REPRODUCTIVE AND DEVELOPMENTAL TOXICITY
OF HIGHLY ENERGETIC COMPOUNDS

IN ZEBRAFISH (Danio rerio)

by

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Dedicated to My Beloved Wife, Madhavi
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ABSTRACT

There has been growing concern in recent years about a variety of toxicants, some of which are highly energetic in nature, that can affect human and ecosystem health. Highly energetic compounds such as perchlorate and hexahydro-1,3,5-trinitro-1,3,5-triazine (known as RDX) have been reported in various environments worldwide. The ecotoxicological impacts of these chemicals are relatively not fully understood. The overall objective of this study was to assess the toxicological effects of these two chemicals on reproduction, development and other activities in a model aquatic organism, the zebrafish (*Danio rerio*).

Perchlorate is a known thyroid-disrupting chemical. Exposure to environmental relevant concentrations of perchlorate caused several pathological alterations in thyroid follicles of zebrafish, which were used to establish novel and sensitive biomarkers of perchlorate exposure (Chapter 1). Namely, angiogenesis and ‘colloidal T₄ ring’ intensity were far more sensitive indicators of thyroid disruption than previous available biomarkers. The developmental toxicity of perchlorate was studied in larval zebrafish (Chapter 2). Exposure to perchlorate at concentrations that inhibited thyroid function affected growth (length) and the sex ratio of exposed populations. Treatment with perchlorate skewed the sex ratio towards females, whereas co-treatment with thyroxine (T₄) skewed the sex ratio towards males. This is the first report for teleost fishes indicating that thyroid hormone is involved in the process of gonadal sex determination and differentiation. An effect of perchlorate on the reproductive performance of zebrafish was also observed in a long-term exposure study (Chapter 3). After 16 weeks of exposure
to perchlorate, a decline was observed in the level of T₄, but not triiodothyronine, in whole-body homogenates of mothers and their embryos. Perchlorate also impaired fecundity (packed-egg volume), although egg diameters were increased and fertilization and hatching rates were unaffected. Analyses of the embryos also indicated impaired growth of jaw-forming cartilages (Meckel’s and ceratohyal).

Relatively little information is available concerning the lethal and sublethal effects of RDX in teleosts. The acute toxicity of RDX was determined for larval zebrafish (Chapter 4). The median lethal concentration of RDX was estimated (96-h LC₅₀, 23-26 ppm), and effects on the surviving fish such as vertebral column deformities and behavioral abnormalities were also documented. The chronic toxicity and bioconcentration potential of RDX were subsequently determined in adult zebrafish (Chapter 5). Long-term exposure to RDX caused severe mortality at a concentration of 9.6 ppm. The bioconcentration factor for RDX was low but slightly increased with time of exposure, from ≤1 at 4 and 8 weeks of exposure to >2 at 12 weeks. The RDX metabolite, MNX, was also found in whole-body extracts. Both RDX and MNX were undetectable in whole-body extracts 2 weeks after the fish were transferred to clean water. Lastly, the effects of RDX at sublethal concentrations on reproduction and egg quality were determined (Chapter 6). Exposure to RDX at the relatively low concentration of 0.5 ppm caused a short-lived increase in fecundity within 2 weeks of the onset of exposures; whereas no effects were noted at the higher concentration tested, 3.2 ppm. Egg fertilization rates and embryo hatching rates were unaffected by parental exposures to RDX.
It is concluded that exposure to environmental perchlorate and RDX can potentially impact the health of teleosts, an important component of the aquatic biota.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Ammonium perchlorate</td>
</tr>
<tr>
<td>BCF</td>
<td>Bioconcentration Factor</td>
</tr>
<tr>
<td>ClO$_4^-$</td>
<td>Perchlorate anion</td>
</tr>
<tr>
<td>DIT</td>
<td>Diiodotyrosine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>Dpf</td>
<td>Day post fertilization</td>
</tr>
<tr>
<td>Dph</td>
<td>Day post hatching</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>Effective concentration</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HPT</td>
<td>Hypothalamic-pituitary-thyroid</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography and mass spectrometry</td>
</tr>
<tr>
<td>LD$_{50}$</td>
<td>Lethal concentration</td>
</tr>
<tr>
<td>LOEC</td>
<td>Lowest-observed-effective concentrations</td>
</tr>
<tr>
<td>MIT</td>
<td>Moniodotyrosine</td>
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<tr>
<td>MNX</td>
<td>Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine</td>
</tr>
<tr>
<td>NIS</td>
<td>Sodium/Iodide Symporter</td>
</tr>
<tr>
<td>NOEC</td>
<td>No-observed-effective-concentration</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered Saline</td>
</tr>
<tr>
<td>PBT</td>
<td>Phosphate-buffered saline containing 0.3% Tween</td>
</tr>
<tr>
<td>PTU</td>
<td>Propylthiouracil</td>
</tr>
<tr>
<td>RDX</td>
<td>Royal demolition explosive</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error mean</td>
</tr>
<tr>
<td>T$_3$</td>
<td>Triiodothyronine</td>
</tr>
<tr>
<td>T$_4$</td>
<td>Thyroxine</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
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<tr>
<td>US EPA</td>
<td>United States Environmental Protection Agency</td>
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INTRODUCTION

