquaria with only one or two surviving fish. Because of these outliers and because otherwise, density varied little (mean \pm SE: 17 ± 0.56 fish/tank on 19 June 2009 (time point near collection)), and other traits (DS, AP and PP) were not affected by density, we did not account for density in the following analyses. Measurements based on digital photos vs. direct measurements on fish were not significantly different with the exception of SL (SL, $t = 23.04$, $df = 563$, $p < 0.0001$; BD, $t = 0.83$, $df = 980$, $p = 0.409$; PS, $t = 1.69$, $df = 901.45$, $p = 0.091$). SL was expected to differ between fish and photos due to the different collection times of samples for each measurement type (i.e., fish measured physically were older) and all other measurements were size corrected. Neither mortality in the first 15 dpf nor survivorship at one year of age significantly differed between controls and fish in different concentrations of perchlorate (One-way ANOVA: $F_{(1,109)} = 0.48$, $p = 0.49$; $F_{(1,109)} = 0.60$, $p = 0.44$, respectively).

The concordance correlation coefficient of repeated measurements for all traits ($n = 30$) ranged from 0.84-0.98 (Table 3.2). The nested ANOVA (side within individual) revealed that the AP had a significant left side bias. The ME was sufficiently large to mask detection of fluctuating asymmetry (Table 3.3). All asymmetry results are presented in supplementary tables (S3.1-S3.4).

3.5.2 Concentration and timing of exposure

The traits measured in this study varied in their response to perchlorate exposure. The SL, PS and PP showed significant trends and are included in Figures 3.4 & 3.5). The measurements for BD, DS and AP showed weak or no response to perchlorate exposure under the study conditions and will only be briefly discussed (Figures 3.4 & 3.5). Overall, concentration of perchlorate was more likely to cause abnormal physical traits than differential timing of exposure.

Body size measurements (SL and BD) varied in their response to perchlorate exposure. Mean SL was decreased compared to controls for fish in the 30 mg/L upshift exposure regime that were continuously exposed starting in the first two weeks post fertilization (Figure 3.4A). Only fish exposed on 0 dpf to 100 mg/L upshift treatment had significantly decreased SL (Figure 3.4B). The mean SL for all downshift treatments were equivalent to controls regardless of perchlorate concentration. There was not a concentration-dependent affect on SL, but the window of effect during early upshift treatments varied.

Compared to controls, mean BD increased in almost all treatments and variance in BD was greater in the 100 mg/L exposed fish than in the 30 mg/L exposed fish (Figure 3.4C & D). Stickleback in the 100 mg/L upshift exposure regime had deeper bodies in the 7,14,21 and 154 dpf treatments but not in 0, 3, 42 and 305 dpf treatments (Figure 3.4C). All fish exposed to 100 mg/L perchlorate in the downshift regime had deeper bodies, with the exception of the 154 dpf treatment. Concentration-dependent trends were not present for BD, but the 100 mg/L downshift regime suggests a critical window between 0 and 154 dpf (Figure 3.4C).

The mean DS was generally decreased with exposure to both concentrations of perchlorate (Figure 4E & F). The only discernible temporal pattern was as the date of rescue

(downshift) increased, mean DS tended to shorten in 100 mg/L exposures. Neither concentration of perchlorate or timing of exposure definitively affected DS.

The mean PS increased in all 100 mg/L exposures and decreased in all 30 mg/L exposures (Figure 3.5A & B). Timing of exposure was not important to PS growth in this long-term study.

The mean response of the AP was variable, but tended to increase with 30 mg/L exposure in the downshift exposure regime. The variance around the mean was greater for 100 mg/L treatments than 30 mg/L treatments (Figure 3.5C & D). Neither perchlorate concentration nor timing of exposure had definitive effects on AP.

The mean PP was decreased in all 100 mg/L exposures with the exception of the 305 dpf upshift treatment (Figure 3.5F). The 30 mg/L concentration did not affect PP phenotype. The 100 mg/L upshift regime suggests PP can be repressed if exposed anytime from fertilization to 154 dpf (Figure 3.5F).

Upshift and downshift exposure regimes differed across all measured bony characters on 0, 3, 14 and 21 dpf for 100 mg/L exposure ($T^2 = 13.0$, $p < 0.01$; $T^2 = 3.92$, $p < 0.01$; $T^2 = 11.07$, $p < 0.001$; $T^2 = 11.36$, $p < 0.001$, respectfully) and 3 and 21 dpf for 30 mg/L exposure ($T^2 = 6.18$, $p < 0.01$; $T^2 = 57.18$, $p < 0.001$, respectfully). The 100 mg/L concentration had a greater effect on dermal bone development than the 30 mg/L concentration, consistent with an expected concentration and response relationship.

3.5.3 Gross abnormalities

With respect to observed gross abnormalities, three control fish were missing all keel plates on the left side. One control fish was missing all plates on the right side but was normal on

the left side. In 30 mg/L perchlorate contaminated water, two individuals were missing keel plates on the left side and one individual was missing a left PS. In 100 mg/L perchlorate contaminated water, one individual had a total of 20 plates on the left and only 4 on the right, another individual was missing all keel plates on the right side and another a left PS. Transparency (lack of dermal pigment and lateral plates) was not observed.

3.6 Discussion

The upshift/downshift experimental design of the present study was intended to detect sensitive windows during development in which perchlorate exposure has the greatest effect on gross morphology of threespine stickleback. The expected result was a reduction in growth and skeletal development with earlier exposure having the greatest potential to have an effect. Our results determined that sodium perchlorate exposure affects gross morphology of stickleback, but with differential responses depending on the character measured. Timing of exposure may be important in the development of some morphological characters.

3.6.1 Body size

Varying concentrations of perchlorate and the timing and duration of exposure affected fish growth (SL and BD). Chronic exposure to 30 mg/L beginning in the first 14 dpf caused fish to have a decreased SL, while in the 100 mg/L exposure group only those exposed on 0 dpf had significantly decreased SL (upshift; Figure 3.4B). This result is consistent with other fish studies in which growth was reduced when chronically exposed to perchlorate [38, 50-53]. There may be a threshold concentration that triggers a physiological change and a compensatory mechanism

could explain the smaller window of sensitivity at 100 mg/L (e.g., fish may eliminate perchlorate at greater rate when exposed to higher concentrations of perchlorate [32]).

Most exposure windows in both 30 mg/L and 100 mg/L produced fish with deeper bodies than controls (upshift and downshift; Figure 3.4C & D). BD increased in all exposure treatments except when exposure began on 0 dpf in 100 mg/L. Being the longest duration of exposure, this is a puzzling result and further study is needed to verify these findings. Hyperplasia due to adenomatous goiter could contribute to a deeper BD measurement and has been demonstrated in previous studies [54]. BD increases with later exposure in 100 mg/L upshift treatments through 14 dpf, but was consistently high for all exposures in 30 mg/L (Figure 3.4C & D). Body size metrics of stickleback responded to perchlorate differently depending on exposure concentration and timing of exposure.

3.6.2 *Aarmor*

The PS demonstrated a concentration-dependent response, with an increase in mean spine length in 100 mg/L and a decrease in 30 mg/L treatments, as compared to control fish (Figure 3.5A & B). Perchlorate exposure (250 mg/L) has previously been shown to cause inhibition of pelvic fin development in zebrafish [35]. This was observed in the present study for 30 mg/L but not 100 mg/L exposures, which may have compensated by a mechanism not activated at 30 mg/L. Additionally, TH deficiency caused abnormal pectoral fin development in Japanese flounder [39] and African barbs [36] and prevented normal caudal fin regeneration in medaka [37]. Brown 2007 [35] observed larger paired fins in 65 dpf zebrafish exposed to potassium perchlorate with late rescue (56 dpf) using T4. Also, Shkil et al. [36] observed some early pectoral girdle developmental effects of hypothyroidism in zebrafish and African barbs, but the

adult morphology was minimally affected. T4 treatment generally causes premature differentiation and abnormalities in pectoral fins in zebrafish [35], barbs [36, 55], goldfish (*Carassius auratus*) [56], chum salmon (*Onchorhynchus keta*) [57], and tilapia (*Oreochromis mossambicus*) [58]. The above studies demonstrate that paired fin development is at least partially controlled by TH and perchlorate exposure may disrupt TH influence if hormone production is severely decreased. The larger PS in stickleback exposed to 100 mg/L corroborates the findings of premature differentiation of paired structures.

The DS in 100 mg/L downshift treatments tended to be longer with earlier rescue (Figure 3.4F). In contrast, when transferred out of perchlorate during the first two weeks, the DS in 30 mg/L treatments was significantly shorter than in control fish. This suggests either that the onset or the rate of character development is altered with perchlorate exposure during the embryonic stage. The difference in concentration response could be that the 100 mg/L early rescue resulted in either early differentiation or compensatory growth as seen in the PS. Further study is needed to determine the underlying mechanisms.

Changes in adult phenotype for perchlorate exposures during only the first 3-7 dpf for BD, PS, DS, PP and AP are puzzling. Given that the embryo's source of T4 and T3 is maternal at this early stage of development [23], and the expression of NIS and formation of thyroid follicles begins sometime between 8 and 11 dpf (J. Postlewait, W. A. Cresko, C. L. Buck and F. A. von Hippel, unpublished data), hypothyroidism via perchlorate is likely not driving these effects. Perchlorate may be altering the timing and/or rate of development of some traits through non-thyroidal pathways as has been demonstrated in gene expression studies [30, 31]. NIS is present in non-thyroidal tissues of vertebrates including mammary, gastric mucosa, salivary glands and

kidney among others [59]. Alternative effects of perchlorate, such as osmotic and oxidative stress, should also be investigated.

3.6.3 Synthesis of results and future work

Threespine stickleback SL was significantly affected by continuous exposure to perchlorate beginning during the first two weeks post fertilization (30 mg/L). In contrast, if fish were rescued within 14 dpf, there was no effect on SL. Perchlorate's effect on SL is likely not developmentally driven, otherwise the fish rescued within 14 dpf would have decreased SL compared to controls. Compensatory growth of characters could be a confounding factor, but were not measured in the current study. Under natural conditions, such reduced growth would have fitness consequences and the timing and duration of exposure should be taken into account when evaluating risk at contaminated sites.

Perchlorate exposure from ambient water causes abnormal development of the stickleback dermal skeleton. Overall, 100 mg/L exposures affected bony characters to a greater degree and with more variance around the mean than 30 mg/L exposures. Comparing all characters, the upshift and downshift exposure regimes differed significantly from each other for four different dpf in 100 mg/L and for two in 30 mg/L treatments, both within the first 21 dpf. This suggests that higher concentrations of perchlorate cause a greater degree of abnormal development and the first 21 dpf represent a critical developmental window for disruption of skeletal traits. Many critical developmental milestones occur within this window (e.g., pigmentation, heart beating, fin development, etc.), including functioning thyroid tissue [60]. Concentration of perchlorate is important in relation to skeletal development in threespine stickleback as previously demonstrated [38].

TH deficiency and TH excess both have effects on individual skeletal structures in fish, ranging from no effect to significant over- or under-development [36, 39]. Shkil et al. [36] concluded that for barbs and zebrafish skeletal elements that originate early, such as appearance of first skull bones and ossification of most vertebral centra, are not TH dependent but that some elements arising later, including neural arches and spines in the vertebral column and fin rays in dorsal and anal fins, are TH dependent. Assuming that the main toxic effect of perchlorate is hypothyroidism caused by disruption of TH homeostasis, this study corroborates that conclusion for some traits. The DS (100 mg/L), PS (30 mg/L) and PP (100 mg/L) were all decreased and develop late in stickleback ontogeny [60, 61] (Figures 3.4 & 3.5). This conclusion is confounded however, by toxicant concentration (as seen in PS [Figure 3.5] and PP [Figure 3.4]), and the potential for compensatory growth, which has been documented on skeletal structures with T4 rescue [35] and potentially non-thyroid tissues effects. Additionally, plasma TH levels are correlated with metamorphosis, season, migration, salinity and sexual maturity in fish [2, 33, 62], and hence thyroid-disrupting compounds such as perchlorate may have complex interactions during fish development [2, 35].

Future work should focus on the first 21 dpf when perchlorate exposure has its greatest apparent effect. Choosing skeletal characters that develop across different stages of ontogeny would provide information on the influence of perchlorate at those particular stages. Age of fish at collection, specific characters measured and timing/duration of exposure should be carefully considered when designing experiments evaluating hypothalamus-pituitary-thyroid axis effects. Including a positive control such as iodide deficiency or another thyrotoxicant could help to distinguish direct from indirect mechanisms of perchlorate on skeletal development. Finally,

given the wide range of phenotypes induced by perchlorate exposure, future work should examine molecular mechanisms that lead to different outcomes.

3.6.4 Conclusion

This study demonstrates the complex relationships involved with perchlorate-induced disruption of TH homeostasis and its influence on skeletal development in stickleback. TH does not have a blanket effect on rate of development [36]. Rather, differences in the rate and/or induction period that cause skeletal abnormalities likely depend on the particular skeletal element, concentration of perchlorate, and age of the fish. The timing of exposure, the physiological status and the species of fish are also likely important. Chronic exposure beginning early in development has the greatest potential for causing deleterious effects on fitness related to growth and skeletal morphology in threespine stickleback. Exposure concentration, timing of exposure and non-thyroidal effects should be considered in future studies.

