THE EFFECTS OF PERCHLORATE EXPOSURE ON A MODEL VERTEBRATE SPECIES: THE THREESPINE STICKLEBACK

By

Richard R. Bernhardt

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Chair, Department of Biology and Wildlife

APPROVED:

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THE EFFECTS OF PERCHLORATE EXPOSURE ON A MODEL VERTEBRATE SPECIES: THE THREESPINE STICKLEBACK

A

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By

Richard R. Bernhardt, B.S.

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Abstract

Few studies have examined the effects of chronic perchlorate exposure during multiple stages of development, and fewer still have analyzed the effects of perchlorate over multiple generations. Perchlorate exposure is known to cause thyrocyte hypertrophy (suggesting glandular stimulation), interference with thyroid hormone synthesis, and ultimately altered levels of circulating thyroid hormones, but whether these effects represent adaptive mechanisms or actual impairment is often debated within the scientific community. This research attempted to clarify whether exposure to environmentally relevant concentrations of perchlorate causes impairment at the organismal level. Ecologically significant endpoints were examined to provide an indication if a contaminated population would be able to sustain itself. Threespine stickleback fish (*Gasterosteus aculeatus*) were exposed to one of eight perchlorate treatments and compared to each other and to fish raised in water without detectable levels of perchlorate (<1.1 μg/L). Patterns are presented for two separate generations ($G_{2,2002}$ and $G_{2,2003}$) of stickleback that were spawned and raised in control or perchlorate treated water and a third generation ($G_{3,2004}$) that was not directly exposed to perchlorate but whose parents ($G_{2,2003}$) were raised from syngamy through sexual maturity in control or perchlorate treated water. When warranted, comparisons are made with their wild-caught parents ($G_{1,2002}$ and $G_{1,2003}$) that were exposed to perchlorate as adults for up to 22 days.

Exposure of mature adult stickleback to perchlorate had no noticeable effect on survival, behavior, or reproductive endpoints. However, chronic exposure of their offspring ($G_{2,2002}$ and $G_{2,2003}$) to perchlorate impaired nearly every aspect of fitness. Aberrant developmental patterns of somatic characters were primarily associated with growth, reproduction, locomotion, anti-predatory structures, and vision. Impaired stickleback ($G_{2,2003}$) that produced offspring in water without detectable levels of perchlorate (<1.1 μg/L) gave rise to offspring ($G_{3,2004}$) without the suite of abnormalities noted among treated fish. These findings suggest that perchlorate exposure during sensitive developmental periods has negative effects on critical life history characteristics of threespine stickleback, but remediation efforts are likely to restore healthy ecosystems.

Effects noted among stickleback provide a useful model for assessing effects that are likely to occur among other contaminated fishes and perhaps to other vertebrates.

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Chapter 1:

Introduction

1.1 Preface

Perchlorate, the primary component of solid rocket propellant (Mendiratta et al., 1996), is well known to competitively inhibit the uptake of iodide into thyroid follicles (NRC, 2005). To date, the majority of research has focused on sub-organismal level effects, such as altered iodide uptake rates, thyroid histology, and thyroid hormone levels. Yet, these endpoints raise debate within the scientific community. For example, some argue that changes to these endpoints following perchlorate exposure represent "adaptive" mechanisms and not necessarily adverse effects (EPA, 2002a; Schwartz et al., 2004). Thus, my research attempts to determine whether exposure to environmentally-relevant levels of perchlorate has adverse effects on ecologically-significant life history characteristics using a model vertebrate species: the threespine stickleback (*Gasterosteus aculeatus*).

1.2 Overview of Key Facts

1.2.1 Perchlorate Introduction and Environmental Chemistry

At a mere 101 Daltons, the perchlorate anion contains only two elements: chlorine and oxygen. Its single chlorine atom is surrounded by four oxygen atoms in a tetrahedral configuration that disperses its electrical field and provides remarkable stability (Urbansky, 1998). It has become a widespread environmental contaminant with the highest concentrations found in proximity to military storage and maintenance facilities that handle solid rocket propellant (Urbansky et al., 2001). When released into the environment, perchlorate readily dissociates from its counter-ion (typically magnesium, ammonium, sodium, or potassium) and enters aqueous solution, where it is highly soluble and stable. Perchlorate persists for several decades under aerobic ground

and surface water conditions (Urbansky and Schock, 1999; Lawrence et al., 2000; Motzer, 2001; Merrill et al., 2003), and under certain conditions, it may persist for substantially longer (e.g. for millennia; Plummer et al., 2006).

Dasgupta et al. (2005) reported that perchlorate can be formed by simulated atmospheric processes, yet the naturally occurring version has a unique isotopic signature that is distinct from anthropogenic perchlorate (Bao and Gu, 2004). Specifically, the $\delta^{17}O$ content of naturally occurring perchlorate is distinctly higher than that of manufactured perchlorate (Bao and Gu, 2004). This characteristic has helped to confirm that the highest levels of environmental perchlorate contamination come from the improper storage and disposal of anthropogenic perchlorate. Natural deposits of perchlorate were previously only known to occur in sodium nitrate deposits of Chilean caliche (which is exported in Chilean fertilizers), in nitrates from Death Valley, California, and in potash deposits from Carlsbad, New Mexico (Ericksen, 1981, 1983; Ericksen et al., 1988; Urbansky et al., 2001), but recent methodological advances are revealing the geographically extensive occurrence of perchlorate at low levels [with notable exceptions, <4 μg/Kg (ppb)] across arid and semi-arid regions of the American Southwest (Rajogopalan et al., 2006; Rao et al., 2007). Certain desert plants, like the prickly pear cactus (*Opuntia spp.*), may contain levels of natural perchlorate reaching 20 mg/Kg (Orris et al., 2007).

1.2.2 Uses of Perchlorate

Anthropogenic perchlorate is found in many common products such as fireworks, gunpowder, explosives, artillery, road flares, and airbag inflation systems (Urbansky, 1998; Urbansky and Schock, 1999). It is an additive in products such as lubricating oils, tanning oils, paints, dyes, fabric fixers, matches, and magnesium batteries (Mendiratta et al., 1996; Urbansky, 1998); it is also used in electroplating, aluminum refining, rubber manufacturing, enamel production, and in certain analytical chemistry procedures (Urbansky, 1998). It has intentionally been added to cattle feed to cause fraudulent weight gain (i.e., water retention) in beef cattle prior to sale and slaughter (Verbeke et al.,

1984; Batjoens et al., 1993), and it has been consumed incidentally when cattle eat contaminated forage items like wheat and alfalfa, which accumulate perchlorate (Jackson et al., 2005). Since perchlorate is found in such a variety of products and has such persistence, it perhaps comes as little surprise that perchlorate is found at elevated levels near perchlorate related facilities.

1.2.3 Widespread Contamination

The extent to which perchlorate contaminates natural watersheds began to be realized in 1997, following the advent of ion chromatography based analytic methods that reduced the limits of detection to 4 μg/L (Motzer, 2001). Prior to 1997, perchlorate could not be detected in simple matrices such as drinking water at levels below 100 μg/L, but detection limits were typically closer to 400 μg/L (Motzer, 2001; Huang and Sorial, 2006). With the development of more sensitive detection techniques (e.g., mass spectrometry, ion chromatography, and High Performance Liquid Chromatography) method detection limits decreased into the parts per trillion range for aqueous media. As a result, the frequency of environmental perchlorate detection rose dramatically. As of March 2005, perchlorate contamination had been reported in 36 states in the United States, ranging in concentration from slightly above method detection limits to as high as 3.7 g/L (parts per thousand) in aqueous media (http://www.epa.gov/fedfac/documents/detection_with_dates_03_25_05.xls). In 2005 perchlorate was reported in the Canadian Great Lakes, marking the first time that perchlorate contamination was identified outside of the United States (Backus et al., 2005).

Perhaps the most significant contamination event occurred in 1997 when perchlorate contamination of the Colorado River was identified. The Kerr-McGee Chemical Corporation in Henderson, Nevada was subsequently identified as the point source (EPA, 2005). Clean-up efforts remain underway, and by July 2005 between 770 and 900 Kg of perchlorate were being removed from the Colorado River every day (EPA, 2005). Nevertheless, the Colorado River is the primary source of drinking water for

approximately 15-20 million Americans (EPA, 2002b) who remain exposed to elevated levels of perchlorate, and contaminated water continues to be used to irrigate important farmlands in many Western states (Sanchez et al., 2005a,b). Beyond the Kerr-McGee incident, the main source of U.S. environmental contamination has come from the production, storage, and elimination of explosives and the solid rocket propellant used by the Department of Defense and the National Aeronautics and Space Administration (Urbansky et al., 1998; Gullick et al., 2001).

Human exposure to perchlorate likely comes from a variety of sources in addition to that which is ingested with drinking water. For instance, detectable levels of perchlorate have been found in fruit, such as tomatoes, cantaloupe, and cucumbers (Yu et al., 2004, Jackson et al., 2005); leafy produce, such as tobacco, lettuce, parsley, basil, and spinach (Ellington et al., 2001, Sanchez et al., 2005a,b; Seyfforth and Parker, 2006); dairy and human breast milk (Kirk et al., 2005); beer and wine (El Aribi et al., 2006), and many other sources (Urbansky et al., 2000; Smith et al., 2004; Sanchez et al., 2005b; Snyder et al., 2006; Krysnitsky et al., 2006). Since Chilean nitrates are the only mineralized nitrogen source authorized by the U.S. Department of Agriculture for use on organically farmed produce (http://www.ams.usda.gov/nop/NOP/standards/ListReg.html), organic produce items often contain higher levels of perchlorate than conventionally farmed produce (Dasgupta et al., 2005; Sanchez et al., 2005). Likewise, drinking bottled water provides no guarantee of getting perchlorate-free water (Snyder et al., 2005).

1.2.4 Known Effects

In its most thoroughly studied mode of action, perchlorate interferes with circulating iodide uptake by blocking sodium-iodide symporters in the thyroid gland; thereby interfering with the initial stages of thyroid hormone (TH) synthesis. It has also been reported to stimulate inorganic iodine efflux in the thyroid gland by an as yet unidentified mechanism (Stanbury and Wyngaarden, 1952; Brabant et al., 1992; Smith et al., 2001; Urbansky et al., 2001; York, 2001; EPA, 2002b). This competitive inhibition

can disrupt the thyroid cascade and cause the production of insufficient levels of thyroid hormones [triiodothyronine (T_3) and thyroxine (T_4)], which typically leads to the up-regulation of thyroid stimulating hormone (TSH) by the anterior pituitary gland and can result in thyrocyte hypertrophy, hyperplasia, angiogenesis, colloid depletion in thyroid follicles, altered TH levels, and goiter (Siglin et al., 2000; York, 2001; Yu et al. 2002; Patiño, 2003; Bradford et al., 2005; Crane et al., 2005, Mukhi et al., 2005). Chronic hypertrophy can lead to hyperplasia, which increases the likelihood that organisms will develop tumors (Capen, 2001). Thus, chronic exposure to perchlorate and exposure during sensitive developmental periods, are likely to have profound consequences on organismal fitness.

1.3 Processes Thyroid Hormones Control

1.3.1 Teleost Model

The threespine stickleback was selected as the model organism for this study. Fish, in general, make excellent models for the study of endocrine disrupting chemicals. Many pollutants end up in aquatic environments, so fish can provide an early indication of environmental degradation. The molecular components of the hypothalamus-pituitary-thyroid axis correspond closely with those of mammals (Blanton and Specker, 2007), and, since all vertebrates have similar adrenal and sex steroid receptors, fish can act as sentinels for effects on other vertebrates (Mattheissen, 2003). Processes such as smoltification, metamorphosis, and sex determination can be particularly susceptible to endocrine disruption and are easily assessed (Matthiessen, 2003). Their sexual development is labile and responds to the presence of endocrine disrupting chemicals, often without causing mortality. This occasionally leads to the production of intersex individuals (i.e., individuals with both testicular and ovarian tissues).

Thorough reviews of teleost thyroid function often highlight the need for further study to fill gaps in understanding (Leatherland, 1982; Kime, 1998). Yet, there is consensus that teleost thyroid hormones have essential roles in many of the same

processes they do in other vertebrates, including in humans. In teleosts, THs modulate osmoregulation, metabolism, temperature tolerance, somatic growth and development, smoltification, gonadal recrudescence, oogenesis, muscular development, skeletal formation, metamorphosis, formation of the gastric organ (stomach), morphogenesis, skin pigmentation, activity levels, and migratory and reproductive behavior (Hurlburt, 1977; Leatherland, 1982; Smith, 1982; Cyr and Eales, 1996; Koumoundouros et al., 1997; Manzon and Youson, 1997; Bentley, 1998; Castonguay and Cyr, 1998; Janz and Weber, 2000; Power et al., 2001; Brown et al., 2004; Crane et al., 2005; Blanton and Specker, 2007).

Thyroid hormone receptors have been reported in the gonads of a variety of teleost species (Kumar et al., 2002), but the action(s) of T_3 on these target cells require further study (Cyr and Eales, 1996). Recent findings, however, have shown that administration of excess T_4 to zebrafish (*Danio rerio*) during embryonic and larval development produces a male-biased sex ratio (Mukhi et al., 2007). This demonstrates that thyroid hormones play a key role in the sexual development of zebrafish. However, comparisons should be made with caution since a sex chromosome has not been identified for zebrafish, and their sexual development appears to be quite labile and influenced by a number of environmental factors (Mukhi et al., 2007).

1.3.2 Human Model

By contrast, the roles of thyroid hormones have been much more thoroughly characterized in humans. They are known to affect processes such as maintenance of basal metabolic rate; carbohydrate, lipid, and protein catabolism/anabolism; neural development and function; cardiovascular function; muscular development and function; growth and development of the skeleton; gastrointestinal motility; and secretion of gastric juices (Porterfield, 1994, 2000; Choksi et al., 2003; Greenspan, 2004; Marieb, 2004; Jameson et al., 2006). They modulate lactation and the female reproductive cycle and promote hydration and glandular secretion to maintain the integument (Marieb, 2004). Thyroid hormones potentiate the actions of epinephrine, growth hormone, and glucagon

and stimulate fatty acid synthesis while enhancing catecholamine-induced lipolysis (i.e., the so-called futile cycling of fatty acid effect; Norris and Carr, 2006).

1.4 My Experiments

With the knowledge that perchlorate alters TH homeostasis and knowledge of which systems THs mediate, a series of experiments were designed to determine if environmentally relevant levels of perchlorate could have detrimental effects on vertebrate development and fitness. Ecologically relevant endpoints that were known or suspected to be influenced by thyroid hormones were of primary interest. When the role of THs in stickleback, or fishes in general, was ambiguous, the role of THs in humans was generalized to find a testable metric applicable to fish. For example, THs are known to play key roles in carbohydrate and lipid catabolism in humans (Marieb, 2004). Therefore, insufficient TH levels are likely to affect the mobilization of energy in stickleback and would presumably impair prolonged swimming performance among fish in higher treatments. The extent of such assumptions was made with caution since the location of hormone receptors, and consequently hormonal function, can vary among taxa. The importance of thyroid hormones on characteristics such as metabolism, for example, vary within the vertebrate lineage, but their contribution to processes such as growth, neural development, and reproduction remain similar in species as seemingly disparate as teleosts and humans (Bentley, 1998). These aspects reinforce the adage that, "it is not the hormones that have evolved but the uses to which they have been put" (Bentley, 1998).

1.4.1 The Stickleback As a Model

Threespine stickleback were selected as the model teleost fish because they offer a number of desirable and sometimes unique characteristics that make them suitable for toxicological studies. They are, for example, one of the few fish whose entire genome has been sequenced and that have genetic sex determination (Griffiths et al., 2000,

Peichel et al., 2004). This averts the need to make assumptions about whether intersex individuals are masculanized females or feminized males (Hahlbeck et al., 2004). Stickleback have unique androgenic, anti-androgenic, and estrogenic protein biomarkers for which sensitive bioassays have been developed (Katsiadaki et al., 2002). These assays provide powerful tools for the determination of potential disruption to sexual development. Wild threespine stickleback are locally available but have a circumpolar distribution, inhabiting both fresh and saltwater habitats. This allows them to serve as *in situ* sentinels of environmental degradation in a variety of conditions and locations. They are extremely robust and can easily be maintained under a variety of conditions, yet their development is sensitive to environmental perturbations. They have well-described and highly ritualized reproductive behaviors that are also subject to environmental alteration. Stickleback are oviparous, which allows detailed monitoring of the timing and success of embryonic development. They have a relatively short reproductive cycle and can be raised in large numbers in a small area (Katsiadaki et al., 2002). This unique suite of characteristics in a single species provides a means for testing an array of endpoints with implications for a variety of vertebrates.

1.4.2 Endpoints / Hypotheses

Analysis of a stickleback's life cycle reveals several critical events that must occur before an individual can reproduce. Being oviparous, it must first emerge from its egg. Like most teleosts, stickleback undergo metamorphosis between their larval and juvenile stages, during which a variety of morphological changes occur (Blanton and Specker, 2007). Having descended from anadromous stock, swimming ability represents a vital component that determines whether stickleback will be able to capture prey, escape predation, and return upstream during their spawning migration. Appropriate courtship and parental care behavior are essential for making nests, attracting mates, spawning, caring for developing embryos, and guarding progeny. Proper gonadal development and gamete viability ensure that fertilization events and fecundity rates allow a population to sustain itself.

These key events were subdivided to produce 15 potentially TH-modulated endpoints with the hypothesis that perchlorate-treated fish were likely to display impairments that were not evident (or less pronounced) among control fish and those exposed to lower perchlorate treatments. These 15 endpoints included: hatchability, growth rates, development/morphology, survivorship, field metabolic rate, swimming performance, gonadal development, nuptial color expression, reproductive behavior, fertility, fecundity, nest-building ability and other reproductive benchmarks, parental behavior, and evidence of transgenerational effects among the unexposed offspring of treated parents. Moreover, toxicokinetic analysis was conducted to determine the amount of perchlorate deposition in whole-body stickleback homogenates.

1.4.3 Generalized Annual Experimental Designs

My research, which spanned five years and involved five generations of threespine stickleback, required a number of separate experiments to assess overall fitness and development. Testing began in 2002 when I collected two-year old, mature, anadromous stickleback ($G_{1,2002}$) from Rabbit Slough, AK, USA (61°32'12"N, 149°15'17"W). They were randomly separated and exposed to perchlorate in one of four nominal experimental groups (Control, and 1.5, 12.0, and Variable$_{(0\text{-}66)}$ mg/L) before being analyzed to test the hypotheses that perchlorate exposure would disrupt reproductive behavior, survivorship, and nuptial coloration. The offspring ($G_{2,2002}$) produced by the $G_{1,2002}$ were raised to 27 weeks of age and used to assess survivorship and developmental morphology resulting from early and extended perchlorate exposure.

A second cohort of adult, anadromous stickleback ($G_{1,2003}$) was captured from the same source population (Rabbit Slough) in 2003 and exposed to perchlorate in one of six nominal experimental groups (Control, and 3.6, Variable$_{(0\text{-}4.5)}$, 30, Variable$_{(0\text{-}60)}$, or 100 mg/L). They were analyzed for evidence of altered metabolic rates, and they produced offspring ($G_{2,2003}$) that were the brood stock and focus of future testing. At 15 weeks of age, a subset of the $G_{2,2003}$ were euthanized for morphological analysis. The remaining $G_{2,2003}$ were raised to sexual maturity at one year of age in control or treated water to test

the hypotheses that perchlorate exposure would alter growth rates, developmental morphology, survivorship, swimming performance, reproductive behavior, color cycling, gonadal development, gamete viability, spawning success, fecundity, and parental care behavior. The offspring ($G_{3,2004}$) produced by the $G_{2,2003}$ were analyzed for hatching success and raised to 25 weeks of age in tap water without detectable levels of perchlorate (<1.1 μg/L), whereupon they were tested and analyzed for evidence that perchlorate induced transgenerational effects to their survivorship or morphology.

The effects noted among the stickleback in this study would be expected to occur among economically significant teleosts, such as salmonids, following accidental or unavoidable exposure to perchlorate. I assume that threespine stickleback respond to perchlorate in a "typical" manner, with perchlorate disrupting thyroid hormone homeostasis as it does in numerous other taxa, including a variety of fishes (Patiño, 2003; Crane et al. 2005), amphibians (Goleman et al., 2002; Miranda et al. 1996; Tietge et al., 2005), birds (McNabb, 2004), and mammals (Siglin et al., 2000; Yu et al. 2002; Choksi, 2003; Baldridge, 2004). If the mode by which perchlorate exerts its effects is indeed through the thyroid gland, then these studies may well inform about potential effects to a wide variety of vertebrates since thyroid hormone structure, their receptors, and often their functions, are both evolutionarily ancient and highly conserved (Bentley, 1998; Norris and Carr, 2006).

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Chapter 2:

Perchlorate Induces Hermaphroditism in Threespine Sticklebacks[1]

2.1 Abstract

Recently, concern regarding perchlorate contamination has arisen in many contexts. Perchlorate has many military, commercial, and domestic applications, and it has been found in milk, drinking and irrigation water, and produce. Perchlorate is harmful at low levels, yet remains unregulated in the United States while the U.S. Environmental Protection Agency attempts to establish acceptable exposure levels. The present study investigated potential reproductive effects of perchlorate on vertebrates using a model fish species, the threespine stickleback (*Gasterosteus aculeatus*). Stickleback were raised from syngamy through sexual maturity in untreated water and in three nominal concentrations of sodium perchlorate-treated water. Perchlorate was found to interfere with the expression of nuptial coloration, courtship behavior, and normal sexual development. Genetic testing revealed that some females were masculinized to the extent that they produced both sperm and eggs, and histological analysis showed that these individuals had intersexual gonads (ovotestes) containing both oocytes and cells undergoing spermatogenesis. *In vitro* fertilizations revealed that those gametes were capable of self- and cross-fertilization. However, crosses using sperm derived from genetic females died during the blastula phase or near the onset of organogenesis. Sperm derived from genetic males produced viable fry when crossed with eggs derived from genetic females in all treatments. To our knowledge, the present study provides the first evidence that perchlorate produces androgenic effects, and is capable of inducing functional hermaphroditism in a nonhermaphroditic vertebrate.

Bernhardt RR, von Hippel FA, Cresko WA. 2006. Perchlorate induces hermaphroditism in threespine sticklebacks. *Environ. Toxicol. Chem.* 25(8):2087-2096.

2.2 Introduction

As of March 2005, perchlorate contamination had been detected in 36 U.S. states (http://www.epa.gov/fedfac/documents/detection_with_dates_03_25_05.xls). Perchlorate is used in many common household and industrial products (http://www.epa.gov/fedfac/documents/perchlorate.htm) and by the U.S. Department of Defense as an oxidizer in solid rocket propellant and artillery. Recent testing has revealed perchlorate contamination ranging from 3.2 to 11.3 μg/L (ppb) in organic and conventional milk from 101 of 104 containers tested in 15 states (http://www.cfsan.fda.gov/~dms/clo4data.html), and from 3,200 to 6,900 ng/g in organic lettuce (http://www.ewg.org/reports_content/rocketlettuce/pdf/wecklabs.pdf). Perchlorate also has been found in tap water (>4 μg/L) and irrigation water (>4 μg/L) derived from the Colorado River (Brechner et al., 2000) and even in human breast milk (0.6 – 92.2 μg/L; mean, 10.5 μg/L; Kirk et al., 2005). Perchlorate is highly soluble in water, can persist unaltered for several decades (Urbansky, 1998), causes deleterious effects at low concentrations (Goleman et al., 2001, 2002; Baldridge et al., 2004), and lacks an enforceable water quality standard while the U.S. Environmental Protection Agency (U.S. EPA) attempts to establish appropriate exposure levels. The most recent (2005) U.S. EPA reference dose is 24.5 μg/L (http://www.epa.gov/iris/subst/1007.htm). Major sources of environmental contamination have come from military storage and disposal practices (Smith et al., 2001), from industrial seepage into the Colorado River (http://www.epa.gov/fedfac/pdf/perch_7th_mnth_rpt.pdf), and to a lesser extent, from Chilean fertilizers (Urbansky et al., 2001).

To determine potential effects of perchlorate on vertebrates, we conducted experiments over a three year period (2002-2004) on a model fish species, the threespine stickleback (*Gasterosteus aculeatus*). The 2002 experiment was designed to understand the effects of perchlorate on morphological development (to be published separately) and provided pilot data to establish the concentrations used in the 2003 and 2004 experiments. This research included a study concerning the effects of perchlorate on

reproduction, during which hermaphroditism was serendipitously discovered. The present study describes the fertility, gamete viability, behavior, and histology of genetically female, intersex fish and the fertility, gamete viability, and nuptial coloration of "overmasculanized," genetically male fish exposed to perchlorate. Perchlorate is a known disrupter of thyroid function, inhibiting the uptake of iodide during the synthesis of triiodothyronine and thyroxine (Smith et al., 2001; Urbansky et al., 2001). Although it has been shown to skew sex ratios with a female bias among developing *Xenopus laevis* (Goleman et al., 2001), its androgenic effects have not been noted previously.

Functionally hermaphroditic (i.e., containing both eggs and sperm capable of fertilization) threespine stickleback have not been described unequivocally in more than 150 years of intense scientific research in Europe, North America, and Asia. In 1959, however, a female stickleback with eyed embryos in her ovaries was photographed and described by a U.S. Fish and Wildlife Service employee at Karluk Lake, Alaska, USA (Greenbank & Nelson, 1959); in the description of their life history, threespine stickleback were described as hermaphroditic. However, testes were misidentified as ovaries, and subsequent histological examination revealed what was described as ovaries (in nongravid fish) to be fatty tissue (Stenger, 1963). Stenger (1963) concluded that the stickleback population in Karluk Lake was not, in fact, hermaphroditic, but since then the original publication occasionally has been cited in the absence of Stenger's follow-up. The eyed embryos were located in the posterior portion of the ovaries, and may have been fertilized by sperm that entered the cloaca with water during a failed spawning attempt (Stenger, 1963).

In 2001, Gercken and Sordyl (2002) described intersex stickleback in northeastern Germany in waters considered to be both heavily and mildly polluted, but no attempts were made to determine the functionality of their gametes. More recently, sex determination in stickleback has been shown to be determined genetically (Avise, 1976; Withler & McPhail, 1985; Griffiths et al., 2000; Peichel et al., 2004). Thus, it is accepted that the threespine stickleback is not a hermaphroditic species.

Endocrine-disrupting chemicals have been used to produce intersex fishes in the laboratory (Katsiadaki et al., 2002; Hahlbeck et al., 2004) and by aquaculturists (Matty, 1985). However, relatively few studies have described masculanized females (Katsiadaki et al., 2002). Nonetheless, Hahlbeck et al. (2004) produced juvenile intersex stickleback (that were genetically determined to be females) by exposing fry at various developmental stages to the synthetic androgen 17α–methyltestosterone. Because Hahlbeck et al. (2004) did not raise these fish to sexual maturity, functional hermaphroditism was not determined. In the present study, we document what is to our knowledge the first identification of perchlorate as a presumptive endocrine disrupter with androgenic properties and the first unequivocal account of functional hermaphroditism among threespine stickleback.

2.3 Materials and Methods

The goal of the present examination was to study potential reproductive effects associated with development in perchlorate-contaminated water. To accomplish this, we exposed wild-caught $G_{0,2003}$ stickleback to different target concentrations of perchlorate (negative controls and 30, 60, and 100 mg/L) during reproduction, raised their offspring ($G_{1,2003}$) in the same concentrations until sexual maturity, and then studied their reproduction as described below.

2.3.1 Animals and Husbandry

Wild-caught, adult, anadromous stickleback were trapped from Rabbit Slough, AK, USA (61°32'12"N, 149°15'17"W) during May and June 2003. They were housed outdoors in 1600 L pools through mid-July under ambient temperature (14-20°C) and photoperiod (18:6-h light:dark, increasing to 20:4-h light:dark before decreasing to 18.5:5.5-h light:dark). All pools were continuously filtered and aerated through biofilters. Each pool contained water with a salinity of approximately 4 g/L, filamentous algae, and sand to be used as nesting material. Adults and juveniles older than 2 months

were fed frozen brine shrimp daily, whereas fry (age, <2 months) were fed a mixture of Golden Pearls 100, *Artemia* food (both from Aquatic Ecosystems, Apopka, FL, USA), and ground brine shrimp daily.

2.3.2 Experimental Groups

Fish from four experimental groups were raised to sexual maturity for reproductive analyses. These experimental groups were distributed among 11 1600 L pools and included the following: negative controls (< the method detection limit of 1.1 μg/L; three replicates), three replicates with 30 mg/L of anhydrous sodium perchlorate (purity, ±99%; EM Science, Cherry Hill, NJ, USA), two replicates with 100 mg/L of sodium perchlorate, and three replicates of a variable treatment. The variable treatment was designed to mimic a 2002 treatment in which perchlorate leached from 3.70g cores of hydroxyl-terminated polybutadiene solid rocket propellant (as might occur in natural waters contaminated by rocket propellant). To that end, sodium perchlorate was added over time until the concentration reached approximately 60 mg/L, after which the concentration was held steady. The only exchange of treated water occurred on October 5, 2003, when the $G_{1,2003}$ generation (see below) were moved indoors and placed into 400 L tanks at the same concentrations in which they had been raised outdoors. With that exception, water was only added to dilute the treatments as perchlorate became more concentrated because of evaporation and to replace water removed during cleaning. These exposure levels were chosen because they approximate (and, in some cases, are much less than) the perchlorate concentrations at a number of contaminated sites (Smith et al., 2001; http://www.epa.gov/fedfac/documents/detection_with_dates_03_25_05.xls).

Baseline perchlorate levels of the tap water used to fill control and treated pools were tested via ion chromatography in tandem with electrospray ionization mass spectrometry (IC-ESI-MS). Following this, temperature and perchlorate concentrations in pools treated with perchlorate were monitored daily using an Acorn 6 perchlorate potentiometer (Oakton, Vernon Hills, IL, USA) with automatic temperature

compensation. The potentiometer was equipped with a perchlorate ion-selective electrode (Cole Parmer, Vernon Hills, IL, USA). To avoid contaminating the negative control water with residual perchlorate, the electrode was thoroughly cleaned before use in the negative controls and was only used on the negative controls for 6 d to take 18 readings. Dissolved oxygen, pH, and salinity were checked every two to three months.

2.3.3 Parental Spawning Protocol

Male nuptial coloration in the Rabbit Slough population is expressed as iridescent blue in the iris and/or reddish throats, mouths, or sides and bluish-gray backs. Nuptially colored males were randomly placed into separate quadrants of the 1600 L pools (four males per pool isolated by netting). Gravid females were randomly introduced into a quadrant and allowed to spawn with the males. Females were immediately removed after successfully spawning or after 20 min if they failed to spawn. Females were introduced in succession until spawning occurred or until 7 d had passed if spawning was unsuccessful. Once a male had spawned, no additional females were provided. After providing 5 d of parental care (12 d post fertilization), adult males were removed from the pools, killed, and stored at -80°C.

