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Perchlorate disrupts embryonic androgen synthesis and reproductive development in threespine stickleback without changing whole-body levels of thyroid hormone

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ABSTRACT

Perchlorate, an environmental contaminant, disrupts normal functioning of the thyroid. We previously showed that perchlorate disrupts behavior and gonad development, and induces external morphological changes in a vertebrate model organism, the threespine stickleback. Whether perchlorate alters these phenotypes *via* a thyroid-mediated mechanism, and the extent to which the effects depend on dose, are unknown. To address these questions, we chronically exposed stickleback to control conditions and to three concentrations of perchlorate (10, 30 and 100 ppm) at various developmental stages from fertilization to reproductive maturity. Adults chronically exposed to perchlorate had increased numbers of thyroid follicles and decreased numbers of thyrocytes. Surprisingly, T_4 and T_3 levels in larval, juvenile, and adult whole fish chronically exposed to perchlorate did not differ from controls, except at the lowest perchlorate dose, suggesting a non-monotonic dose response curve. We found no detectable abnormalities in external phenotype at any dose of perchlorate, indicating that the increased number of thyroid follicles compensated for the disruptive effects of these doses. In contrast to external morphology, gonadal development was altered substantially, with the highest dose of perchlorate causing the largest effects. Perchlorate increased the number both of early stage ovarian follicles in females and of advanced spermatogenic stages in males. Perchlorate also disrupted embryonic androgen levels. We conclude that chronic perchlorate exposure may not result in lasting adult gross morphological changes but can produce lasting modifications to gonads when compensation of T_3 and T_4 levels occurs by thyroid follicle hyperplasia. Perchlorate may therefore affect vertebrate development *via* both thyroidal and non-thyroidal mechanisms.

1. Introduction

Thyroid hormones (THs) are important for nearly every aspect of vertebrate growth and development (Power et al., 2001; Jugan et al., 2010). Consequently, environmental contaminants affecting TH production and use have wide-ranging impacts. Contaminants that interfere with the vertebrate hypothalamic–pituitary–thyroid (HPT) axis have clear deleterious effects, particularly in aquatic organisms (Power et al., 2001; Milnes et al., 2006; Carr and Patiño, 2011). A large number of abnormal phenotypes have been ascribed to thyroid-disrupting chemicals, including altered body size and shape (Lawrence et al., 2005; Bernhardt et al., 2011), disrupted immune function (Capps et al., 2004), modified bone development (Stevens et al., 2000; Bernhardt et al., 2011), and changes in behavior and gonad masculinization or feminization in threespine stickleback (*Gasterosteus aculeatus*) and zebrafish (*Danio rerio*), respectively (Bernhardt et al., 2006; Mukhi and Patiño, 2007; Bernhardt and von Hippel, 2008; Sharma and Patiño, 2013). The biological mechanisms of contaminant-induced phenotypic abnormalities are largely unknown, however. In particular, interactions of the HPT axis with other endocrine axes and developmental pathways are still poorly understood in many vertebrates, although it is clear that early developmental disruption of thyroid signaling would be deleterious in mammalian (Préau et al., 2014) and non-mammalian vertebrates (Morvan Dubois et al., 2006; Préau et al., 2014). Consequently, the full suite of developmental and reproductive abnormalities caused by HPT disruption

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is still unknown for most contaminants. In addition, the dose–response curves, critical developmental windows of exposure, and physiological endpoints to assess the effects of many HPT disruptors have not been well defined in any vertebrate.

To better understand the biological mechanisms of contaminant-induced abnormalities we examined the hormonal and morphological effects of a known HPT antagonist, perchlorate (ClO_4^-), on thyroidal, musculoskeletal, and reproductive development in a vertebrate model species, the threespine stickleback, at environmentally relevant concentrations (Urbansky, 1998; Goleman et al., 2002). Perchlorate is a persistent, widespread environmental contaminant and a potent endocrine disruptor with documented pathological effects on TH production in humans (US EPA, 2002; Blount et al.,, 2006b). The perchlorate anion acts at the thyroid follicle to block iodide uptake *via* competitive inhibition of the sodium-iodide symporter (NIS, alias *SLC5A5*; Eskandari et al., 1997; Wolff, 1998), thereby impairing sequestration of iodide, an ion necessary for synthesis of TH. Perchlorate appears in ground and surface waters throughout the United States (Trumpolt et al., 2005) at levels up to 4000 ppm (Urbansky, 1998). Recent studies detected perchlorate in breast milk of pregnant and lactating women (Kirk et al., 2005; Pearce et al., 2007), and perchlorate was ubiquitously detectable in the urine of 22,000 pregnant women in Wales and Italy (Pearce et al., 2010). Amazingly, all 2800 participants in a recent study had perchlorate in their urine, with levels ranging from 0.00019 to 0.160 ppm; children had the highest calculated exposure (Blount et al., 2006a, 2006b).