3.7 Acknowledgments

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Table 3.1: Number of individuals (combing three replicates) for each measurement by perchlorate exposure treatment. Upshift exposures began in control water and were moved to contaminated water. Downshift exposures were the reciprocal of upshift exposures. Some treatments had no usable fish (NA).

dpf ^a	Exposure	100 mg/L						30 mg/L					
		SL ^b	BD ^c	PS ^d	DS ^e	PP ^f	AP ^g	SL	BD	PS	DS	PP	AP
Control	Upshift	106	203	203	106	103	79	106	203	203	106	103	79
	Downshift	106	203	203	106	103	79	106	203	203	106	103	79
Day 0	Upshift	NA	15	15	0	0	0	NA	15	14	0	0	0
	Downshift	11	21	21	11	11	6	32	47	45	32	32	23
Day 3	Upshift	13	28	28	13	13	7	NA	15	15	0	0	0
	Downshift	34	49	19	34	34	32	18	28	27	18	18	9
Day 7	Upshift	NA	16	16	0	1	0	NA	15	15	0	0	0
	Downshift	25	40	40	25	25	20	23	38	37	23	23	22
Day 14	Upshift	18	33	33	18	18	14	1	16	15	1	1	0
	Downshift	16	31	31	16	15	9	9	24	23	9	9	4
Day 21	Upshift	12	27	27	12	12	7	6	21	21	6	6	6
	Downshift	22	22	22	22	22	18	16	31	31	16	16	14
Day 42	Upshift	11	21	20	11	11	6	11	26	26	12	12	11
	Downshift	18	33	33	18	18	13	9	24	23	9	9	5
Day 154	Upshift	30	45	44	30	30	28	6	21	19	6	6	6
	Downshift	17	31	31	17	17	13	5	20	18	5	5	0
Day 305	Upshift ^h	6	21	21	6	6	0	18	33	33	18	18	16

^adpf- days post fertilization, ^bSL- standard Length, ^cBD- body depth, ^dPS- pelvic spine, ^eDS- dorsal spine, ^fPP- posterior plate height, ^gAP- anterior 6 height ^hOnly upshift exposure regime was performed at 305 dpf.

Table 3.2: Concordance of morphometric measurements for bilateral traits of threespine stickleback. The smaller value was divided the by the larger value and averaged for all traits to determine the strength of correlation between repeated measures; n = 30.

Trait	Concordance
Standard Length	0.98
Body Depth	0.96
Dorsal Spine	0.90
Pelvic Spine L	0.94
Pelvic Spine R	0.94
Posterior Plate L	0.84
Posterior Plate R	0.86
Anterior Plate L	0.94
Anterior Plate R	0.94

Table 3.3: Two-way mixed model ANOVA of Side (fixed) by Individual (random). A significant Side X Individual P value would indicate measurement error variance is sufficiently smaller than between side variance to allow detection of trait asymmetry [47]. A significant Side P value indicates directional asymmetry. n = 30

Trait	Side		Individual		SideXIndividual	
	F	P	F	P	F	P
Pelvic Spine	2.151	0.153	23.433	<0.001	1.176	0.292
Posterior Plate	1.681	0.198	3.424	<0.001	0.469	0.986
Anterior Plate	9.981	0.004	14.4	<0.001	1.32	0.181

3.10 Figures

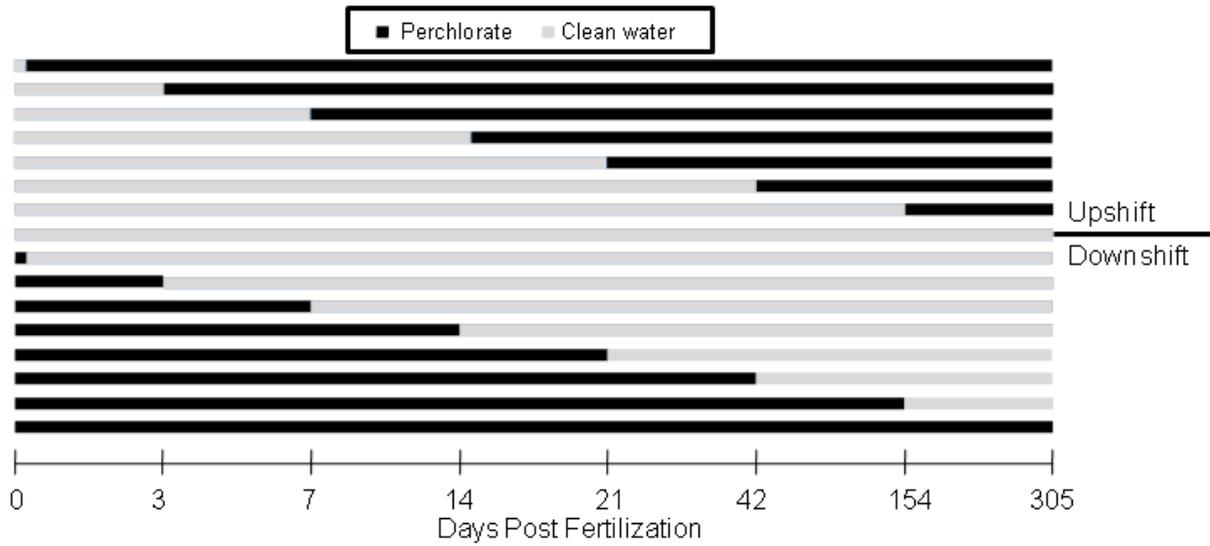


Figure 3.1: Perchlorate exposure regime. This was used for both 30 and 100 mg/L exposure with three replicates at each dpf. Zero dpf downshift fish were exposed during fertilization and for 15 minutes post fertilization before their transfer to clean water.

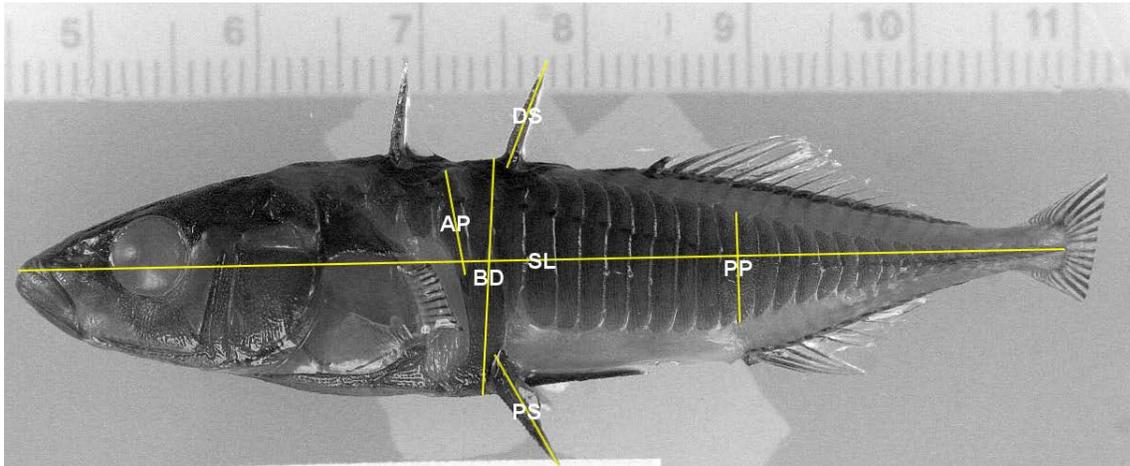


Figure 3.2: Morphometric measurements made on stickleback. Photo courtesy of Jeff Colgren (UAA). Pectoral and caudal fins have been clipped on this individual. Dorsal spine length (DS), Anterior plate height (AP), Body depth (BD), Standard Length (SL), Pelvic spine length (PS) and Posterior plate height (PP).

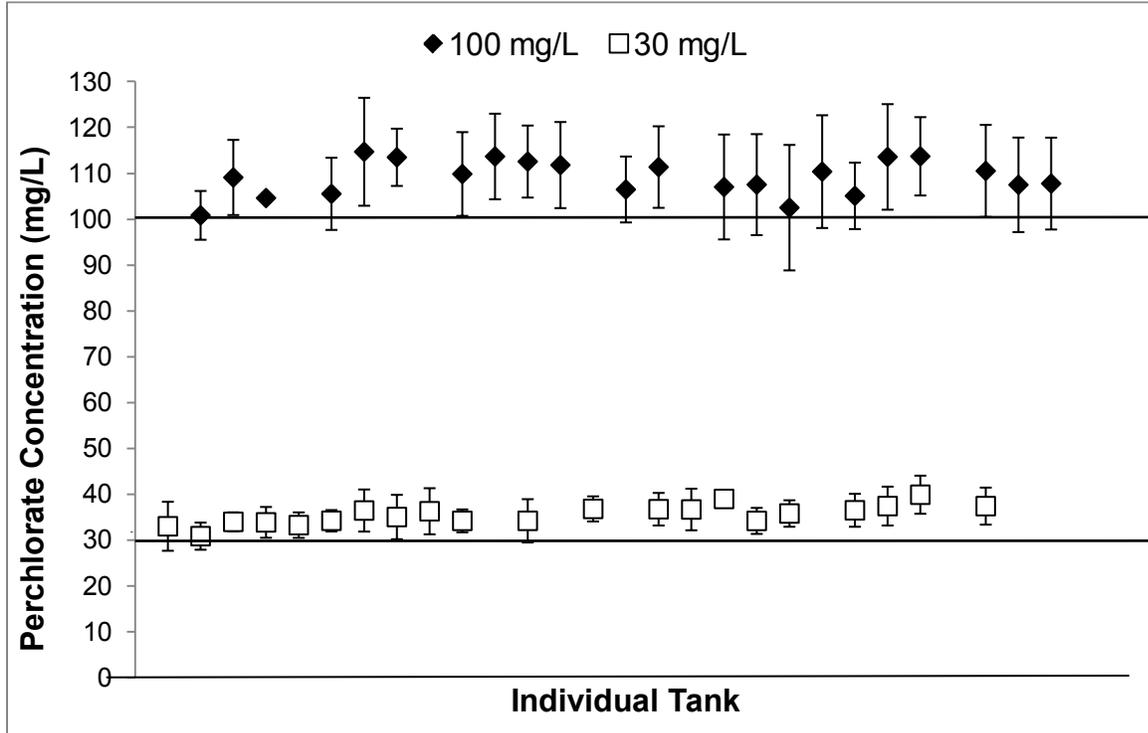


Figure 3.3: Mean \pm SE perchlorate concentrations in individual aquaria over the course of the experiment. Each point represents one aquarium.

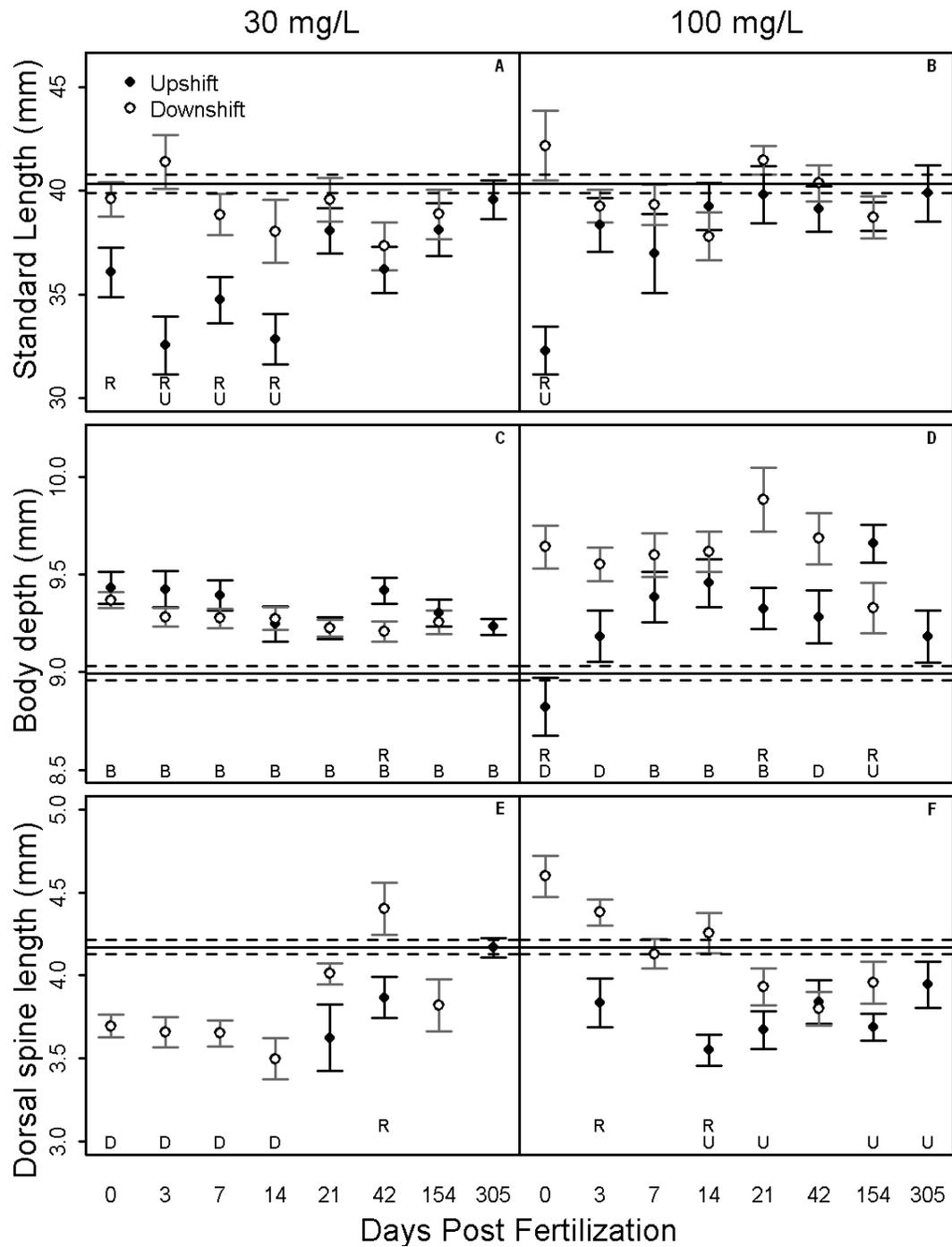


Figure 3.4: Mean \pm SE of standardized trait lengths of stickleback in each treatment. Upshift = fish began in clean water and were transferred to contaminated water. Downshift = reciprocal of upshift. Significant differences at $\alpha < 0.05$ indicated by: (U) between upshift and control, (D) between downshift and control, (B) both upshift and downshift compared to control, (R) between upshift and downshift regimes. The solid horizontal line is the control mean and the dashed lines are the SE around the control mean.