2.3.4 G_1 Husbandry and Reproduction

The offspring (G_1 generation) continued to be raised outdoors under ambient temperature (20° declining to 8°C) and photoperiod (20:4-h light:dark, declining to 11:13-h light:dark) through October 5, 2003. At this point (age, 15 weeks), 50 fish from each of the four treatments (controls and 30, 60, and 100 mg/L) were transferred indoors. To assess the effects of perchlorate on fertility and reproductive behavior, these offspring were maintained in 400 L tanks under simulated natural photoperiod at 17 to 19°C until they reached sexual maturity the following spring. Although sexual maturity occurred at one year of age in our laboratory conditions, most stickleback from the source population reproduce at two years of age.

At approximately one year of age, stickleback showing male nuptial coloration (n = 10, 6, 2, and 0, for the controls, 30, 60, and 100 mg/L experimental groups, respectively) were assumed to be males and isolated in individual 38 L aquaria containing perchlorate-free water. Gravid females were segregated according to experimental group and collectively remained in their 400 L holding tank (controls, 30, 60, or 100 mg/L). The remaining fish of unknown sex were isolated in 40 L aquaria without perchlorate (n = 0, 4, 8, and 10 for the controls and the 30, 60, and 100 mg/L treatments, respectively), until a total of 10 aquaria per experimental group each housed a single fish (either a nuptially colored male or a fish of unknown sex; n=40 isolated fish and aquaria). Lower survivorship among fish exposed to higher concentrations of perchlorate (see Results, section 2.4) led to fewer nuptially colored fish from which to choose. Extra fish were isolated and later killed.

Nesting material consisting of dried, filamentous algae and sand was added to each of the 40 aquaria. Biofilters were placed in each aquarium, and the water was continually filtered and aerated. The water in the aquaria was maintained at a salinity of 4 g/L and was not exchanged. Each fish isolated in a 40 L aquarium was scored daily for body and eye color.

From May 29 to June 18, 2004, females from like treatments were placed individually into one of the 40 L aquaria and allowed 10 min to spawn. The female was immediately removed after successfully spawning or at the end of the 10 min period if spawning was unsuccessful. Gravid females continued to be introduced into each aquarium daily until either a successful spawning occurred or three weeks of courtship activity had passed. Once a male had successfully spawned, no additional females were provided. Courtship, spawning, and parental care behaviors were videotaped for analysis.

2.3.5 Gamete Viability

To determine whether males and females from any or all experimental groups were capable of producing viable gametes, we conducted *in vitro* fertilizations. Three males from a given experimental group were dissected; their testes were macerated, mixed homogeneously with water in a Petri dish, and stored on ice for 10 to 50 min until this procedure had been completed for males from each of five experimental groups (controls; 30, 60, and 100 mg/L; and wild-caught Rabbit Slough fish, the source population for the present study). Next, the eggs from three females per experimental group (controls; 30, 60, and 100 mg/L; and wild-caught Rabbit Slough females) were removed, mixed homogeneously, and evenly distributed between five Petri dishes per treatment ($n = 25$ dishes). Two to three droplets of sperm per treatment were added and mixed with the eggs from each treatment so that all combinations of sperm and eggs were achieved in the 25 Petri dishes. The eggs were housed in a low temperature incubator (Fisher Scientific, Hampton, NH, USA) at 20°C and monitored at least daily for evidence of micropyle formation, blastula formation, organogenesis, and hatching.

Three fish from two treatments (one fish from the 30 mg/L treatment and two fish from the 100 mg/L treatment) displayed both a gravid appearance and characteristic male courtship behavior (see *Results*). The two fish from the 100 mg/L treatment were dissected, and evaluation by light microscopy revealed the presence of both eggs and motile sperm. *In vitro* fertilizations were conducted to assess their gamete viability. In each case, testicular material was removed, macerated, and mixed in approximately 10 drops of water. Next, the eggs were removed and separated into two Petri dishes. Eggs and sperm also were removed from a wild-caught male and female from Rabbit Slough in a similar manner. By adding the sperm to the eggs, three crosses were made for each hermaphrodite: between hermaphrodite eggs and wild male sperm (H♀ x RS♂), between hermaphrodite sperm and wild female eggs (H♂ x RS♀), and self-fertilization with hermaphrodite sperm and eggs (H♂ x H♀). Five randomly selected females from each

experimental group (n = 20 total) also were dissected for evidence of structural hermaphroditism.

2.3.6 Genotypic Sex Determination

To establish if the hermaphrodites were masculinized females or feminized males, we examined each fish using a polymerase chain reaction (PCR) analysis to determine their genotypic sex. A total of five control males that exhibited male nuptial coloration and courtship behavior and six control females that became gravid were tested along with the three hermaphrodites. We also tested additional fish from each experimental group (n = 43 total). Pectoral fins were taken from each fish and digested overnight with ProteinaseK (Acros Organics, Somerville, NJ, USA), and DNA was extracted using standard Phenol-Chloroform-Isoamyl techniques. The PCR was performed using the Ga1 Forward and Reverse primers (Ga1F 5′-CTTCTTTCCTCTCACCATACTCA-3′;Ga1R 5′-AGATGACGGGTTGATAAACAG-3′) as reported by Griffiths et al. (2000). The PCR reactions were carried out in 20 µl volumes containing 20 pmol of each primer, 200 µM of each deoxyribonucleotide triphosphate, 100 to 150 ng of target DNA, 0.5 U *Taq* DNA polymerase, 2.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 8.0). The thermocycler conditions were 94° C for 6 min, followed by 37 cycles of 94° C for 40 sec, 44° C for 40 sec, and 72° C for 50 sec, followed by a final extension at 72° C for 10 min. These primers produce fragments of two sizes, approximately 370 and 600 bp, with characteristic male and female XY (both bands) and XX (only the 600 bp band) genotypes, respectively. Genotypes were visualized by electrophoresis on a 2% agarose gel. The female-specific band is larger than the male-specific, and it serves as an internal control for the bias of PCR for amplification of smaller products. In addition, we ran the PCR independently three times on each fish, and the results of the control fish were compared to their known sexual phenotype (ovaries or testes).

2.3.7 Histological Analysis

The gonads of both untreated male and female fish were sectioned and compared to a genotypically female stickleback suspected of hermaphroditism from the highest perchlorate treatment (100 mg/L) to determine if perchlorate affected gonadal structure. The control fish were from the same Rabbit Slough population as the parents of the treated fish. Stickleback were fixed for 36 h in Bouin-Hollande solution (4.0% acetic acid, 4.0% formaldehyde, 4.0% picric acid, 2.5% copper(II) acetate, and 1% distilled water), then rinsed and stored in 70.0% ethanol. The samples were then dehydrated in pure ethanol and embedded in paraffin for sectioning. Transverse sections (5μm thickness) were cut through the trunk portion of each fish, starting approximately at the pectoral fin and moving posteriorly to the anal fin. The sections were stained in haematoxylin and eosin. Slides were examined by light microscopy under a variety of magnifications, and digital images of sections were taken using a SPOT camera (Diagnostic Instruments, Sterling Heights, MI, USA) mounted to the scope. Analysis of the gonads from the control male involved identification of spermatogenic cells in lobules, and analysis of those from the control female involved identification of the follicular envelope and oocytes, with characteristic yolk granules and vacuoles. In the sections of the treated genotypic female, intersexual gonads were identified by the presence of several oocytes as well as cells that appeared to be similar to those undergoing spermatogenesis in the lobules of the control male.

2.3.8 Statistics

The Statistical Package for Social Sciences (Ver 11.5; SPSS, Chicago, IL, USA) was used for all statistical procedures. Differences between groups were analyzed using a Kruskall-Wallis test; statistics were two-tailed.

2.4 Results

The results of the daily perchlorate readings from the Oakton Acorn 6 perchlorate potentiometer are shown in Fig. 2.1. The mean perchlorate concentration of the negative control water based on the 18 Acorn 6 readings was 0.15 mg/L (SE = 0.01). The more precise perchlorate measurements of the negative control water from the IC-ESI-MS (n = 21 samples) revealed the perchlorate concentration to be less than the method detection limit of 1.1 µg/L (Dodds et al., 2004).

Our group demonstrated that perchlorate becomes deposited in whole-body stickleback homogenates in proportion to their levels of exposure (Dodds et al., 2004). Adult, wild-caught, male stickleback were analyzed in the year 2002 after they had been exposed to control or perchlorate-treated water for up to 22 d. Perchlorate was not detected at or above the IC-ESI-MS limits of quantification (1.1µg/L) in any of the 19 control tissue samples tested (Dodds et al., 2004). Fish housed in perchlorate-treated water with a mean concentration of 1.59 mg/L (n = 21 daily potentiometer readings, SE = 0.04 mg/L) had a mean tissue concentration of 0.66 mg/L (IC-ESI-MS; n = 17 fish exposed on average for 17.7 d, SE = 0.06 mg/L). Fish in a mean concentration of 12.15 mg/L (n = 22 daily potentiometer readings, SE = 0.28 mg/L) had a mean tissue concentration of 4.27 mg/L (IC-ESI-MS; n = 15 fish exposed on average for 18.0 d, SE = 0.44 mg/L). Those in a treatment with an increasing perchlorate concentration that began at zero and reached 18.4 mg/L at harvest (potentiometer; $y = 0.69x + 3.96$, $r^2 = 0.90$) had a mean tissue concentration of 5.03 mg/L (IC-ESI-MS; n = 17 fish exposed on average for 19.0 d, SE = 0.40 mg/L).

Perchlorate exposure was found to interfere with the expression of male nuptial coloration among fish raised in perchlorate (G_1 generation; Fig. 2.2). Nuptial coloration was only rarely and weakly expressed among perchlorate-exposed fish: only five of 24, two of 22, and 0 of 13 fish in the 30, 60, and 100 mg/L treatments, respectively, showed nuptial coloration. Therefore, the sex of most treated fish could not reliably be determined before genetic testing, which was conducted post-mortum. Because of the

ambiguity of their sex and impaired survival among treated fish (Fig. 2.3), fewer treated males were available from which to select for behavioral analysis. These factors led to a small number of genetic females being isolated into 40 L aquaria as if they were males (n = 0, 2, 3, and 2 for controls and the 30, 60, and 100 mg/L treatments, respectively).

All control males (n = 10) expressed normal nuptial coloration, displayed normal courtship behavior (e.g., nest building, territoriality, biting introduced females, zigzagging, leading, and fanning), and eight of 10 control males ultimately spawned. Most treated males ignored gravid females and failed to display appropriate courtship behavior, and spawning success among treated fish was much lower than that for controls: four of eight males, two of seven males, and none of eight males successfully spawned in the 30, 60, and 100 mg/L treatments, respectively. All ten control males built nests, but only six of eight, three of seven, and none of eight males in the 30, 60, and 100 mg/L treatments, respectively, built nests.

The combined onset of their ripe appearance and display of male-typical behavior gave the initial indication of potential hermaphroditism among three of the treated fish (one fish in the 30 mg/L and two fish in the 100 mg/L). Although all three failed to make nests, each became territorial and performed male courtship displays, such as biting, zigzagging, and assuming aggressive, head-down postures when females were introduced into their aquaria. They also attempted to lead females, just as courting males do. Introduced females responded to these "male" courtship displays by assuming the head-up posture typical of receptive females and by following the hermaphrodite as it attempted to lead.

Genotypic testing of fish from all experimental groups revealed that fish with discrete testes were genotypically male, and fish that developed discrete ovaries were genotypically female. The genotypic sex also matched the phenotypic sex in each of the 11 fish tested from the control group. The three known hermaphrodites were found to be genetic females. Dissections provided further evidence of their masculinization. Hermaphrodites lacked discrete testes but had a thin layer of macroscopically amorphous

testicular tissue with sparse melanophore deposition (Fig. 2.4), but their eggs did not become fertilized *in vivo*.

Histological sections of a genotypically female hermaphrodite in the 100 mg/L treatment provided evidence for intersexual gonads. Comparisons between sections of the control male and female and the treated genotypic female showed the treated female contained gonads that were a chimera of ovarian and testicular tissues (ovotestes; Fig. 2.5). In particular, the gonads of the treated female clearly contained oocytes, as evidenced by yolk granules and vacuoles, follicular envelopes, and ovarian cavities around the oocytes. We would not have expected to find mature eggs because they had been stripped from the hermaphroditic females for *in vitro* fertilizations before the fish were sectioned for histology. Structures similar to individual testis lobules also were present. Under higher magnification, these structures had morphological and staining patterns consistent with the presence of spermatogenic cells. This is particularly evident when comparing the higher-magnification sections from the control male to those from the treated female (Fig. 2.5).

The putative hermaphrodite in the 30 mg/L treatment was the first to become gravid, and *in vitro* fertilizations were not performed on this fish. When the testicular material from the two hermaphrodites in the 100 mg/L treatment was removed and macerated in a Petri dish with water, motile sperm became evident. The two freshly killed hermaphrodites in the 100 mg/L treatment had testicular tissue with motile sperm. However, dissections of other fish that had been stored at -80° C (including the putative hermaphrodite in the 30 mg/L treatment) for months after death failed to clearly reveal the number of females that had become structurally hermaphroditic. These fish had been thawed and refrozen repeatedly for analysis during their storage, which led to poor preservation of their gonadal tissue and a lack of shimmering, motile sperm. Therefore dissections could not be used as a reliable, stand-alone indicator of hermaphroditism.

The *in vitro* fertilizations of the two fish in the 100 mg/L treatment provided the final evidence of their functional hermaphroditism. In all three crosses from both

hermaphrodites in the 100 mg/L treatment, identification of the micropyle (Fig. 2.6) and separation of the chorion from the vitelline membrane (Fig. 2.7) revealed that motile sperm had fertilized the eggs.

Development continued in all three crosses for both hermaphrodites to at least the blastula phase, beyond which organogenesis concluded only for crosses between hermaphrodite eggs and wild male sperm. Thus, each cross using a hermaphrodite's sperm resulted in embryonic death during the blastula phase or near the onset of organogenesis. Embryonic development continued to progress in the crosses between hermaphrodite eggs and wild male sperm, and fry hatched in both cases.

In vitro fertilizations using gametes from randomly chosen fish not suspected of being hermaphroditic revealed that fish of both sexes from all experimental groups were capable of producing viable gametes. Twenty-four of these 25 *in vitro* fertilizations resulted in fry successfully hatching from their eggs. The only exception involved a cross between a control female and a male in the 30 mg/L treatment. During the initial series of crosses, none of the eggs from control females became fertilized because they recently had spawned naturally with isolated control males and only contained immature oocytes. When they reclutched two weeks later, a second series of *in vitro* fertilizations were performed using mature eggs from the control females and sperm from control males, males in the 60 and 100 mg/L treatments, and Rabbit Slough males. No males in the 30 mg/L treatment remained alive from which to acquire sperm, but all other combinations produced fry. It therefore was determined that males and females from each experimental group were capable of producing viable gametes and that sperm from each experimental group could fertilize eggs from any group. As a percentage of eggs that were fertilized using *in vitro* techniques, differences in hatching success were statistically significant (Kruskal Wallis test, $\chi^2 = 13.462$, df = 3, p = .004), but perhaps not biologically significant. Control fish, wild fish, and those exposed to 100 mg/L perchlorate had similar hatching success while stickleback exposed to 30 and 60 mg/L perchlorate had significantly lower hatching success. These findings are based on eggs

fertilized *in vitro* and discount the effects that parental behaviour and survival following fertilization may have.

Perchlorate exposure also produced an overmasculinizing effect among fish that were genetically determined to be males (Fig. 2.8). A dose-dependent relationship became evident as fish exposed to higher perchlorate concentrations grew larger testes than controls or those exposed to lower perchlorate concentrations (Kruskall-Wallis test, $\chi^2 = 16.249$, $df = 3$, $p=0.001$). Melanophore deposition on the testes was much sparser on males exposed to higher concentrations of perchlorate (Fig. 2.9).

2.5 Discussion

All developing vertebrates pass through a stage with undifferentiated tissue capable of producing either ovaries or testes, but teleosts fish have no spatial distinction between these types of tissues. With the correct dose of estrogens or androgens at the correct time and for the correct duration, these intermingled, undifferentiated gonadal primordia can be induced to form either distinct ovaries or testes, despite the genotype of the fish (Matty, 1985; Hahlbeck et al., 2004). Endocrine-disrupting chemicals can lead to atypical gonadal formations (Patiño, 1997; Gerken & Sordyl, 2002; Hahlbeck et al., 2004). For stickleback, exogenous hormones have the strongest influence on gonadal development during their first 14 d post-hatch. Gonads are less responsive to exogenous hormonal treatment beyond 14 d, but a lower occurrence of intersexuality can be induced with estrogenic, but perhaps not androgenic, hormones administered between 14 and 60 d post-hatch (Matty, 1985; Hahlbeck et al., 2004). Therefore, briefly exposing adult stickleback to perchlorate would not be expected to produce the same type of hermaphroditic changes as observed in our research.

Those who have studied the reproductive effects of perchlorate on fishes (Patiño, 1997; Park et al., 2005) generally have exposed adults to perchlorate and then analyzed the mass or volume of eggs produced and/or the gonadosomatic index (GSI) of females. Patiño et al. (2003) found no effects on the spawned egg volume at 18 mg/L, but spawned

egg volume became negligible after four weeks of exposure to 677 mg/L. Conversely, Park et al. (2005) found that perchlorate had a stimulatory effect on GSI, egg/embryo mass, and fecundity in some of the females exposed to 1, 10, and 100 mg/L. To our knowledge, the present study is the first to analyze the gonadal histopathology of fish that were exposed to perchlorate and the effects of perchlorate exposure during early development on gonads. Our findings suggest that gonadal histopathology as well as male GSI may be more sensitive biomarkers of effect for perchlorate exposure than previous reproductive endpoints focused on female fecundity and female GSI.

In the present study, chronic exposure to sodium perchlorate at nominal concentrations of 30, 60, and 100 mg/L from syngamy through sexual maturity induced functional hermaphroditism in a vertebrate with genetically controlled sex determination (Peichel et al., 2004). Perchlorate produced functional hermaphrodites by masculanizing genetically female threespine stickleback. Histological examination revealed hermaphrodite gonads to be a chimera of ovarian and testicular material. Treated male stickleback exhibited marked testicular hypertrophy in a dose-dependent manner, with fish exposed to higher perchlorate concentrations developing larges testes. Thus, these findings give the initial indication that perchlorate produces androgenic effects (either directly or indirectly) and that perchlorate is capable of inducing functional hermaphroditism in a nonhermaphroditic vertebrate. Those findings raise the possibility that the androgenic effects of perchlorate could occur in other species of vertebrates as well.

Spawning success also was reduced in a dose-dependent manner. The differences in spawning success between treated and control fish appeared to be related, in part, to the lack of proper behavior, poor expression of nuptial coloration (Fig. 2.2) among treated males, and apparent inability of hermaphroditic stickleback to build nests. These reproductive anomalies would pose a formidable barrier to recruitment in wild fish exposed to perchlorate.

Gamete viability appeared to have been uncompromised among sperm-producing males or egg-producing females for all treatments. However, all four crosses using sperm derived from genetic females died either during the blastula stage or near the onset of organogenesis, whereas crosses using the hermaphrodites' eggs and sperm derived from genetic males produced viable fry. Because cell division beyond the blastula stage is regulated by the developing embryo, perchlorate may have produced lethal mutations in the hermaphrodites' sperm that led to early embryonic death. Alternatively, the sperm, although capable of fertilizing the eggs, may have incompletely contributed DNA to the egg, thus making an aneuploid or even haploid embryo, as has been produced using protocols developed for zebrafish (*Danio rerio*; Streisinger et al., 1981). Stickleback eggs also can be induced to develop haploid embryos when fertilized with sperm that has been irradiated to cross-link male DNA (W. Cresko, University of Oregon, Eugene, OR, USA, unpublished data).

Overall, survivorship was poor among treated fish (Fig. 2.3), suggesting that many fish are incapable of tolerating the stresses induced by chronic perchlorate exposure. Two perchlorate median lethal concentration (LC50) estimates have been made for zebrafish embryos/larvae. Patiño et al. (2003) cited unpublished data corresponding to a 5 d LC50 for ammonium perchlorate of 529 mg/L. Liu et al. (2005) determined the 96-h LC50 for sodium perchlorate to be 1401.2 mg/L and suggested that the higher lethality reported by Patiño et al. (2003) may have been the result of ammonium toxicity. Park et al. (2005) determined the 5 d LC50 for sodium perchlorate to be 404 mg/L using mosquitofish (*Gambusia holbrooki*) larvae. Additional 96 h LC50 estimates have been calculated for rainbow trout (*Onchorynchus mykiss*), fathead minnow (*Pimephales promelas*), and bluegill sunfish (*Lepomis macrochirus*), corresponding to 2,100, 1,655, and 1,470 mg/L, respectively (EA Engineering, Science & Technology, 1998; Dean et al., 2004; www.epa.gov/ncea/perchlorate/references2/documents/44908.pdf).

If a dose-response relationship exists between exposure concentrations and the range and severity of deleterious effects, as the present study indicates, then only a small fraction of those exposed to low doses would display pathological conditions (i.e., those that are more vulnerable; Klaasen, 2001). Additionally, limited sample sizes may mask potential outcomes at the margins of normalcy when using lower doses. Therefore, doses up to 100 mg/L were chosen to explore the range of deleterious effects that may occur among more vulnerable individuals at lower contaminant levels and among less vulnerable individuals at higher levels. The experimental levels of perchlorate used in the present study are far above the U.S. EPA 2005 reference dose of 24.5 μg/L. However, our concentrations are not only environmentally relevant but also below levels that have been detected in the groundwater of seven U.S. states (AL, AR, CA, MD, MO, NV, and TX; http://www.epa.gov/fedfac/documents/detection_with_dates_03_25_05.xls).

The present study demonstrates that the negative effects of perchlorate are not limited to thyroid function, and its androgenic effects may affect other vertebrates as well. We would not expect hermaphroditism to be induced in any mammal, but other disruptions of reproductive function are possible. The primary sex determination gene at the head of the pathway seems to vary across species, but many of the downstream genes and pathways in sex determination, such as *Sox9* and Doublesex- and MAB3-Related Transcription Factor (DMRT) genes, seem to be conserved across species, including stickleback (Cresko et al., 2003). Thus, if the androgenic mode of action for perchlorate in stickleback is downstream in the pathway, mechanistic studies in this species may well inform us about possible effects in humans and other mammals (Marshall-Graves & Shetty, 2001).

The threespine stickleback is uniquely suited for research concerning disruption of sex steroids, because it has both male- and female-specific biomarkers that can be easily and rapidly measured by enzyme-linked immunosorbant assays (Katsiadaki et al., 2002; Nilsen et al., 2004). Reproductive males produce a glue protein called spiggin

from the kidneys, which is used to glue together nesting material, and the reproductive female produces the egg-yolk protein vitellogenin. Exposure to androgenic compounds can cause the female kidney to produce spiggin, whereas estrogenic compounds can cause the male to produce vitellogenin (Katsiadaki et al., 2002).

The mechanisms of action that induced hermaphroditism in the present study remain unclear. However, hermaphroditism among treated females and oversized testes among treated males suggest that perchlorate has an androgenic endocrine-disrupting role in addition to its known thyroid-disrupting properties. With the knowledge that perchlorate is capable of inducing hermaphroditism, a sensitive assay, such as that described by Katsiadaki et al. (2002), could be combined with structural studies of reproductive tissues and genetic testing to explore perchlorate's mode of action. Studies should examine whether the masculinization response is mediated through direct activation of androgen receptors or by indirect means, such as alteration of steroid metabolism. In vitro examination of female or immature male stickleback kidney cell cultures after exposure to perchlorate may reveal whether perchlorate is capable of directly activating androgen receptors and, hence, inducing transformation of the stickleback kidney from an osmoregulatory organ into a spiggin-producing organ for nest construction (see, e.g., De Ruiter & Wendelaar-Bonga, 1985). The impaired ability of treated males to build nests may have been caused, in part, by a lack of spiggin production, which warrants further investigation. A replication of the present study should include histological analysis of the overmasculanized testes to determine if spermatogenesis and testicular morphology (e.g., sperm ducts or unusual cavities as described in Hahlbeck et al. 2004) have been affected. Sperm analysis could reveal whether quantity, viability, and motility have been affected. Because the viability of any species ultimately depends on its ability to reproduce, the significance of perchlorate contamination should not be downplayed.

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2.8 Figures

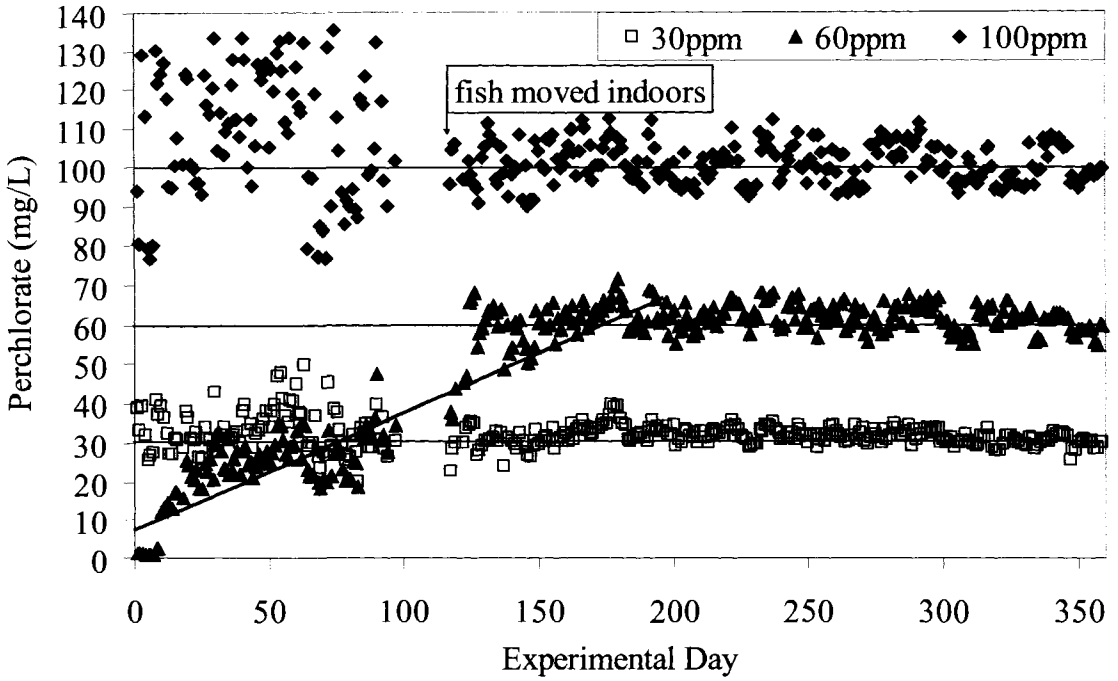
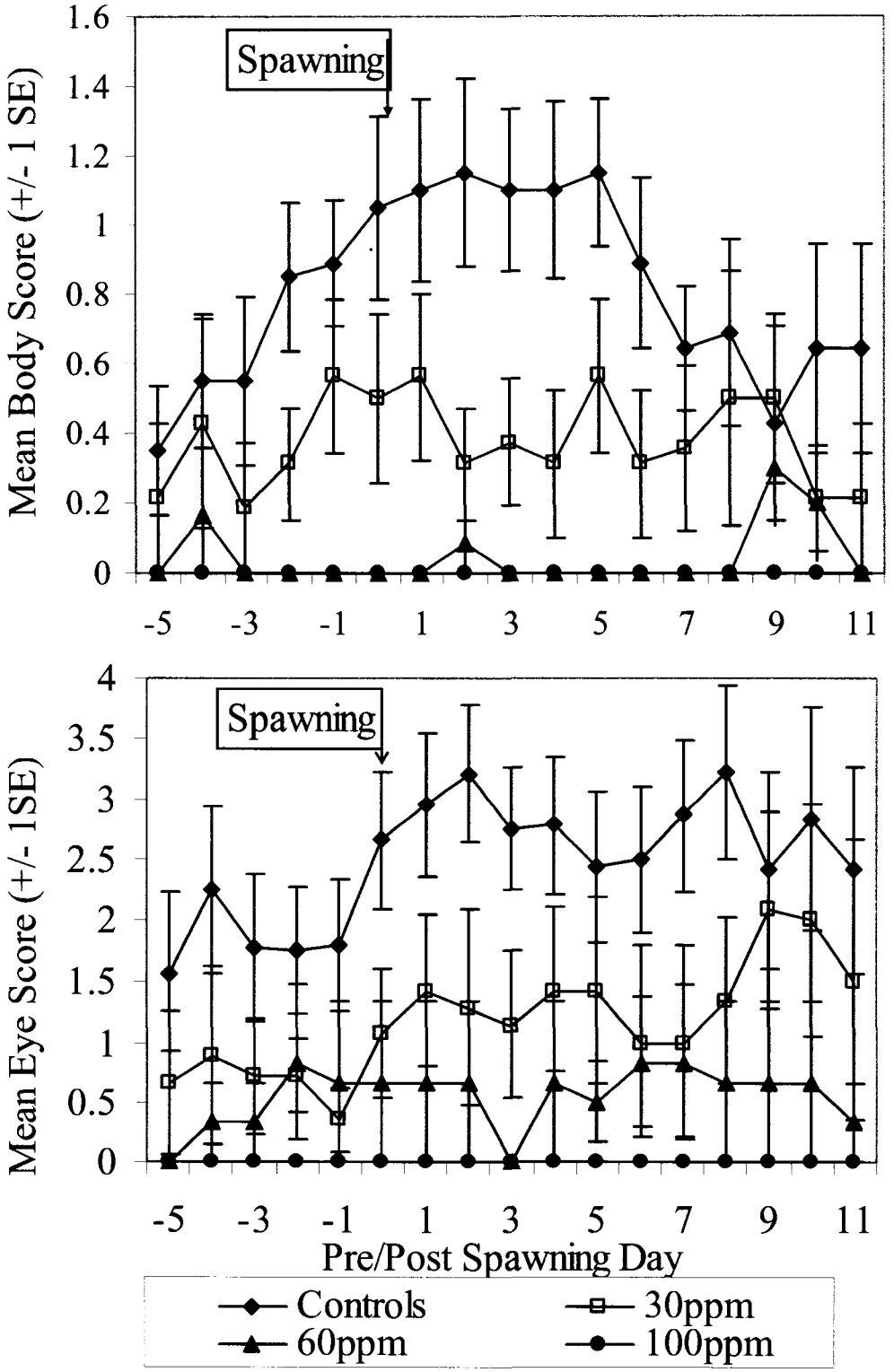


Fig. 2.1 Daily Perchlorate Readings From the Acorn 6 Potentiometer. Readings were more variable when the fish were housed outdoors, where temperatures fluctuated more than indoors. At day 100 the potentiometer broke, and was replaced three weeks later with the same model. This period is indicated by a gap in readings. The entire dataset revealed that the nominal 30 mg/L treatment had a mean perchlorate concentration of 32.00 mg/L (n=334, SE=0.20), and the 100 mg/L treatment had a mean perchlorate concentration of 102.92 mg/L (n=334, SE=0.57). The nominal 60 mg/L treatment mimicked the increasing perchlorate concentration noted in the year 2002, as perchlorate leached from solid rocket propellent cores ($y_{2002}=0.32x + 12.04$, $r^2=0.92$ vs $y_{2003}=0.31x + 9.44$, $r^2=0.88$). The 2003 nominal 60 mg/L treatment reached a maximum concentration near 60 mg/L about halfway through the experiment, after which the readings remained relatively stable (mean, 61.80 mg/L; n=185; SE=0.19). Values for the target dose of 0 (negative controls) were less than the method detection limit of the ion chromatograph in tandem with electrospray ionization mass spectroscopy (1.1 µg/L) (Matty, 1985).