Perchlorate produces deleterious effects in other vertebrates similar to those seen in humans. For example, perchlorate negatively affects amphibian health – and can lead to death – at environmentally relevant concentrations of 200–500 ppm (Goleman et al., 2002). Perchlorate exposure in teleost fishes leads to a wide array of altered phenotypes, some of which suggest disruption to development outside of the HPT axis (Crane et al., 2005; Bernhardt et al., 2006; Bernhardt and von Hippel, 2008; Sharma and Patiño, 2013). Effects of chronic perchlorate exposure on stickleback include abnormal lateral plate development, decreased swimming performance, slower growth rates, and reduced pigmentation (Bernhardt et al., 2006, 2011; Bernhardt and von Hippel, 2008). Perchlorate also appears to act *via* an unknown mechanism to alter gonad development and sex determination in teleosts, a finding not predicted to occur solely *via* thyroid disruption (Bernhardt et al., 2006; Mukhi and Patiño, 2007; Sharma and Patiño, 2013); the HPT axis, however, has been implicated in effects on reproductive development and function in teleosts (Carr and Patiño, 2011; Flood et al., 2013). Developmental exposure to perchlorate skews the sex ratio towards female in zebrafish (Mukhi and Patiño, 2007; Sharma and Patiño, 2013), a species in which various strains are reported to have different genetic bases for sex determination (Bradley et al., 2011; Anderson et al., 2012). Perchlorate masculinizes the gonad in male and female stickleback, in addition to increasing the gonadal-somatic index in male stickleback (Bernhardt et al., 2006). In some cases, perchlorate exposure causes genotypically female stickleback to become functional hermaphrodites, leading us to hypothesize that perchlorate has androgenic effects (Bernhardt et al., 2006).

Multiple lines of evidence suggest that the effects of perchlorate could be widespread throughout the body. TH receptors occur in most cells (Hulbert, 2000; Power et al., 2001), and perchlorate-induced changes in thyroid hormone production therefore have the potential for widespread disruption of numerous tissues regulated by the HPT axis. In addition, studies of the specific effects of perchlorate on circulating or whole body TH concentration are often contradictory. For example, Mukhi et al., (2005) found no significant effect of 12 weeks of ammonium perchlorate exposure on whole-body T_4 concentrations in zebrafish, but in a subsequent study these authors reported that T_4 concentrations were significantly decreased while T_3 concentrations remained unchanged after 16 weeks of exposure (Mukhi and Patiño, 2007). A similar study in mosquitofish (*Gambusia affinis*), however, demonstrated no dose-dependent relationship in T_4 levels in response to various levels of perchlorate exposure after 2, 10 or 30 days (Bradford et al., 2005). Conflicting results across studies of HPT target pollutants may occur because the widespread effects of TH toxins interact with the specifics of experimental conditions such as length of exposure, age, species and genotype of the animal exposed, and dose of toxin to produce discordant results (reviewed by Carr and Patiño (2011)). Because the shape of the dose–response curve for perchlorate has not been rigorously determined across various life stages or broadly across vertebrate phylogeny, and because different studies have used various end points as indicators of HPT function, it is difficult to determine the scope of perchlorate's effects on vertebrate development.

Taken together, these results indicate that two key hormone signaling pathways, the HPT axis and the hypothalamic–pituitary–gonadal (HPG) axis, which regulates production of reproductive steroids, are likely disrupted by perchlorate (Bernhardt et al., 2006; Carr and Patiño, 2011; Sharma and Patiño, 2013). Establishing a perchlorate dose–response curve for a variety of phenotypes related to the HPT and HPG axes is an important step in understanding whether early developmental exposure to perchlorate affects vertebrates solely through thyroidal mechanisms. We hypothesized that perchlorate may alter development of thyroid tissues as well as reproductive tissues through cross-talk among signaling pathways or through interference with normal cell physiology during development. In particular, sodium-iodide symporters (NIS) are located in the gonads of vertebrates (Bermudez, 2008; Russo et al., 2011), and a reasonable mechanistic hypothesis is that perchlorate directly affects gonad tissues through interactions with these symporters. Because perchlorate has been shown to be behaviorally and morphologically masculinizing in stickleback, we investigate possible effects of perchlorate on androgen levels.

To better understand the effects of perchlorate on the HPT and HPG axes, we performed a large, replicated, and long-running experiment on developing stickleback at a variety of chronic and environmentally relevant perchlorate exposures. We examined intermediate and end-point effects of perchlorate exposure by mapping changes of endocrine profiles as well as alterations to morphology and gonadal development. We studied stickleback because it is a widely used aquatic model of ecology, behavior, evolution, endocrine disruption, and genetics (Bell and Foster, 1994; Gravenmier et al., 2005; Cresko et al., 2007; von Hippel, 2010). This small fish is a differentiated gonochorist with genotypic sex determination (Hahlbeck et al., 2004; Kitano et al., 2007; Lewis et al., 2008). Stickleback live in a variety of marine, brackish and freshwater habitats throughout much of the Northern Hemisphere (Bell and Foster, 1994), including in polluted sites. Stickleback are therefore a good model species for investigations of environmental contaminants that might affect human sexual differentiation and development and thyroid function.

In the current study, we found no abnormalities in external phenotype at any dose of perchlorate, but we did discover a significant increase in the number of thyroid follicles in stickleback. Surprisingly, whole fish T_4 and T_3 levels were not different from controls, except at the lowest dose (10 ppm). Perchlorate induced an increase of early stage follicular ovarian follicles in females, and enlargement of the testes and increased occurrence of mature stage sperm in males with increasing dose in both juveniles and adults. Perchlorate also disrupted embryonic androgen levels. We conclude that perchlorate-induced perturbations to the thyroid throughout development may not result in lasting