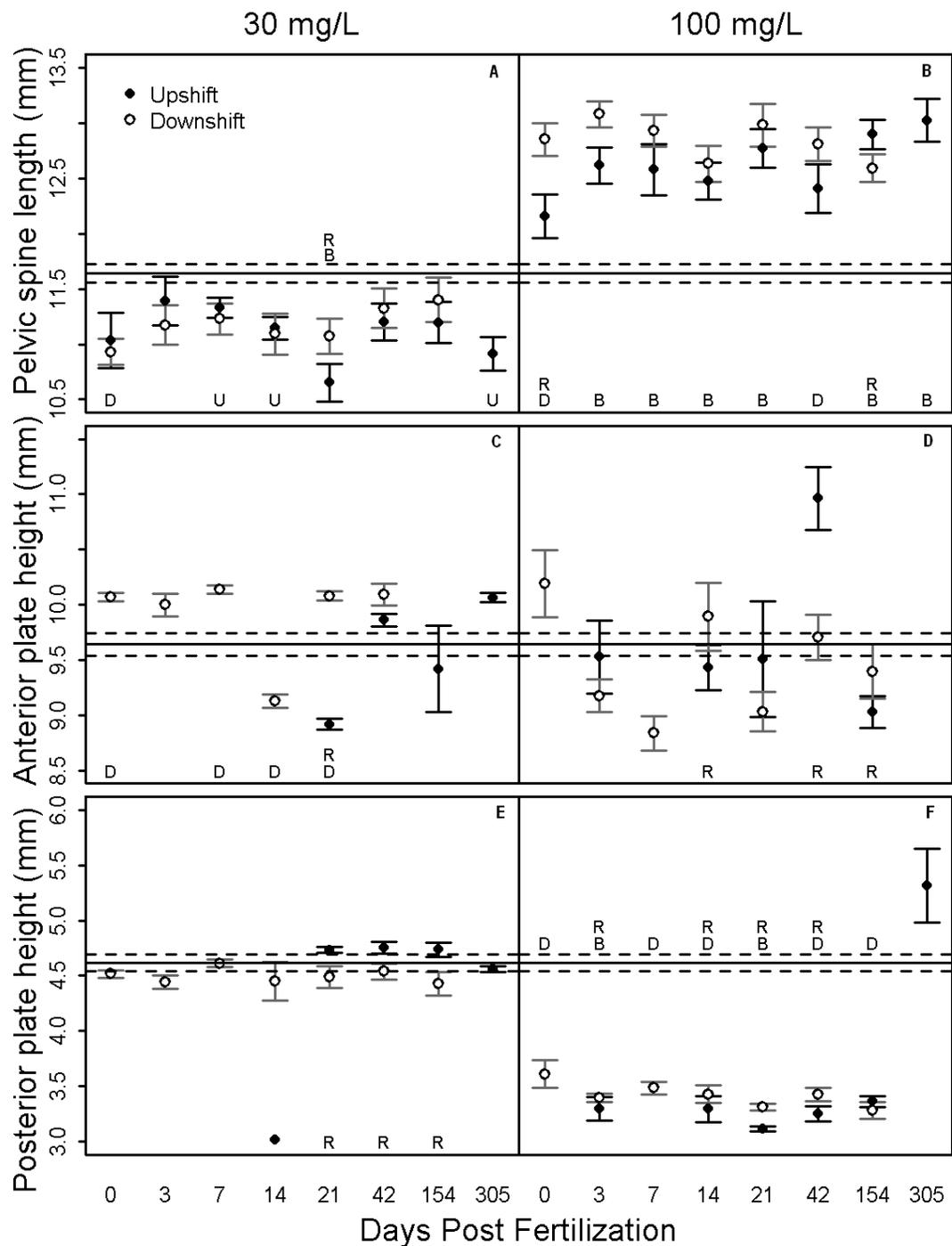


Figure 3.5: Mean \pm SE of standardized trait lengths of stickleback in each treatment. Upshift = fish began in clean water and were transferred to contaminated water. Downshift = reciprocal of upshift. Significant differences at $\alpha < 0.05$ indicated by: (U) between upshift and control, (D) between downshift and control, (B) both upshift and downshift compared to control, (R) between upshift and downshift regimes. The solid horizontal line is the control mean and the dashed lines are the SE around the control mean.

3.11 Supplementary Material

Table S 3.1: Asymmetry data (R-L) for three bilateral skeletal armor traits of threespine stickleback exposed to 30 mg/L perchlorate in the upshift exposure regime (see text).

30mg/L Trait	Upshift dpf ¹	n	Mean ²	SE ³	P ⁴	Skewness	Kurtosis	S-W ⁵
Pelvic Spine	Control	203	-0.002	0.005	0.985	-0.146	0.148	0.747
	0	14	-0.003	0.025	0.814	1.614	2.858	0.005
	3	15	-0.023	0.018	0.173	-2.005	4.171	0.001
	7	15	-0.015	0.014	0.266	0.147	-0.238	0.281
	14	16	-0.015	0.026	0.567	-0.364	-0.510	0.885
	21	21	-0.015	0.023	0.500	-0.187	-0.754	0.925
	42	26	0.032	0.018	0.156	0.677	-0.190	0.101
	154	21	-0.008	0.018	0.729	-0.361	0.441	0.362
305	33	0.007	0.019	0.685	0.379	-0.198	0.620	
Anterior Plate	Control	79	-0.030	0.005	0.000	-0.176	-0.585	0.537
	0	0	NA	NA	NA	NA	NA	NA
	3	0	NA	NA	NA	NA	NA	NA
	7	0	NA	NA	NA	NA	NA	NA
	14	0	NA	NA	NA	NA	NA	NA
	21	6	-0.017	0.011	0.020	0.021	-2.141	0.230
	42	10	-0.041	0.016	0.040	1.344	0.727	0.014
	154	6	0.016	0.021	0.048	-0.020	-2.111	0.273
305	16	-0.013	0.014	0.215	-1.352	2.109	0.018	
Posterior Plate	Control	103	0.061	0.009	<0.001	0.308	-0.049	0.105
	0	0	NA	NA	NA	NA	NA	NA
	3	0	NA	NA	NA	NA	NA	NA
	7	0	NA	NA	NA	NA	NA	NA
	14	1	0.132	NA	NA	NA	NA	NA
	21	6	0.127	0.020	0.029	-0.032	-1.918	0.651
	42	11	0.128	0.032	0.015	1.394	1.473	0.009
	154	6	0.235	0.051	0.047	0.034	-1.999	0.414
305	18	0.045	0.025	0.219	-1.336	2.195	0.025	

¹Days post fertilization; ²Mean of Right-Left trait size; ³Standard Error of Right-Left trait size; ⁴p-value of one sample t-test, $\mu=0$; ⁵p-value of Shapiro-Wilk normality test

Table S 3.2: Asymmetry data (R-L) for three bilateral skeletal armor traits of threespine stickleback exposed to 30 mg/L perchlorate in the upshift exposure regime (see text).

30mg/L dpf ¹	Downshift Treatment	n	Mean ²	SE ³	P ⁴	Skewness	Kurtosis	S-W ⁵
Pelvic Spine	Control	203	-0.002	0.005	0.985	-0.146	0.148	0.134
	0	46	-0.047	0.015	0.271	1.041	2.246	0.005
	3	28	-0.022	0.020	0.569	-0.515	1.343	0.130
	7	38	-0.050	0.015	0.108	0.392	-0.382	0.487
	14	24	-0.031	0.026	0.609	1.018	0.451	0.023
	21	31	-0.065	0.014	0.189	0.218	1.625	0.132
	42	24	-0.070	0.019	0.215	-0.397	-0.825	0.200
	154	20	-0.009	0.025	0.799	0.270	-0.626	0.563
	305	NA	NA	NA	NA	NA	NA	NA
Anterior Plate	Control	79	-0.030	0.005	0.000	-0.176	-0.585	0.874
	0	23	0.023	0.010	0.146	-0.705	-0.277	0.110
	3	8	-0.002	0.010	0.947	-0.124	-2.081	0.043
	7	22	-0.026	0.010	0.071	-0.296	-0.688	0.866
	14	4	-0.036	0.014	0.361	-0.527	-1.866	0.293
	21	14	0.001	0.011	0.963	0.603	-1.180	0.086
	42	5	0.027	0.015	0.445	-0.019	-2.067	0.704
	154	0	NA	NA	NA	NA	NA	NA
	305	NA	NA	NA	NA	NA	NA	NA
Posterior Plate	Control	103	0.061	0.009	<0.001	0.308	-0.049	0.105
	0	32	0.024	0.014	0.234	0.310	-0.562	0.145
	3	17	-0.003	0.019	0.895	1.206	2.890	0.010
	7	23	0.043	0.022	0.167	0.454	-0.943	0.272
	14	9	0.012	0.016	0.886	-0.249	-1.404	0.689
	21	16	0.003	0.015	0.825	0.312	-0.296	0.822
	42	9	0.003	0.016	0.987	0.043	-1.334	0.847
	154	5	0.051	0.016	0.167	-0.623	-1.348	0.408
	305	NA	NA	NA	NA	NA	NA	NA

¹Days post fertilization; ²Mean of Right-Left trait size; ³Standard Error of Right-Left trait size; ⁴p-value of one sample t-test, $\mu=0$; ⁵p-value of Shapiro-Wilk normality test

Table S 3.3: Asymmetry data (R-L) for three bilateral skeletal armor traits of threespine stickleback exposed to 100 mg/L perchlorate in the upshift exposure regime (see text).

100mg/L Trait	Upshift dpf ¹	n	Mean ²	SE ³	P ⁴	Skewness	Kurtosis	S-W ⁵
Pelvic Spine	Control	203	-0.002	0.005	0.985	-0.146	0.148	0.134
	0	15	-0.071	0.028	0.025	-0.628	-0.328	0.376
	3	28	0.005	0.023	0.978	0.076	-0.915	0.686
	7	16	0.024	0.035	0.365	0.438	-1.269	0.126
	14	33	-0.050	0.015	0.003	-0.140	1.382	0.061
	21	27	-0.040	0.021	0.073	-1.146	0.435	0.003
	42	20	-0.012	0.024	0.780	-0.510	1.257	0.237
	154	44	-0.040	0.016	0.026	-0.190	-0.500	0.592
	305	21	-0.036	0.022	0.140	-0.015	1.129	0.195
Anterior Plate	Control	79	-0.030	0.005	0.000	-0.176	-0.585	0.874
	0	0	NA	NA	NA	NA	NA	NA
	3	7	0.019	0.009	0.232	0.035	-1.678	0.930
	7	0	NA	NA	NA	NA	NA	NA
	14	14	-0.009	0.019	0.755	-0.362	-1.227	0.488
	21	7	-0.043	0.020	0.358	0.850	-0.641	0.203
	42	6	-0.052	0.018	0.169	-0.065	-2.035	0.421
	154	28	0.012	0.015	0.485	-0.393	-0.842	0.434
	305	0	NA	NA	NA	NA	NA	NA
Posterior Plate	Control	103	0.061	0.009	<0.001	0.308	-0.049	0.105
	0	0	NA	NA	NA	NA	NA	NA
	3	13	0.062	0.036	0.147	0.162	-0.945	0.295
	7	1	0.063	NA	NA	NA	NA	NA
	14	18	0.048	0.022	0.105	0.173	-1.051	0.195
	21	12	0.043	0.021	0.188	-0.034	-1.654	0.235
	42	11	0.087	0.017	0.003	-0.491	-0.589	0.725
	154	30	0.009	0.018	0.686	0.384	0.042	0.636
	305	6	0.013	0.015	0.637	-0.381	-1.495	0.827

¹Days post fertilization; ²Mean of Right-Left trait size; ³Standard Error of Right-Left trait size; ⁴p-value of one sample t-test, $\mu=0$; ⁵p-value of Shapiro-Wilk normality test

Table S 3.4: Asymmetry data (R-L) for three bilateral skeletal armor traits of threespine stickleback exposed to 100 mg/L perchlorate in the downshift shift exposure regime (see text).