Fig. 2.2 Body and Eye Color Cycling of Nuptial Males. Mean color scores show that few treated males expressed nuptial coloration, and those that did lacked the color intensity shown by control fish. The 100 mg/L males failed to develop any nuptial coloration. Body scoring criteria were as follows: 0 = No nuptial coloration; 1 = Light orange coloration in/around the mouth or throat (ventrally); 2 = Orange remains on the mouth and is present on the operculum, but not posterior to it; 3 = Light orange coloration along body posterior to the operculum (easily seen). Fish have light or patchy bluish-grey coloration on their back. Eye scoring criteria were as follows: 0 = No color; 1 = Light blue on dorsal side only; 2 = Light blue on dorsal and ventral sides only; 3 = Dark blue on dorsal and ventral sides, with light blue on anterior and posterior; 4 = Eyes completely the same shade of blue; 5 = Iris of eyes are completely blue with an iridescent or glowing appearance.


Spawning
Mean Body Score (+/- 1 SE)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-5 -3 -1 1 3 5 7 9 11
Spawning
Mean Eye Score (+/- 1SE)
4
3.5
3
2.5
2
1.5
1
0.5
0
-5 -3 -1 1 3 5 7 9 11
Pre/Post Spawning Day
Controls
30ppm
60ppm
100ppm

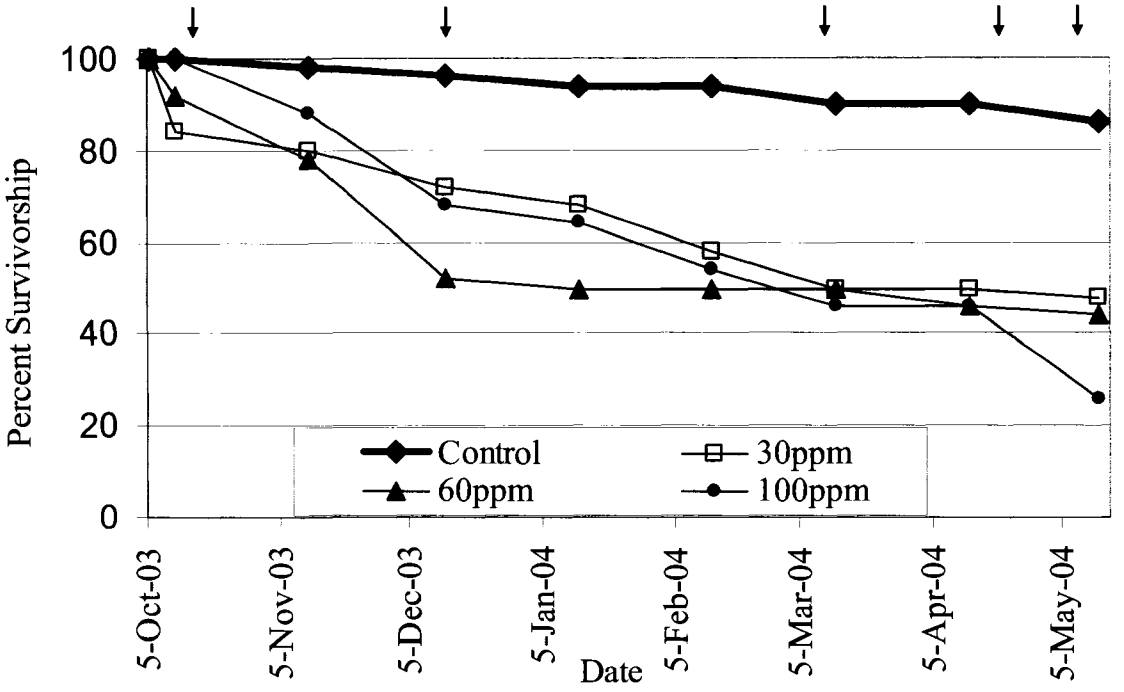


Fig. 2.3 Survivorship Curves By Experimental Group. Arrows represent handling/stressful events, such as periodic measuring or relocation.

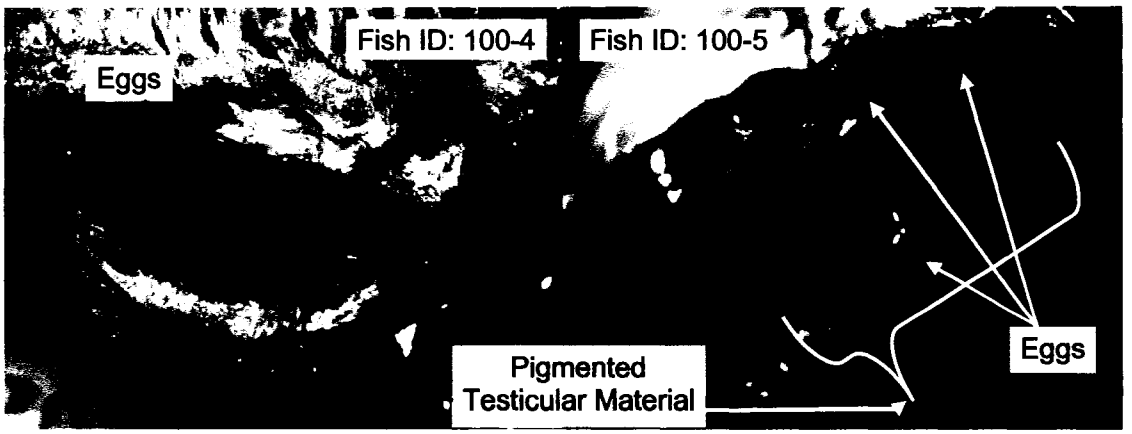


Fig. 2.4 Pigmented, Sperm-Producing Testicular Material. This material was located distally from ripe ovaries. Discrete testes were not produced.

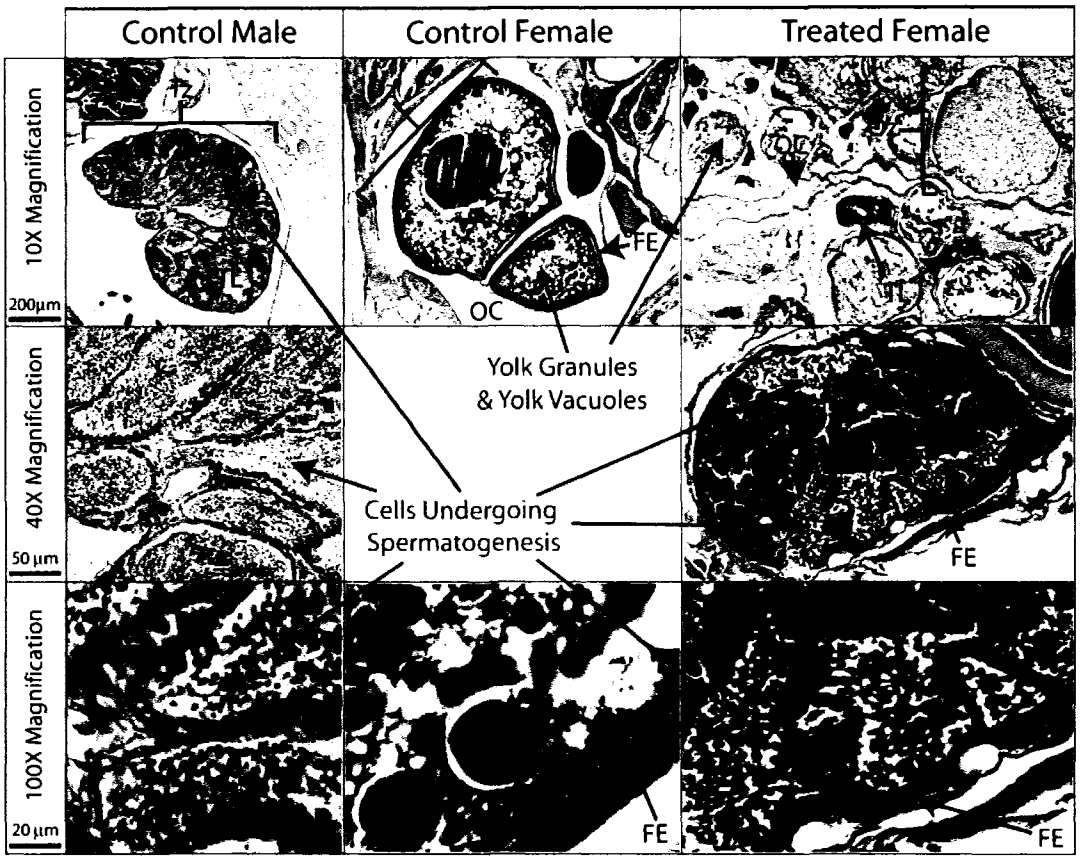


Fig. 2.5 Gonadal Histology. This figure shows haematoxylin and eosin-stained sections through untreated male and female fish with a treated, hermaphroditic female shown at right. The left three panels show male histological features, including the entire testis (Tz), testis lobules (TL), and sperm-producing cells, stained dark blue. The center panel shows a section of a developing oocyte (Oz) in a control female, including dark red-staining yolk granules and clear yolk vacuoles. The right panels show intersexual gonads of a genotypically female fish, which appears to contain a mixture of both types of tissues, including oocytes, ovarian cavity (OC), and what appear to be sperm lobules. The higher-magnification image in the bottom right corner exhibits darkly staining sperm-producing cells, similar to what is seen in the control male testis (lower left). The histological work indicates that the intersexual gonad is a chimaera of ovarial and testicular tissue.

Fig. 2.6 Penetration of Eggs. Formation of the micropyle (arrows) provided the first evidence that fertilization occurred for all three crosses with both hermaphrodites tested. H♂ = hermaphrodite sperm; H♀ = hermaphrodite eggs; RS♂ = sperm from wild Rabbit Slough (Alaska, USA) male; RS♀ = eggs from wild Rabbit Slough female.

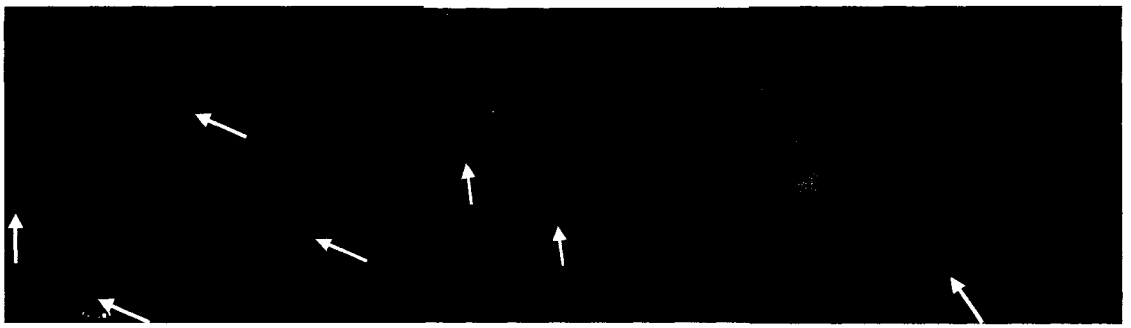


Fig. 2.7 Chorion Separation and Cleavage. After fertilization, separation of the chorion from the vitelline membrane (white arrows) and initial cleavage (black arrows) occurred in all three crosses for each hermaphrodite. H♂ = hermaphrodite sperm; H♀ = hermaphrodite eggs; RS♂ = sperm from wild Rabbit Slough (Alaska, USA) male; RS♀ = eggs from wild Rabbit Slough female.

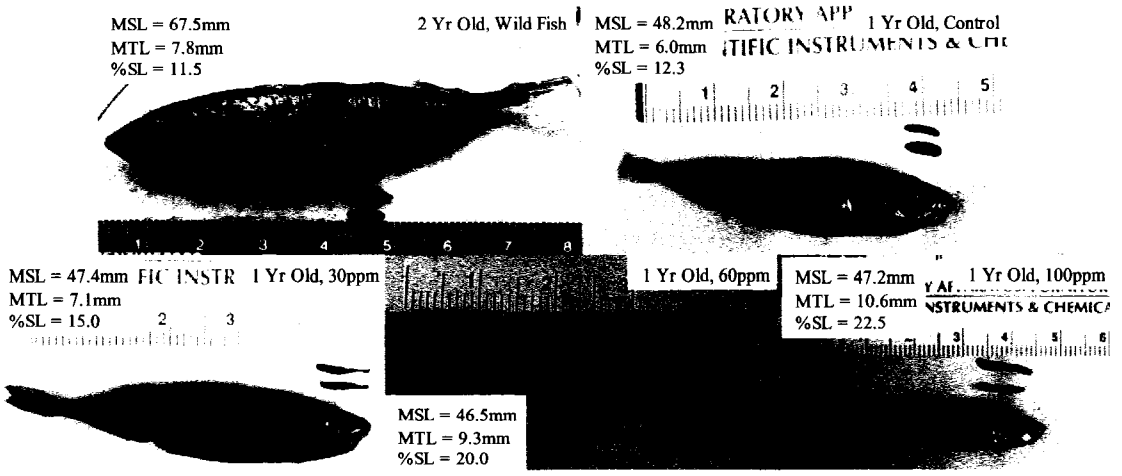


Fig. 2.8 Testicular Length By Experimental Group. As the perchlorate concentration increased, so did the length of testes. Mean testicular length (MTL) for two-year-old, wild-caught males was 7.8 mm (n=8; SE=0.24; top left). One-year-old control fish had an MTL of 6.0 mm (n=7, SE=0.54; top right). One-year-old fish in the 30 mg/L treatment had an MTL of 7.1 mm (n=7, SE=0.35; bottom left). One-year-old fish in the 60 mg/L treatment had an MTL of 9.3 mm (n=7, SE=0.21; bottom center), and one-year-old fish in the 100 mg/L treatment had an MTL of 10.6 mm (n=6, SE=0.22; bottom right). MSL = Mean Standard Length. %SL = (MTL / MSL).

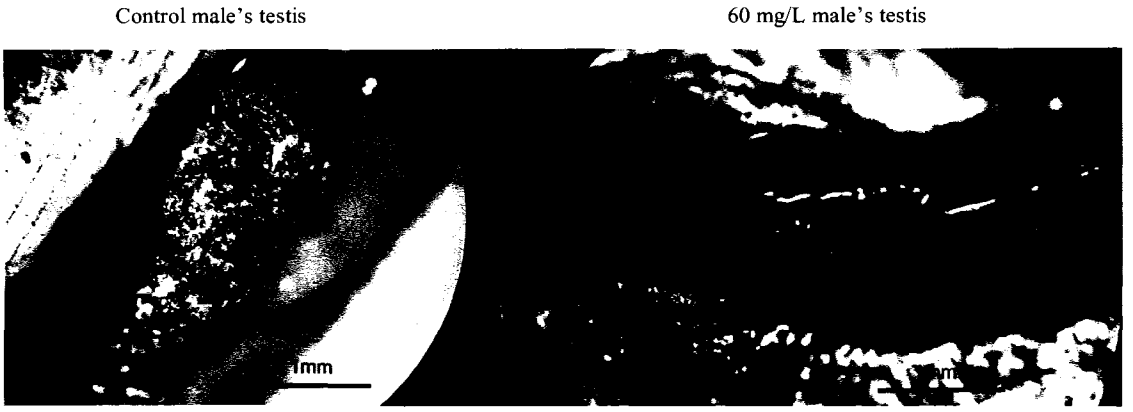


Fig. 2.9 Testicular Melanophore Density. As the perchlorate concentration increased, the density of testicular melanophores decreased.

Chapter 3:

Chronic Perchlorate Exposure Impairs Stickleback Swimming Performance and Reproductive Behaviour[1]

3.1 Summary

We describe behavioural changes in two generations of threespine stickleback (*Gasterosteus aculeatus*) exposed to environmentally relevant concentrations of perchlorate. The first generation ($G_{0,2002}$) was exposed as two-year-old adults to perchlorate in experimental groups ranging in concentration from less than the method detection limits (<1.1 ppb) to 18.6 ppm for up to 22 d during their courtship, spawning, egg guarding, and first five days of fry guarding. No differences were noted in the behavior or reproductive output of these fish that were exposed as adults. However, perchlorate exposure from fertilization through sexual maturity caused widespread effects in the second generation ($G_{1,2003}$), which was spawned and raised through sexual maturity in one of four nominal experimental groups (0, 30, and 100 ppm, and a "variable" treatment that progressively increased from <1.1 ppb to approximately 60 ppm perchlorate). Dose-dependent effects were found during the $G_{1,2003}$'s swimming and behavioural evaluations, including higher mortality rates among treated fish following stressful events. Perchlorate-exposed fish had higher failure rates during swimming trials and failed at lower flow rates than control fish. A number of treated fish exhibited erratic and uncontrolled swimming behaviour. In a dose-dependent manner, progressively fewer males completed benchmark metrics, such as nest building, spawning, nursery formation, or fry production. Fewer males from higher treatments courted females, and those that

[1]Bernhardt RR and von Hippel FA. 2008. Chronic perchlorate exposure impairs stickleback reproductive behaviour and swimming performance. *Behaviour.* 145:527-559.

did initiated courtship later and had a reduced behavioral repertoire compared to fish from lower treatments. The lowest observed adverse effect level (LOAEL) for swimming performance, reproductive behaviour, survivorship, and recruitment was 30 ppm perchlorate (our lowest $G_{1,2003}$ treatment), and near complete inhibition of reproductive activity was noted among males raised in 100 ppm perchlorate. A small number of treated $G_{1,2003}$ females were isolated in aquaria, and some performed reproductive behaviour typical of males, such as biting, leading, and zig-zagging in the presence of gravid females. These findings have profound implications for recruitment in wild fish populations exposed to perchlorate, and suggest that perchlorate may disrupt behaviour in other vertebrates as well.

3.2 Introduction

Perchlorate (ClO_4^-) is found in many common consumer products, such as road flares, fireworks and automobile air bags (http://www.epa.gov/fedfac/documents/perchlorate.htm) and is used as an oxidizer in solid rocket propellant and artillery (Mendiratta et al., 1996).

Perchlorate contamination is widespread across the United States. For example, as of March 2005 perchlorate contamination had been detected in 36 U.S. states (http://www.epa.gov/fedfac/documents/detection_with_dates_03_25_05.xls). In 2005 perchlorate was reported in the Canadian Great Lakes, marking the first time that perchlorate contamination was identified outside the United States (Backus et al., 2005).

Recent testing has revealed perchlorate contamination ranging from 3.2 to 11.3 ppb (µg/l) in organic and conventional milk from 101 of 104 containers tested in 15 U.S. states (http://www.cfsan.fda.gov/~dms/clo4data.html), and in organic lettuce with concentrations ranging from 3,200 to 6,900 ng/g (http://www.ewg.org/reports_content/rocketlettuce/pdf/wecklabs.pdf). Perchlorate has also been found in tap (>4 ppb) and irrigation (>4 ppb) water derived from the Colorado

River (Brechner et al., 2000), and even in human breast milk ranging from 0.6 to 92.2 ppb (mean = 10.5 ppb; Kirk et al., 2005).

Perchlorate is highly soluble in water, can persist unaltered for several decades (Urbansky, 1998), causes deleterious effects at low concentrations (Goleman et al., 2001, 2002; Baldridge et al., 2004), and lacks an enforceable water quality standard in the U.S. while the U.S. Environmental Protection Agency (U.S. EPA) attempts to establish appropriate exposure concentrations. The U.S. EPA's most recent (2005) reference dose is 24.5 ppb (http://www.epa.gov/iris/subst/1007.htm). Major sources of environmental contamination in the U.S. have come from military storage and disposal practices (Smith et al., 2001), from industrial contamination of the Colorado River (http://www.epa.gov/fedfac/pdf/perch_7th_mnth_rpt.pdf), and to a lesser extent from Chilean fertilizers (Urbansky, 2001).

The effects of perchlorate on thyroid histology and thyroid hormone synthesis are well established (Miranda et al., 1996; York et al., 2001a, b, 2003; Patiño et al., 2003; McNabb et al., 2004; Bradford et al., 2005; Crane et al., 2005; Park et al., 2006). Perchlorate is an endocrine disrupting toxicant known to interfere with thyroid hormone production by competitively inhibiting the ability of sodium-iodide symporters to transport iodide into thyroid follicles, which in turn reduces thyroid hormone synthesis (NRC, 2005). Many studies describe histological effects of perchlorate exposure, such as hypertrophied thyroid follicular cells (York et al., 2001a, b; Patiño et al., 2003; McNabb et al., 2004; Bradford et al., 2005; Park et al., 2006), but few have addressed the effects of perchlorate on ecologically relevant life history characteristics, such as reproductive behaviour (York et al., 2001b, 2003; Crane et al., 2005). What few studies exist primarily document the number of mounting attempts during mice reproduction (York et al., 2001a) or fertility/fecundity parameters (York et al., 2001b; Wibe et al., 2002; Patiño et al., 2003).

Using a model fish species, the threespine stickleback (*Gasterosteus aculeatus*), we previously documented that some treated females became masculanized to the point of functional hermaphroditism (Bernhardt et al., 2006). Additionally, we found that

genetic males developed hypertrophied testes in a dose-dependent manner. These findings suggest that perchlorate produces a variety of effects that are difficult to explain solely on the basis of impaired thyroid hormone homeostasis. Furthermore, reproductive and behavioural functions are likely to be impaired if either thyroid hormone synthesis is altered or fish are masculinized during development.

In this chapter we describe how perchlorate exposure affects a variety of behavioural characteristics in stickleback. We report on swimming performance, nest-building, courtship, and parental care activities, as well as resilience to stress in two generations of fish exposed to perchlorate. We show that perchlorate affects all of these endpoints, each of which contributes significantly to the two major components of lifetime fitness, namely, survivorship until breeding age and reproductive output. Our data demonstrate that perchlorate not only has immediate effects, but that exposure during key developmental windows has delayed effects that are only expressed at reproductive maturity. Such delayed effects would not be noticed during acute toxicity experiments. Our findings make clear that this pervasive pollutant has different suites of immediate and long term effects on stickleback specifically, and likely on many other vertebrates as well.

3.3 Material and Methods

Reproductive behavior was measured in two generations of stickleback, wild caught adults ($G_{0,2002}$) and laboratory-reared adults ($G_{1,2003}$); the swimming performance of $G_{1,2003}$ fish was also tested. Details of experimental conditions and treatment groups are provided in Table 3.1. Exposure concentrations were chosen because they approximate (and, in some cases, are much less than) perchlorate concentrations at a number of contaminated sites (Smith et al., 2001; http://www.epa.gov/fedfac/documents/detection_with_dates_03_25_05.xls).

3.3.1 Perchlorate Measurements

Baseline perchlorate concentrations in the tap water used to fill all pools and aquaria were tested via ion chromatography in tandem with electrospray ionization mass spectrometry (IC-ESI-MS; Dodds et al., 2004). Temperature and perchlorate concentrations were monitored daily using an Oakton Acorn 6 perchlorate potentiometer with automatic temperature compensation (Oakton, Vernon Hills, IL, USA). The potentiometer was equipped with a perchlorate-ion-selective electrode (Cole Parmer, Vernon Hills, IL, USA). To avoid contaminating the negative control water, the electrode was thoroughly cleaned prior to use and only used on the negative controls on six days to take 18 readings in 2003. Dissolved oxygen, pH, and salinity were checked every two to three months.

3.3.2 Swimming Trials

Swimming performance was assessed between 18 and 25 May, 2004 for 102 of the $G_{1,2003}$ fish that survived to sexual maturity ($N = 43$, 24, 22, and 13 for controls, 30, 60, and 100 ppm fish, respectively). Two additional fish appeared moribund and were not tested during the swimming trials. Water without detectable concentrations of perchlorate (<1.1 ppb; 17.5-18.0° C) was used during all swimming trials. Mean body length [mbl; 47.5 ± 3.2 mm (1 sd), $N = 104$] did not differ significantly between treatments [Analysis of Variance (ANOVA, $F_{3,104} = 1.4$, $p = .242$)] when the $G_{1,2003}$ were tested in the flume, so mean body lengths per second was determined to be an appropriate measure of flow rate.

Fish remained in the flume for up to 58 minutes at a time. The first 10 minutes allowed a fish to acclimate to slowly flowing water [flow rate = 1.6 mbl/s = 7.6 cm/s]. Fish that rested or became trapped against the downstream barrier during the initial 10 minute acclimation period were not considered to have failed and were immediately removed from the barrier and coaxed approximately 0.5 m upstream.

Following the acclimation period, swimming ability was quantified for up to 38 consecutive minutes (28 minutes for a "stepwise" test, followed by 10 minutes for an "endurance" test) by measuring the swimming performance of a single fish at a time. During the stepwise test, the flume's flow rate was increased by increments of approximately 0.37 mbl/s (1.7 cm/s) at two minute intervals. This stepwise process continued for a maximum of 28 minutes as long as the fish avoided becoming trapped against the mesh barrier at the downstream end of the swim chamber or until the flow rate reached 6.4 mbl/s (28.1 cm/s). Fish that maintained position for two minutes at each flow rate through 6.4 mbl/s were considered to have passed the stepwise swimming portion and began the endurance test. During endurance testing, the flow rate was increased to the maximum (6.5 mbl/s; 28.5 cm/s), for up to 10 additional minutes. If a fish successfully resisted the maximum flow rate for 10 minutes, it was considered to have passed the endurance test.

The final 10 minutes of every swim trial consisted of a 'cool-down' period at the original acclimation speed (1.6 mbl/s) in order to facilitate O_2 exchange across the gills, reduce lactic acid buildup (Wood et al., 1983), and avoid the Root effect (massive offload of oxygen from hemoglobin at low pH and reduced overall O_2 carrying capacity) in exhausted fish. Neither resting nor trapping bouts were recorded during the cool-down period; likewise success and failure were not considered.

If a fish became trapped against the downstream mesh at any point during the stepwise or endurance testing and was unable to free itself within three seconds, or if a fish rested its tail against the mesh five times at a single flow rate, it was considered to have failed the trial. At that point the flow rate was quickly reduced to the original acclimation speed (1.6 mbl/s), and the fish was freed from the mesh with a pair of forceps. The velocity of failure was recorded, and the fish entered the cool-down period previously described. Fish that failed the stepwise swimming test did not participate in the endurance test.

On 22 June 2006, flow rates from three local, Alaskan streams [Anchor River (59°46'30"N, 151°51'50"W), Deep Creek (60°01'41"N, 151°41'00"W), and Rabbit

Slough (61°32'12"N, 149°15'17"W)] with anadromous threespine stickleback runs were measured. Measurements occurred approximately 1 km upstream from their confluence with Cook Inlet, using a Sigma Sport flow meter model FP101 (Global Water, Gold River, CA, USA) in order to provide a rough estimate of flow rates that returning stickleback might encounter en route to their spawning grounds.

For convenience, we divided reporting of mortality into two time frames: before reaching sexual maturity, and following sexual maturity. Flume testing represented a convenient reference point separating these periods.

3.3.3 Spawning Protocol

The same basic protocol for introducing females to isolated males and videotaping reproductive behaviour was used in 2002 ($G_{0,2002}$ wild-caught adults) and 2004 ($G_{1,2003}$ lab-raised adults). For brevity, we describe how this was done for the $G_{1,2003}$ fish and discuss differences in techniques for the $G_{0,2002}$ following this description.

At approximately one year of age, following the conclusion of swimming trials, $G_{1,2003}$ stickleback showing male nuptial coloration (N = 10, 6, 2, and 0, for the controls, 30, 60, and 100 ppm experimental groups, respectively) were assumed to be males and isolated in 38 l aquaria containing tap water without detectable concentrations of perchlorate. Lower survivorship among fish exposed to higher concentrations of perchlorate (Bernhardt et al., 2006) led to fewer nuptially colored fish from which to choose. Water was maintained at 4 parts per thousand (‰) salinity and continually filtered and aerated with Azoo biofilters (Aquatic Ecosystems, Apopka, FL, USA); it was not exchanged.

Gravid females were segregated according to experimental group and collectively remained in their 400 l holding tank (controls, 30, 60, or 100 ppm). The remaining fish of unknown sex were isolated, as above, in 38 l aquaria without detectable concentrations of perchlorate (N = 0, 4, 8, and 10 respectively for controls, 30, 60, and 100 ppm treatments). In this way, a total of 10 aquaria per experimental group each housed a

single fish (either a nuptially colored male or a fish of unknown sex; $N = 40$ individually housed fish and aquaria). Extra fish were separated and later euthanized.

Males that died prior to the onset of mating trials were replaced as long as additional fish suspected of being males were available from the same treatment. After subtracting individually housed females and adding replacement males, a total of 10, 8, 8, and 11 males for the control, 30, 60, and 100 ppm experimental groups, respectively, were isolated in 38 l aquaria and given the opportunity to reproduce, yet not all of these males survived to perform reproductive behaviour. Videotapes of behaviour were made for all surviving males, including all ten control males, six of eight 30 ppm males, five of eight 60 ppm males, and six of 11 100 ppm males. The remaining males died prior to making a nest.

All fish were maintained on a natural photoperiod. Nesting materials consisting of dried, filamentous algae, and a 90mm Petri dish filled with sand were added to each of the aquaria. Females were introduced to the aquaria of individually housed presumptive-males without nests at two day intervals to stimulate nest building.

From May 29 to June 18, 2004, single gravid $G_{1,2003}$ females from like experimental groups were placed into a male's aquarium and allowed 10 min to spawn. The female was removed either after successfully spawning or at the end of the 10 min period if spawning was unsuccessful. If, however, females remained within a nest at the end of the 10 minute period, they were not removed until they emerged. Gravid females continued to be introduced into each aquarium daily until either a successful spawning event occurred or three weeks of courtship activity had passed. No additional females were provided to a male after he successfully spawned. Courtship, spawning, and parental care behaviours were videotaped for analysis.

Because sample sizes were small, $G_{1,2003}$ females were used for more than one spawning attempt. *In vitro* fertilizations were performed as described in Bernhardt et al. (2006) to compare hatching success between treatments. Following behavioural testing, the $G_{1,2003}$ were euthanized with an overdose of tricane methane sulfonate (MS-222).

The $G_{0,2002}$ spawning protocol differed in four respects. A plentiful supply of colorful, two-year old $G_{0,2002}$ males ensured that only males were placed in each of the 80 aquaria. Nominal experimental groups differed between the $G_{0,2002}$ and $G_{1,2003}$ trials (Table 3.1). $G_{0,2002}$ females were not used for more than one spawning trial, and the $G_{0,2002}$ were euthanized in liquid nitrogen.

To test whether differences in fanning rates were related to morphological differences, we measured pectoral fin length (base to the distal end of the longest fin ray) and standard length on the $G_{1,2003}$ after being euthanized (17-22 June 2004).