100mg/L Trait	Downshift Dpf ¹	n	Mean ²	SE ³	P ⁴	Skewness	Kurtosis	S-W ⁵
Pelvic Spine	Control	203	-0.002	0.005	0.985	-0.217	0.343	0.134
	0	21	0.015	0.015	0.766	-1.009	1.692	0.055
	3	49	-0.011	0.012	0.574	0.671	0.226	0.144
	7	40	-0.033	0.019	0.403	0.076	-0.244	0.846
	14	31	-0.022	0.009	0.688	-0.202	-0.262	0.916
	21	22	-0.022	0.015	0.380	0.030	-0.865	0.685
	42	33	-0.004	0.018	0.870	0.062	-0.414	0.599
	154	31	-0.005	0.025	0.544	1.646	3.682	0.001
	305	NA	NA	NA	NA	NA	NA	NA
Anterior Plate	Control	79	-0.030	0.005	0.000	-0.190	-0.163	0.874
	0	11	-0.012	0.013	0.645	-0.925	-0.697	0.085
	3	34	-0.045	0.013	0.003	0.007	-0.629	0.779
	7	25	0.016	0.016	0.479	0.569	-0.252	0.142
	14	15	-0.011	0.019	0.706	0.018	-1.439	0.747
	21	22	0.001	0.019	0.967	0.498	-0.134	0.707
	42	18	-0.005	0.012	0.818	0.001	-1.217	0.751
	154	17	0.019	0.015	0.458	0.060	-1.012	0.996
	305	NA	NA	NA	NA	NA	NA	NA
Posterior Plate	Control	103	0.061	0.009	<0.001	0.308	-0.049	0.105
	0	6	0.058	0.022	0.124	0.704	-0.497	0.466
	3	32	0.094	0.015	<0.001	0.333	-0.861	0.171
	7	20	0.055	0.015	0.005	0.908	0.057	0.032
	14	9	0.008	0.011	0.597	-0.675	0.045	0.216
	21	18	0.003	0.021	0.839	0.185	-1.176	0.258
	42	13	0.013	0.021	0.647	-0.118	-1.118	0.855
	154	13	-0.037	0.021	0.337	-0.087	-1.523	0.187
	305	NA	NA	NA	NA	NA	NA	NA

¹Days post fertilization; ²Mean of Right-Left trait size; ³Standard Error of Right-Left trait size; ⁴p-value of one sample t-test, $\mu=0$; ⁵p-value of Shapiro-Wilk normality test

Chapter 4: Critical developmental windows for histomorphologic changes of fish thyroid and gonad tissues due to sodium perchlorate exposure¹

4.1 Abstract

Perchlorate, a common aquatic contaminant, is well known to disrupt homeostasis of the hypothalamus-pituitary-thyroid axis in vertebrates. Circulating thyroid hormones are important initiators of many developmental processes and maintain metabolic homeostasis. Fish are susceptible to perchlorate exposure via gill, integument, and oral routes. This study utilizes the threespine stickleback species model to determine if perchlorate has varying morphological effects on thyroid and gonad tissues during certain windows of development. Fish were variably exposed to perchlorate contaminated water (30 and 100 mg/L) starting at 0, 7, 14, 21, 42, 154 or 305 days post fertilization until approximately one year old. A reciprocal treatment (contaminated water switched to untreated water) was conducted concurrently. Perchlorate exposure caused morphological changes in thyroid and gonad tissues that were concentration and temporally dependent. Gonadal maturity was delayed and sex ratio was biased toward males with chronic exposure to perchlorate.

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4.2 Key Words

Gasterosteus aculeatus; endocrine disruption; thyroid hormone; development; fish; histology

4.3 Introduction

The perchlorate ion is a common aquatic contaminant in the United States (U.S.) and has been classified as a contaminant of concern by the U.S. Environmental Protection Agency (EPA), which has committed to setting drinking water limits under the Clean Water Act [1]. The Department of Defense (DoD) uses a majority of the commercially available perchlorate for ammunition and solid rocket propellant and most contamination originates from former military and manufacturing sites [2-4]. Naturally produced perchlorate salts do persist at low levels in arid regions of the world such as Antarctica, the Atacama Desert in Chile and the Southwestern U.S. [5-7]. Perchlorate salts are highly water soluble and the ion is kinetically stable, nonreactive and resistant to adsorption in solution [2, 8]. These properties make perchlorate highly mobile and readily available to aquatic organisms which are exposed through ambient water (via gills, mouth and integument) and/or ingested food [9, 10]. The ubiquity and persistence of this known endocrine disruptor in water sources make it a cause for concern for aquatic organisms and humans. Understanding the adverse effects of exposure throughout an organism's lifecycle (fertilization to maturation) is crucial to definitively elucidating fitness and population level consequences.

The toxic effects of perchlorate stem from its structural and ionic similarity to iodide which give it the ability to competitively inhibit the uptake of iodide through the basolateral membrane of thyroid follicle cells at the sodium/iodide symporter (NIS) [11, 12]. It effectively inhibits the synthesis of thyroid hormones (TH: thyroxine, T4 and triiodothyronine, T3) which

are essential regulators of metabolism, growth, development and metamorphosis in vertebrates [11-13]. Lack of iodide can halt production of T4 and can lead to hypothyroidism and changes in thyroid tissue such as follicle cell hypertrophy, proliferation of follicles and surrounding tissue, increased vasculature, and reduced colloid [13-16].

Perchlorate has been shown to interfere with development and fitness of species in multiple taxa (see Kendall and Smith, 2006 [17] for review). Studies utilizing zebrafish (*Danio rerio*) [15, 18-21], eastern mosquito fish (*Gambusia holbrooki*) [22, 23], threespine stickleback (*Gasterosteus aculeatus*) [24-26], fathead minnows (*Pimephales promelas*) [27, 28], and molly fish (*Poecilia sphenops*) [29] have all found detrimental effects on development and/or reproduction from perchlorate exposure. These detrimental effects include: reduced growth rates [21, 26, 28, 30-33], reduced reproductive output [18, 24, 25, 33], skewed sex ratio [30], skeletal abnormalities [26], abnormal thyroid histomorphology [15, 18, 21, 22, 28, 31, 33, 34] and intersex gonads [24]. Perchlorate causes a female bias in the sex ratio of zebrafish [30, 35], while evidence of masculinization was found for stickleback [24]. A critical window for sex determination in stickleback exposed to sex steroids was determined to be within the first 14 days post hatch (dph) [36]. Lewis et al. [37] found that sex determination in stickleback was due to proliferation of primordial germ cells in females and no proliferation in males before 11 days post fertilization (dpf). Perchlorate is known to cause developmental abnormalities and has the potential to cause perturbations during those sensitive early developmental periods.

The current study utilizes threespine stickleback to evaluate effects of perchlorate exposure on thyroid and gonad tissues at varying durations and time points during development. Changes in thyroid tissue histomorphology are a sensitive endpoint to some endocrine disruptors that directly alter iodide disposition such as perchlorate [38]. Due to the importance of TH during

development, the masculinizing effects of perchlorate on stickleback, and the early sex determination in stickleback, we hypothesized that perchlorate exposure during early development and metamorphosis (through 21 dpf) would cause histological changes to thyroid tissue such as thyrocyte hypertrophy, colloid depletion and follicle hyperplasia, as well as abnormal appearing gonads and male-biased sex ratios.

4.4 Materials and Methods

4.4.1 Experimental design

In a chronic static renewal test, fish were exposed to sodium perchlorate (> 98% purity, Sigma-Aldrich, St. Louis, MO, USA) contaminated (30 mg/L or 100 mg/L) or control water beginning at fertilization. In order to determine critical developmental windows of exposure, fish were moved from control water to contaminated water (upshift) or from contaminated water to control water (downshift) on 0, 3, 7, 14, 21, 42, 154 and 305 dpf. Once reaching sexual maturity at approximately one year old, stickleback were collected and processed for histological analysis (thyroid and gonads) as well as gross morphologic assessment and survival (reported separately; chapter 3).

4.4.2 Fish collection and husbandry

Stickleback were collected from Rabbit Slough (61.534° N, 149.266° W) in the Matanuska-Susitna Valley, Alaska on 4 June 2008. Rabbit Slough fish were chosen to represent the ancestral marine ecotype and *G. aculeatus* exhibit little genetic divergence regionally [39, 40]. Fish were kept in outdoor pools filled with de-chlorinated city water with 3 g/L Instant Ocean[®] added. A mass cross using eggs from 40 females and sperm from 40 males was

performed on 10 June, 2008. Reverse osmosis purified water with Instant Ocean[®] added to 4 g/L was used as the embryo medium. Contaminated water was produced by adding sodium perchlorate dried in an oven at 90° C before weighing, at 30 and 100 mg/L. Clutches of eggs from the 40 females were combined for randomization and divided into 38 Petri dishes (100 X 20mm) with a previously determined water treatment (control: no perchlorate added, 30 or 100 mg/L of perchlorate) and fertilized with mixed sperm from the 40 males. Each Petri dish was then subdivided into three Petri dishes with approximately 100 embryos per Petri dish for three replicates of each treatment. Embryos were incubated at 20 ± 0.5° C. Water was changed daily and dead embryos removed for the first 10 dpf.

By 10 dpf, most embryos had hatched and were transferred to 56.8L aquaria (60cm X 31cm X 32cm) aerated with AZOO[®] multi sponge filters (65 mm diameter). The aquaria began with 6L of water due to the small size of the fish. Water level was proportionally increased as the fish grew to maintain fish density at appropriate conditions (1L water per 1cm fish). Water changes (15% of total volume) were carried out biweekly and as needed. Two to three ml of Bacta-pur[®] N3000 (IET-Aquaresearch Ltd., Quebec, Canada) were added to each tank once a week to limit nitrates. Reverse osmosis purified water was added weekly to compensate for evaporative loss. A YSI photometer model 9100 (Yellow Springs Instrument Co., Yellow Springs, OH, USA) was used to periodically check salinity (4-5 g/L), pH (7.0-8.0) and Ammonia (<2 mg/L total nitrogen); no abnormal levels were detected. Perchlorate concentrations in aquaria were measured with an Acorn Ion 6 meter (Oakton Instruments, Vernon Hills, USA) with a perchlorate ISE electrode (Cole-Parmer, Vernon Hills, IL, USA). Stickleback fry (<2 months old) were fed live brine shrimp and a mixture of Golden Pearls 100 (a commercial larval food), *Artemia* food (both from Aquatic Ecosystems, Apopka, FL, USA), and frozen ground

brine shrimp (*Artemia* sp.; Brine Shrimp Direct, Ogden, UT, USA). Juvenile to adult fish were fed frozen brine shrimp daily. Perchlorate intake from food sources was assumed to be negligible. The photoperiod in the lab was adjusted weekly to mimic natural conditions for Anchorage, Alaska. Mean temperature in the aquaria throughout the experiment was $13.5 \pm 0.5^\circ$ C.

4.4.3 Histology

At sexual maturity (approximately one year old) fish were collected for histological analysis. Fish were euthanized with an overdose of MS-222 (Argent Chemical Laboratories, Richmond, WA, USA) and a digital photograph was taken of both sides of each fish. The caudal fin was removed and preserved in 95% undenatured ethanol. The abdomen was opened with a scalpel before placing the carcass in Dietrich's solution [41]. After fixing for 2-5 days, fish were preserved in 70% isopropanol. Fish were then dehydrated in a graded series of ethanol using an auto processor, embedded in TissuePrep-2 Embedding Media (Fisher Scientific, PA, USA), coronally sectioned at 5 μ m thickness and stained with hematoxylin and eosin. Stained sections were embedded with Cytoseal XYL (Richard-Allan Scientific, MA, USA) and a cover slip was added. Thyroid histopathology was examined for 194 adult stickleback. The gonads of 282 adult fish (males=145, females=137) were also examined.

4.4.4 Thyroid hormone extraction and ELISA

A subset of adult fish was collected for TH analysis including fish from control (n = 7), 100 mg/L 154 dpf downshift (n = 4), and 100 mg/L 3 dpf upshift (chronic; n = 6) treatments. Fish were collected as above, but not placed in fixative and were stored frozen whole at -80° C

until hormone analysis. THs were extracted from whole-body homogenates as outlined in Crane et al. (2004) [42], with the following modifications: the final extract was stored dry at -80°C until the day of the assay. Each sample was reconstituted with 330 µL of EIA buffer (0.1M PBS, 0.15M NaCl, 0.1% BSA, pH 7.4). T4 was assayed in duplicate using 25 µL per well (Total T4 EIA, MP Biomedicals, Santa Ana, CA) and T3 was assayed in triplicate using 38 µL per well (Total T3 EIA, MP Biomedicals). Both EIAs were validated using tests of parallelism and standard addition. Intra-assay variation was 6.9% for T3 and 3.6% for T4. Inter-assay variation was 12.2% and 13.0% for T₃ and T₄, respectively.

4.4.5 Imaging and histomorphology measurements

Micrographs of the assessed target tissues were generated using a Leica DM4500B microscope, Leica DFC420C microscope camera and Leica Application Suite imaging software (Leica Microsystems, Wetzlar, Germany). Five measurements were made on micrographs using ImageJ [43] to quantify thyroid tissue morphology. All follicles were counted on the section with the greatest amount of visible thyroid tissue. Subsequently, four follicles per section were randomly chosen and the following measurements made: 1) area of colloid, 2) area of follicle (lumen, not including follicle cells), 3) circularity of follicle ($4\pi \times \left(\frac{Area}{Perimeter^2}\right)$), 4) cell height of four follicle cells at approximately the 12, 3, 6 and 9 o'clock positions, and 5) area of follicle with no apparent colloid (follicle area - colloid area). Means of these measures were calculated for each fish. In addition, the colloid was characterized qualitatively including the evaluation of depletion, texture and density. The degree of small vessels surrounding thyroid follicles (angiogenesis score) was determined using control histological sections as a baseline and

assigning a score to each section as similar to control or as slight, moderate, or severe angiogenesis (Figure 4.1).

The stage of maturity was determined for gonads of 4-6 fish from each treatment. Stages (1-4) of all oocytes visible on a slide were counted. Stages used were modified from Sokołowska and Kulczykowska [44]: early (their 1 and 2), mid (3 and 4), late (5 and 6), and mature (7 and 8) (Figure 4.2 & Suppl. Table S4.1). Testicular lobules tended to be homogenous in their maturity state and were given one score. Stages for testicular lobules were also modified from Sokołowska and Kulczykowska: early (their 6 and 7), intermediate (1 and 2), late (3 and 4), and sexual phase with little to no spermatozoa present in tubules (5) (Figure 4.2 & Suppl. Table S4.1).

4.4.6 Genotypic sexing

Genomic PCR was used to determine the genetic sex of 146 individual stickleback (control, n = 63; 30 mg/L, n = 43; 100 mg/L, n = 40) of which the phenotypic sex was known from visual inspection of histology slides. DNA was extracted from caudal fins. Primers used were: Ga1F: CTTCTTTCCTCTCACCATACTCA and Ga1R: AGATGACGGGTTGATAAACAG. The PCR conditions were as follows: 94 °C for 3 minutes, 94 °C for 45 seconds, 36 cycles of 44 °C for 45 seconds, 72 °C for 45 seconds and 72 °C for 10 minutes. The samples were run on a 1% agarose gel.