3.3.4 Behavioural and Statistical Analysis

Each fish was videotaped with a Sony (Sony Corp., Tokyo, Japan) DCRTRV-17 or DCRTRV-25 digital video recorder for a minimum of 10 minutes per day. If fertilization occurred, the male was videotaped for an additional 10 minutes to permit analysis of his initial parental care activities. Aquaria were positioned end to end in rows, and each aquarium was surrounded with cardboard on three sides to prevent neighboring fish from interacting. To ensure filming was conducted without bias, lists of random numbers were generated daily to dictate the sequence of filming.

Behavioural events were scored by a single person (RRB) from video tapes using "The Observer" behavioural analysis software (Noldus Information Technology, Wageningen – The Netherlands). Three courtship videos were randomly chosen to be re-analyzed to test for concordance between scoring attempts. Event recording was determined to be highly reliable (<1% variation in total duration of each behaviour for all three concordance analyses).

The male behaviours of interest, as described by van Iersel (1953) and Wootton (1976), included: "carrying" sand or vegetation to, from, or independent of the nest; "boring" into the nest; "gluing" at the nest; "fanning" the nest; "creeping through" the nest; "biting" the female; "zig-zagging" toward the female; "leading" or attempting to lead the female; "dorsal pricking," which consists of the male using his dorsal spines to

prick the female's abdomen during courtship; and "quivering" along the female's caudal peduncle to stimulate egg deposition within the nest. Finally, an "intermediate" category was used to include all behaviour neither previously mentioned, nor essential to nest building, courtship, or parental care. These behavioural categories were both mutually exclusive and comprehensive.

After analyzing all of the courtship videos, the total number of behaviours performed per individual was recorded. Next, the mean number of courtship behaviours was calculated per treatment and compared across treatments. Thus, if a fish performed only two behaviours during one courtship attempt and two behaviours during a second attempt, including one new behaviour, then the individual fish was considered to have performed three distinct behaviours during its courtship testing. This number (3) was then added to the total number of courtship behaviours performed by the other males in its experimental group and divided by the number of active males to determine the mean number of courtship behaviours for a given experimental group.

To determine if perchlorate affected readiness or ability to court, the time until onset of each of the courtship behaviours was quantified. Since some males spawned on their first attempt, and others took multiple attempts, the mean time until onset of each behaviour was determined for each individual. Group means were then calculated and used for comparisons.

The Statistical Package for Social Sciences (Version 11.5; SPSS, Chicago, IL, USA) was used for all statistical procedures. Nonparametric statistics were used when data were unevenly distributed, when variances were not homogeneous, and/or when sample sizes were small ($n \leq 16$ per group). Statistics were two-tailed, and values are reported as mean ± 1 standard error (SE) unless otherwise noted. Because only two 60 ppm males spawned, multiple Mann-Whitney U tests were used to compare spawning success and timing (Shaw & Wheeler, 1985), but Kruskal-Wallis tests were used when there were between three and 16 observations (Shaw & Wheeler, 1985). One-way ANOVAs were used for normally distributed data with sample sizes above 16

observations per group and compared with non-parametric statistics for these same parameters; in all cases the results were consistent.

3.4 Results

3.4.1 Perchlorate Measurements

Background concentrations of perchlorate in the source water were found to be below the method detection limit of ion chromatography in tandem with electrospray ionization mass spectrometry (<1.1 ppb). The following measures in the ppm range were performed with Acorn 6 potentiometers. In 2002, aquaria receiving nominal perchlorate concentrations of 1.5 and 12 ppm had measured mean perchlorate concentrations of 1.5 (n=55, SE=0.02) and 12.0 ppm (n=55, SE=0.13), respectively. In 2003, aquaria with nominal perchlorate concentrations of 30 and 100 ppm had measured mean perchlorate concentrations of 32.0 (n=334, SE=0.20) and 102.9 ppm (n=334, SE=0.57), respectively. The nominal 60 ppm treatment mimicked the increasing perchlorate concentration noted in 2002 as perchlorate leached from solid rocket propellant cores ($y_{2002} = 0.32x - 12.04$, $r^2 = 0.92$ vs. $y_{2003} = 0.31x - 9.44$, $r^2 = 0.88$). It reached a maximum concentration near 60 ppm about halfway through the experiment, after which the readings remained relatively stable (mean = 61.8 ppm, $N = 185$, SE = 0.19).

The mean perchlorate concentration of the negative control water based on the 18 Acorn 6 readings was 0.15 ppm (SE 0.01). More reliable analysis of the water in the negative control experimental group also was conducted by ion chromatography in tandem with electrospray ionization mass spectrometry ($N = 21$ samples). This technique revealed the water to have less than the method detection limit of 1.1 ppb perchlorate (Dodds et al., 2004).

3.4.2 $G_{0,2002}$ - Reproductive Benchmarks

Neither the success rate (Kruskal-Wallis, $\chi^2 = .61$, df = 3, $p = .893$) nor the timing (ANOVA, $F_{3,76} = 1.94$, $p = .131$) of wild-caught adult males ($G_{0,2002}$) making nests varied

across experimental groups in 2002. Likewise, all 80 of the $G_{0,2002}$ males were recorded fertilizing a clutch of eggs within 7 days of one another. Thus, there were no differences between experimental groups in spawning success (Kruskal-Wallis, $\chi^2 = 3.05$, df = 3, p = .384) or in the timing of spawning events (ANOVA, $F_{3,76} = 0.93$, $p = .430$). Of the fish that spawned ($N = 20$ per treatment), fewer made nurseries (hole torn into nest that exposes newly hatched fry and enhances the exchange of gases; $N = 17$, 19, 20, and 18 for Controls, 1.5 ppm, 12 ppm, and the variable treatment, respectively). Experimental group did not affect nursery making success (Kruskal-Wallis, $\chi^2 = 3.44$, df = 3, $p = .329$) or timing of nursery production (Kruskal-Wallis, $\chi^2 = 6.99$, df = 3, $p = .072$). Most males that made nurseries also produced fry ($N = 16$, 19, 20, and 17 for Controls, 1.5, 12, and the variable treatment, respectively), and whether or not fry were produced was not influenced by experimental group (Kruskal-Wallis, $\chi^2 = 5.31$, df = 3, $p = .150$); nor was the timing of fry production affected (ANOVA, $F_{3,68} = 1.31$, $p = .280$).

3.4.3 $G_{1,2003}$ - Swimming Performance

A clear dose-dependent relationship was evident in the swimming trials as fish from higher perchlorate treatments experienced progressively lower success rates than those from lower treatments and control fish (Fig. 3.1): 76% of control fish successfully completed the stepwise swimming trials, compared to 67%, 32%, and 8% of the 30, 60, and 100 ppm fish, respectively. The velocity at which fish failed the stepwise trials reflected a similar dose-dependent relationship. Control, 30, 60, and 100 ppm fish that failed did so at significantly different (Kruskal-Wallis, $\chi^2 = 16.59$, df = 3, $p < .001$) mean velocities (± 1SE) of: 5.8 MBL/s (±1.5), 5.3 (±1.6), 4.8 (±0.23), and 3.7 (±1.4), respectively. The same dose-dependent relationship was generally noted among the fish that passed the stepwise trials and participated in the endurance trials with 79% of control fish successfully completing the endurance trials compared to 69%, 43%, and 100% ($N = 1$) of the 30, 60, and 100 ppm fish, respectively. Differences in success rates between treatments were significant in the stepwise test ($\chi^2 = 26.14$, df = 3, $p < .001$). Overall, differences for the endurance tests were not significant (Kruskal-Wallis $\chi^2 = 3.66$, df = 2,

$N = 56$, $p = .160$), but post-hoc comparisons approached significance between the control and 60 ppm groups (Mann-Whitney $U_{control - 60\ ppm}$), $N = 40$, $Z = -1.91$ $p = .056$).

In field studies, the mean flow rates of 15, 46, and 110 cm/s were recorded in Rabbit Slough, Anchor River, and Deep Creek, respectively. These flow rates equate to 3.2, 9.7, and 21.3 mbl/s, respectively, illustrating that the flow rates used in the swimming trials were below those recorded at two of the three stickleback bearing streams. However, flow rates taken from the field should be viewed with caution as both seasonal and geospatial variations occur. These flow rates provide an index of resistance that fish may encounter during their upstream migration, though stickleback are likely to find more favorable flow rates as they negotiate the stream.

Flume success rates alone are an incomplete measure of swimming performance since mortality rates following the swimming trials were quite different among experimental groups (Kruskal-Wallis, $\chi^2 = 11.33$, df = 3, $N = 39$ males, $p = .010$). Post-flume mortalities, 0-22 days later, included zero control males, four 30 ppm males, six 60 ppm males, and seven 100 ppm males. Tetany occurred in at least one of these fish within 24 h of flume testing, with ruptured blood vessels apparent at the base of its pectoral fins.

Although standard lengths were similar across treatments (see Methods), lengths of pectoral fins were significantly different (Kruskal-Wallis, $\chi^2 = 18.80$, df = 3, $N = 26$, $p < .001$). Bonferroni multiple comparison tests showed that the pectoral fins of the controls and 30 ppm fish were similar in size [means = 16.44 mm (±0.47), $N = 7$ and 15.34 mm (±0.66), $N = 6$, respectively] but significantly larger than the pectoral fins of the 60 and 100 ppm fish [means = 11.72 mm (±0.63), $N = 6$ and 12.61 mm (±0.45), $N = 7$, respectively], which were also similar in size to one another.

3.4.4 $G_{1,2003}$ - Courtship

Unlike control males, several treated males displayed brief, erratic, twitchy, and seemingly uncontrolled swimming patterns that might be characterized as seizures.

Many "seizures" preceded or accompanied fleeing behaviour from the female during courtship and often ended when the male swam into the glass. Fish with aberrant swimming patterns include: zero of ten control fish, four of eight 30 ppm fish, three of seven 60 ppm fish, and two of eight 100 ppm fish. Only one of the fish that had these unusual swimming patterns died prior to the end of the experiment.

Failure to perform any courtship behaviours during at least one spawning attempt increased in frequency in a dose-dependent manner (Kruskal-Wallis, $\chi^2 = 9.12$, df = 3, $N = 27$, $p = .028$; Fig. 3.2). Additionally, treated males that participated in courtship displayed a smaller behavioural repertoire (performed fewer behaviours) in a dose-dependent manner (Fig. 3.3).

The length of time between a female's introduction and the onset of 11 male-typical behaviours was analyzed for those fish that performed courtship displays (Table 3.2). Of these, only biting differed significantly between treatments, with control males initiating courtship by biting females sooner than treated males in a dose-dependent manner ($N = 26$ first biting means, $\chi^2 = 8.24$, df = 3, $p = .041$). Although differences in the onset of other behaviours were not found between treatments, it should be noted that fewer 100 ppm males courted (Fig. 3.2), and those that did performed few courtship activities (Fig. 3.3, Table 3.2). Hence, the 100 ppm fish were different by virtue of their omission of behaviours.

Control fish spent the greatest percentage of time carrying, boring, gluing, and biting during courtship compared to all treated fish (Fig. 3.4), and the percentage of time spent conducting these behaviours decreased in a dose-dependent manner, although differences were not statistically significant after the 100 ppm males were excluded (Kruskal-Wallis, df = 2, $N = 21$, $p > .05$ for all). The 100 ppm fish always spent the least percentage of time engaged in courtship behaviours and none managed to spawn successfully. Control males spent the lowest percentage of time conducting non-courtship-related intermediate behaviours (72%), followed by the 30 and 60 ppm males (77% each). The 100 ppm fish spent the greatest percentage of time conducting intermediate behaviours (98%). Variations in the proportion of time spent in the

intermediate category were statistically significant (Kruskal-Wallis test, $\chi^2 = 9.324$, df = 3, $N = 27$, $p = .025$) when all four experimental groups were compared, but not significant when the 100 ppm males were excluded (Kruskal-Wallis test, $\chi^2 = 0.16$, df = 2, $N = 21$, $p = .699$).

3.4.5 $G_{1,2003}$ - Parental Care

Because none of the 100 ppm males spawned, they conducted no parental care and have been excluded from these analyses. Fry normally hatched after 6 days post fertilization (dpf; range = 5-7) in 2002 at a mean water temperature of 18°C and after 7 dpf (range = 6-8) in 2004 at a mean water temperature of 14°C. On parental care days one through seven, the two 60 ppm males provided the most parental care (total percent of time spent fanning, boring, carrying, and gluing) and spent the least amount of time conducting non-parental care-related "intermediate" activities compared to control (Mann-Whitney $U_{(60\ ppm - control)}$, $N = 66$, $Z = -2.89$, $p = .004$) and 30 ppm males (Mann-Whitney $U_{(60\ ppm - 30\ ppm)}$, $N = 32$, $Z = -2.58$, $p = .008$). On parental care days 1-6, the 60 ppm fish always spent the greatest percentage of time fanning (Fig. 3.5).

$G_{1,2003}$ fish exposed to perchlorate showed delays and sequentially worse success rates for nest building (Fig. 3.6). Perchlorate-exposed fish also showed progressively lower success for spawning, nursery production, and fry production compared with controls (Table 3.3). Although not statistically significant (Kruskal-Wallis, df = 2, $N = 19$, $\chi^2 = 2.065$, $p = 0.356$), there was a trend for perchlorate-treated fish to build nests later than control fish (Fig. 3.6). The timing of fertilization events was not significantly different between treatments (Mann-Whitney $U_{(control - 30\ ppm)}$, $N = 14$, $Z = -.81$, $p = .419$; Mann-Whitney $U_{(control - 60\ ppm)}$, $N = 11$, $Z = -1.32$, $p = .188$; and Mann-Whitney $U_{(30 - 60\ ppm)}$, $N = 7$, $Z = 1.37$, $p = .171$). A total of zero, two, three, and five males from the control, 30, 60, and 100 ppm experimental groups died following the swim tests, bringing the number of control, 30, 60, and 100 ppm males that survived through the end of the spawning trials to ten, six, five, and six, respectively. All but one of these mortalities occurred prior to a fertilization event. Although the remaining male successfully

attracted a mate and fertilized her eggs, he died three dpf. His lack of parental care resulted in the majority of his eggs becoming infected with fungus and dying prior to hatching. Two of the eggs hatched 7 dpf, but both fry died one day later.

Fecundity rates in terms of the number of eggs produced per female did not differ between females exposed to 30, 60, or 100 mg/L perchlorate. However, control females produced more eggs than treated females (Kruskal-Wallis test, $\chi^2 = 11.645$, df = 3, p = .009), even though their mean standard length and mass did not differ at sexual maturity. Half of the treated males that managed to spawn (30 and 60 ppm, only) eventually produced fry, compared to 75% of control fish (Table 3.3). Differences in hatching success, based on *in vitro* fertilizations, were statistically significant (Kruskal-Wallis test, $\chi^2 = 13.462$, df – 3, p = .004), but perhaps not biologically significant since control, wild, and 100 ppm females had similar hatching success while 30 and 60 ppm females had significantly lower hatching success.

3.4.6 $G_{1,2003}$ - Behaviour of Hermaphrodites

DNA sex markers were used to determine the genetic sex of the $G_{1,2003}$ fish suspected of being hermaphrodites, as well as of a sample of presumptive males and females. Two genetically-female 100 ppm fish were confirmed to be functional hermaphrodites via *in vitro* fertilizations of their eggs and sperm and through histological examination of their gonads (Bernhardt et al., 2006); these two fish also performed male courtship behaviors in the presence of gravid females (Table 3.4). In addition to these known hermaphrodites, two 30 ppm genetic females were suspected hermaphrodites because they also performed male courtship behaviors when exposed to gravid females (Table 3.4). None of the genetically female hermaphrodites made nests, even though they performed "male" courtship displays. Introduced females often responded to these "male" courtship displays by assuming the head-up posture typical of receptive females and by following the hermaphrodite as it attempted to lead. Two of the 60 ppm females died before encountering a gravid female, and the third remained inactive throughout the

encounter. This third 60 ppm female died before encountering another gravid female; therefore, it is unknown if any of the 60 ppm females were hermaphroditic.

While hermaphroditic females (presumed and confirmed) showed a limited male courtship repertoire, their levels of inactivity were comparable to those for males exposed to 100 ppm perchlorate. Differences between the time spent engaged in male courtship behavior by hermaphrodites and 100 ppm males were not statistically significant (Mann-Whitney U tests, all p's >.05). However, the trend was for the hermaphrodites to spend slightly more time engaged in a given courtship state while courting introduced females (means $_{(100\ ppm\ males\ vs.\ hermaphroditic\ females)}$ for leading = 1.1 s vs. 1.4 s and for zig-zagging = 0.9 s vs. 2.1 s).

3.5 Discussion

Our study demonstrates that chronic perchlorate exposure during development is capable of dramatically altering the behaviour of threespine stickleback. Subchronic perchlorate exposure (<22 d) of adult stickleback did not significantly alter any of the measured variables (i.e., survivorship, reproductive behaviour, fertility, or spawning success). Conversely, chronic exposure (~1 year) from syngamy through sexual maturity impaired survivorship and caused numerous reproductive behavioural abnormalities, which increased in frequency with higher exposure concentrations of perchlorate and ultimately reduced reproductive success. A no adverse effect level was not determined because significant behavioural effects were found even among fish in the lowest treatment of 30 ppm. These chronically exposed $G_{1,2003}$ fish will be the focus of the discussion.

3.5.1 Swimming Performance

Swimming performance was impaired in a dose-dependent manner (Fig. 3.1), suggesting that fish exposed to perchlorate would be less capable of capturing prey, evading predators, and returning to their spawning grounds. Anadromous stickleback

have been shown to rely on pectoral fin rowing for several hours at a time at velocities exceeding five body lengths per second (Taylor and McPhail, 1986). The flow rates used in this experiment are well below those measured in two of three local streams with anadromous stickleback runs. Therefore, the flow rates ($\leq$ 6.5 mbl/s) and test durations ($\leq$ 58 minutes) chosen to assess swimming performance are ecologically relevant. This suggests fish exposed to perchlorate would be less successful at returning to suitable spawning habitat. Furthermore, Mesa et al. (1994) demonstrated that poor fast-start swimming ability and reduced endurance among fishes leads to increased susceptibility to predation, which further reduces the likelihood of survival to reproductive age and would expose predators to dietary perchlorate.

Perchlorate-exposed fish suffered higher mortality following stressors such as exhaustive exercise, relocation, and handling while out of the water for <10 seconds (Bernhardt et al., 2006). Exhaustive exercise causes plasma concentrations of lactate, Na^+, Cl^-, plasma proteins, and haemoglobin to rise as plasma hydrates white muscle tissue to offset intracellular lactic acid production (Wood et al., 1983). Recovery depends on lipid oxidation (Richards et al., 2002). Since THs affect osmoregulation (Leatherland, 1982) and lipid catabolism (Marieb, 2004), perchlorate-treated fish would be expected to display reduced recovery rates. Moreover, LaRoche et al. (1966) demonstrated that radiothyroidectomized fish had marked hemolysis attributable to erythrocyte fragility compared with control fish. Such results may help to explain higher mortality rates among treated fish following swimming trials. In addition to the higher mortality among perchlorate-treated fish prior to sexual maturity, mortality rates were elevated among males in a dose-dependent manner during their 33 days of isolation for courtship and parental care.

Both thyroid hormones and serotonin can have a direct stimulatory effect on spontaneous locomotion, and prenatal reductions of thyroid hormone can lead to lower levels of serotonin in the adult (Leatherland, 1982; Castonguay & Cyr, 1998; Weis et al., 2001). Reduced thyroid hormone levels during development have also been shown to affect spontaneous activity via reduced production of seratonergic neurotransmitters,

which in turn leads to sluggishness (Castonguay and Cyr 1998; Weis et al., 2001). Mummichogs (*Fundulus heteroclitus*) living in PCB-, DDT-, and heavy metal-contaminated water had reduced thyroid hormone production, and demonstrated sluggish swimming performance, poor prey capture, and poor predator avoidance abilities (Smith & Weis, 1997). These factors are all likely to contribute to the production of lethargic adults with impaired swimming ability.

Chemicals, such as perchlorate, that inhibit thyroid hormone production can damage or impair nervous system development, since thyroid hormones have critical roles during neurogenesis (Porterfield, 2000). Neuronal outgrowth and migration are dependent upon microtubule synthesis, which is regulated by thyroid hormones (Nunez et al., 1991). Thyroid hormones also regulate nerve growth factor production (Oh et al., 1991). Animals with experimentally induced hypothyroidism show decreased axonal and dendritic arborization, fewer nerve terminals, and a variety of other neural impairments (Porterfield & Hendrich, 1993) and hence might be expected to have altered neurons associated with behaviour. Deficiencies in proper neuromuscular development and function may explain the impaired swimming performance and reproductive behaviour noted in the 30, 60, and 100 ppm perchlorate-treated fish.

LaRoche et al. (1966) noted that radiothyroidectomized rainbow trout had neuromuscular anomalies as indicated by seizures, which confer a poor degree of coordination. These fish often displayed differences in swimming performance and "inefficient swimming motions resembled those of tadpoles." These inefficient swimming movements appeared to be related to improper neuromuscular coordination and occasional and unpredictable seizures, which involved violent flexions of the body (LaRoche et al., 1966). Such seizures were observed among perchlorate-treated stickleback, possibly implicating impaired neuronal development in the aberrant swimming and reproductive behaviours.

3.5.2 Reproductive Behaviour

Whoriskey and FitzGerald (1994) categorized male stickleback reproductive success into three major components: nest building; the number of females the male can attract, and hence the number of eggs he can acquire; and the ability to provide appropriate parental care. All three of these components were impaired among stickleback chronically exposed to perchlorate, resulting in a dose-dependent reduction in reproductive success: 60% of controls produced fry, while 20% of 30 ppm, 10% of 60 ppm, and none of the 100 ppm fish produced fry. Although behavioural inhibition was noted among 30 ppm and 60 ppm fish during courtship (Fig. 3.3), there appears to be a point over which exposed fish are unable to produce nests or perform appropriate reproductive behaviours. This point for complete inhibition of reproductive behaviour was between 60 and 100 ppm.

The courtship phase in threespine stickleback normally begins when the male creeps through his nest (Wootton, 1976). Yet, nest-building was delayed in perchlorate-treated fish in a dose-dependent manner (Fig. 3.6), which would shorten the effective breeding season for wild stickleback. Delayed nest building can translate into the male attracting fewer or no females, leading to reduced reproductive success (Mori, 1993). Douthwaite et al. (1981) determined that exposure to endosulfan (a xenoestrogenic chemical) caused *Tilapia rendalli* to build fewer nests than unexposed fish, thereby reducing reproductive output.

Chronic perchlorate exposure also caused $G_{1,2003}$ males to court less often (Fig. 3.2) and perform fewer courtship behaviours in a dose-dependent manner (Fig. 3.3). These factors contributed to the poor spawning success and reduced reproductive output of treated males (Table 3.3). When treated males performed courtship behaviours, the timing to onset of a given behaviour after a female's introduction differed only for biting (Table 3.2), which initiates courtship in this population. Furthermore, $G_{1,2003}$ males treated with $\geq$ 60 ppm perchlorate (and some treated with 30 ppm) bit females with less vigor/intensity than control fish (Bernhardt pers. obs.).

The O_2 consumption and CO_2 production of embryos following fertilization is initially minimal (van Iersel, 1953). At this time, the water and gasses within the nest are rapidly exchanged, and the O_2 demands of the zygotes are quickly met after only a few moments of fanning (van Iersel, 1953). The initial bout of fanning following fertilization may help to distribute sperm throughout the eggs to ensure their fertilization. Metabolic demands steadily rise as the embryos mature, and elevated CO_2 levels have been shown to increase fanning activity in stickleback (Sevenster, 1961). Consequently, the male typically devotes more time and energy toward fanning the nest through at least day five post fertilization at 20°C (van Iersel, 1953; von Hippel, 2000; Pall et al., 2002; Pall et al., 2005). After day 5 or 6, less fanning is needed because the male tears a hole into the top of his nest to make a nursery that further promotes gas exchange, before the embryos hatch into fry (van Iersel, 1953). Thus, prolonged bouts spent fanning the nest are normally unnecessary immediately following fertilization, but males devote more time toward fanning as the embryos mature, until he makes a nursery.

This pattern of increased fanning activity during embryonic development was noted among control and treated fish (Fig. 3.5), but fish exposed to 60 ppm perchlorate spent significantly more time fanning until the eggs began to hatch on day 7. The most parsimonious explanation may be that the smaller pectoral fins of the 60 ppm males displaced less water within the nest and therefore required more fanning effort to adequately ventilate the nest. This would have caused the 60 ppm males to fan more in response to elevated CO_2 levels within the nest. However, the plethora of behavioural differences makes it difficult to discount the possibility that perchlorate exposure interfered with parental behaviour via altered thyroid hormone levels and impaired neural development/function (Porterfield & Hendrich, 1993; Porterfield, 2000), via altered brain aromatase levels, or by interference with hormonal metabolic pathways. It may also be possible that increased fanning among 60 ppm males was an artifact of small sample size.

While a degree of parental care is necessary to remove dead eggs and those covered with fungus (van Iersel, 1953) and to deliver O_2 and remove CO_2 from the nest, the control fish clearly demonstrated that they are capable of caring for the developing

embryos with less energy investment in terms of fanning (Fig. 3.5), and possibly boring, gluing, and carrying. Less energy expenditure in parental care not only conserves energy that can be devoted toward future courtship and spawning, but also reduces the conspicuousness of the nest to nest predators. Stickleback males that are better able to conceal their nests are more likely to be undetected by roving groups of cannibalistic stickleback and are more likely to hatch their eggs (FitzGerald, 1993).

Several hormones normally regulate sexual development, sexual physiology, and sexual behaviour, and prodigious cross-talk occurs between various endocrine axes. Interference with one is likely to cause concomitant effects among others (Matthiessen, 2003), yet individual hormones have been linked to specific reproductive behaviours. For example, prolactin (PRL) has been implicated in the control of parental care in vertebrates, including fishes (Slijkhuis et al., 1984). A functional role for PRL in the control of fanning behaviour in sticklebacks was confirmed by Pall et al. (2004), who demonstrated that PRL administration increased fanning behaviour in nesting males. Bell (2001) determined that gonadal steroids have contrasting effects on conspecifics oriented and nest oriented behaviours during stickleback courtship. Specifically, high plasma 11-ketotestosterone (11-KT) levels were positively correlated with conspecific-oriented behaviours (e.g., aggression toward males and courtship toward females) and negatively correlated with nest-oriented behaviours. Conversely, elevated estradiol levels increase nest-oriented activity at the expense of courtship or defense. Pall et al. (2002) also found contrasting behavioural responses to 11-KT levels between the courtship and parental care phases of threespine stickleback, with levels of 11-KT falling during the transition from the courtship phase into the parental care phase.

Perchlorate exposure masculinized developing stickleback in terms of producing male fish with hypertrophied testes and genetically-female hermaphrodites (Bernhardt et al., 2006). This masculinization of male and female gonads likely occurred during early development when sexual differentiation was underway, but concomitantly altered hormone production may affect reproductive behaviour at sexual maturity. We found that control males were the most active courters while 60 ppm males were the most active

during the parental care phase. In other words, perchlorate-treated fish showed less conspecific-oriented behaviour during courtship and more nest-oriented activity during parental care. These findings would be characteristic of fish with lower 11-KT levels and/or higher prolactin or estradiol levels. Therefore, perchlorate's role(s) as a masculinizing agent are not easily reconciled with the reduced courtship activity and increased parental care among the 60 ppm males, suggesting perchlorate does not mimic endogenous androgens. Smaller pectoral fins that are less efficient at water and gas exchange may have incidentally increased the fanning activity of 60 ppm males, though this does not exclude the possibility of altered hormonal and neural function.

Perchlorate exposure may also have induced a stress response that contributed to differential mortality and the inhibition of courtship behaviours. Stress effects were most clearly evident among 100 ppm fish following disturbance. Stress leads to elevated levels of circulating glucocorticoids, depressed levels of circulating gonadotropins and sex steroids, as well as increased mineralocorticoids (Carr and Norris, 2006). Since thyroid hormones and gonadotropic hormones are known to act together during gonadal formation, impaired production of either or both of these hormones is likely to impair sexual development. Virtually every process influenced by gonadotropic hormones, from courtship behaviour to fertilization, was inhibited by perchlorate exposure. Inhibition may have resulted from organizational rather than activational effects since subchronically-exposed $G_{0,2002}$ adults showed no behavioural inhibition (though exposure concentrations only ranged up to 18.6 ppm) while $G_{1,2003}$ fish that were chronically exposed to 30, 60, and 100 ppm perchlorate from early development through sexual maturity showed marked behavioural inhibition. The hormonal bases of perchlorate's actions are likely to be a productive avenue for future research.

3.5.3 Behaviour of Hermaphrodites

Individually housed intersex females (genetically-female hermaphrodites in the 30 and 100 ppm treatments) courted introduced, gravid females. However, 30 ppm and 60 ppm females that were housed collectively by treatment in female pools managed to

spawn naturally when introduced to territorial males from the same treatment. Some or all of these treated females may have been hermaphrodites, though this was not tested. In the two instances when gamete viability of hermaphroditic fish was tested (Bernhardt et al., 2006), the eggs fertilized by sperm from a hermaphrodite died prior to hatching. This raises the question of whether a treated, hermaphroditic female may have fertilized a portion of her own eggs before the male fertilized the rest. If so, it would have contributed to the poor hatching success noted among the 30 and 60 ppm clutches that arose from natural spawning events since the results of *in vitro* fertilization testing revealed that eggs fertilized by a hermaphrodite's sperm die before hatching. Whether perchlorate-exposed hermaphroditic females would be more likely to establish a territory in the wild and attempt to court other females, respond to male courtship displays, or both, remains unclear.

The presence of motile sperm in female stickleback exposed to perchlorate (Bernhardt et al., 2006) suggests that the steroidogenic lobule-boundary cells (homologous with Interstitial cells of Leydig) may have been functional and producing enough testosterone to support sperm development, or that perchlorate altered the metabolism of testosterone (either directly or indirectly) from the gonads and/or from precursors made in the adrenal cortex. Elevated testosterone levels, altered hormone metabolism, and/or altered brain aromatase activity in genetically-female hermaphrodites may help to explain their "masculine" courtship displays. Yet, none of the females exposed to perchlorate produced nests, suggesting that if testosterone levels were changed, the degree was limited.

Female stickleback exposed to testosterone are capable of making spiggin (the male glue protein produced by the kidneys and used for nest building; Katsiadaki et al., 2002), and a small proportion of gonadectomised female stickleback treated with the synthetic androgen 17α-methyltestosterone build rudimentary nests (Wai & Hoar, 1963). The females that built nests in Wai & Hoar's (1963) study showed most of the relevant behavioural patterns associated with nest building, but the organization of these patterns was insufficient to produce a proper nest. Their nest pit was shallow; there was little

boring; and although gluing occurred, the vegetation was not neatly glued together so that the end product was merely a flat mass of vegetation lying in a shallow pit. It is therefore unlikely that the intersex gonads were capable of producing sufficient testosterone to induce complete behavioural sex reversal, or capable of completely transforming the kidneys into a spiggin-producing organ (Guderly, 1994).

Administration of precise concentrations of exogenous sex steroid hormones during specific developmental windows is a common procedure in fish aquaculture to produce monosex populations (Donaldson & Hunter, 1982, Matty, 1985). The resultant sex-reversed fish are fertile and function as the phenotypic sex, including the display of behaviours typical of their phenotypic sex (Hunter & Donaldson, 1983; Pandian and Sheela, 1995; Piferrer, 2001). Complete sex reversal (fish of one genotypic sex with only the gonads of the other sex), however, has not been reported among wild fish exposed to xenobiotics (Kime, 1998). Rather, such fish develop intersex gonads (ovotestes), which is consistent with the effects of chronic perchlorate exposure (Bernhardt et al., 2006).

Mukhi et al. (2007) demonstrated that administration of perchlorate to zebrafish (*Danio rerio)* during the larval-juvenile developmental period produced a female-biased sex ratio, and supplemental thyroid hormone administration produced a male-biased sex ratio. This finding demonstrates that thyroid hormones play a key role in the sexual development of at least some fish. However, comparisons between the sexual development of zebrafish and stickleback should be made with caution since a sex chromosome has not been identified for zebrafish, and their sexual development appears to be labile and influenced by a number of environmental factors including dissolved oxygen content (Shang et al., 2006), temperature (Uchida et al., 2004) and others (Strussmann and Nakamura, 2002).

It is often assumed, and the majority of available evidence supports the conclusion, that genetic males are typically feminized into hermaphrodites by contaminants rather than genetic females being masculinized. However, this can only be confirmed by determining the genetic sex of intersex individuals, and DNA sex markers have only been developed for a few species, including the stickleback. Nevertheless,

based on current knowledge, the endocrine-disrupting effects of perchlorate are rare in that perchlorate exposure is capable of invoking masculinization during development (Bernhardt et al., 2006).

This study demonstrates that there are delayed costs of perchlorate exposure that would not be detected in standard acute or subchronic toxicity testing and that perchlorate exposure during early development poses risks that may only become apparent at sexual maturity. Poor swimming ability (Fig. 3.1) would decrease the likelihood of avoiding predators, catching prey, and reaching suitable spawning habitat. Inhibition of nest construction and courtship behaviour (Fig. 3.2, 3.3, & 3.6) and failure to complete reproductive benchmark metrics (Table 3.3) suggest that fish capable of reaching their spawning grounds would be unlikely to build a nest, attract mates, or produce fry. Diminished resilience in the face of disturbance would further reduce survivorship and contribute to lower recruitment among contaminated fish. The effects of perchlorate on the two major components of lifetime fitness, namely survivorship until breeding age and reproductive output, are unlikely to be limited to the stickleback.

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3.8 Figures

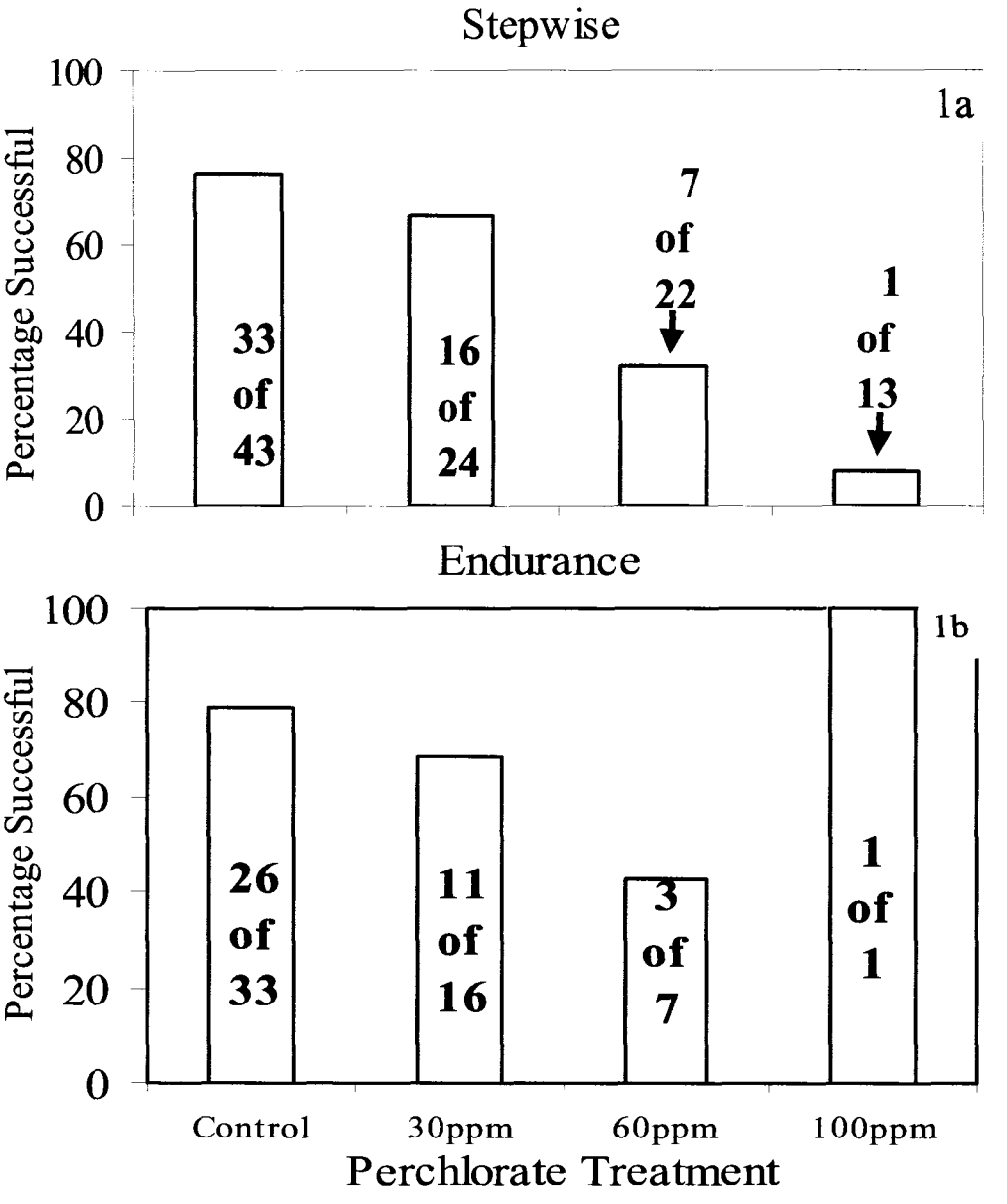

Fig. 3.1 Swim Trial Results. 1a) Stepwise increment testing. Swimming performance degraded in a dose-dependent manner during the stepwise swim trials. 1b) Endurance testing. Swimming performance also degraded in a dose-dependent manner during endurance testing with the exception of the 100 ppm treatment, which shows the results of the sole 100 ppm fish that passed both the stepwise and endurance trials.

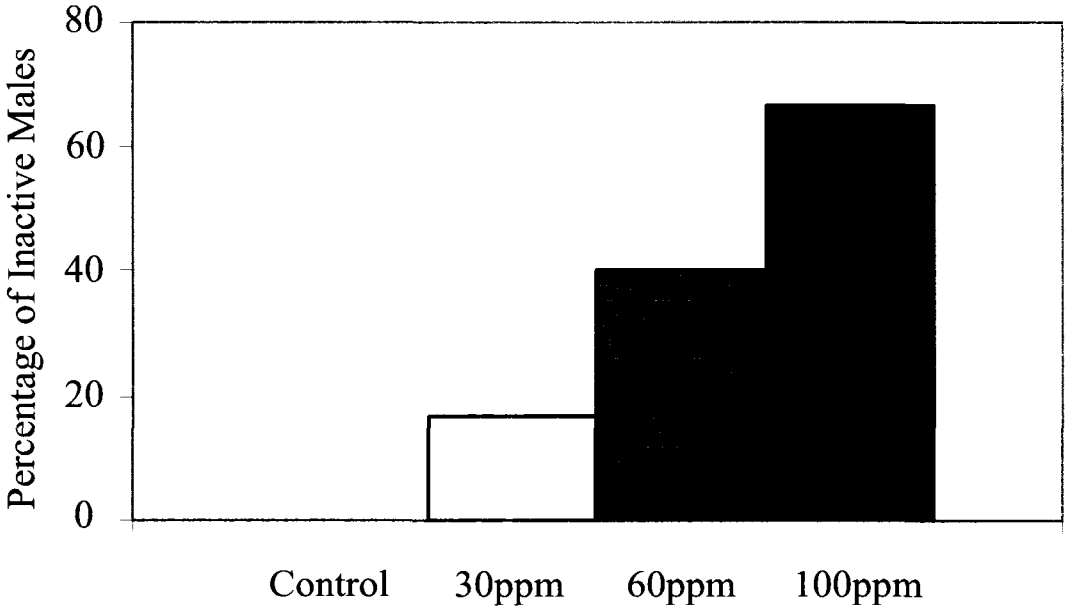

Fig. 3.2 Inactive Males During Courtship. Males from higher perchlorate treatments failed to court introduced females during at least one of their courtship trials more frequently than males from lower treatments. These data exclude males that died prior to courtship trials.

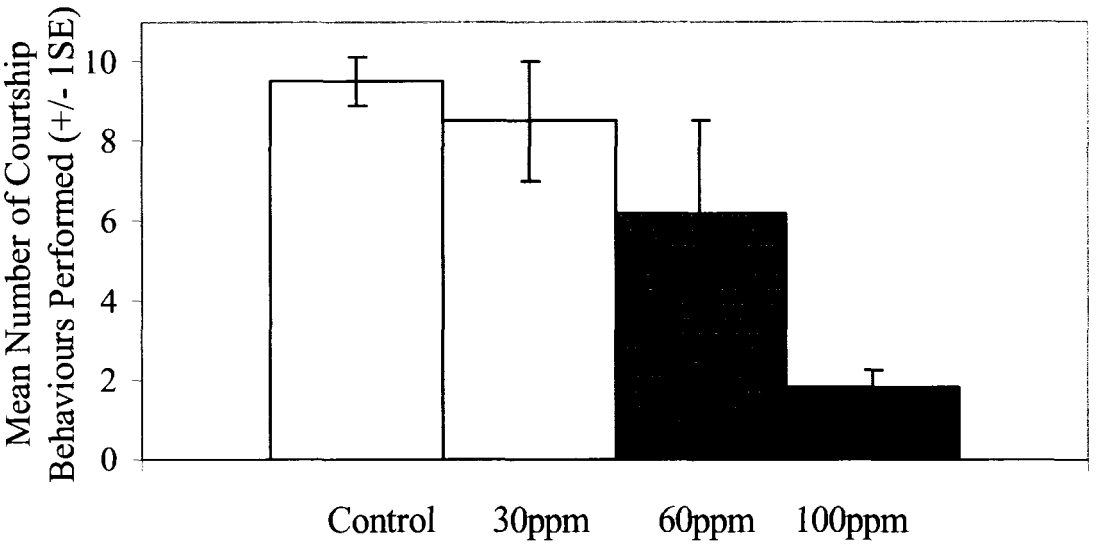

Fig. 3.3 Behavioural Repertoire. The behavioural repertoire of courting males (± 1SE) shows statistically significant differences (Kruskal-Wallis, df = 3, N = 27 fish, χ^2 = 10.77, p = .013) with males in higher perchlorate treatments performing fewer behaviours. Behaviours must have occurred at least once during an individual's spawning trials.

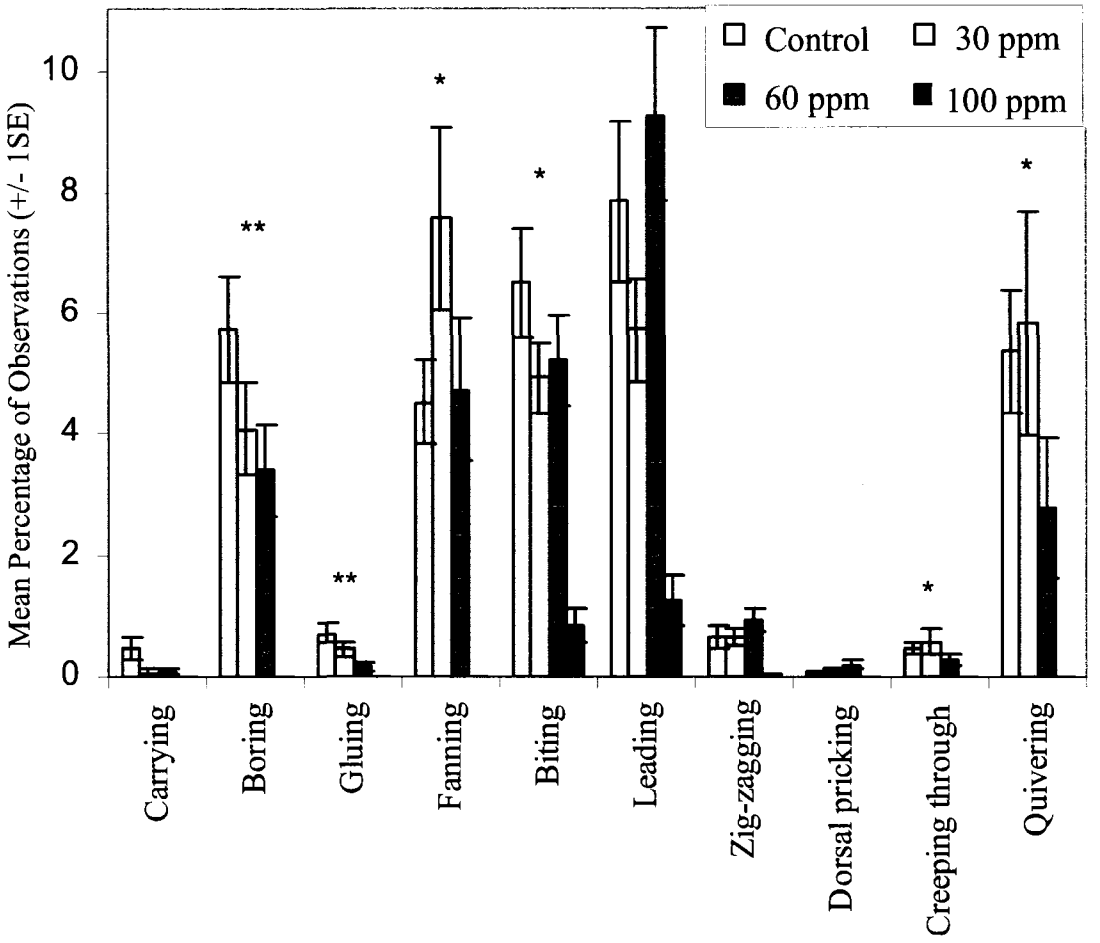

Fig. 3.4 Courtship Activity Budget. The mean percentages of time spent engaged in each behaviour during courtship observations are shown. Time spent in the "intermediate" category is not depicted in this figure in order to highlight the other categories. Statistically significant differences (Kruskal-Wallis test, df = 3, $N = 27$) between treatments are depicted with asterisks (* $p < .05$, ** $p < .01$).

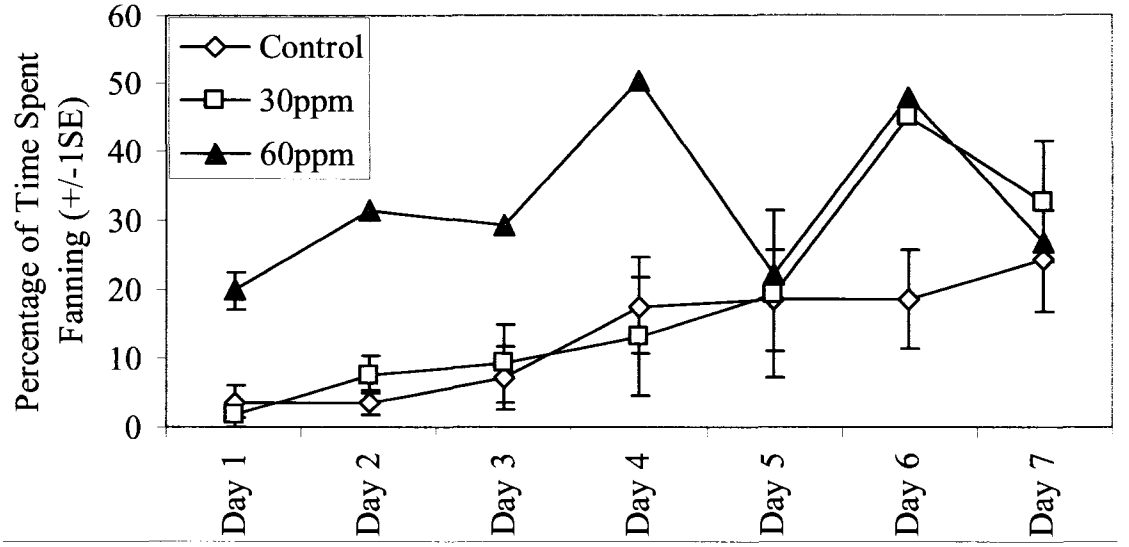

Fig. 3.5 Parental Care Fanning Comparisons. Seven day parental care analysis shows the mean duration spent fanning from parental care day 1 (the day of fertilization) through day 7 (generally the day of hatching). Although two 60 ppm males spawned, only one was recorded per day after the first day.

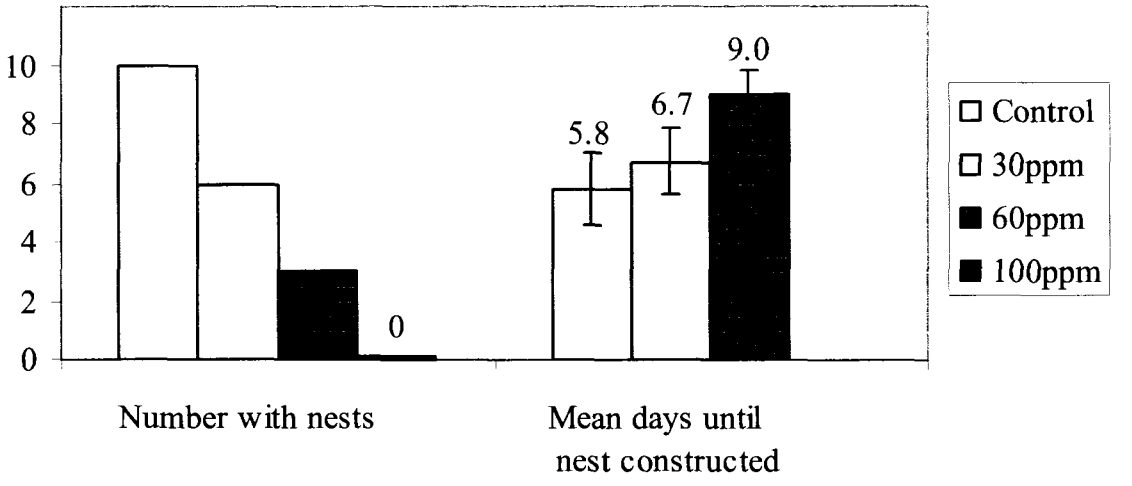

Fig. 3.6 Nest-Building Analysis. Fewer treated males produced nests in a dose-dependent manner, and those that did took longer to create their nests. Shown are means (± 1 SE).

3.9 Tables

Table 3.1 Experimental Groups and Treatment Conditions. Pedigree and treatment groups are shown for three generations of stickleback exposed to perchlorate.

Generation	Experimental group[1]	Length of exposure	Notes and developmentmental stage at exposure	Perchlorate source	Measured
$G_{0,2002}$	Control[2]	14-22 days	Wild-caught adults trapped May-June 2002[3,4]; tested during nest-building, courtship and parental care	n/a	Behavior
	1.5 ppm	↓	↓	Sodium perchlorate[5]	↓
	12 ppm	↓	↓	Sodium perchlorate	↓
	Variable 0-18.6 ppm[6]	↓	↓	Solid rocket propellant	↓
$G_{0,2003}$	Control	30 days	Wild-caught adults trapped May-June 2003[7]; Parents of $G_{1,2003}$[8]	n/a	Behavior not analyzed
	30 ppm	↓	↓	Sodium perchlorate	↓
	Variable 0-33 ppm	↓	↓	Sodium perchlorate	↓
	100 ppm	↓	↓	Sodium perchlorate	↓
$G_{1,2003}$[9]	Control	One year	Syngamy through reproductive maturity at 1 year[10]	n/a	Behavior & swimming performance
	30 ppm	↓	↓	Sodium perchlorate	↓
	Variable 0-60 ppm[11]	↓	↓	Sodium perchlorate	↓
	100 ppm	↓	↓	Sodium perchlorate	↓

[1] For all experimental groups, adults and juveniles >2 months old were fed frozen brine shrimp (*Artemia sp.*) daily, while fry <2 months old were fed daily a mixture of Golden Pearls 100, *Artemia* food (both from Aquatic Ecosystems, Apopka, FL, USA), and ground brine shrimp.

[2] No perchlorate added (less than the method detection limit of 1.1 ppb).

3 Wild-caught fish were obtained in Rabbit Slough, Alaska (61°32'12"N, 149°15'17"W) from an anadromous run.

[4] After capture, these fish were acclimated to captivity in 400 l indoor pools [4ppt (g/l) salinity] for approximately two weeks. These adults were then isolated in 38 l aquaria (also 4 ‰ salinity). Males that failed to build a nest were replaced with surplus males until every aquarium (n = 80) contained a single male with a nest.

[5] Anhydrous sodium perchlorate with a purity of 99% or greater (EM Science, Cherry Hill, NJ, USA).

[6] Perchlorate leached from dissolving 3.70 g cores of hydroxyl-terminated polybutadiene (HTPB) solid rocket propellant, a simulation of what might occur in fresh water polluted by unburned rocket propellant.

[7] After capture, the $G_{0,2003}$ fish were housed outdoors in 1600 l pools through mid-July under ambient temperature (water temperature = 14-20°C) and photoperiod (18:6 h [light:dark] increasing to 20:4 h before decreasing to 18.5:5.5). All pools were continuously filtered and aerated through biofilters. Pools in each of the four experimental groups contained water with approximately 4 ‰ salinity, filamentous algae, and sand to be used as nesting material.

[8] Nuptially colored $G_{0,2003}$ males were randomly placed into separate quadrants of the 1600 l pools (four males per pool separated by netting). Gravid $G_{0,2003}$ females were randomly introduced into a quadrant and allowed to spawn with males. Females were immediately removed after successfully spawning, or after 20 min if they failed to spawn. Females were introduced in succession until spawning occurred or until 7 d had passed if spawning was unsuccessful. Once a male had spawned, no additional females were

provided. After caring for the embryos until hatching, and then providing five additional days of parental care (generally 12 dpf), adult males were removed from the pools, euthanized, and stored at -80°C.

[9] The $G_{1,2003}$ were raised outdoors in 1600 l pools under ambient temperature (20°-8°C) and photoperiod (20:4 h [light:dark] declining to 11:13 h) through October 5, 2003. At this point (15 weeks of age) 50 fish from each of the four treatments (controls, 30, 60, and 100 ppm) were transferred indoors into 400 l pools at the same concentrations in which they had been raised outdoors. This is when the only exchange of treated water occurred. With that exception, water was only added to dilute the treatments as perchlorate became more concentrated due to evaporation and to replace water removed during cleaning. Treated water lost during cleaning was replaced with water containing the appropriate mass of perchlorate crystals to restore the proper water level and perchlorate concentration in each tank. These $G_{1,2003}$ fish were maintained in 400 l tanks under simulated natural photoperiod at 17-19°C until they reached sexual maturity the following spring.

[10] Although sexual maturity occurred at one year of age in our laboratory conditions, most stickleback from the source population reproduce at two years of age (Furin & von Hippel, unpublished data).

[11] The variable treatment was designed to mimic the 2002 treatment when perchlorate leached from HTPB solid rocket propellant. To that end, sodium perchlorate was added to the variable treatment at a rate that matched the changing concentration in 2002 until its concentration reached approximately 60 ppm, after which the concentration was held steady.

Table 3.2 Timing of Behavioural Interactions. Mean (± 1 SE) duration in seconds until onset of each male's behaviour after a female's introduction. Kruskal-Wallis (K-W) tests were used since sample sizes were small ($n \leq 10$ fish per treatment), and only biting differed significantly between groups. Individual fish means were used to calculate experimental group means. Empty cells in the 100ppm column indicate these behaviours were not performed by fish in this treatment.

	Controls	30ppm	60ppm	100ppm	Statistics (K-W)
Biting	46.0 (21.1)	54.7 (19.5)	99.2 (57.7)	165.3 (35.1)	χ^2=8.24, df=3, p=.041
Leading	134.9 (31.2)	133.6 (40.8)	133.8 (60.6)	276.1 (126.6)	χ^2=1.26, df=3, p=.747
Zig-zagging	218.5 (44.6)	167.5 (44.4)	195.5 (42.5)	318.6 (288.9)	χ^2=0.80, df=3, p=.849
Dorsal pricking	471.5 (174.8)	165.6 (93.1)	291.5 (59.6)		χ^2=1.39, df=2, p=.498
Boring	330.9 (43.5)	201.8 (55.0)	254.8 (36.5)		χ^2=3.10, df=2, p=.213
Fanning	275.7 (46.6)	248.8 (73.1)	218.7 (7.2)		χ^2=0.03, df=2, p=.984
Gluing	555.9 (125.0)	298.9 (83.9)	269.8 (242.2)		χ^2=1.74, df=2, p=.419
Creeping through	294.6 (71.6)	292.2 (116.3)	368.7 (87.1)		χ^2=1.06, df=2, p=.590
Quivering	461.9 (97.8)	394.8 (90.7)	285.5 (86.4)		χ^2=0.63, df=2, p=.730
Fertilizing	890.8 (172.7)	791.9 (298.8)	518.5 (360.6)		χ^2=0.89, df=2, p=.640

Table 3.3 2004 Benchmark Completion. Values reflect the percentage of individually housed males that succeeded at completing each benchmark metric. When the $G_{1,2003}$ matured in 2004, marked differences were apparent in their abilities to complete benchmark reproductive metrics. Control males had the greatest success for each metric, and there was a clear dose-response relationship with fish in higher treatments always performing worse than controls and those in lower treatments.

	Controls (% of 10)	30 ppm (% of 8)	60 ppm (% of 8)	100 ppm (% of 11)
Nest Building	100	75	38	0
Successful Spawning	80	50	25	0
Nursery Production	70	25	13	0
Fry Production	60	25	13	0

Table 3.4 Behavioural Repertoire of Isolated Genetic Females. Individually housed females from the 30 and 100 ppm treatments performed male-typical courtship displays when a gravid female was introduced into their aquaria. "X" indicates that a given masculine courtship behaviour was performed by the female on at least one courtship trial. "n/a" indicates that a fish could not have performed the activity because it was already dead. Head down represents the hermaphrodites that assumed a masculine head down position, and "Percent Intermediate" represents the mean percent (± 1SE) of time that each female spent conducting non-courtship-related activities. 100-4 and 100-5 were confirmed to be functionally hermaphroditic.

Fish ID	Head down	Biting	Leading	Zig-zagging	Percent "Intermediate"	Comments
30-2	X	X	X		89.6	Died after one courtship trial
30-7	X	X	X		94.2 (2.0)	Euthanized after spawning trials
60-1	n/a	n/a	n/a	n/a	n/a	Died before courting
60-3					100	Died after one courtship trial
60-4	n/a	n/a	n/a	n/a	n/a	Died before courting
100-4	X	X	X	X	99.8 (0.2)	Died after seven courtships trials
100-5	X	X	X	X	95.3 (1.9)	Euthanized after spawning trials

Chapter 4:
Chronic Perchlorate Exposure Causes Morphological Abnormalities in Developing Stickleback[1]

4.1 Abstract

Few studies have examined the effects of chronic exposure to perchlorate during growth and development, and fewer still have analyzed the effects of perchlorate over multiple generations. We describe morphological and developmental patterns for two separate generations ($G_{1,2002}$ and $G_{1,2003}$) of threespine stickleback (*Gasterosteus aculeatus*) that were spawned and raised in perchlorate treated water and a third generation ($G_{2,2004}$) that was not directly exposed to perchlorate but whose parents ($G_{1,2003}$) were raised from syngamy through sexual maturity in perchlorate treated water. Both G_1 generations displayed a variety of abnormalities, including impaired formation of calcified traits, slower growth rates, aberrant sexual development, poor survivorship, and reduced pigmentation that allowed internal organs to be visible. Yet these conditions were ameliorated when the offspring of contaminated fish ($G_{2,2004}$) were raised in untreated water, suggesting that surviving populations can recover following remediation of perchlorate-contaminated sites.

4.2 Introduction

Perchlorate is used by the National Aeronautics and Space Administration and the U.S. Department of Defense (DoD) as an oxidizer in the solid propellant of rockets, missiles, and the space shuttle, and in artillery and other explosives (Greer et al., 2002). It is also found in many common household and industrial products (Urbansky et al.,

[1]Bernhardt RR, von Hippel FA, O'Hara TM. Chronic perchlorate exposure causes morphological abnormalities in developing stickleback. *Environ. Toxicol. Chem.* (Under review).

2001; Greer et al., 2002). Natural deposits of perchlorate were previously only known to occur in sodium nitrate deposits of Chilean caliche (which is exported in Chilean fertilizers), in nitrates from Death Valley, California, and in potash deposits from Carlsbad, New Mexico (Ericksen, 1981; Ericksen, 1983; Ericksen et al., 1988; Urbansky et al., 2001), but recent methodological advances are revealing the geographically extensive occurrence of perchlorate at low levels [with notable exceptions, <4 μg/Kg (ppb)] across arid and semi-arid regions of the American Southwest (Rajagopalan et al., 2006; Rao et al., 2007). Higher levels of environmental pollution have come from industrial contamination of the Colorado River (EPA, 2002) and from military storage and disposal practices Smith et al., 2001). In 2005 perchlorate was reported in the Canadian Great Lakes, marking the first time that perchlorate contamination was identified outside the United States (Backus et al., 2005). As of March 2005, perchlorate contamination has been reported in 36 of the United States (http://www.epa.gov/fedfac/documents/known_perchlorate_releases_in_the_us_09_23_2004.xls). Perchlorate contamination has been detected in organic and conventional milk ranging from 1.3-11.3 μg/L (ppb) in 101 of 104 containers from 15 states (http://www.cfsan.fda.gov/~dms/clo4data.html), in both conventional and organic lettuce with levels ranging from 129 to 6,900 μg/kg (http://www.ewg.org/reports_content/rocketlettuce/pdf/wecklabs.pdf), and in bottled water (Snyder et al., 2005; http://www.cfsan.fda.gov/~dms/clo4data.html). Perchlorate has been found in tap (>4 μg/L) and irrigation (>4 μg/L) water derived from the Colorado River (Lamm & Doemland, 1999; Brechner et al., 2000; http://www.epa.gov/safewater/ucmr.html) and in human breast milk ranging from 0.6 – 92.2 μg/L (mean = 10.5 μg/L) Kirk et al., 2005).

Perchlorate is highly soluble in water (Motzer, 2001), typically remains unaltered for several decades in solution (Urbansky, 1998; Flowers & Hunt, 2000), can persist for millennia under certain conditions (Plummer et al., 2006) causes deleterious effects at low levels (Goleman et al., 2001; Goleman et al., 2002; Baldridge et al., 2004), and remains unregulated while the U.S. Environmental Protection Agency (EPA) attempts to

establish maximum exposure levels. The EPA's most recent (2005) reference dose is 24.5 μg/L (http://www.epa.gov/iris/subst/1007.htm#reforal).