4.4.7 Data analysis

Normality of dependent variables was determined using the Shapiro-Wilk test. Area measurements and number of follicles were positively skewed and were log transformed

($\log(1+x)$) to achieve normality. Pearson's product-moment correlation was used to determine the relationships between colloid area, follicle area and no colloid area. A multivariate analysis of variance test (MANOVA) was used to evaluate interactions and significance between two continuous dependent variables (number of follicles and area of colloid) and four categorical independent variables (concentration, dpf, upshift/downshift, and tank). The proportion of all fish examined for gonad maturity with the most mature stage (stage 3 in males and stage 4 in females) present in individuals was used as an indicator of reproductive maturity due to insufficient sample sizes to detect differences in timing of exposure. The 0, 3, 7, and 14 dpf treatment groups in the upshift exposure regime were combined to increase sample size and examine only fish exposed for the longest durations and beginning during early development. Temporal differences between treatments (dpf) within each exposure regime and concentration were analyzed using ANOVA. Pair-wise comparisons of treatment and control fish were achieved with a TukeyHSD multiple comparisons test. Contingency tables were evaluated with a Fisher Exact Test or Chi-square test as appropriate. When the assumptions for parametric tests were violated, the Kruskal-Wallis test was used. All analyses were considered significant when $p < 0.05$ and were performed using R (version 3.0.1, [45]).

4.5 Results

4.5.1 Thyroid hormone levels

There were no significant differences between controls and either 100 mg/L 154 dpf downshift and 100 mg/L 3 dpf upshift treatment groups for whole body homogenate T4 and T3 (Kruskal-Wallis: T4, $\chi^2 = 1.98$, $p = 0.37$; T3, $\chi^2 = 0.49$, $p = 0.78$; Figure 4.3). As expected, T4

levels were consistently higher than T3 levels (mean = 2.99 ± 2.2 ng/g and 0.88 ± 0.32 ng/g, respectively; t-test: $t = -8.27$, $df = 17$, $p < 0.0001$).

4.5.2 *Thyroid histomorphology*

Thyroid tissue from fish exposed to control conditions had large, colloid filled follicles surrounded by a layer of squamous or cuboidal epithelial cells (Figure 4.4A&C). Stickleback chronically exposed to perchlorate exhibited a proliferation of small follicles (hyperplasia), hypertrophy of the follicle cells and colloid depletion (Figure 4.4B,D,E&F). A proliferation of small blood vessels (angiogenesis) was evident in many of the chronically treated fish compared to controls (Figure 4.4E).

The area measurements of the follicle and colloid were highly correlated (Pearson's product-moment correlations, $df = 192$), follicle area and colloid area, $r = 0.97$, $p < 0.0001$; follicle area and no colloid area, $r = 0.98$, $p < 0.0001$; and colloid area and no colloid area, $r = 0.92$, $p < 0.0001$). To avoid redundancy and because other measurements characterize the follicle, area of colloid was chosen to represent these measurements in further statistical analyses. All independent variables except tank were significant in the comprehensive MANOVA: concentration of perchlorate: (number of follicles; $p = 0.0032$, colloid area; $p < 0.0001$), upshift or downshift exposure regime: (number of follicles; $p < 0.0001$, colloid area; $p < 0.0001$), and dpf of exposure or rescue: (number of follicles; $p = 0.0229$, colloid area; $p < 0.0001$). There was a significant interaction between the upshift/downshift regime with both dpf and concentration ($p < 0.0001$, $p = 0.0057$, respectively) which was anticipated due to the experimental design. Sex was a significant factor in cell height with males having significantly

greater hypertrophy of thyrocytes than females regardless of treatment ($t = -6.11$, $df = 144$, $p < 0.0001$). All other thyroid metrics were not significantly different between sexes.

The number of follicles was significantly greater in the 100 mg/L and 30 mg/L upshift regimes than in the control treatment when exposures were initiated <42 dpf (Figure 4.5). The ANOVA for the 100 mg/L downshift treatments indicated significant differences present (Table 4.1), but there was no definitive pattern and no post-hoc significant differences. The area of colloid was significantly reduced in both upshift and downshift exposure regimes (Table 4.1, Figure 4.6). Colloid area was reduced in the upshift 100 mg/L and 30 mg/L when exposure began < 42 dpf. The upshift and downshift regimes had opposing trends as would be expected due to total duration effects of perchlorate exposure (Figure 4.6).

The angiogenesis score significantly changed in all exposure regimes (Table 4.1, Figure 4.7). In 30 mg/L upshift treatments all but 154 and 305 dpf had significantly greater proliferation than controls while in 100 mg/L upshift, all treatments were greater than controls. Upshift and downshift exposure regimes trended oppositely (Figure 4.7). Rescue beginning at 14 dpf and later resulted in more angiogenesis in 30 mg/L downshift exposures while rescue at 42 dpf and later resulted in more angiogenesis in 100 mg/L downshift treatments (Figure 4.7).

Height of follicle cells was affected by perchlorate exposure (Table 4.1). In the 100 mg/L upshift exposure regime the ANOVA was significant (Table 4.1) but only the 0 dpf treatment had significantly greater cell height compared to controls and there was a negative trend with cell height decreasing as the dpf of exposure increased. The 30 mg/L downshift exposure regime had significantly greater cell height in 7 and 42 dpf treatments, but there was no clear trend. Circularity ranged from 0.68 to 0.96 with 1.0 being a perfect circle. The control group mean was 0.83 ($n = 48$). The 100 mg/L upshift exposure regime had significantly more circular follicles in

the 14, 21 and 42 dpf treatments. Neither timing of exposure nor concentration of perchlorate conclusively affected follicle shape. Most treatments exhibited greater qualitative colloid descriptors (depletion, grainy texture and decreased density) than controls regardless of concentration or timing of exposure. There was a significant concentration-dependent reduction in colloid in fish exposed to 100 mg/L beginning in the first 14 dpf (Table 4.2).

4.5.3 Gonad maturity and sex ratio

The maturity score was equivalent among the four earliest dpf treatments, in which all stages were observed in both males and females (Kruskal-Wallis test, $df = 3$: Females; 30 upshift, $H = 5.97$, $p = 0.113$, 100 upshift, $H = 1.80$, $p = 0.616$, Males; 30 upshift, $H = 1.97$, $p = 0.579$, 100 upshift, $H = 1.30$, $p = 0.729$). The proportion of late stage testes and oocytes significantly decreased with chronic exposure to perchlorate in both exposure concentrations (Table 4.2). Additionally, sex ratio (identified by gonadal inspection) significantly shifted toward males in a concentration dependent manner (Table 4.2). Control stickleback had a sex ratio of 0.62:1 males:females, while the stickleback in 100 mg/L had a sex ratio of 2.08:1, or 2 males for each female. Genotypic sex matched phenotypic sex in all but three individuals; one control fish was phenotypically male but genetically female, two fish exposed to 100 mg/L perchlorate were phenotypically opposite of their genetic sex (one male and one female).

4.6 Discussion

The present study utilized timing and varying durations of exposure to two perchlorate concentrations (compared to control) over a one year period to determine sensitive windows during development in which perchlorate exposure has the greatest effect on threespine

stickleback. Thyroid tissue, sex ratios and gonad maturity were sensitive to chronic exposure that began during early stages of development. The upshift/downshift experimental design illuminated critical windows when perchlorate exposure caused histomorphological changes to thyroid tissue. In contrast, whole body T4 and T3 levels did not vary between control and treated fish (Figure 4.3). This is consistent with other studies using zebrafish [15], mosquitofish [22], fathead minnow [28] and stickleback (J. Postlewait, W. A. Cresko, C. L. Buck and F. A. von Hippel, unpublished data). Thyroid tissue morphology may be more sensitive than whole body TH levels to perchlorate exposure due to direct effects on follicle cells, the complexity of HPT-axis feedback mechanisms, and TH storage [14, 38].

Hyperplasia of follicles has previously been described as a useful biomarker for perchlorate exposure in fish [18, 21] and this study confirmed its utility for threespine stickleback. It was the most obvious histomorphological response of the thyroid. The total number of follicles increased within a critical window between 0 and 154 dpf (Figure 4.5). There was not an obvious early threshold dpf, but chronic exposure beginning anytime within 42 dpf caused histomorphological responses (Figures 4.5). Exposing fish to perchlorate after 42 dpf resulted in no detectable hyperplasia. We conclude those fish neither developed hyperplasia nor were able to compensate and recover by the sampling time. Hyperplasia in all downshift treatments were not significantly different from controls indicating that hyperplasia is not permanent and complete recovery is possible. The degree of hyperplasia was concentration-dependent (Figure 4.5).

Area of colloid (highly correlated with area of follicle) was significantly reduced. The critical window of sensitivity was within the first 42 dpf, again with no early threshold. The area of colloid in downshift treatments tended to be reduced, but there was no significant temporal

trend. Recovery from reduced area of colloid occurs, but not as readily as recovery from hyperplasia (compare downshift in Figures 4.5 & 4.6). The response of colloid area was independent of concentration of perchlorate (Figure 4.6). The probable reason for reduced colloid area is the lack of large follicles in chronically exposed fish (Figure 4.4A & B). Given that the number of follicles increases in perchlorate exposed individuals, the total area of colloid may not differ from control individuals. A more detailed histomorphological study could elucidate the relationships between follicle size, follicle number and total colloid area in fish exposed to a variety of perchlorate concentrations.

The circularity of follicles was variable and effects did not correlate with temporal differences in timing of exposure to perchlorate. A concentration-dependent relationship was not detected. Conclusions from gross inspection of follicles include consistently circular small follicles in exposed fish and elongated to circular large follicles in control fish (compare Figures 4.4A & B), but a difference in shape was not statistically significant. Schmidt et al. [21] detected semi-quantitative changes in follicle shape (in and out-foldings) in zebrafish exposed to 500 and 5000 $\mu\text{g/L}$ perchlorate.

Mean hypertrophy of thyrocytes increased in some treatments, particularly in 100 mg/L perchlorate, but there were no statistically supported trends. The extended exposure length may have allowed for recovery from hypertrophy in this study. Increased cell height has routinely been found in other fish studies with perchlorate exposure periods of 90 days or less (zebrafish [15, 18, 21, 30, 31, 33, 34], mosquitofish [22, 32], fathead minnow [28]). In zebrafish, hypertrophy is less sensitive than follicle number [21] and colloid depletion [18, 21, 33], but the opposite is true for African-clawed frogs (*Xenopus laevis*) [46]. In zebrafish, complete recovery from hypertrophy was documented in less than 12 weeks after a 12 week exposure period [15]

and less than 15 days after a 37 day exposure period [31]. Hypertrophy of follicle cells has been suggested to be proportional to thyroid stimulating hormone (TSH) secretion [13, 38].

Colloid condition varied by individual and was not reliable for detecting temporal effects of exposure to perchlorate. Colloid appears to recover perchlorate perturbations relatively quickly in fish (e.g., within four weeks in zebrafish [15]). Combining results from fish exposed to perchlorate beginning in the first 14 dpf (upshift) demonstrated a concentration-dependent response in the percent occurrence of colloid depletion (Table 4.2). Chronically exposed stickleback had reduced colloid with perchlorate exposure, consistent with previous studies [18, 21, 30, 31].

Of the metrics employed in this study, angiogenesis had the most defined critical window with exposure between 7 and 154 dpf and 21 and 305 dpf for 30 and 100 mg/L, respectively (Figure 4.7). Stickleback exposed to perchlorate anytime within those developmental windows expressed significantly increased angiogenesis. The 100 mg/L downshift exposed fish were able to recover when rescued up to 21 dpf while paradoxically, 30 mg/L exposed fish recovered when rescued up to 7 dpf (Figure 4.7). Once angiogenesis occurred, it was permanent within the timeframe of this study. In the upshift exposure regime, no matter when fish were placed in 100 mg/L perchlorate, they developed angiogenesis. The higher concentration stimulated a greater response, even when exposed as adults 60 days before sample collection. Angiogenesis can occur quickly as demonstrated in zebrafish which displayed increased angiogenesis after only two weeks of exposure to 90, 1131 and 11,480 $\mu\text{g/L}$ perchlorate [15].

The timing of perchlorate exposure, especially in the upshift regime, appeared to contribute more to inducing histomorphological changes to the thyroid than the perchlorate concentration. The stark differences between upshift treatments <42 dpf and 154 dpf in area of

colloid (30 and 100 mg/L), number of follicles (30 and 100 mg/L) and angiogenesis (30 mg/L) provide convincing evidence that exposure to perchlorate during development alters the HPT-axis in stickleback. Fish exposed on 154 and 305 dpf were statistically indistinguishable from control fish (Figures 4.5 & 4.6).

Most important developmental milestones in threespine stickleback occur by 42 dpf [47]. Thyroid tissue in stickleback appears 8-11 dpf (J. Postlethwait, W. A. Cresko, C. L. Buck and F. A. von Hippel, unpublished data). Some point between 42 and 154 dpf the critical window “closes” and thyroid tissue is not permanently altered by exposure to perchlorate. Because adult thyroid tissue is normal when exposed to perchlorate after 42 dpf, it may be assumed that adult stickleback thyroid tissue is able to compensate for the disruption caused by the perchlorate ion. This could be due to: 1) a decreased demand for TH post development and metamorphosis, 2) altered TSH levels due to other stimuli (neural, hormonal, external, etc.) or 3) physiological adjustments to the HPT-axis to provide adequate TH that were not detected in this study. Greater sensitivity to perchlorate due to increased demand for TH in larvae and fry [38] may contribute to the shift at 42 dpf. Lack of observed angiogenesis in fish rescued early may be due to the presence of TH of maternal origin and lack of functioning thyroid follicles.