Many acute toxicity studies have been conducted on a variety of vertebrates, showing that environmentally-relevant concentrations of perchlorate (i.e., levels found in ground and surface water) affect the vertebrate thyroid gland and alter circulating thyroid hormone (TH) levels. Perchlorate delivered via water has been shown to reduce plasma TH concentrations among fish (Patiño et al., 2003; Crane et al., 2005), amphibians (Miranda et al., 1996; Goleman et al., 2002), birds (McNabb et al., 2004), and mammals (Yu et al., 2002; Choksi et al., 2003; Baldridge et al., 2004), including humans (Choksi et al., 2003). Altered thyroid $^{125}I^-$ uptake rates have been observed in as little as 11 hours after oral administration of perchlorate to Sprague-Dawley rats (*Rattus norvegicus*) (Yu et al., 2002). It disrupts the thyroid cascade by competitively inhibiting iodide uptake (Yu et al., 2002) and has been reported to stimulate the discharge of inorganic iodine from the thyroid gland by an as yet unidentified mechanism (Stanbury & Wyngaarden, 1952; Wolff, 1998; Brabant et al., 1992; Smith et al., 2001; Urbansky et al., 2001; http://www.epa.gov/safewater/ucmr.html). These conditions can lead to the production of insufficient levels of THs [triiodothyronine (T_3) and thyroxine (T_4)]. This in turn, typically leads to the up-regulation of thyroid stimulating hormone (TSH) by the anterior pituitary that can result in thyroid hypertrophy, hyperplasia, angiogenesis, colloid depletion, and goiter (York et al., 2001; Yu et al., 2002; Patiño et al., 2003; Bradford et al., 2005; Crane et al., 2005). Chronic thyroid hypertrophy can lead to hyperplasia, which increases the likelihood that organisms will develop tumors (Premdas & Eales, 1976; Capen, 2001).

Proper thyroid hormone levels are also essential for proper development since they mediate a wide range of developmental processes. In fishes, several physiological processes are believed to be at least partially under the influence of thyroid hormones, including: metamorphosis; formation of the gastric organ (stomach); morphogenesis; temperature tolerance; skeletal and somatic growth; muscular development; calcification;

locomotor activity; behavioral activity (including migration and reproduction); osmoregulation; lipid, carbohydrate, protein, and vitamin metabolism; smoltification; ovarian maturation and oogenesis; gonadal recrudescence; reproduction; integumentary silvering and melanophore function (La Roche et al., 1966; Premdas & Eales, 1976; Hurlburt, 1977; Leatherland, 1982; Smith, 1982; Cyr & Eales, 1996; Koumoundouros et al., 1997; Manzon & Youson, 1997; Bentley, 1998; Castonguay & Cyr, 1998; Janz & Weber, 2000; Power et al., 2001; Brown et al., 2004; Crane et al., 2005; Blanton & Specker, 2007). Goleman et al. (2001) demonstrated that perchlorate exposure altered sex ratios in post-metamorphosed *Xenopus laevis* with a bias toward females. Mukhi et al. (2007) demonstrated that administration of perchlorate to zebrafish (*Danio rerio)* during larval-juvenile development produced a female-biased sex ratio at 43 days post-fertilization (dpf), and perchlorate exposure with supplemental thyroid hormone administration produced a male-biased sex ratio. This finding demonstrates that altered thyroid hormone levels play a key role in the sexual development of teleosts. However, Bernhardt et al. (2006) demonstrated that chronic perchlorate exposure masculanized genotypic female threespine stickleback (*Gasterosteus aculeatus*) to the point that they produced both functional sperm and eggs.

The physiological responses of a variety of fish species have been analyzed following perchlorate exposure, including fathead minnows (*Pimephales promelas*; Crane et al., 2005), zebrafish (Patiño et al., 2003; Mukhi & Patiño, 2007), and Eastern mosquitofish (*Gambusia holbrooki*; Bradford et al., 2005; Park et al., 2006). These studies illustrate that fish respond in a "typical" manner to perchlorate exposure (i.e., all species have displayed thyroid follicular hypertrophy, hyperplasia, and altered thyroid hormone levels). Therefore, we assume that similar effects occur in threespine stickleback exposed to perchlorate and have chosen to examine whether endpoints typically mediated by thyroid hormones are affected by chronic perchlorate exposure. We employ the threespine stickleback as our model organism to test the hypothesis that developmental abnormalities become more prevalent and more severe as the concentration of perchlorate exposure increases. We also test for transgenerational

effects by analyzing whether stickleback raised in water lacking detectable concentrations of perchlorate (<1.1 μg/L) are affected by parental exposure.

4.3 Materials and Methods

4.3.1 Methodology Overview

To determine how exposure to sublethal concentrations of perchlorate [sodium perchlorate; Sigma Aldrich Batch: 12802 TA, and ammonium perchlorate dissolving from 3.70 g cores of hydroxyl-terminated polybutadiene (HTPB) propellant; Air Force Research Laboratory, Edwards Air Force Base, California] affects growth and development, we conducted a series of experiments on the threespine stickleback between 2002 and 2004. Two separate cohorts of sexually mature threespine stickleback ($G_{0,2002}$ and $G_{0,2003}$) were captured from Rabbit Slough, Alaska (61°32'12"N, 149°15'17"W) as they returned to spawn (see below). These fish were exposed to perchlorate for approximately three weeks while they spawned and tended their young. Their offspring ($G_{1,2002}$ and $G_{1,2003}$) were raised in control [< the method detection limits (MDL) of 1.1 μg perchlorate/L] or perchlorate-treated water for approximately six and four months, respectively, prior to euthanasia and morphometric analysis.

A subset of the $G_{1,2003}$ was raised to sexual maturity at one year of age in control (<1.1 μg/L) or perchlorate-treated water, and their standard lengths (SL), pectoral fin lengths, and body pigmentation were reanalyzed. Upon reaching sexual maturity, these fish were allowed to reproduce in 38 L aquaria containing water without detectable levels of perchlorate (<1.1 μg/L; one male per aquarium). Therefore, gametogenesis for the $G_{1,2003}$ occurred in perchlorate-treated water, but oviposition and fertilization occurred in untreated water. The resulting $G_{2,2004}$ fish were maintained in water without detectable levels of perchlorate until 25 weeks of age (from ~8 June – 28 December, 2004), at which time they were euthanized and stored at -80°C until their morphology could be analyzed for evidence of transgenerational effects.

4.3.2 Animals and Husbandry

Sexually mature, anadromous threespine stickleback return to Rabbit Slough, Alaska every spring between May and July. Wild-caught adults from this population were trapped as they returned to spawn in 2002 and 2003 and provided the brood stock from which all of the offspring in this study were derived. These fish were housed in 400 L pools, 1600 L pools and/or 38 L aquaria containing fortified tap water (3-4 g/L Instant Ocean sea salt) that was continuously filtered and aerated through Azoo biofilters (Aquatic Ecosystems, Apopka, FL, USA). All 38 L aquaria contained one 65mm biofilter, nesting material, and a sandy substrate; 400 L and 1600 L pools each contained two 150mm biofilters.

Adult fish were acclimated to captivity in water lacking detectable levels of perchlorate (<1.1 μg/L) for approximately three weeks before being added to perchlorate treated water. Daily observations were made throughout the experiment for viability and behavioral abnormalities. Dead and moribund fish were removed and recorded upon detection. Temperature and perchlorate concentrations were monitored daily using an Acorn 6 potentiometer (Oakton, Vernon Hills, IL, USA) with perchlorate ion-sensitive electrode (Cole-Parmer, Vernon Hills, IL, USA). Dissolved oxygen, pH, and salinity were checked every two to three months. After the perchlorate treatments were established in 2002 the water was not exchanged. In 2003, the only exchange of treated water occurred on 5 October, 2003 (experimental day 120) when a subset of the $G_{1,2003}$ fish were moved indoors and placed in 400 L tanks at the same perchlorate concentrations as they had been raised outdoors. On 21 May 2004 (experimental day 349), $G_{1,2003}$ males were removed from their treated water and isolated in individual 38 L aquaria containing fortified tap water (2 g/L Instant Ocean without perchlorate added) in order to produce the $G_{2,2004}$ fish.

Adults and juveniles (>2 months old) were fed frozen brine shrimp daily (Brine Shrimp Direct, Ogden, UT, USA), while fry (<2 months old) were fed a mixture of

Golden Pearls 100, Artemia food (both from Aquatic Ecosystems), and ground brine shrimp. During cleaning, water was siphoned into 19 L buckets at approximately 3 week intervals in order to remove debris and uneaten food from the bottom of the tanks. After the debris settled to the bottom of the buckets, the perchlorate-treated water lacking debris was returned to the tanks. Perchlorate-treated water lost during routine aquarium maintenance was replaced with fresh perchlorate-treated water to maintain the desired perchlorate concentrations. With those exceptions, water was only added to the treatments to offset evaporative water loss.

4.3.3 2002 Experimental Groups

In 2002, all husbandry and experiments were conducted indoors in 38 L aquaria under simulated natural photoperiod [18:6 h (light:dark) increasing to 20:4 h and then decreasing to 18.5:5.5]. Four experimental groups were distributed among 80 38 L aquaria, consisting of: "negative controls" (< the method detection limits of 1.1 μg/L; 20 replicates), and nominal perchlorate concentrations of 1.5 mg/L, 12.0 mg/L, and a variable$_{(0\text{-}66mg/L)}$ HTPB treatment, which contained 3.70 g cores of Hydroxyl-terminated polybutadiene (HTPB) solid rocket propellant from which ammonium perchlorate leached out over time (20 replicates each). The perchlorate concentration of the variable$_{(0\text{-}66mg/L)}$ HTPB treatment gradually increased from <MDL on experimental day one to approximately 18.6 mg/L between experimental days 14-22 when the adult males ($G_{0,2002}$) were euthanized. At this time, each male had completed five days of fry guarding. The perchlorate concentration continued to rise to a maximum of 66.2 mg/L before the $G_{1,2002}$ were euthanized on 22 December 2002 (experimental day 201) at approximately 6 months of age. This treatment was referred to as the variable$_{(0\text{-}66\ mg/L)}$ HTPB treatment and simulated the conditions that might occur if chunks of solid rocket propellant fell into isolated water bodies following a failed launch attempt.

4.3.4 2003 Experimental Groups

Because overall survivorship was poor in 2002, we repeated the morphological analysis in 2003 with additional perchlorate concentrations. The concentrations in 2003 were chosen to bracket those used in 2002. Wild-caught adult males ($G_{0,2003}$) in six experimental groups were distributed among 15 1600 L outdoor pools, and each pool was partitioned into four quadrants with one adult male per quadrant. The six experimental groups consisted of controls (<MDL of 1.1 µg/L; three pools/12 quadrants), and nominal perchlorate concentrations of 3.6 mg/L (three pools/12 quadrants), variable$_{(0\text{-}4.5)}$ mg/L (one pool/four quadrants), 30 mg/L (three pools/12 quadrants), variable$_{(0\text{-}60mg/L)}$ (three pools/12 quadrants), and 100 mg/L (two pools/eight quadrants). The experiment took place under ambient photoperiodic (same as described for $G_{1,2002}$, above) and temperature (14-20°C) conditions through mid July.

In 2003, sodium perchlorate was gradually added to the variable$_{(0\text{-}60mg/L)}$ experimental group to mimic the increasing concentrations recorded in 2002 as perchlorate leached from the 3.70 g cores of HTPB solid rocket propellant. This pseudo-replicate was designed to determine whether the dramatic effects noted among the $G_{1,2002}$ variable$_{(0\text{-}66\ mg/L)}$ HTPB treatment were likely due to perchlorate itself or to the binding matrix in HTPB cores, or some combination. The concentration in the variable$_{(0\text{-}60mg/L)}$ treatment increased from <MDL on 1 June 2003 to 60 mg/L by 14 October 2003 (experimental day 129). The fifteenth pool contained the partially spent HTPB cores from the 2002 trials, causing its perchlorate concentration to increase from undetectable levels on 1 June 2003 to 4.5 mg/L by 22 August 2003 (experimental day 76) as ammonium perchlorate continued to leach from the cores.

4.3.5 $G_{1,2003}$ Husbandry

After providing five days of fry guarding (generally 11-12 days post-fertilization), adult $G_{0,2003}$ males were removed from their pools and euthanized. Their offspring

($G_{1,2003}$) remained outdoors in their treatment groups under ambient photoperiod [20:4 h (light:dark) declining to 11:13 h] and temperature (20°C declining to 8°C) through 5 October 2003. When the $G_{1,2003}$ reached 15 weeks of age, only 200 fish from four experimental groups could be brought indoors due to space limitations. Fifty fish each from the control group and the 30, variable$_{(0\text{-}60)}$, and 100 mg/L groups were selected. These fish provided an opportunity to assess survival rates while being raised to sexual maturity. They continued to be maintained under simulated natural photoperiod throughout the winter but were kept between 17 and 19°C until they reached sexual maturity the following spring. Their offspring ($G_{2,2004}$) were used to assess potential transgenerational effects from parental perchlorate exposure.

4.3.6 Morphological Analysis

By comparing the absolute difference in lengths of bilateral characters in 2002, fluctuating asymmetry was analyzed. In all years, bilateral characters were summed to produce a total length for each character, which then was treated as a single character. This allowed a maximum of 39 comparisons to be analyzed between treatments (Fig. 4.1 and Table 4.1). Fluctuating asymmetry was not analyzed in 2003 or 2004, leaving 29 and 18 comparisons to be made in these respective years. Differences in the number of characters analyzed per year reflect streamlining based on observations made during previous years. In addition to analyzing the characters shown in Fig. 4.1, a fish was considered to have a completely armored ring if the pelvic girdle, anterior lateral plates, and second dorsal spine complex formed an overlapping calcified ring entirely around the fish. A fish was considered to be transparent when vertebrae, ribs, internal tissues or organs were externally visible. Total pelvic score was determined by adding one point for each bilateral component of the pelvic girdle, including the two ascending processes, two anterior processes, two posterior processes, and the two pelvic spines (Bell et al., 1993). Therefore, total pelvic scores could range from zero to eight.

Characters were divided into functional groups. In 2002, these functional groups included miscellaneous traits (i.e. standard length, body mass, body depth, body width, and sum of orbit lengths), calcified traits (i.e., first, second, and third dorsal spines, pelvic girdle length, sum of keel lengths, sum of ascending branch lengths, sum of anterior lateral plate lengths, sum of opercular lengths, sum of opercular depths, sum of pelvic spine lengths, sum of anal plate lengths, total number of lateral plates, total number of keel plates, and total pelvic score), fin related characters (i.e., dorsal fin length, anal fin length, sum of pectoral fin length, number of dorsal fin rays, number of anal fin rays, and total number of pectoral fin rays), traits analyzed for fluctuating asymmetry as indicated by differences between the left and right sides of each individual (i.e. number of lateral plates, the number of pectoral fin rays, keel lengths, the number of keel plates, pectoral fin lengths, ascending branch lengths, anterior lateral plate lengths, opercular lengths, opercular depths, orbit lengths, pelvic spine lengths, and anal plate lengths), and dichotomous traits (i.e. transparent flesh and completely developed armored ring).

Functional groupings were identical in 2003, except that fluctuating asymmetry was not analyzed, anal spine length was added to the group of calcified characters, and dorsal fin height was added to the group of fin-related characters. In 2004, functional groups and their characters included miscellaneous traits (i.e. standard length and body depth), calcified traits (i.e. first dorsal spine, second dorsal spine, third dorsal spine, pelvic girdle length, sum ascending branch length, sum anterior lateral plate length, sum pelvic spine length, sum anal plate length, total number of lateral plates, total number of keel plates, and total pelvic score), fin related characters (i.e. dorsal fin length, sum pectoral fin length, and number of dorsal fin rays), and dichotomous traits (i.e. transparent flesh and completely formed armored ring). The proportion of significant characters within functional groups in Tables 4.1, 4.2, and 4.3 was used to determine if a functional group was affected since several relatively high p-values are often stronger evidence against the null hypothesis than one moderately low value (Garcia, 2004).

In addition to making comparisons of untransformed measurements and the occurrence or outright loss of characters, all continuous measurements (i.e. those involving lengths or mass) were transformed to reveal how large a character would have been if all fish grew to be the same SL, using the regression method described by von Hippel and Weigner (von Hippel & Weigner, 2004). In short, the mean SL for all fish (i.e. global standard mean length) was determined; regressions were performed between SL and each of the continuous morphometric characters that were measured on each fish by experimental group; residuals for each individual were isolated for each character; the character size plus or minus the residual was scaled along treatment-specific regression lines to reveal how large the character would have been if all fish grew to the same SL (i.e. to the global standard mean length); the residuals were then added to the scaled character and compared between treatments. This technique minimizes differences in character lengths that are the result of differences in SL and is conceptually similar to an ANCOVA, except that it lacks a covariate and accommodates heterogeneous slopes by adjusting each character along treatment-specific regression lines to the global standard mean length before making comparisons across experimental groups.

4.3.7 2003 Condition Indices

Condition indices for every surviving $G_{1,2003}$ fish were periodically assessed. These included SL measurements, transparency, gravidity, proper swimming behavior, the presence of fungal infections, and survivorship within treatments. Swimming ability was assessed daily during feeding. Dead and moribund fish were removed upon detection and photo censuses of the $G_{1,2003}$ fish were conducted monthly. The SL of each $G_{1,2003}$ fish was measured five times between 30 August 2003 (experimental day 84) and 10 June 2004 (experimental day 369). On each occasion, all surviving $G_{1,2003}$ fish were removed from their tanks, measured with Fowler Sylvac digital calipers (Model S 235 PAT; Newton, MA, USA), and placed in separate 19 L containers with the same

concentration of perchlorate from which they came. After all fish from a single treatment had been measured they were returned to their original pools.

4.3.8 Statistical Analyses

Prior to conducting statistical comparisons, homogeneity of variances was analyzed using Levene's tests, and data normalcy was tested using Kolmogorov-Smirnov tests. Kruskal Wallis tests were used when violations to normalcy, homogeneity of variances, or low sample sizes ($n < 20$) occurred with continuous data. Frequency data, such as the percent of transparent fish and the percent with complete armored ring development were compared using Chi squared tests. When data were normally distributed with homogeneous variances and sample sizes were ≥ 20, data were analyzed using analysis of variance (ANOVA) tests. Bonferroni multiple comparison tests revealed which groups differed when ANOVAs indicated overall significance, and Dunnett's tests followed significant Kruskall-Wallis tests. An analysis of covariance (ANCOVA) was used to determine the significance of perchlorate concentration (factor) and fish density (covariate) on growth (dependent variable) of the $G_{1,2003}$. Pearson correlation coefficient matrices were made for each year to determine the degree of correlation between each character. SPSS v. 15.0 was used for all statistical procedures, and all tests were two-tailed.

To reduce the likelihood of committing type I errors, α values were adjusted downward to equal the probability of having one false positive in the number of annual tests plus one. This is a more conservative approach than using an α value of .05 but less restrictive than using a Bonferroni correction, which would inflate the likelihood of committing type II errors to an unacceptable level. For example, the greatest number of statistical tests ($n = 39$) were run in 2002, so an α of .025 (i.e. 1 / 40 = .025) was deemed to be the most appropriate measure of significance in 2002. Likewise, 29 tests were run in 2003, and a significance value of .038 (i.e. 1/30 = .038) was adopted. A similar

correction in 2004 would have caused the α value to rise above .05; therefore the α value was not adjusted.

4.4 Results

4.4.1 Perchlorate Measurements

Analysis of the negative control water was conducted by ion chromatography in tandem with electrospray ionization mass spectrometry (n = 21 samples). Spectrophotometric analysis revealed that the level of background perchlorate in untreated tap water used in all experimental groups was less than the limits of detection (<1.1ppb; Dodds et al., 2004). The mean perchlorate concentration of the negative control water based on the 18 Acorn 6 readings was 0.15 mg/L (SE 0.01), yet this value is less than the lowest calibration standard used (1 mg/L) and is less reliable than the spectrophotometric analysis. The results of the daily perchlorate readings from the Acorn 6 perchlorate potentiometer show that the nominal 1.5 mg/L treatment from 2002 had a mean perchlorate concentration of 1.5 mg/L (n = 55, SE = .02) while the nominal 12.0 mg/L treatment had a mean perchlorate concentration of 12.0 mg/L (n = 55, SE = .13). The perchlorate concentration of the nominal variable$_{(0\text{-}66mg/L)}$ HTPB treatment progressively increased (y_{2002}=0.32x + 12.04, r^2=0.92) from <1.1 µg/L on experimental day zero to a maximum concentration near 66 mg/L on experimental day 108 (20 September 2002). Beyond day 108, the readings remained relatively stable (mean = 61.80 mg/L, n=185, SE=0.19) through the conclusion of the experiment on experimental day 170 (21 November 2002).

In 2003, the Acorn 6 potentiometer showed that the control treatment had a mean perchlorate concentration of 0.16 mg/L (n = 14, SE = 0.01); the nominal 1.6, variable$_{0\text{-}4.5}$, 30, and 100 mg/L treatments had mean perchlorate concentrations of 1.6 mg/L (n = 85, SE = 0.05), 2.2 mg/L (n = 85, SE = 0.08), 32.0 mg/L (n = 334, SE = 0.20), and 102.9 mg/L (n = 334, SE = 0.57), respectively. In 2003, the nominal variable$_{(0\text{-}60mg/L)}$ treatment, which mimicked the increasing perchlorate concentration noted in 2002, reached a

maximum concentration of 71.8 mg/L on experimental day 179 (3 December 2003) with a mean perchlorate concentration of 51.12 mg/L (n=324 readings, SE = ±1.00 mg/L). After day 179, the readings remained relatively stable ($mean_{2003}$ = 61.8 mg/L, n = 185, SE = 0.19). The rates of increase between 2002 and 2003 were similar ($y_{2002} = 0.32x - 12.04$, $r^2 = 0.92$ vs. $y_{2003} = 0.31x - 9.44$, $r^2 = 0.88$).

4.4.2 Untransformed Morphometric Results

In 2002, fish raised from syngamy through 5.5 months of age in negative control water (<MDL of 1.1 µg/L), 1.5 mg/L, or 12.0 mg/L treatments showed significant developmental differences in 9 of 39 morphological characteristics (Table 4.1). It is noteworthy, however, that the two surviving fish from the $variable_{(0\text{-}66\ mg/L)}$ HTPB treatment were excluded from these statistical analyses. They are, however, shown in Fig. 4.2 to provide an indication of their developmental patterns, which were frequently outside the range noted among the other treatments. For example, both fish from the $variable_{(0\text{-}66\ mg/L)}$ HTPB treatment were the only transparent fish; they were the only fish to lack keel plates; they had fewest and smallest lateral plates, and both lacked a completely formed armored ring.

High mortality rates in 2002 resulted in few survivors and reduced our ability to draw definitive conclusions. Therefore, the experiments of 2003 repeated and expanded upon the 2002 experiments with larger sample sizes. In 2003, fish raised from syngamy to 15 weeks of age showed highly significant statistical differences ($p<.0001$) in developmental patterns for 27 of 29 morphological characters (Fig. 4.3 and Table 4.2). The dose-dependent relationship noted in 2002 was replicated in 2003 as a greater proportion of fish from higher treatments displayed transparency, incomplete keel plate development, fewer and smaller lateral plates, incomplete armored ring formation, and smaller fins and spines (Tables 4.1 and 4.2 and Fig. 4.2 and 4.3).

The $G_{2,2004}$ that were produced as a result of *in vitro* fertilizations during fertility testing (Bernhardt and von Hippel, 2008) were combined with those that were produced

via natural spawning and were maintained in water without detectable levels of perchlorate (<1.1 μg/L) to test for transgenerational effects. Early mortality reduced the surviving $G_{2,2004}$ sample sizes, and by the time they were euthanized in December, 2004 only 32, 20, and 7 of the $G_{2,2004}$ remained from the control, 30, and variable$_{(0\text{-}60)}$ mg/L experimental groups (i.e., their parent's treatment groups), respectively. From these, 20, 20, and 7 $G_{2,2004}$ progeny from the control, 30, and variable$_{(0\text{-}60)}$ mg/L experimental groups, respectively, were tested for morphological differences.

The $G_{2,2004}$, all unexposed to perchlorate, showed growth patterns typical of fish being raised at different densities, but atypical of stickleback affected by perchlorate. Statistically, the progeny of fish exposed to control water and 30 mg/L perchlorate did not differ from each other (Fig. 4.4 and Table 4.3), but both groups were generally smaller than the unexposed $G_{2,2004}$ progeny of fish that had been raised in the variable$_{(0\text{-}60)}$ mg/L treatment (Fig. 4.4). Although 4 of 18 characters were significantly different between the $G_{2,2004}$ groups, the progeny of fish from the variable$_{(0\text{-}60)}$ mg/L group were larger than the progeny of fish exposed to control and 30 mg/L perchlorate. None of these five characters showed highly significant differences ($p < .001$) between experimental groups (Table 4.3). None of the $G_{2,2004}$ fish showed incomplete calcification, transparency, or impaired developmental patterns, either. However, one anomalous developmental event occurred on one of the $G_{2,2004}$ progeny from the variable$_{(0\text{-}60)}$ mg/L group. This fish developed a fourth dorsal spine (Fig. 4.5).

Since multiple characters were measured on each individual every year, variations in most character measurements showed significant intra-annual correlations within functional groups (Tables 4.1, 4.2, and 4.3), as would be expected. In fact, only two characters (total number of pectoral fin rays and total pelvic score) were not significantly correlated (all p's > .05) with more than 25% of the other characters across functional groups. However, comparisons were made between treatments, not between characters within treatments, so correlations between the remaining characters do not violate

assumptions of independency. Moreover, autocorrelation between characters did not translate into correlated significant findings (Tables 4.1 and 4.3).

4.4.3 Standardized Morphometric Results

For $G_{1,2002}$ fish, after the continuous morphometric characters were transformed to remove effects of differences in SL, 7 of 32 characters showed statistically significant differences between treatments (Table 4.1). As was the case with the "untransformed" morphometric analysis, the two surviving fish in the variable$_{(0\text{-}66\ mg/L)}$ HTPB treatment were excluded from these statistical analyses. After removing the effects of different standard lengths from the 2003 treatments, statistically significant differences were found in developmental patterns for 24 of 25 continuous characters; all but two of these 24 characters showed highly significant differences ($p<.0001$; Tables 4.1, 4.2, and 4.3).

4.4.4 Growth Rates

When the $G_{1,2002}$ were measured in November 2002, there were significant mass differences between fish of different experimental groups (Kruskal-Wallis, $\chi^2 = 8.0$, df = 3, $p = .046$). The control fish were significantly larger than the 1.5 mg/L fish (Dunnett's t-test, $p = .041$) and the 12.0 mg/L fish (Dunnett's t-test, $p = .049$). More detailed analysis of growth rates occurred during the 2003 experiments (Fig. 4.6). Control fish reached their maximum SL faster than treated fish, but all fish ultimately reached similar maximum standard lengths (ANOVA, $F_{3,100} = 1.418$, $p = .242$).

4.4.5 Density Effects

To elucidate whether perchlorate concentration or fish density has a stronger effect on growth, comparisons were made between fish raised at different densities and perchlorate concentrations. Prior to conducting the ANCOVA, preliminary analysis evaluating the homogeneity-of-slopes assumption indicated that the relationship between

density (covariate) and SL (dependent variable) differed significantly as a function of the perchlorate concentration (independent variable; $F_{3,224} = 59.67$, $MSE = 451.43$, $p < .0001$, partial $\eta^2 = .444$). Furthermore, Levene's homogeneity of variances testing demonstrated that variances were not homogenous ($F_{5,228} = 4.792$, $p < .001$). Nevertheless, the ANCOVA was highly significant ($F_{5,227} = 36.89$, $MSE = 495.46$, $p < .0001$). The strength of the relationship between the perchlorate concentration and SL was strong, as assessed by a partial η^2, with perchlorate concentration accounting for 45% of the variance in SL when density of the stickleback was held constant at 253 fish per pool. Since both ANCOVA assumptions were violated, the statistical results were interpreted as being "suggestive." However, examination of the four instances when $G_{1,2003}$ fish were raised in lower perchlorate concentrations and higher densities provided further evidence of perchlorate's role in growth inhibition. In every instance, fish maintained at higher density and lower perchlorate concentration grew to be larger than those raised in higher perchlorate treatments at lower densities (Table 4.4). These results support the assessment that the concentration of perchlorate had a greater effect on stickleback growth than did fish density, within the respective ranges of the present study.

4.4.6 Gross Developmental Abnormalities

Several gross developmental abnormalities were noted among perchlorate treated fish (Fig. 4.5). Ventral and dorsal bulges developed on 3%, 12%, and 42% of fish exposed to 30, variable$_{(0\text{-}60)}$, and 100 mg/L, respectively, but were absent from control fish. Exopthalmia (bulging eyes) was noted in treatments ≥ 30mg/L. All fish exposed to 12.0 mg/L or greater either lacked lateral plates, or their lateral plates were abnormally small and poorly calcified. Control fish had 9-10 keel plates on each side of their caudal peduncles, while 50% of 30 mg/L fish, 82% of variable$_{(0\text{-}60)}$ mg/L fish, and 95% of 100 mg/L fish completely lacked these plates. This is despite the fact that their wild-caught parents had opaque flesh, complete lateral plate development, and a full complement of keel plates.

Subsamples of 27-36 $G_{1,2003}$ fish per treatment were analyzed for transparency at 35 weeks of age (experimental day 262). None of the 36 control fish were transparent, but four of 29 (13.8%), 19 of 25 (76%), and 24 of 26 (92.3%) of the 30, variable$_{(0\text{-}60)}$, and 100mg/L fish, respectively, were transparent. In fact, many treated fish remained transparent at sexual maturity, allowing vertebrae, air sacs, digestive tracts, kidneys, and ribs to be seen externally (Fig. 4.6). Treated fish failed to produce normal nuptial coloration (Bernhardt et al., 2006).

4.5 Discussion

Both generations of stickleback ($G_{1,2002}$ and $G_{1,2003}$) that were raised in perchlorate-treated water displayed abnormalities that increased in frequency and severity in a dose-dependent manner (Fig. 4.2, 4.3, and 4.5). The $G_{2,2004}$ stickleback, which were not directly exposed to perchlorate but were the offspring of perchlorate-treated parents (excluding control fish), did not show endpoints characteristic of perchlorate exposure, suggesting a lack of perchlorate-related transgenerational effects (Fig. 4.4). However, one of the seven $G_{2,2004}$ progeny of fish from the variable$_{(0\text{-}60\ mg/L)}$ experimental group developed an extra dorsal spine (Fig. 4.5), which is a rare event. Whether this was a perchlorate-related transgenerational effect or a random mutation is unclear, but we did not observe this abnormality on any other fish, nor have we observed a fourth dorsal spine on any of the thousands of wild-caught stickleback that we have analyzed from the source population (Rabbit Slough, Alaska).