It is likely that differences between exposure concentrations were caused by greater competitive inhibition of iodide uptake in the higher concentration resulting in less T4 synthesis leading to less negative feedback at the hypothalamus and/or pituitary. This would stimulate TSH release which is known to stimulate thyroid tissue hyperplasia and follicle cell hypertrophy [38, 48]. In fathead minnows exposed to methimazole (a thyrotoxin blocking TH synthesis) the mRNA thyrotropin β -subunit was elevated in pituitary tissue [49]. The concentrations of perchlorate in this study are within the environmentally relevant range [38, 50-52] and similar

responses have been described at lower concentrations for zebrafish [21, 34] and mosquitofish [22].

The heterogeneous thyroid tissue in freshwater fish is more dynamic than mammalian glandular thyroid and has been hypothesized to go through a histophysiological cycle [14, 53]. If this cycle is accelerated due to perchlorate exposure, this could explain the reduction in colloid area (fewer large colloid filled follicles) and proliferation of small follicles more suited to concentrate iodide [53]. Additionally, small follicles may have different sensitivities to colloid depletion than large follicles, which could explain the lack of consistent results for colloid depletion in this study. Age and species of experimental animals and duration of exposure may affect the sensitivity of endpoints measured due to species differences and the complex feedback pathways of the HPT-axis [14, 38].

The proportion of late stage gonads decreased in a concentration dependent manner in male stickleback chronically exposed to perchlorate (Table 4.2). The late stage of oocytes responded more strongly to 30 mg/L than 100 mg/L, but both were reduced (Table 4.2). Perchlorate may alter the rate of gametogenesis in both sexes. Sharma and Patiño [35] describe a greater proportion of late stage testes in 60 dpf male zebrafish along with a greater proportion of early stage oocytes in female zebrafish exposed to 100 mg/L sodium perchlorate. The temporal differences between the present study and Sharma and Patiño [35] makes comparisons difficult, but together these studies provide convincing evidence that perchlorate can delay gamete maturation in some fish.

Whole body thyroid hormone levels were not altered by perchlorate exposure, suggesting that perchlorate may have extrathyroidal effects on gonads. Further investigation is needed to determine if perchlorate has direct or indirect effects on gonad maturation. Alteration of sex

steroid metabolism could also cause perturbation in gonad development. Interactions between thyroidal and reproductive endocrine systems have been previously reviewed [14, 54-56].

The phenotypic and genotypic sex ratio was skewed toward males in fish exposed continuously beginning in the first 14 dpf (Table 4.2). This is in agreement with Lewis et al.'s [37] finding of threespine stickleback sex determination within approximately 11 dpf and also Bernhardt et al.'s [24] finding that perchlorate has a masculinizing effect on threespine stickleback. Bernhardt et al. [24] observed hypertrophy of testes in males and ovotestes in some genotypic females chronically exposed to 100 mg/L perchlorate. Intersex gonads were not observed in the present study, and complete sex reversal was ruled out as an explanation for the biased sex ratio by genotypic sexing. Differential survival of males in the 100 mg/L treatments is likely the cause of the observed altered sex ratio.

The bias towards males in this study is in contrast to a female-biased sex ratio in zebrafish. Mukhi et al. [30] and Sharma and Patiño [35] found a female bias with perchlorate exposure and a male bias with T4 supplementation. Sex determination in teleosts is highly variable and both genetic and environmental factors can influence the outcome [57]. The direction of sex bias is likely related to species differences in sex determination and further investigation of the underlying mechanisms could help to determine specific tissue targets and temporal sensitivities to perchlorate on a species specific basis. Mechanistic studies would also inform potential risks of perchlorate exposure to human sexual development.

The opposing trends of exposure regimes for area of colloid and angiogenesis score combined with the absence of effect post 42 dpf in upshift treatments demonstrate the importance of timing of exposure. Short term exposure studies can detect acute responses of the thyroid tissue (e.g. [16, 20, 22, 31]), and determine thyroid status during developmentally critical

stages, but do not necessarily translate into long term effects and consequences to fitness of the individual or to the population (such as changes in sex ratios) [58]. Chronic exposures can evaluate fitness endpoints and multigenerational effects (e.g. [24-26]), but cannot detect temporal sensitivities to the contaminant under study. The plasticity and variation between species of the HPT-axis system eludes straight forward causal relationships, so species should be considered on an independent basis with the goal of synthesizing mechanisms of toxicity across taxa.

Knowledge of temporal sensitivities over key lifecycle stages to contaminants is valuable. For example, management of waterways and wastewater discharge can be maximized to protect sensitive species during critical developmental windows. Exposure experiments examining the critical windows over all potentially sensitive life stages (e.g., organogenesis, metamorphosis, and gonadal recrudescence) can provide surprising and critical knowledge about the effects of contaminants.

In summary, the experimental design of this study successfully detected critical windows of sensitivity to perchlorate exposure for thyroid histological biomarkers. Hyperplasia, colloid area and angiogenesis were sensitive during development. Recovery occurred readily for hyperplasia, somewhat for colloid area and not at all for angiogenesis. The proportion of late stage testes and oocytes decreased and sex ratio was significantly biased toward males in fish chronically exposed to perchlorate. Long-term exposure studies that assess contaminant effects at various stages of development provide novel information to characterize risk to aquatic organisms and management of resources. Future work should focus on the specific time points and targets of perchlorate toxicity, including molecular mechanisms of action. Specifically, the first 21 dpf are critical in stickleback and perturbations during that time could have fitness and

population consequences. Additionally, lower concentrations of perchlorate should be evaluated for effects on thyroid histomorphology and influence on sex ratio and gonad maturity.

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4.8 References

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Table 4.1: Results of Analysis of Variance tests of each thyroid measurement within each exposure regime and concentration. Significant differences between timing of exposure (dpf of shift) was explored with these tests. Concentrations: 30 = 30 mg/L perchlorate, 100 = 100 mg/L perchlorate. Exposure regime: Downshift = fish began in contaminated water and were moved to clean water on the given day post fertilization (dpf), Upshift = fish began in clean water and were moved to contaminated water on the given dpf.

	30 upshift	30 downshift	100 upshift	100 downshift
Characters	F/df/p value	F/df/p value	F/df/p value	F/df/p value
Area of colloid	13.1/8,71/<0.0001	4.91/7,74/0.0001	24.25/8,82/<0.0001	7.32/7,77/<0.0001
Area of follicles	11.98/8,71/<0.0001	4.7/7,74/0.0002	21.09/8,82/<0.0001	7.12/7,77/<0.0001
Total number of follicles	7.13/8,71/<0.0001	NS	10.45/8,82/<0.0001	2.5/7,77/0.0232
Thyocyte cell height	NS	2.33/7,74/0.033	2.96/8,82/0.0058	NS
Angiogenesis score	35.79/8,71/<0.0001	21.01/7,74/<0.0001	25.53/8,82/<0.0001	8.43/7,77/<0.0001
Circularity	NS	NS	2.72/8,82/0.0105	NS
Area without colloid	10.19/8,71/<0.0001	4.16/7,74/0.0006	10.45/8,82/<0.0001	5.91/7,77/<0.0001

Table 4.2: Percentage of late stage testes (stage 3) and oocytes (stage 4), sex ratio and percent occurrence of depleted colloid in threespine stickleback exposed to perchlorate continuously beginning on 0, 3, 7, and 14 days post fertilization as combined data (upshift). Parentheses contain actual counts and asterisk indicates significant difference from control (Fishers Exact Test: $p < 0.05$)

	Male	Female	Sex ratio (M/F)	% occurrence depleted colloid
Control	42.0 (10/24)	41.0 (16/39)	0.62	12.5 (6/48)
30 mg/L	11.1 (2/18)*	8.0 (2/25)*	0.72	31.3 (5/16)
100 mg/L	3.7 (1/27)*	23.1 (3/13)	2.08*	50.0 (12/24)*

4.10 Figures

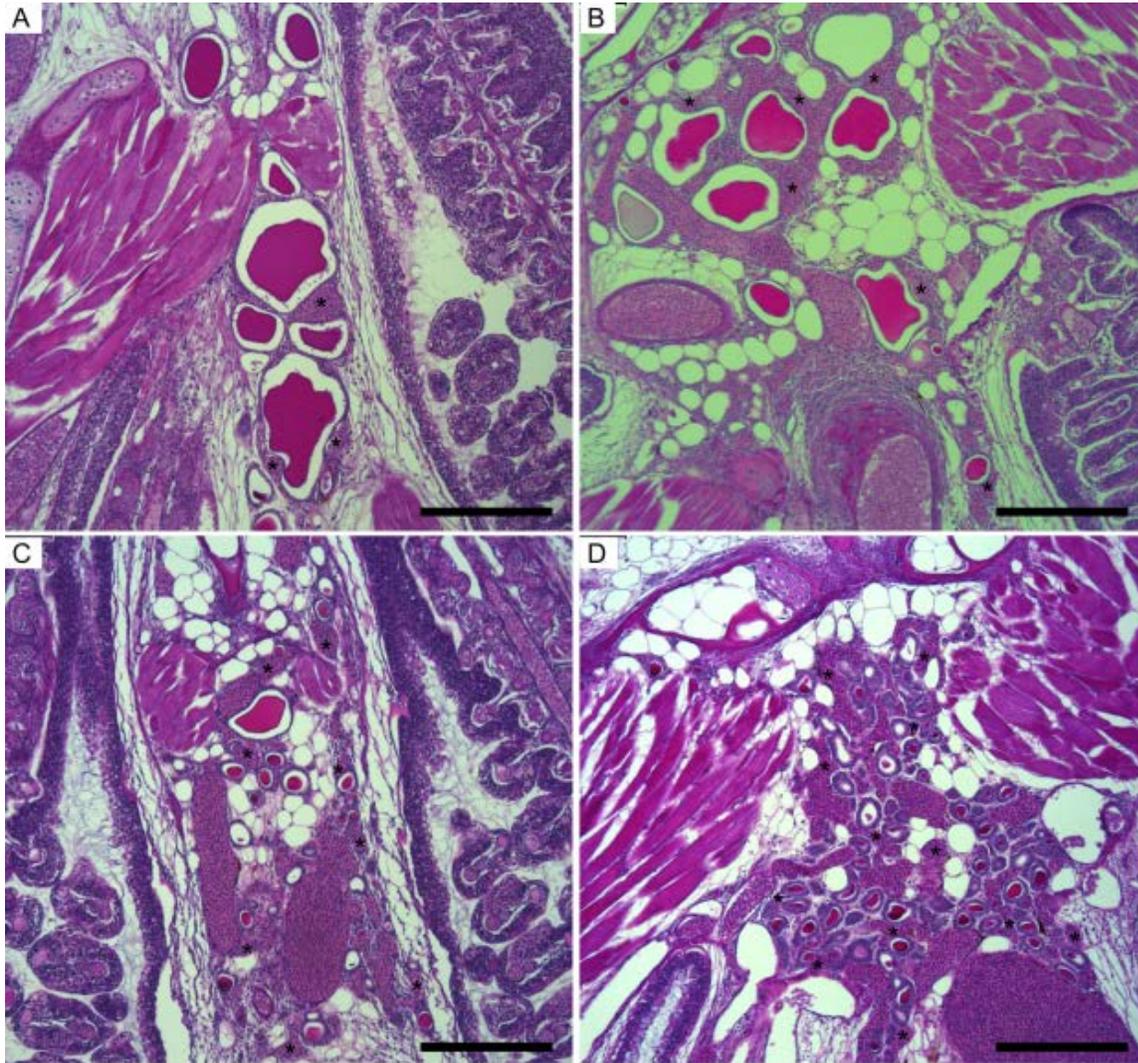


Figure 4.1: Photomicrographs of threespine stickleback thyroid tissue showing typical angiogenesis scores of normal (A), mild (B), moderate (C), or severe (D). Asterisks indicate blood vessels. Scale bar = 200 μ m.

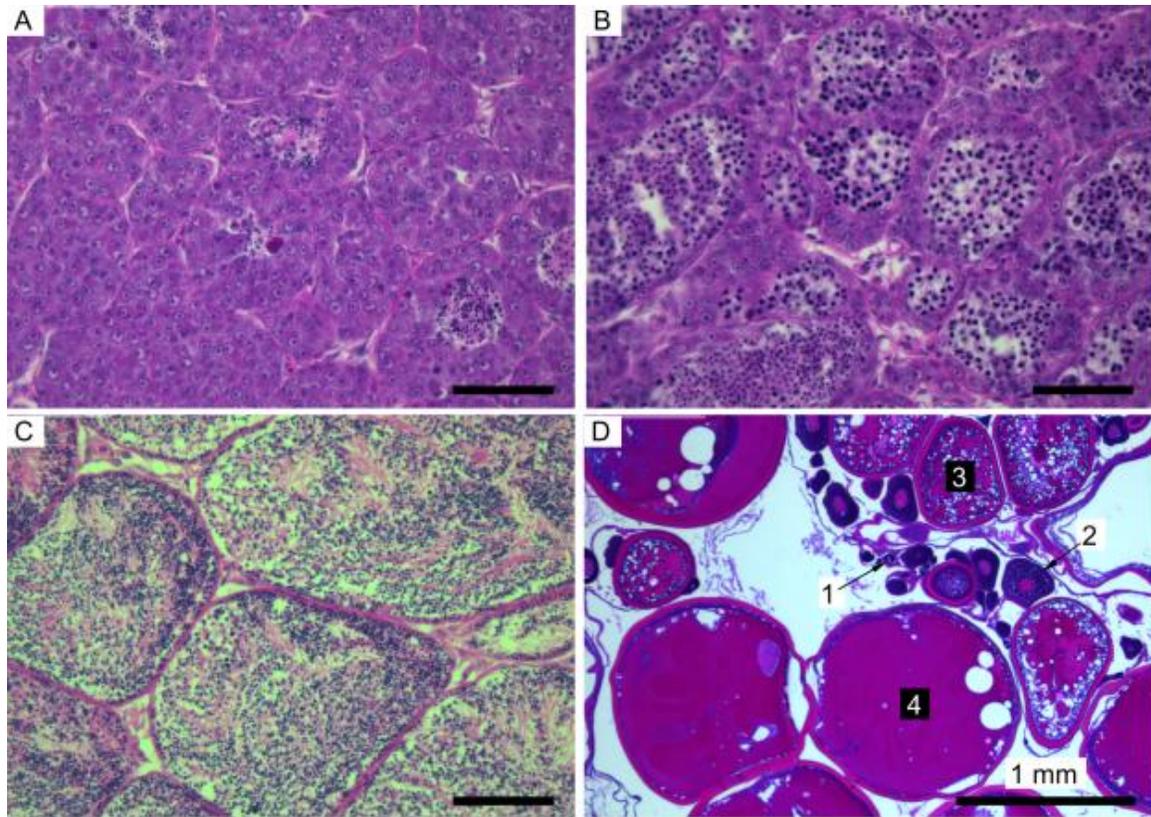


Figure 4.2: Photomicrographs of threespine stickleback gonads. Testes were scored for maturity stage as: early (A), intermediate (B), or late (C). Ovaries contained oocytes at various stages (D) and were scored using four stages: early (1), mid (2), late (3) and mature (4). See supplementary table S4.1 for descriptions of maturity stages. Scale bar in A, B and C = 50 μ m.

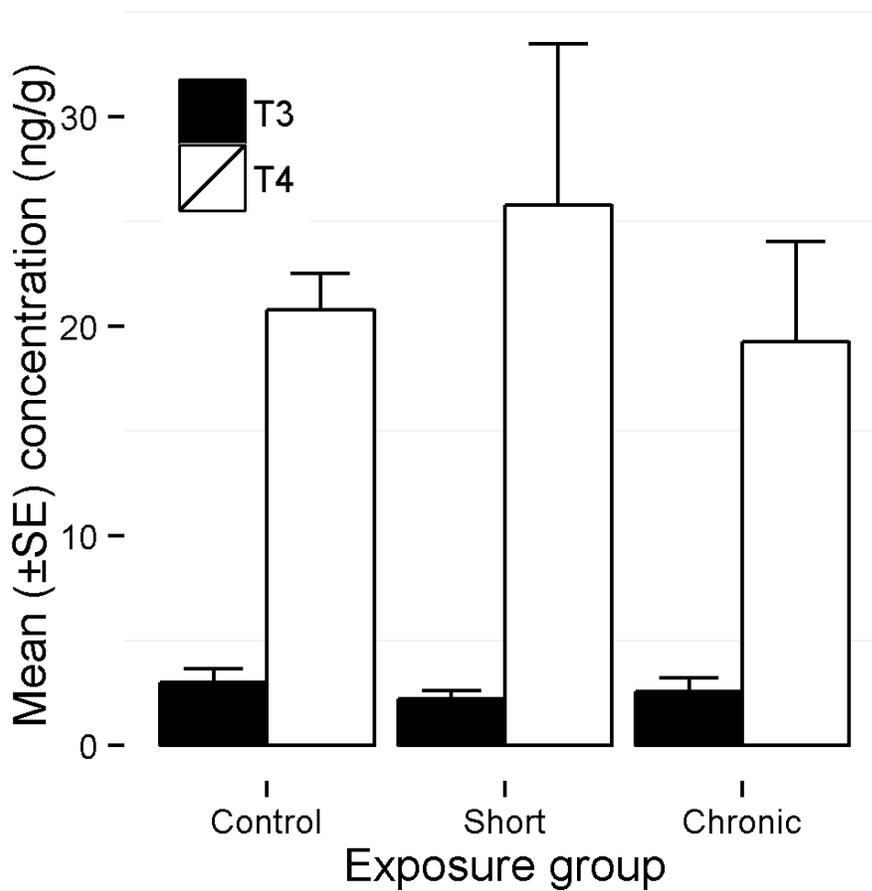


Figure 4.3: Whole body homogenate tissue concentration of thyroxine (T4) and triiodothyronine (T3) in threespine stickleback exposed to 100 mg/L perchlorate either chronically beginning at 3 dpf (chronic) or beginning at fertilization and rescued at 154 dpf (short). Fish were collected at one year.

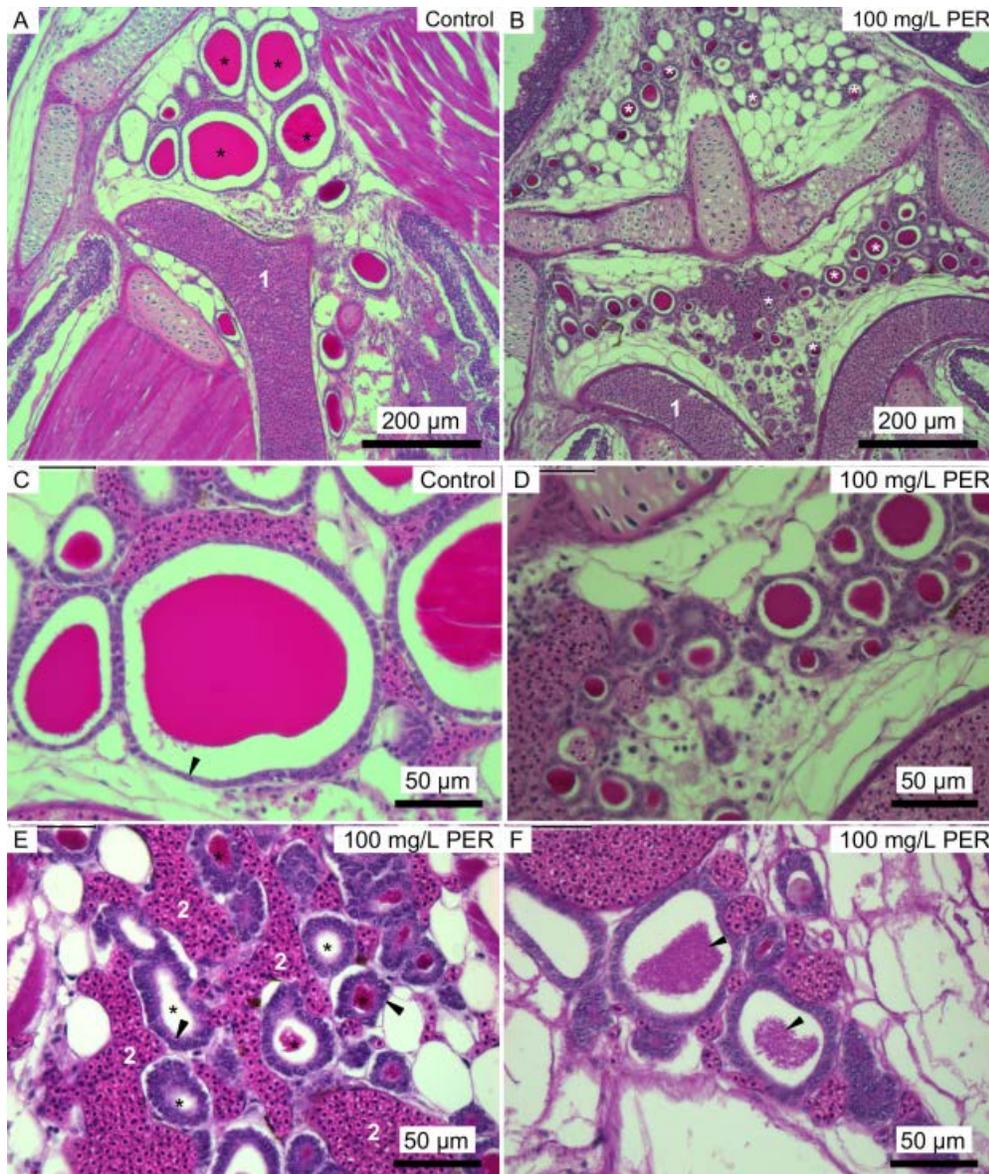


Figure 4.4: Photomicrographs of threespine stickleback thyroid tissue from control water (A & C) and 100 mg/L perchlorate exposures (B, D, E & F). Asterisks indicate follicles, 1= brachial artery, 2 = blood vessels. Some perchlorate exposed fish exhibited thyrocyte hypertrophy (arrows in E) compared to control thyrocytes (arrow in C) and grainy colloid (arrows in F). Note the proliferation of small follicles in exposed fish (B) compared to control fish (A).

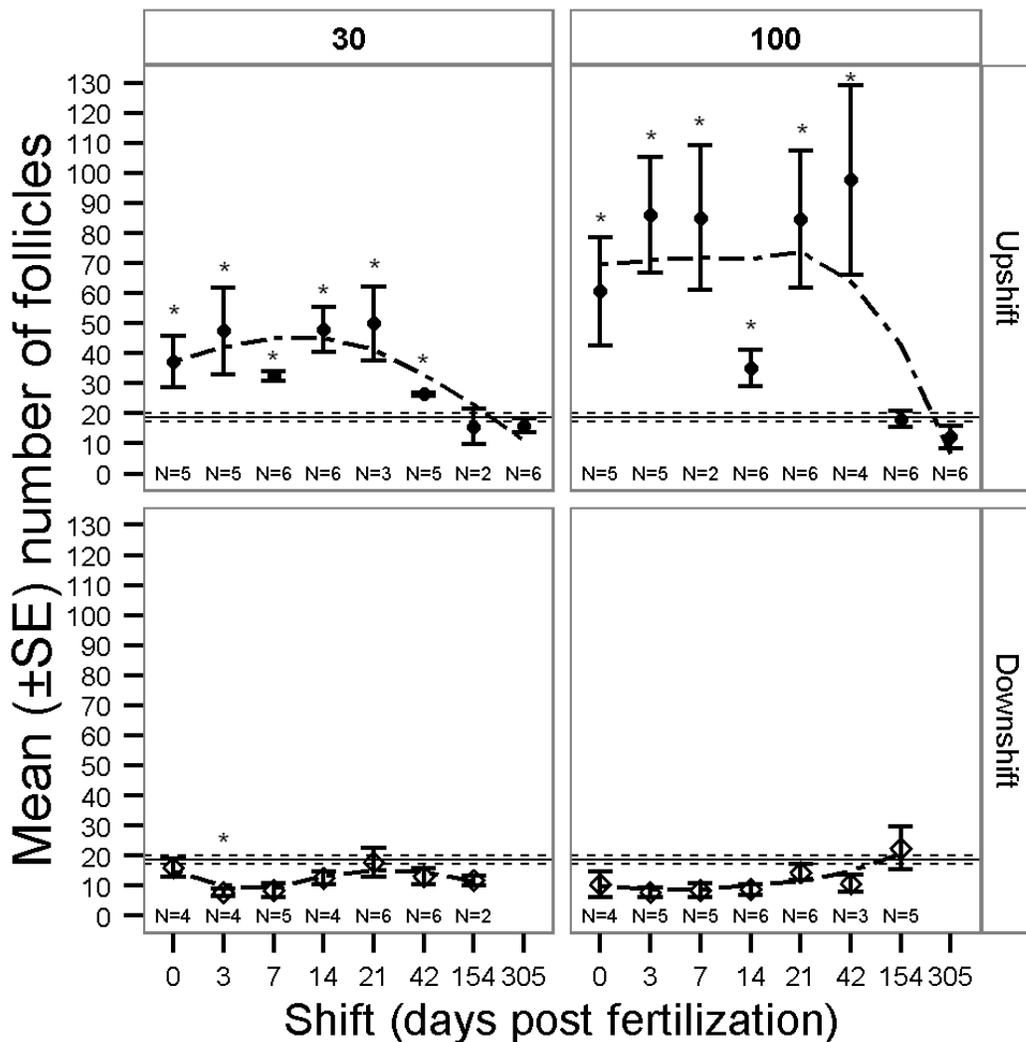


Figure 4.5: Mean (\pm SE) number of thyroid follicles in slides from perchlorate exposed threespine stickleback. 30 = 30 mg/L perchlorate, 100 = 100 mg/L perchlorate. Exposure regime: Downshift = fish began in contaminated water and were moved to clean water on the given day post fertilization (dpf), Upshift = fish began in clean water and were moved to contaminated water on the given dpf. Trend line is a LOESS curve and is included only for visualization of trends. ANOVA was used to compare treatments within each panel (concentration and exposure regime). The solid horizontal line is the control group mean and dashed lines are the SE of the control group. Asterisks indicate a significant difference from control ($\alpha = 0.05$).