Size differences by experimental group were apparent among the $G_{2,2004}$ (Fig. 4.4), but these did not appear to be perchlorate-related transgenerational effects ($G_{1,2003}$) since the progeny of fish in the variable$_{(0\text{-}60)}$ mg/L experimental group were larger than the offspring of the other groups. Instead, these effects were likely the result of different fish densities among the $G_{2,2004}$ experimental groups, since fish raised at lower densities grew faster and larger than those at higher densities. The progeny of fish exposed to 30 mg/L perchlorate are statistically similar to control fish, and the progeny of fish in the

variable$_{(0\text{-}60)}$ mg/L treatment were larger than both, which is contrary to what would have been expected if parental perchlorate exposure imposed dose-dependent transgenerational effects.

Fish in higher perchlorate treatments experienced higher mortality (Bernhardt et al., 2006), and were therefore raised at lower densities between 15 and 52 weeks of age. Fish maintained at lower densities typically grow larger and/or faster than those raised at higher densities (Ewing & Ewing, 1995; Rose et al., 2001; Ellis et al., 2002; Bolasina et al., 2006; Huntingford et al., 2006) even, as in our study, when food is not a limiting factor (Rose et al., 2001). The present study suggests that the effects of density on growth may be less important than the perchlorate concentration in which they were maintained. Specifically, stickleback maintained at higher densities and lower perchlorate concentrations were larger than those raised at lower densities and higher perchlorate concentrations (Table 4.4). Though assumptions were violated, our ANCOVA analysis also suggests that perchlorate reduced growth when the effects of density were held constant. These two analytical methods both imply that perchlorate may have been a more important determinant of growth than density. However, an interaction study specifically designed to analyze these factors would be necessary to definitively tease these apart.

Analyses of size-adjusted data demonstrated that perchlorate also affected the relative size of characters (Tables 4.1, 4.2, and 4.3). Therefore, size differences for many characters were not due solely to fish from higher perchlorate concentrations being smaller. Rather, perchlorate exposure caused the relative size of many traits to be proportionally smaller.

Overall our findings demonstrate that fish raised in higher perchlorate treatments showed impaired development, slower growth, and smaller characters, with the exception of the $G_{1,2003}$ 3.7 mg/L fish, which were larger than expected (Fig. 4.2 and 4.3 and Table 4.4). Several characters showed evidence of reduced calcium deposition, and nearly every measured characteristic on fish from higher treatments was smaller than on fish from

lower treatments or controls. Many characters were absent or had incomplete expression among fish from higher treatments. Thus, the trends noted among the few surviving $G_{1,2002}$ were reinforced by the $G_{1,2003}$ testing. These findings reflect what have become "typical" dose-related trends for animals exposed to perchlorate during development. Crane et al.'s (2005) 10 and 100 mg/L perchlorate-treated fathead minnows (*Pimephales promelas*) also had significantly lower wet body mass and standard lengths compared to controls and those exposed to 1 mg/L perchlorate. Goleman et al. (2002) found that growing *Xenopus laevis* exposed to 425 mg/L ammonium perchlorate had a reduced snout to vent length after only 16 days of exposure. However, evidence of hormesis, an overcompensation to insult resulting in overdevelopment or overexpression, has occasionally been reported among organisms exposed to approximately 1 mg/L perchlorate (Siglin et al., 2000; McNabb et al., 2004; Crane et al., 2005; Mukhi et al., 2007). Therefore, we cannot rule out the possibility that the larger $G_{1,2003}$ exposed to 3.7 mg/L perchlorate displayed a hormetic effect, but their lower density almost certainly contributed to their enhanced growth.

Another prominent trend in the present study was for calcified traits to be underdeveloped among perchlorate-treated fish (Fig. 4.5 and Tables 4.1, 4.2, and 4.3). Dose-dependent effects were revealed with fish in higher treatments having smaller (or missing) calcified traits (Tables 4.1, 4.2, and 4.3). Reduced calcium deposition is known to be associated with chronic hypothyroidism among fishes (La Roche et al., 1966). Likewise, Crane et al. (2005) found that fathead minnows exposed to 10 and 100 mg/L ammonium perchlorate for 28 days had smaller scales than controls or those exposed to 1 mg/L. Srivastava (1960a, b) showed that thyroxine administration stimulates phosphate uptake from aquarium water, which may reflect an intensification of calcification and/or protein synthesis.

Robust calcification of characters such as lateral plates, spines, and the pelvic girdle on threespine stickleback reduce the risk of predation and may help in the acquisition and maintenance of breeding territories (Wootton, 1976). A robust armored

ring (including anterior lateral plates, pelvic girdle, pelvic spines, and dorsal spines) poses a formidable defensive barrier to gape-limited predators, such as many piscivorous fish (Reimchen, 1994).

Previous experiments have shown that both pectoral fin and lateral plate formation are linked with swimming performance in stickleback (Schlichting, 1960; Webb, 1982). In fact, Bergstrom (2002) used freshwater stickleback to demonstrate that the number of lateral plates was negatively correlated with swimming velocity and attributed these findings, in part, to less drag among fish with fewer lateral plates. Thus, when all else is equal, one might expect to find enhanced swimming performance among stickleback with fewer and smaller lateral plates. However, stickleback exposed to higher perchlorate concentrations generally performed worse in swimming trials than those exposed to a lower perchlorate concentration or controls (Bernhardt & von Hippel, 2008). These findings suggest that lateral plate reductions are not linked to the impaired swimming performance noted among perchlorate treated fish. Poor swimming performance may have been associated with abnormalities in their shape (Fig. 4.5), sub-optimal energy metabolism or muscular formation associated with altered TH levels (Marieb, 2004), or even reduced size of the pectoral fins (Table 4.3).

When Taylor and McPhail (1985) compared resident, freshwater stickleback with smaller pectoral fins to anadromous stickleback with larger fins, they found that the resident freshwater morphotypes were only able to maintain swimming speeds of 5 mbl/s for between 12 and 24 minutes before failing to resist the current. Longer pectoral fins provide more thrust per stroke than shorter pectoral fins (Blake, 1981), and allow stickleback to achieve greater velocity with fewer fin beats. Therefore, fish with smaller pectoral fins have to expend more energy than fish with larger pectoral fins in order to achieve the same velocity. Abnormally small fins and reduced armor development would have also affected key components of fitness, such as vulnerability to predation, migratory success, and parental care behavior (Bernhardt & von Hippel, 2008).

The present study reveals that perchlorate-exposed stickleback displayed impaired silvering and remained transparent at phenotypic maturity, with organs and vertebrae clearly visible (Fig. 4.5). Although a greater percentage of stickleback in the nominal variable$_{(0\text{-}60)}$ mg/L treatment had opaque flesh, compared to the 30 and 100 mg/L treatments, the concentration of the nominal variable$_{(0\text{-}60)}$ mg/L treatment was less than 30 mg/L until the fry were approximately five weeks of age (30 July 2003), long after the point that integumentary silvering normally occurs. Therefore, the proportion of fish with normal flesh (i.e., opaque and silvery) actually decreased in a dose-dependent manner (Fig. 4.3).

Transparency may be linked to impaired thyroid function, but researchers have shown that altered thyroid function can affect the integumentary silvering and melanophore function (i.e., darkening) of different organisms in different ways. Silvering of teleosts normally occurs when thyroxine and/or TSH stimulates integumentary purine deposition (specifically guanine and hypoxanthine; Dales & Hoar, 1954; Chua & Eales, 1971; Premdas & Eales, 1976; Leatherland, 1982). Goitrogens, such as thiourea and perchlorate, are known to inhibit thyroxine production, thereby preventing integumentary silvering [thiourea: (Chua & Eales, 1971); perchlorate: (Smith, 1982;Crane et al., 2005). Crane et al. (2005) showed that fathead minnows exposed to 10 and 100 mg/L ammonium perchlorate for 28 days also retained the transparency typical of their larval form. Mukhi et al. (2007) demonstrated that zebrafish treated with 100 and 250 mg/L perchlorate exhibited reduced integumentary silvering. Both of these studies (Crane et al., 2005; Mukhi et al., 2007) also demonstrated that perchlorate exposure interfered with thyroid function as indicated by follicular hypertrophy and hyperplasia, colloidal depletion, and altered T_4 levels.

Others studying hypothyroidism note that T_4-deficient fish and frogs often become increasingly melanistic (e.g., Fortune, 1960; Wright & Lerner, 1960; La Roche et al., 1966; Leatherland, 1982). Using thiourea to induce hypothyroidism in rainbow trout fry (*Onchorhynchus mykiss*), Fortune (1960) demonstrated that fish with impaired thyroid

function became considerably darker than those with intact thyroid glands. Wright and Lerner (1960) demonstrated that hypothyroid frogs became melanistic, but T_4-supplementation had an antagonistic effect, producing frogs with a pallid appearance. LaRoche et al. (1966) used ^{131}I to thyroidectomize fish and found that those with radio-ablated thyroid glands were often intensely darker than intact fish, which resulted from an increased concentration of pigment cells of the skin in all regions of the body.

The studies describing hypothyroid, melanistic organisms seem to contradict our hypothesis that perchlorate exposure impairs thyroid function, which then causes transparency. However, T_4 has been shown to affect the pigmentation of different species in different ways (Leatherland et al., 1977). The transparency of our perchlorate-treated stickleback as well as the reduction of pigmentation on the testes of genotypic male stickleback exposed to perchlorate (Bernhardt et al., 2006), suggest that perchlorate inhibits melanogenesis or may be melanocytotoxic. Most depigmenting agents act by destroying epidermal melanocytes (Kasraee et al., 2004). They are often substrates of tyrosinases and/or peroxidases that give rise to intracellular cytotoxic species that subsequently kill pigment cells when metabolized (Kasraee, 2002). Such cytotoxicity is not specific against melanocytes (Kasraee et al., 2004). This mechanism may not be applicable, however, since mammalian studies show that Sprague-Dawley rats (*Rattus norvegicus*) metabolize less than 0.1% of doubly-labeled perchlorate *in vivo* (Yu et al., 2002). The remainder is excreted unchanged. Conversely, chemicals can lighten tissue by inhibiting melanogenesis, often by interfering with peroxidase activity (Kasraee, 2002), which in turn can suppress the immune system (Balazs et al., 1986). Whether perchlorate causes transparency (Crane et al., 2005; Bernhardt et al., 2006; Mukhi et al., 2007) by inhibiting melanogenesis or by being melanocytotoxic is unknown, but this is a topic worthy of mechanistic study to determine if the mechanism by which perchlorate disrupts pigmentation is of concern to human health.

LaRoche et al. (1966) found that retarded gonadal development among radiothyroidectomized fish was attributed to a chronic state of hypothyroidism. Although

retarded gonadal development might be expected among fish with impaired thyroid function, the intersex gonads in genotypic female stickleback produced by perchlorate exposure (Bernhardt et al., 2006) suggests interference with developmental processes beyond the control of thyroid hormones. In fact, we found that perchlorate-treated genotypic male stickleback developed markedly enlarged testes in a dose-dependent manner (Bernhardt et al., 2006). Other studies have also shown that perchlorate has direct effects on animal tissues that appear to be extrathyroidal, such as renal flow, muscular function, and maintenance of bone (Yen et al., 1973; Moonga et al., 1991; Huang, 1998).

4.6 Conclusion

Every threespine stickleback that was chronically exposed to ≥ 12.0 mg/L of sodium perchlorate displayed morphological abnormalities at phenotypic maturity (Fig. 4.1, 4.2, and 4.5 and Tables 4.1, 4.2, and 4.3). Aberrant patterns of somatic characters were primarily associated with reproduction, locomotion, calcification, underdeveloped anti-predatory structures, and vision. In 2002, fish exposed to a nominal 1.5 mg/L perchlorate treatment developed many characters that were significantly smaller than on control fish, but survivorship to phenotypic maturity was poor (n = 8). This pattern was not replicated in 2003 among fish exposed to either 3.6 or 4.5 mg/L perchlorate, but fish exposed to higher perchlorate concentrations showed growth retardation in a concentration dependent manner. The qualitatively similar, but more dramatic effects noted in 2003, reinforce the assessment that the 2002 findings were not the result of autocorrelation and chance. Genotypic female fish exposed to ≥ 30 mg/L perchlorate became masculanized, and some developed intersex gonads, showing both structural and functional hermaphroditism (Bernhardt et al., 2006). Many fish exposed to ≥ 30 mg/L perchlorate failed to become silver, allowing vertebrae and internal organs to be visible. The abnormalities noted among these fish illustrate the profound morphological changes induced by chronic exposure to perchlorate. However, impaired stickleback that

reproduced in water without detectable levels of perchlorate (<1.1 μg/L) produced offspring without the suite of abnormalities noted among perchlorate-treated fish. This suggests that surviving populations can restore themselves following remediation of perchlorate-contaminated sites.

4.7 Acknowledgement

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4.9 Figures

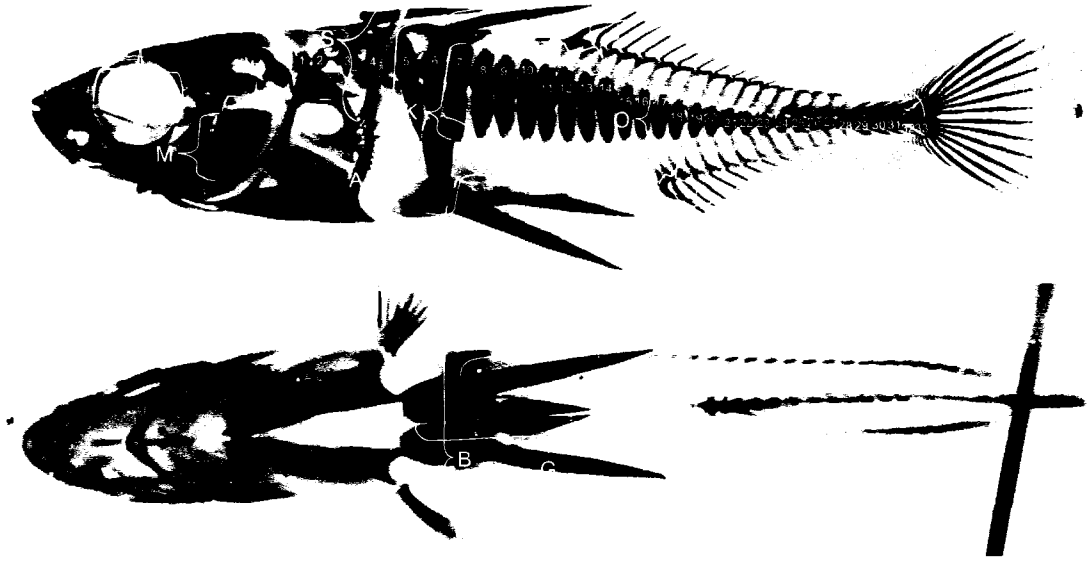

Fig. 4.1. Morphological Landmarks. The 45 characters that were measured on each of the $G_{1,2002}$ and $G_{1,2003}$ fish are shown. A=body depth, B=body width, C=orbit length, D=1st dorsal spine length, E=2nd dorsal spine length, F=3rd dorsal spine length, G=pelvic girdle length, H=anal spine length, I=keel length, J=ascending branch length, K=anterior lateral plate length, L=opercular length, M=opercular depth, N=pelvic spine length, O=anal plate length, #1 - #33 = number of lateral plates, #24 - #33 = number of keel plates, P=dorsal fin length, Q=dorsal fin height, R=anal fin length, S=pectoral fin length, T=number dorsal fin rays, U=number anal fin rays, V=number pectoral fin rays. The total pelvic score was determined by adding J, N, and the two components represented by G (anterior and posterior processes of the pelvic girdle) for each side of the fish. Since these traits are bilateral, pelvic scores ranged from 0 to 8. An armored ring was considered to be completely developed when the calcified structures represented by E, 6, J, and G overlapped one another without gaps. See text for the criteria used to determine transparency.

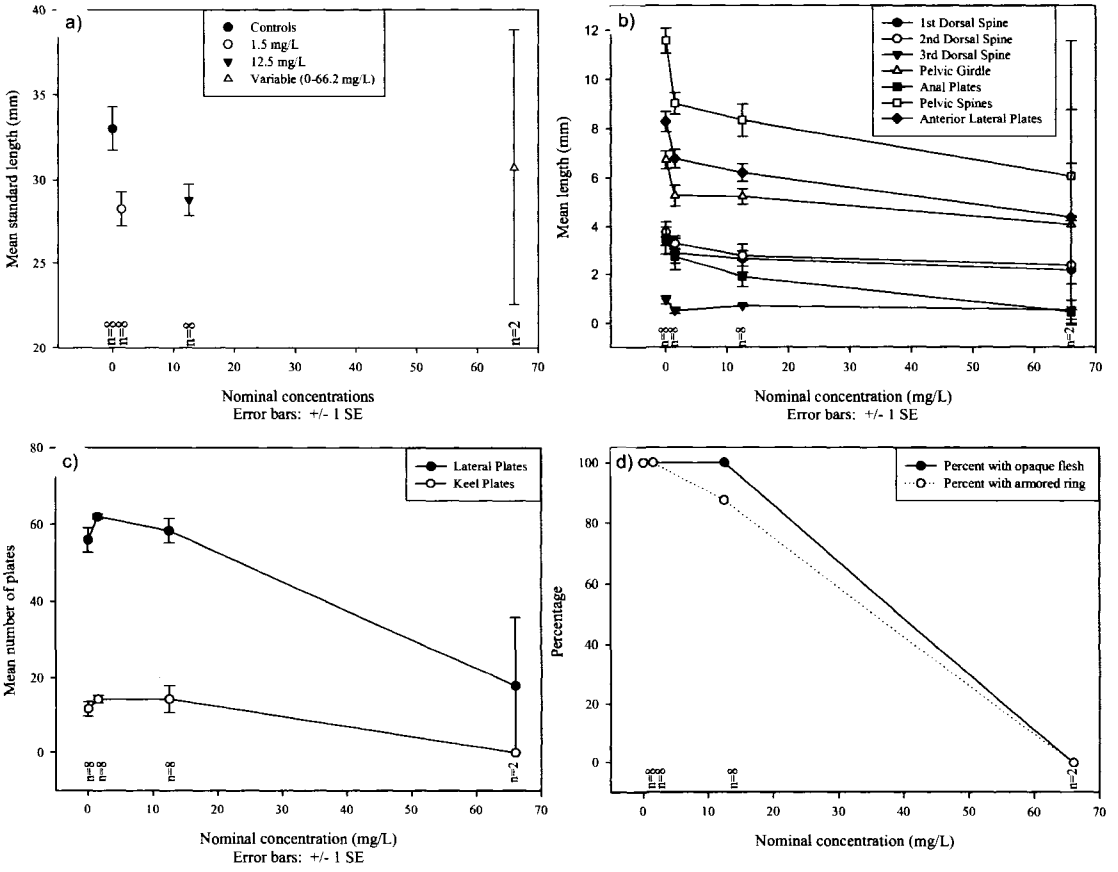

Fig. 4.2. Typical Developmental Patterns for the $G_{1,2002}$ Fish. a) mean standard length by experimental group; b) mean length of select calcified structures by experimental group; c) mean number of lateral and keel plates by experimental group; and d) percent of fish with opaque flesh or armored ring by experimental group. Control stickleback typically grew to be larger with more robust features than stickleback exposed to perchlorate. Only stickleback in the variable$_{(0\text{-}66\ mg/L)}$ HTPB treatment were transparent and completely lacked keel plates.

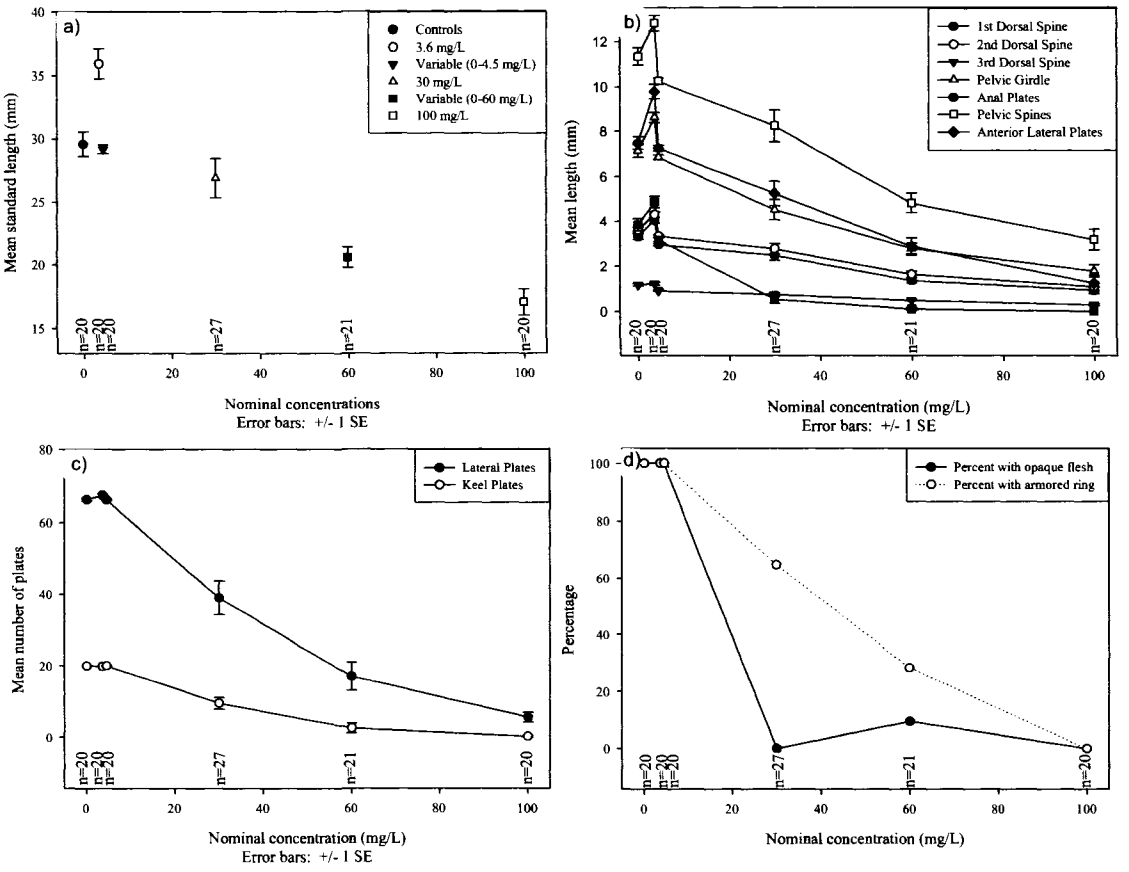

Fig. 4.3. Typical Developmental Patterns for the $G_{1,2003}$ Fish. a) mean standard length by experimental group; b) mean length of select calcified structures by experimental group; c) mean number of lateral and keel plates by experimental group; and d) percent of fish with opaque flesh or armored ring by experimental group. The trends noted in 2002 were repeated in 2003 with dose-dependent reductions in growth patterns. The occurrence of transparency and incomplete development of armored rings at phenotypic maturity showed dose-dependent responses. Whether the magnified growth patterns of the fish exposed to 3.6 mg/L perchlorate represent evidence of hormesis or differential growth associated with unequal population densities is unclear.

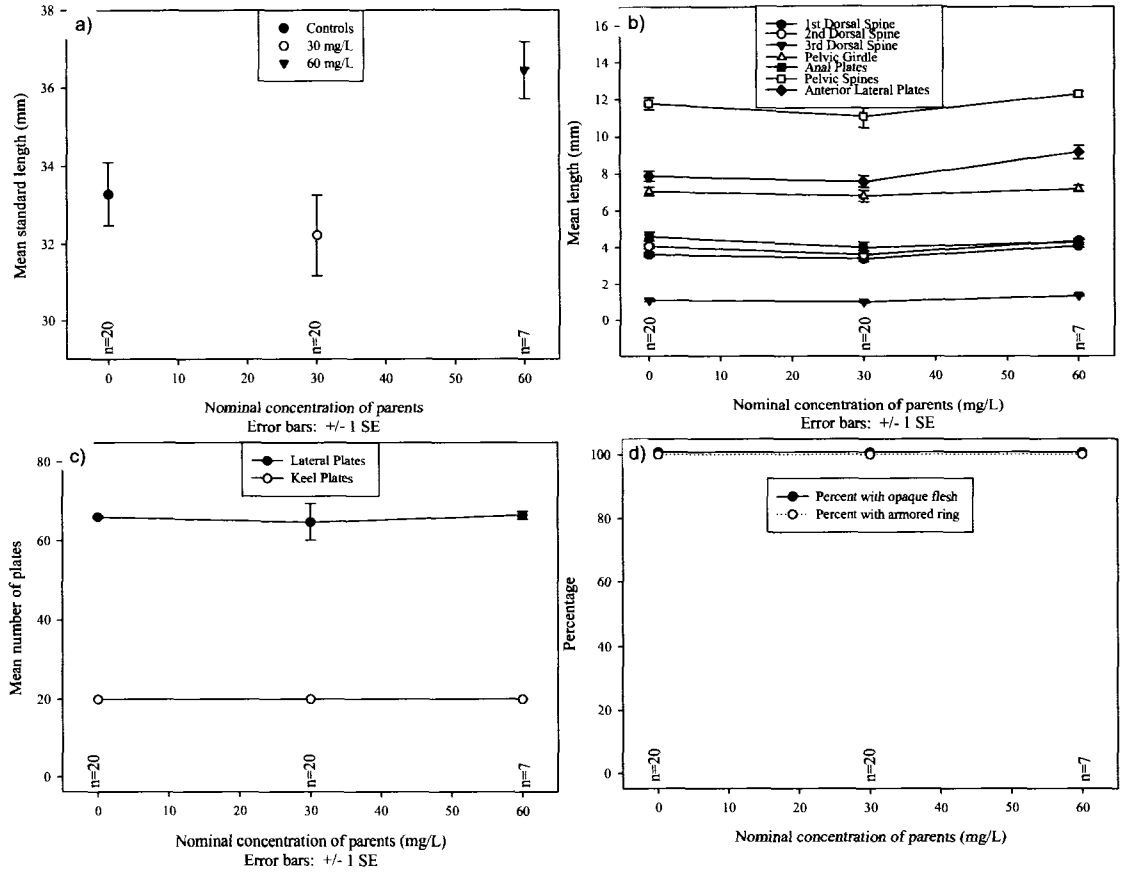

Fig. 4.4. Typical Developmental Patterns for $G_{2,2004}$ Fish. a) mean standard length by experimental group; b) mean length of select calcified structures by experimental group; c) percent of fish with opaque flesh or armored ring by experimental group; and d) mean number of lateral and keel plates by experimental group. The $G_{2,2004}$ are categorized according to the nominal exposure of their parents. The progeny of fish exposed to 30 and variable$_{(0\text{-}60)}$ mg/L perchlorate show none of the aberrant patterns noted among their perchlorate-treated parents. When differences occurred, the $G_{2,2004}$ progeny of fish from the variable$_{(0\text{-}60\ mg/L)}$ experimental group were larger than fish in the other treatments, probably reflecting the effects of unequal fish densities.

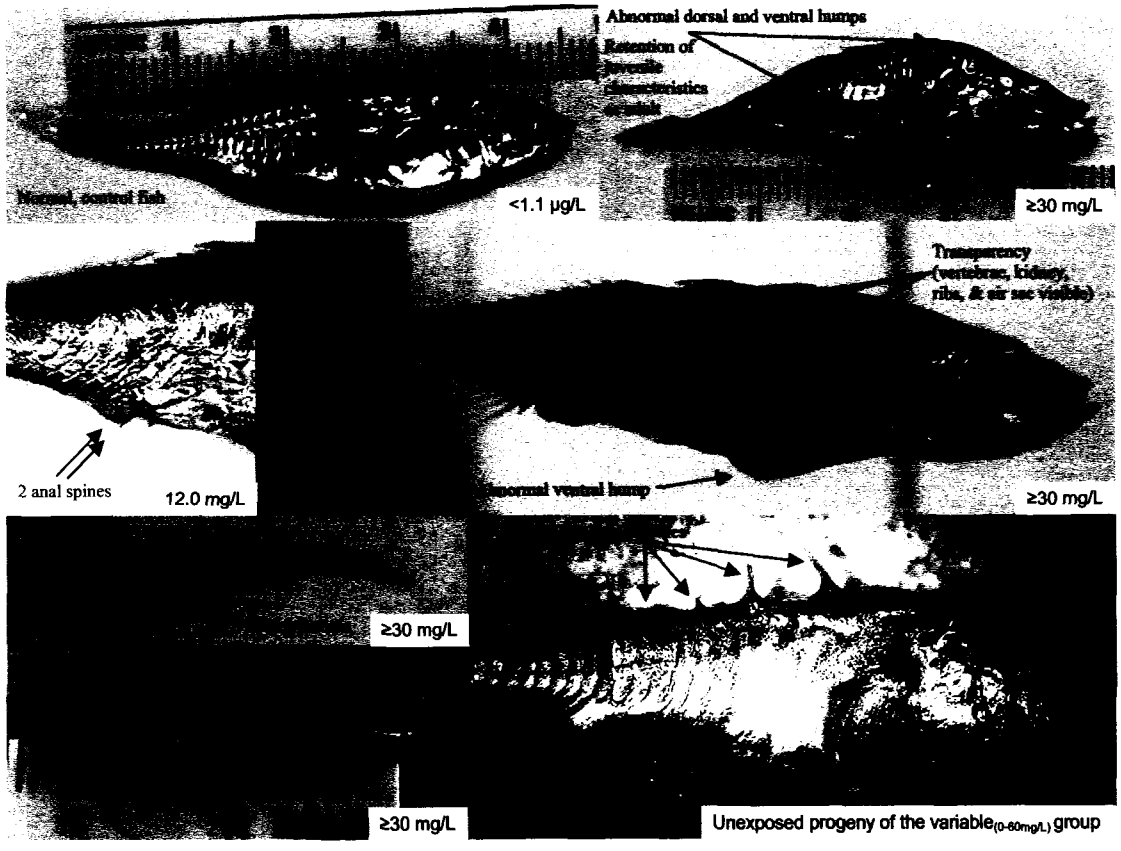

Fig. 4.5. Gross Developmental Abnormalities. Perchlorate clearly disrupted body pigmentation, lateral plate development, body shape, and appeared to express itself in features such as exopthalmia and possibly duplicated spines.

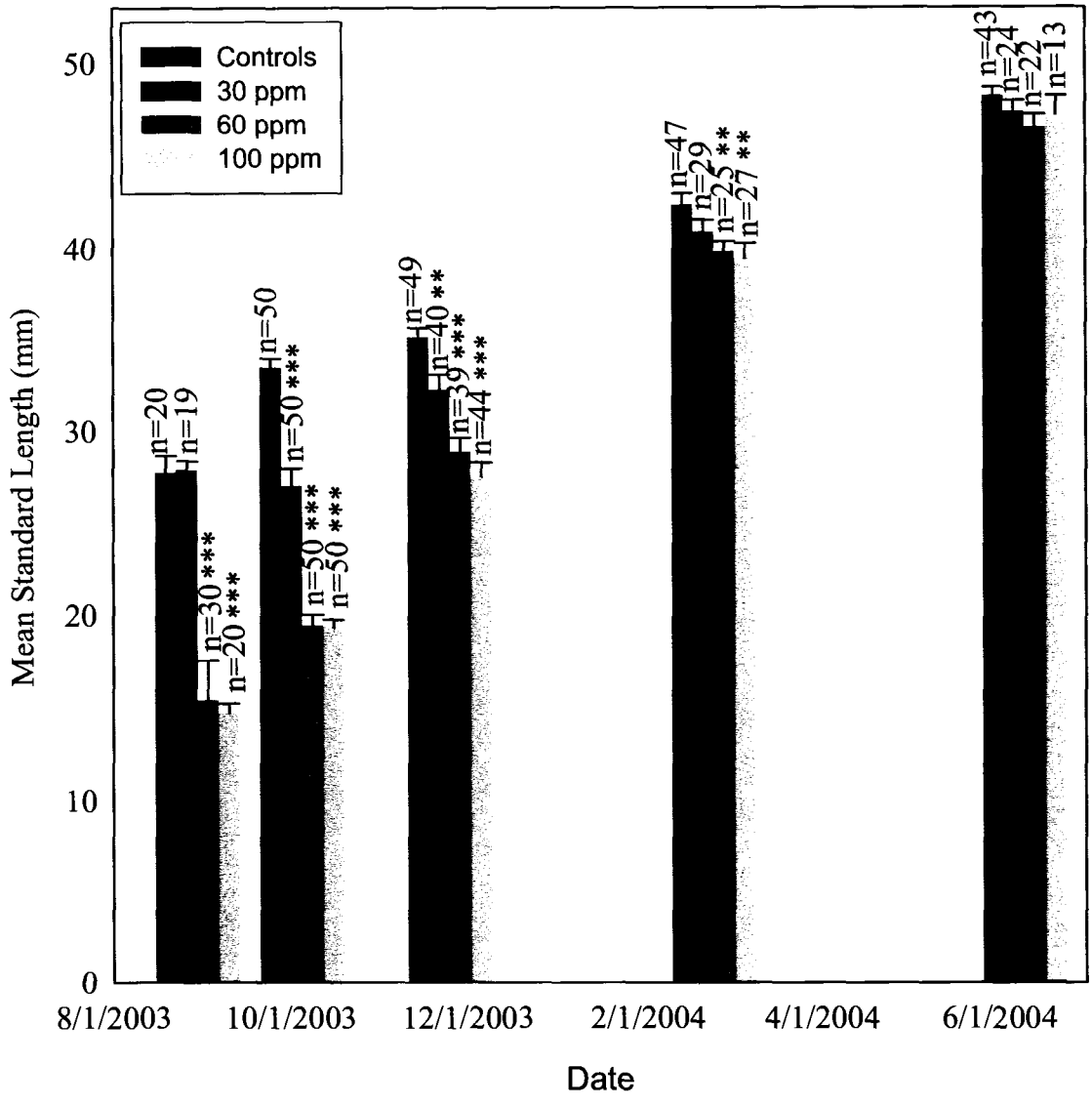

Fig. 4.6. Growth of $G_{1,2003}$. Asterisks represent the degree of significance between each treatment and control stickleback (* $p < .05$, ** $p < .01$, and *** $p < .001$). Rapid growth among fish in the variable$_{(0\text{-}60)}$ and 100 mg/L perchlorate treatments demonstrate a "release effect" between 5 October and 24 February because densities were standardized to 50 fish per 400 L tank on 5 October. Yet, treated fish in the 30, variable$_{(0\text{-}60)}$, and 100 mg/L treatments suffered higher mortality rates and subsequently developed at lower densities than the control fish

4.10 Tables

Table 4.1 $G_{1,2002}$ Morphometric Characteristics. Of particular note are the high correlation coefficients between SL and each character. Each provides an indication of the reliability of transformed character values at a common SL. The low correlation coefficients and scarcity of significant findings from fluctuating asymmetry analysis suggested that additional efforts in 2003 and 2004 were unwarranted. High correlation coefficients show that by measuring a handful of characters in each of the functional groups, one can make meaningful inferences about the degree of variation between treatments without measuring as many traits. An α value of 0.025 was adopted for the 2002 analyses, and significant differences were identified for 9 of 39 untransformed characters and for 7 of 32 transformed characters in nominal treatments ranging from <1.1 µg/L to 12 mg/L. Asterisks represent the degree of significance for the correlation between each character and standard length (* $p < .05$ and ** $p < .01$).

	Characters[a]	2002 r^2 with SL	2002 Untransformed K-W χ^2 / df / p-values	2002 Standardized K-W χ^2 / df / p-values
Miscellaneous	Standard length 4 of 4	1	**7.805 / 2 / .020**	
	Body mass 4 of 4	0.967**	**8.852 / 2 / .012**	1.505 / 2 / .471
	Body depth 4 of 4	.937**	5.855 / 2 / .054	1.295 / 2 / .523
	Body width 4 of 4	.801**	**7.421 / 2 / .024**	**9.785 / 2 / .008**
	Sum orbit length 4 of 4	.843**	**8.930 / 2 / .012**	**8.540 / 2 / .014**
Calcified	1st dorsal spine 12 of 13	.761**	5.267 / 2 / .072	3.920 / 2 / .141
	2nd dorsal spine 12 of 13	0.815**	6.983 / 2 / .030	**9.765 / 2 / .008**
	3rd dorsal spine 10 of 13	.643**	3.850 / 2 / .146	1.935 / 2 / .380
	Pelvic girdle length	.852**	**8.685 / 2 / .013**	1.565 / 2 / .457
	Sum Keel length 10 of 13	0.395	2.445 / 2 / .294	3.345 / 2 / .188
	Sum ascending branch length 12 of 13	.865**	7.085 / 2 / .029	3.555 / 2 / .169
	Sum anterior lateral plate length 12 of 13	.837**	**10.535 / 2 / .005**	5.435 / 2 / .066
	Sum opercular length 10 of 13	.923**	4.603 / 2 / .100	5.615 / 2 / .060
	Sum opercular depth 10 of 13	.918**	3.378 / 2 / .185	3.120 / 2 / .210
	Sum pelvic spine length 12 of 13	.832**	**10.460 / 2 / .005**	**7.505 / 2 / .023**
	Sum anal plate length 12 of 13	.608**	4.536 / 2 / .104	5.04 / 2 / .080
	Total # of lateral plates 10 of 13	0.262	5.149 / 2 / .076	5.149 / 2 / .076
	Total # of keel plates 3 of 13	0.173	.967 / 2 / .617	
	Total Pelvic Score 0 of 13		.000 / 2 / 1.000	
Fin-related	Dorsal fin length 4 of 5	.826**	1.865 / 2 / .394	**9.915 / 2 / .007**
	Anal fin length 4 of 5	.863**	**12.252 / 2 / .002**	
	Sum pectoral fin length 3 of 5	.813**	**7.917 / 2 / .019**	1.115 / 2 / .573
	# dorsal fin rays 3 of 5	.394*	4.108 / 2 / .128	
	# anal fin rays 4 of 5	.589**	2.661 / 2 / .264	2.661 / 2 / .264
	Total # of pectoral fin rays 0 of 5	0.116	0.388 / 2 / .823	0.388 / 2 / .823
Fluctuating Asymmetry	Δ # L&R lateral plates 1 of 11	-0.077	2.516 / 2 / .284	2.516 / 2 / .284
	Δ # L&R pectoral fin rays 0 of 11	-0.249	0.575 / 2 / .750	0.575 / 2 / .750
	Δ L&R keel lengths 0 of 11	0.086	1.427 / 2 / .490	3.120 / 2 / .210
	Δ # L&R keel plates 0 of 11	-0.07	1.954 / 2 / .376	1.954 / 2 / .376
	Δ L&R pectoral fin lengths 0 of 11	-0.013	1.567 / 2 / .457	.245 / 2 / .885
	Δ L&R ascending branch lengths 0 of 11	-0.268	.015 / 2 / .993	0.125 / 2 / .939
	Δ L&R anterior lateral plate lengths 0 of 11	0.107	1.097 / 2 / .578	3.120 / 2 / .210
	Δ L&R opercular lengths 0 of 11	0.183	1.472 / 2 / .479	1.635 / 2 / .442
	Δ L&R opercular depths 0 of 11	0.368	3.668 / 2 / .160	3.345 / 2 / .188
	Δ L&R orbit lengths 0 of 11	-0.171	.011 / 2 / .994	**15.860 / 2 / <.001**
	Δ L&R pelvic spine lengths 0 of 11	-0.068	.865 / 2 / .649	**15.765 / 2 / <.001**
	Δ L&R anal plate lengths 0 of 11	0.254	1.833 / 2 / .400	5.180 / 2 / .075
Dichoto-mous	Transparent Flesh 1 of 1		.000 / 2 / 1.000	
	Armored ring completely formed 1 of 1		2.000 / 2 / .368	
	Significance Summary		**9 of 39**	**7 of 32**

[a] Superscripts indicate the number of significant correlations a character has with other characters in its functional group.

Table 4.2. $G_{1,2003}$ Morphometric Characteristics. Differences in morphological traits between treatment groups are shown for the $G_{1,2003}$. An α value of 0.038 was adopted for the 2003 analyses, and significant differences were identified for 27 of 29 untransformed characters and for 24 of 25 transformed characters in nominal treatments ranging from <1.1 µg/L to 100 mg/L. Asterisks represent the degree of significance for the correlation between each character and standard length (* $p < .05$ and ** $p < .01$).

	Characters [a]	2003 r^2 with SL	2003 Untransformed K-W F / df / p-values	2003 Standardized K-W F / df / p-values
Miscellaneous	Standard length [4 of 4]	1**	**82.013 / 5 / <.0001**	
	Body mass [4 of 4]	0.92**	**95.355 / 5 / <.0001**	**74.398 / 5 / <.0001**
	Body depth [4 of 4]	0.982**	**78.258 / 5 / <.0001**	**28.762 / 5 / <.0001**
	Body width [4 of 4]	0.948**	**71.495 / 5 / <.0001**	**45.421 / 5 / <.0001**
	Sum orbit length [4 of 4]	0.945**	**79.965 / 5 / <.0001**	**72.798 / 5 / <.0001**
Calcified	1st dorsal spine [13 of 14]	0.95**	**89.930 / 5 / <.0001**	**94.643 / 5 / <.0001**
	2nd dorsal spine [14 of 14]	0.941**	**87.991 / 5 / <.0001**	**91.330 / 5 / <.0001**
	3rd dorsal spine [13 of 14]	0.892**	**85.844 / 5 / <.0001**	**89.296 / 5 / <.0001**
	Pelvic girdle length [13 of 14]	0.925**	**91.927 / 5 / <.0001**	**77.253 / 5 / <.0001**
	Anal spine length [14 of 14]	0.919**	**78.358 / 5 / <.0001**	**83.929 / 5 / <.0001**
	Sum Keel length [13 of 14]	0.86**	**87.127 / 5 / <.0001**	**101.491 / 5 / <0.001**
	Sum ascending branch length [14 of 14]	0.98**	**76.800 / 5 / <.0001**	**68.160 / 5 / <.0001**
	Sum anterior lateral plate length [13 of 14]	0.944**	**90.828 / 5 / <.0001**	**83.926 / 5 / <.0001**
	Sum opercular length [13 of 14]	0.986**	**81.658 / 5 / <.0001**	**69.114 / 5 / <.0001**
	Sum opercular depth [13 of 14]	0.984**	**83.004 / 5 / <.0001**	**75.883 / 5 / <.0001**
	Sum pelvic spine length [13 of 14]	0.944**	**87.171 / 5 / <.0001**	**74.745 / 5 / <.0001**
	Sum anal plate length [13 of 14]	0.79**	**103.441 / 5 / <.0001**	**78.943 / 4 / <.0001**
	Total # of lateral plates [13 of 14]	0.817**	**95.503 / 5 / <.0001**	**98.985 / 5 / <.0001**
	Total # of keel plates [13 of 14]	0.771**	**106.432 / 5 / <.0001**	**80.213 / 4 / <.0001**
	Total Pelvic Score [3 of 14]	0.169	5.050 / 5 / .410	
Fin-related	Dorsal fin length [5 of 6]	0.978**	**77.029 / 5 / <.0001**	8.220 / 5 / .145
	Dorsal fin height [5 of 6]	0.952**	**71.192 / 5 / <.0001**	**61.350 / 5 / <.0001**
	Anal fin length [5 of 6]	0.968**	**79.169 / 5 / <.0001**	**12.572 / 5 / .028**
	Sum pectoral fin length [5 of 6]	0.888**	**97.389 / 5 / <.0001**	**96.658 / 5 / <.0001**
	# dorsal fin rays [5 of 6]	0.681**	**53.149 / 5 / <.0001**	**100.779 / 5 / <.0001**
	# anal fin rays [5 of 6]	0.611**	**34.096 / 5 / <.0001**	**72.172 / 5 / <.0001**
	Total # of pectoral fin rays [0 of 6]	0.004	9.589 / 5 / .088	**50.449 / 3 / <.0001**
Dichotomous	Transparent Flesh [1 of 1]	0.59**	**112.817 / 5 / <.0001**	
	Armored ring completely formed [1 of 1]	0.754**	**81.334 / 5 / <.0001**	
	Significance Summary		27 of 29	24 of 25

[a] Superscripts indicate the number of significant correlations a character has with other characters in its functional group.

Table 4.3 $G_{2,2004}$ Morphometric Characteristics. Differences in morphological traits between $G_{2,2004}$ experimental groups are shown. All $G_{2,2004}$ were raised in water without detectable levels of perchlorate, but their parents were raised from fertilization to sexual maturity in control or perchlorate-treated water. Differences were considered significant when $\alpha \leq 0.05$ in 2004. Significant differences were identified for 4 of 18 untransformed characters and for 7 of 13 transformed characters. Differences were due to the offspring of the $G_{1,2003}$ fish in the variable$_{(0\text{-}60)}$ mg/L treatment being larger, which was likely due to lower densities in their aquaria. Asterisks represent the degree of significance for the correlation between each character and standard length (* $p < .05$ and ** $p < .01$).

	Characters[a]	2002 r^2 with SL	2004 Untransformed K-W F / df / p-values	2004 Standardized K-W F / df / p-values
Misc.	Standard length 1 of 1	1	**6.717 / 2 / .035**	
	Body depth 1 of 1	0.951 **	5.151 / 2 / .076	1.111 / 2 / .574
Calcified	1st dorsal spine 9 of 10	0.925 **	**7.271 / 2 / .026**	**8.244 / 2 / .016**
	2nd dorsal spine 9 of 10	0.895 **	5.906 / 2 / .052	**7.120 / 2 / .028**
	3rd dorsal spine 9 of 10	0.848 **	**9.357 / 2 / .009**	**6.967 / 2 / .031**
	Pelvic girdle length 9 of 10	0.924 **	5.151 / 2 / .076	**13.366 / 2 / .001**
	Sum ascending branch length 9 of 10	0.896 **	4.408 / 2 / .110	**6.247 / 2 / .044**
	Sum anterior lateral plate length 9 of 10	0.899 **	**6.857 / 2 / .032**	0.409 / 2 / .815
	Sum pelvic spine length 10 of 10	0.892 **	0.657 / 2 / .720	.498 / 2 / .780
	Sum anal plate length 9 of 10	0.844 **	3.174 / 2 / .205	**7.554 / 2 / .023**
	Total # of lateral plates 9 of 10	0.561	0.635 / 2 / .728	4.815 / 2 / .090
	Total # of keel plates 10 of 10		0.000 / 2 / 1.000	
	Total Pelvic Score 2 of 10	0.089	0.000 / 2 / 1.000	
Fin-related	Dorsal fin length 2 of 2	0.843 **	4.154 / 2 / .125	3.590 / 2 / .166
	Sum pectoral fin length 2 of 2	0.87 **	.080 / 2 / .961	**12.115 / 2 / .002**
	# dorsal fin rays 2 of 2	0.383 *	2.222 / 2 / .329	0.424 / 2 / .809
Dichotomous	Transparent Flesh 1 of 1		0.000 / 2 / 1.000	
	Armored ring completely formed 1 of 1		0.000 / 2 / 1.000	
	Significance Summary		**4 of 18**	**7 of 13**

[a] Superscripts indicate the number of significant correlations a character has with other characters in its functional group.

Table 4.4 Effects of Perchlorate Concentration and Fish Density on SL. Fish raised in treatments with higher perchlorate concentrations and lower densities are smaller than those raised at lower perchlorate concentrations and higher densities, suggesting that the concentration of perchlorate may have a greater effect on growth than fish density. For example, fish from tank 4 exposed to as high as 60 mg/L perchlorate and raised at a density of 0.182 fish/L were larger than fish from tank 15 exposed to 100 mg/L perchlorate and raised at a density of 0.166 fish/L. Fish in tank 4 were exposed to the same perchlorate treatment as fish in tank 11 [variable$_{(0\text{-}60)}$ mg/L treatment], but tank 11 had a higher density of fish and produced smaller fish.

Tank	$[ClO_4^-]$ (mg/L)	Number of Fish	Density (Fish / Liter)	Mean Standard Length (mm)
15	100	265	0.166	16.5
4	0-60	291	0.182	20.5
11	0-60	343	0.214	18.22
12	30	294	0.184	24.05

Chapter 5:
Conclusion

5.1 Summary of Findings

To assess the effects of perchlorate exposure on fitness and development, critical life history endpoints were analyzed for threespine stickleback that were either maintained in a control group or one of eight perchlorate treatments. My research shows that fish exposed to environmentally relevant concentrations of perchlorate grow, develop, behave, and perform in a manner that is impaired in comparison to fish maintained in water lacking detectable levels of perchlorate. In fact, chronic perchlorate exposure, beginning at fertilization, interfered with nearly every measured component of fitness and development between conception and reproductive maturity. Yet, the timing of exposure to perchlorate appears to be as important as the dosage itself. The multi-generational study design that was employed showed that adults and juveniles respond to perchlorate exposure in vastly different ways and that many effects may be initiated early during sensitive developmental windows, but do not express toxicity until the organism reaches maturity. Adult stickleback exposed for up to 30 days to the same concentrations as their progeny showed none of the behavioral, anatomical, coloration, or survivorship differences as their offspring. Likewise, when progeny of malformed and impaired stickleback were fertilized and raised in water without detectable levels of perchlorate, they showed none of the impairments noted among their perchlorate-treated parents. These findings reinforce the notion that both the magnitude and timing of exposure to endocrine disrupting contaminants should be considered when assessing potential adverse effects since many organizational modifications do not become apparent until later in life (Guillette et al., 1995).

From a life history perspective, the first challenge that a young stickleback faces is early development and emergence from its egg. As a percentage of eggs that were fertilized using *in vitro* techniques, differences in hatching success were statistically significant, but perhaps not biologically significant. Control fish, wild fish, and those

exposed to 100 mg/L perchlorate had similar hatching success while stickleback exposed to 30 and 60 mg/L perchlorate had significantly lower hatching success. These findings are based on eggs fertilized *in vitro* and discount the effects that parental behaviour and parental survival following fertilization may have on hatching success. For example, one of the two males that spawned naturally in water containing 60 mg/L perchlorate died following fertilization. His lack of parental care resulted in the death of his clutch, reinforcing the notion that perchlorate exposure would have deleterious effects on hatching success in a natural setting.

The next challenges facing young stickleback are growth and development. Growth was impaired in a concentration-dependent manner through nine months of age, with fish exposed to the highest concentrations of perchlorate growing slower than those in control water and lower treatments. Rapid growth is particularly significant for young fishes, since many piscivores are size-selective. Thus, fish that remain small for longer periods remain vulnerable to gape-limited piscivores and have a greater likelihood of being eaten before reproduction. However, perchlorate treated fish reached the same size as control fish at one year of age (sexual maturity). Somatic growth later in life is not adaptive for semelparous fishes like stickleback because it requires energy reserves that would normally be devoted toward reproduction (see also fecundity discussion, below; Cyr and Eales, 1996).

Both generations of stickleback ($G_{1,2002}$ and $G_{1,2003}$) that were chronically exposed to $\geq$ 12.5 mg/L sodium perchlorate developed abnormalities (i.e. not reflective of control fish or the wild-caught parents from the source population) that increased in frequency and severity in a concentration-dependent manner. Aberrant developmental patterns were primarily associated with reproduction, locomotion, growth, formation of anti-predatory structures, and vision. All stickleback exposed to $\geq$ 12.5 mg/L perchlorate either lacked, or expressed abnormally small and poorly calcified spines, lateral plates, and fins. Those exposed to $\geq$ 30 mg/L perchlorate exhibited concentration-dependent increases in transparency with internal organs and bones clearly visible. The frequency of fish without keel plates also increased in a concentration-dependent manner, starting at $\geq$ 30mg/L perchlorate.

Only two of the $G_{1,2002}$ fish that were exposed 3.70 g cores of dissolving solid rocket propellant survived to phenotypic maturity, so they were excluded from statistical analyses. Differences were apparent for 9 of 39 morphological tests between the remaining $G_{1,2002}$ experimental groups (control, 1.5, and 12.5 mg/L perchlorate). Twelve of these tests were conducted to determine if fluctuating asymmetry was affected before bilateral traits were totaled to compare the remaining 27 anatomical characters. Fluctuating asymmetry was not affected by perchlorate exposure in 2002 and was not analyzed in 2003 or 2004. Among the $G_{1,2003}$, developmental abnormalities were common with 27 of 29 morphological characters showing significant differences after bilateral characters were summed.

Impaired stickleback that produced offspring in water without detectable levels of perchlorate (<1.1 μg/L) produced offspring without the suite of abnormalities noted among their parents. However, the $G_{2,2004}$ were maintained at unequal densities since fewer $G_{1,2003}$ in higher treatments reproduced. Thus, progeny of fish exposed to 60 mg/L perchlorate (the highest treatment in which fish managed to spawn) were raised at lower densities and generally grew to be larger than control fish or the progeny of fish exposed to 30 mg/L perchlorate. This pattern is contrary to the trends noted among the two previous generations of stickleback that were directly exposed to perchlorate ($G_{1,2002}$ and $G_{1,2003}$) and probably reflects the effects of unequal population density (Ewing and Ewing, 1995; Ellis et al., 2002) rather than a transgenerational effect from parental perchlorate exposure. Yet, one anomalous developmental event occurred among the $G_{2,2004}$. One of the progeny of fish exposed to 60 mg/L perchlorate developed a fourth dorsal spine, which raises the question of whether this was a random mutation or a perchlorate related transgenerational effect since anal spine duplication was noted among one of the $G_{1,2002}$ fish exposed to 12.5 mg/L perchlorate.

Overall, survivorship to sexual maturity was poor among treated fish (Fig. 2.3), suggesting that many fish are incapable of tolerating the stresses induced by chronic perchlorate exposure. Fish exposed to higher concentrations of perchlorate suffered greater mortality, in a concentration-dependent manner, than those exposed to lower treatments and control fish. Mortality was particularly high following stressors such as

exercise, relocation, and handling while out of the water for <10 seconds (Bernhardt et al., 2006). In addition to the concentration-dependent mortality rates noted prior to sexual maturity, elevated mortality rates during their 33 days of reproductive isolation were unmistakably linked to perchlorate exposure. These findings demonstrate that perchlorate-treated fish are less resilient than control fish that experience the same stressors.

A study involving zebrafish (*Danio rerio*) demonstrated that developing larvae were much more susceptible to the toxic effects of perchlorate than were juveniles (Liu et al., 2005). Therefore, overall mortality would presumably be much higher than a simple extrapolation of my findings would suggest since I began to quantify survivorship on juveniles at 15 weeks of age. In addition to its immediate effects on fitness, differential mortality rates left progressively fewer fish and lower population densities (i.e., fish per liter of water) in the higher treatments. Fish maintained at lower densities typically grow faster and larger than those maintained at higher densities. Therefore, the differential growth rates, previously mentioned, are even more significant since stickleback maintained in higher perchlorate concentrations at lower population densities should have grown to be larger than those maintained in lower perchlorate concentrations and higher densities, if density was the most important factor. Yet, the opposite results noted in my study suggest that perchlorate exposure may have had a greater impact on growth than density. However, my studies were not designed to characterize this interaction since periodic mortalities altered the densities at irregular intervals. Therefore, further study would be required to validate this observation.

The testing of swimming performance revealed biologically significant differences that would be likely to affect the fitness of contaminated fish in a wild population (Bernhardt and von Hippel, 2008). For anadromous stickleback, such as those from the source population, the natal spawning migration is a critical event that determines whether fish that survive to sexual maturity will get the opportunity to court and potentially reproduce. However, swimming performance also provided an indication whether wild, contaminated fish would be able to capture elusive prey and escape predation. During the stepwise testing, when the flow rate was increased at two minute

intervals, control fish clearly outperformed those exposed to perchlorate in a concentration-dependent manner. Only fish that successfully completed the stepwise testing participated in the ten minute long stamina testing. The results of the stamina testing also showed concentration-dependent responses for stickleback in the control, 30 mg/L and 60 mg/L experimental groups. The sole fish exposed to 100 mg/L perchlorate that passed the stepwise testing also passed the stamina testing, but this achievement is probably not representative of the typical swimming performance of fish chronically exposed to 100 mg/L perchlorate.

Once stickleback reach suitable spawning habitat, males must accomplish a series of events that influence reproductive success, such as: making a nest; developing appropriate nuptial coloration to attract mates; performing courtship displays; fertilizing eggs; and caring for their young, which includes fanning embryos to deliver oxygenated water and remove excess carbon dioxide from the nest, rearranging the nest to make a nursery shortly before hatching, and protecting the embryos and fry. Each of these components was analyzed and found to be impaired in a concentration-dependent manner (Bernhardt and von Hippel, 2008).

Nest-building success was strongly influenced by experimental group with 100% of control males making a nest compared to 75%, 38%, and 0% of the males exposed to 30, 60, and 100 mg/L perchlorate, respectively. Of the few treated males that performed courtship displays, the number of behaviours performed by these males declined as their level of perchlorate exposure increased. Their limited behavioral repertoire contributed to the lack of spawning success noted among treated males. While 80% of control males spawned successfully, only 50%, 25%, and 0% of males exposed to 30, 60, and 100 mg/L perchlorate managed to spawn. Nuptial coloration has also been shown to influence mate choice among wild stickleback (Rowland, 1994), yet perchlorate exposure interfered with the ability of sexually mature males to express vivid nuptial coloration. The degree of color inhibition was concentration-dependent.

Seven perchlorate treated $G_{1,2003}$ females were isolated in aquaria, and four performed reproductive behaviour typical of males, such as biting, leading, and zig-zagging in the presence of gravid females. Two of the other isolated females died before

being given a courtship opportunity, and the remaining female died after a single courtship event, during which it did not actively court an introduced female. Although introduced females occasionally responded to the courtship displays of resident females by assuming receptive, head-down postures and following the resident females, none of the resident females managed to build a nest or spawn.

Development in perchlorate-treated water had a masculinizing effect on the gonads of both male and female stickleback. Genetic testing and histological analysis revealed that female stickleback were masculanized to the extent that they developed ovotestes (i.e., a chimera of sperm- and egg-producing cells in the same organ) and produced both sperm and eggs. *In vitro* fertilization attempts proved that gametes from a single female donor were capable of self- and cross-fertilization, demonstrating that genetically female fish had become functionally hermaphroditic. Concentration-dependent testicular hypertrophy was noted among genetically male stickleback with control males having the smallest testes and males exposed to 100 mg/L having the largest.

Additional *in vitro* fertilization testing showed that fish from all experimental groups were capable of producing fertile gametes. More specifically, sperm derived from genetic males produced viable fry when crossed with eggs derived from genetic females. However, the sperm derived from genetic females was capable of fertilizing eggs, but the embryos died between the blastula phase and the onset of organogenesis. Neither sperm motility nor morphology was analyzed.

None of the males exposed to 100 mg/L perchlorate managed to spawn, so they did not conduct parental care and were excluded from parental care analysis. Surprisingly, the two males that spawned from the 60 mg/L treatment spent the greatest percentage of time providing parental care, but only one made a nursery and produced fry. The percentage of time spent providing parental care did not differ between the eight control males and four 30 mg/L males that spawned. Yet, 88% (seven of eight) of the control males with fertilized eggs made nurseries, and 86% (six of seven) of those with nurseries produced fry. Only half of the parental males exposed to 30 mg/L perchlorate (two of four males) made nurseries, and both of them produced fry. Therefore, males

exposed to 60 mg/L perchlorate provided the most parental care, but control males had the greatest recruitment rates in terms of the number and percentage of males that produced fry.

Fecundity rates in terms of the number of eggs produced per female did not differ between females exposed to 30, 60, or 100 mg/L perchlorate. However, control females produced more eggs than treated females, even though their mean standard length and mass did not differ from treated females at sexual maturity. This may suggest that stickleback which were exposed to perchlorate and allocated energy toward somatic growth later in life did so at the expense of reproductive output. Alternatively, thyroid hormone levels are known to be associated with gonadal recrudescence and reproductive success (Cyr and Eales, 1996; Power et al., 2001; Blanton and Specker, 2007). Therefore, these findings would be predictable if thyroid hormone homeostasis was indeed impaired.

Extrapolation outside the range of measurements must be made with caution since environmental factors, such as pH, temperature, salinity, dissolved oxygen and ionic content may all affect toxicity. However, the implications of these findings in an ecological context are likely to be profound. If, for example, eggs were deposited in water contaminated by similar concentrations of perchlorate, the fry would presumably hatch without difficulty. Following hatching, they would likely suffer reduced growth rates, leaving them vulnerable to a wider variety of size-selective piscivores. Survivorship, and in particular, resilience following disturbance would be severely reduced among exposed fish. Concentration-dependent degradation of swimming ability suggests that contaminated fish would be less successful at capturing prey, avoiding predation, and returning upstream during their natal migration. Many males that reach suitable spawning habitat would fail to build a nest, and a portion of those that made nests would fail to display the appropriate courtship behavior to attract a mate. Even males with proper displays would lack vivid nuptial coloration, making spawning success even less likely. A portion of the females would be masculanized and produce sperm capable of self-fertilization, but incapable of generating viable offspring. Contaminated females would produce fewer eggs than uncontaminated fish, and those that managed to

become fertilized by sperm from a genetic male would be cared for by males that would risk exposing the nest to predation by their excessive fanning. Impairment of any one of these characteristics is likely to diminish reproductive success, but taken together, these findings suggest that perpetually contaminated populations may not be able to sustain themselves. However, the lack of transgenerational effects among the $G_{2,2004}$ suggest that remediation efforts are likely to restore ecosystem health if a portion of the contaminated population survives and is able to reproduce in unspoiled water.

5.2 Recommendations for Future Study

Collectively, these studies reveal a number of issues that are likely to provide fertile ground for future research. Studies should examine whether the masculinization response reported in chapter two is mediated through direct activation of androgen receptors or by indirect means, such as alteration of steroid metabolism. Histological examination of the hypertrophied testes would help to determine if spermatogenesis and testicular morphology are affected (e.g., sperm ducts or unusual cavities as described in Hahlbeck et al. 2004). Likewise, analysis of sperm motility, morphology, and volume would indicate if fertility is enhanced, as suggested by studies involving a variety of vertebrates with transient pre-pubertal hypothyroidism (Jannini et al., 1995) or if the testes are simply larger without commensurate increases in sperm production. The differential mortality rates following swim testing, reported in chapter three, provide a tantalizing but unsubstantiated link to differential stress responses according to perchlorate treatment, and the hormonal bases of perchlorate's actions (including extrathyroidal endpoints and aromatase activity levels) are likely to be a productive avenue for future research.

5.3 References

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