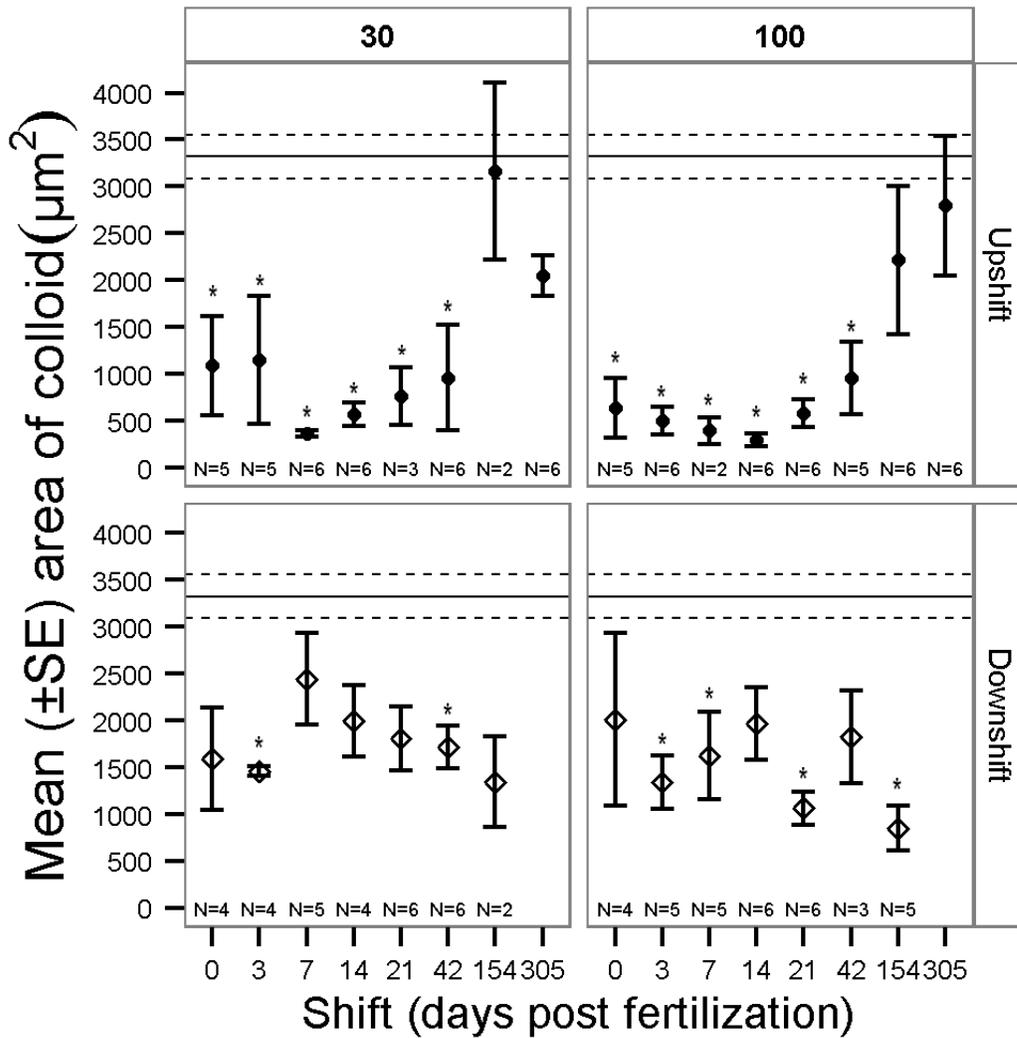


Figure 4.6: Mean (\pm SE) area of colloid in four follicles for perchlorate exposed threespine stickleback. 30 = 30 mg/L perchlorate, 100 = 100 mg/L perchlorate. Exposure regime: Downshift = fish began in contaminated water and were moved to clean water on the given day post fertilization (dpf), Upshift = fish began in clean water and were moved to contaminated water on the given dpf. Trend line is a LOESS curve and is included only for visualization of trends. ANOVA was used to compare treatments within each panel (concentration and exposure regime). The solid horizontal line is the control group mean and dashed lines are the SE of the control group. Asterisks indicate a significant difference from control ($\alpha = 0.05$).

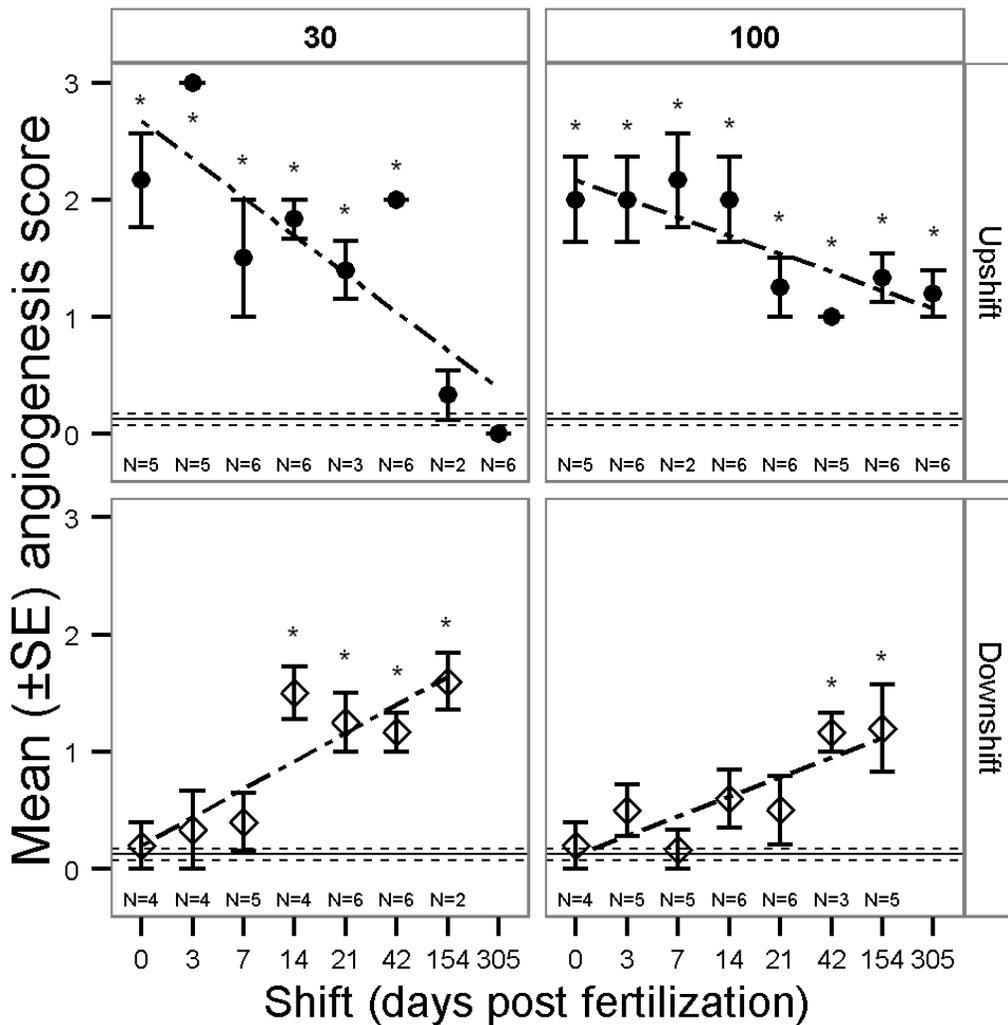


Figure 4.7: Mean (\pm SE) angiogenesis score for perchlorate exposed threespine stickleback. 30 = 30 mg/L perchlorate, 100 = 100 mg/L perchlorate. Exposure regime: Downshift = fish began in contaminated water and were moved to clean water on the given day post fertilization (dpf), Upshift = fish began in clean water and were moved to contaminated water on the given dpf. Trend line is the regression line and is included only for visualization purposes. ANOVA was used to compare treatments within each panel (concentration and exposure regime). The solid horizontal line is the control group mean and dashed lines are the SE of the control group. Asterisks indicate a significant difference from control ($\alpha = 0.05$).

4.11 Supplementary Material

Table S 4.5: Gonad maturity stages

Stages used in this study	Characteristics (from Sokołowska & Kulczykowska, 2006 [44])	Sokołowska & Kulczykowska stages
Oocytes: Stage 1	Previtellogenic: small, round follicles. Large nucleus with nucleoli along periphery or over the surface of the nucleus. The cytoplasm stains dark violet.	Stages 1 & 2
Oocytes: Stage 2	Vitellogenesis begins and small vacuoles appear beginning on the periphery and moving toward the center as they increase in size. Oocytes become more opaque and enlarge as more cytoplasm is added.	Stages 3 & 4
Oocytes: Stage 3	Vacuoles occupy all areas of cytoplasm. The oolemma becomes a conspicuous eosin stained band. Yolk vesicles begin to accumulate in the periphery as eosin stained circles. Oocytes continue to enlarge.	Stages 5 & 6
Oocytes: Stage 4	Yolk fills most of oocyte as a homogenous mass. Oocytes are large with conspicuous oolemma.	Stages 7 & 8
Testes: Stage 1	Seminiferous tubules almost entirely filled with spermatogonia. Primary and secondary spermatocytes begin to appear.	Stages 6 & 7
Testes: Stage 2	Seminiferous tubules filled with spermatocytes and spermatids. Single spermatogonia present. Spermatozoa range from small to large numbers in the center part of the tubules. Spermatocytes and spermatids in “cysts” along the periphery of tubules filled with spermatozoa.	Stages 1 & 2
Testes: Stage 3	Tubules filled with spermatozoa. Interstitial tissue becomes well developed. Few to no “cysts” of spermatocytes and spermatids.	Stages 3 & 4

Chapter 5: General Conclusions

5.1 Overall conclusion

The goals of this dissertation were to expand the knowledge of the impact of perchlorate on aquatic vertebrates (fish), particularly during development. The commitment of the U.S. Environmental Protection Agency (EPA) to regulate perchlorate will undoubtedly necessitate the evaluation and possibly remediation of aquatic resources throughout the U.S. Characterization of the degree of impact and critical developmental time points that are sensitive to exposure for various aquatic organisms are required for the risk assessment process to be ecologically relevant.

5.2 Hypotheses and findings

Hypothesis 1: Perchlorate does not biomagnify and water and food routes of exposure will contribute to tissue concentration additively (Chapter 2).

Findings: Results confirm that perchlorate **does not biomagnify** as predicted based on its high water solubility. Presumably, most of it is eliminated via urine and/or gills. Interestingly, **perchlorate is not additive in concentration** as tissue concentrations exceeded an additive (water and food routes) response relationship in the lowest exposure concentration tested (10 mg/L). This is most likely due to the higher tissue concentrations in the GIT.

Hypothesis 2: Gross morphological characters respond equally for the control, 30 and 100 mg/L perchlorate exposures in all time windows and durations, respectively (Chapter 3).

Findings: The **differential response related to time and duration of exposure to perchlorate for skeletal traits** found in the present study corroborates the findings of other studies examining hypothyroid effects on skeletal development in fish [1, 2]. Additionally some trait responses were concentration-dependent. These data provide evidence that the effects of perchlorate on skeletal development are likely a result of thyroidal disruption, perhaps due to variation in initiation or rate of development which is controlled by TH. The results show that perchlorate causes abnormal development of phenotypic characters that are important to the survival (armor) and fitness (body size) of stickleback.

Hypothesis 3: Thyroid histological biomarkers respond equally for the control, 30 and 100 mg/L perchlorate exposures in all time windows and durations, respectively (Chapter 4)

Findings: The upshift/downshift experimental design used in this study successfully elucidated critical windows during development that were sensitive to perchlorate exposure. Thyroid tissue clearly responded to perchlorate as has been shown in many other vertebrates. These data show that early exposure, less than 42 dpf, compromise the HPT-axis in stickleback. Equally important is that exposure to environmentally relevant concentrations at the time points tested after 42 dpf do not cause changes in thyroid histomorphology. The critical developmental window detected coincides with the timeframe of stickleback development [3]. This dissertation provides convincing evidence that the developmental period is susceptible to perchlorate and HPT-axis perturbation.

Hypothesis 4: Sex ratio and gonadal maturity respond equally for the control, 30 and 100 mg/L perchlorate exposures in all time windows and durations, respectively (Chapter 4).

Findings: The results of chapter 4 indicate that either sexual determination is significantly influenced by perchlorate exposure or that perchlorate causes higher mortality in females than males. Fish in control treatments had a slight female bias, while stickleback raised in perchlorate switched to a substantial male bias. This study supports results of our previous work that found masculinization effects of perchlorate [4]. This is an exciting and highly relevant result because androgenic compounds are rarely detected in contaminants studies. Populations with skewed sex ratios may have reduced fitness due to loss of mating opportunities. Along with changes in sexual determination or differential survival, perchlorate likely alters sexual differentiation by delaying gonadal maturation. This could have reproductive effects such as mismatches of male and female readiness to spawn during the breeding season.

5.3 Toxicity testing

Current toxicity testing practices may not be adequate to evaluate toxic effects of contaminants, or at the very least, not as efficiently as they should. In light of the upshift/downshift experimental design used in this study, developmental windows should be explored for integration into standard assays. The information gained would likely outweigh the added cost in animals and time, especially if there is prior knowledge of developmental timing which would allow the strategic targeting of developmental processes. If a toxicant only has a detrimental effect during a certain window of development, it will be most efficient to work within that window [5]. Treatments, waste products and sample sizes could be reduced once windows of sensitivity are established. The information gained would have direct, real world utility for resource managers who could mitigate the impact of contaminants by timing their release. For

example, managers could delay release of wastewater effluent into a river for certain durations to protect early development of a sensitive species of fish [6].

Route of exposure should also be given more attention for toxicity testing for aquatic organisms. As this study demonstrated, even hydrophilic chemicals can accumulate unexpectedly and the routes of exposure are relevant. Ambient water is often not the sole source of exposure, as standard testing sometimes assumes. A case in point is that perchlorate is bioaccumulated by some plants and herbivorous organisms could potentially be exposed to higher concentrations via food than water, and in turn the herbivores will be eaten by carnivores or omnivores. Initial testing could include both water and food routes of exposure and then a decision made for subsequent testing.

5.4 Future research

Future work should determine the toxicokinetics of perchlorate and describe NIS expression in ecologically and economically important aquatic species to better understand how perchlorate interacts with tissue type and potentially concentrates in tissues. Higher resolution upshift/downshift experiments could better pinpoint the sensitive timeframe of action and help elucidate the specific developmental process being targeted and ascertain critical timepoints of exposure for transcriptomic studies. This may also shed light on whether or not the toxicity of perchlorate is strictly through its effect on thyroid tissue, or whether extrathyroidal disruption also occurs. The use of positive controls and thyrotoxicants with different targets of toxicity (e.g., thyroperoxidase or deiodinases) would also aid in elucidating the mode of action. Future research should also determine the mechanisms of masculinization caused by perchlorate exposure and thus what other chemicals may act via this mechanism.

5.5 References

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