Industrialization, urbanization and fast population growth have contributed to the degradation of the environment. Except for some natural calamities, anthropogenic activities are the major cause for environmental degradation. Control of pollution and prevention of environmental degradation have been subjects of major debates for many decades. Every year hundreds of new chemicals enter into the market as food additives, industrial reagents, pesticides, insecticides or as therapeutic drugs (Lave, 1990; Barsano and Thomas, 1992). A large portion of these chemicals enter the environment as waste either deliberately or unknowingly. These substances have many adverse effects on biotic communities. They either cause acute mortality of organisms or have sublethal long-term physiological effects (Baker and Landrigan, 1991). Organisms are affected by these contaminants directly via different routes of exposure (air, water, and food chain).

In recent years, there has been growing concern about the effects on human and ecosystem health of a variety of toxicants that are highly energetic (explosive) in nature. Highly energetic compounds such as perchlorate and hexahydro-1,3,5-trinitro-1,3,5-triazine (commonly known as RDX) have been reported in various environments in the United States and other countries (US EPA, 2002; Urbansky, 2002; Walsh and Jenkins, 1992). These two compounds have been primarily used by the military as fuels in rockets and missiles (perchlorate) or as munitions (RDX). Due to their widespread use, these chemicals eventually end up in the environment. Ecotoxicological impacts of these chemicals are relatively poorly understood. For a better ecological risk assessment, better
knowledge is needed about the lethal and sublethal toxicity of these chemicals. Current knowledge of the toxicity of these chemicals is described in the following sections.

Perchlorate

Perchlorate (CAS 7790-98-9) is a highly oxidized form of chlorine (Figure a). For more than 50 years, perchlorate has been used in the manufacture of ammunitions, missiles, and matchsticks and it also has application in industries like leather processing, electro-plating, aluminum refining, rubber manufacturing, and in paints and enamels production (US EPA, 1998). Perchlorate salts (ammonium, sodium, potassium) are quite soluble in water, and therefore these chemicals come out of military installations along with effluent water and find their way into reservoirs, rivers or other natural water bodies (Urbansky, 2002; Tipton et al., 2003). Perchlorate is found in drinking water in more than 30 states in North America and has been an issue of human health and ecological risk assessment (US EPA, 2002).

Figure a: Chemical structure of perchlorate (www.perchlorate.org)
Thyroid function and effects of perchlorate

Thyroid hormones are synthesized and stored inside the thyroid follicles. The thyroid follicles are spherical in shape and consist of a single layer of epithelial cells in periphery surrounding an extracellular lumen containing proteinaceous colloid. In tetrapods thyroid tissue forms a distinct gland whereas in most teleosts is scattered diffusely in the basibranchial region (Brown et al., 2004). In the first step during thyroid hormone synthesis, thyroid follicular cells concentrate iodide against a strong electrochemical gradient. This is an energy dependent process that requires the Na⁺/I⁻ symporter for the transport of I⁻ from the outer environment into the thyroid follicular cell (Tronko et al., 1993) and subsequently secreted into the colloidal space possibly by an apical membrane protein pendrin (Royaux et al., 2000). Iodide is then oxidized by the enzyme, thioperoxidase that requires H₂O₂ for its action. The next step is the iodination of tyrosine, otherwise known as organification of iodide (Chopra et al., 1978). In this step, oxidized iodide reacts with tyrosyl residues in thyroglobulin and forms monoiodotyrosine (MIT) and diiodotyrosine (DIT). Thyroglobulin is a large iodinated and glycosylated protein that is synthesized in the basal portion of the cell and moves to the follicular lumen into which it is secreted. Thyroglobulin is then stored in the extracellular colloid. Thyroglobulin hydrolysis is stimulated by thyroid-stimulating hormone (TSH) produced in the pituitary gland and delivered to the thyroid via the circulation (Larsen, 1982). This hydrolysis yields the thyroid hormones triiodothyronine (T₃) and thyroxine (T₄). Two DIT molecules couple to form T₄ whereas the coupling of MIT and DIT forms T₃ within the thyroglobulin molecule (Granner, 1999). The stored thyroglobulin can supply T₃ and
T₄ for several weeks in a healthy individual. The circulating levels of T₃ and T₄ regulate TSH production from the pituitary by means of a negative feedback mechanism. Thyroid hormone synthesis, metabolism and thyroid disruption by environmental contaminants with specific reference to teleosts has been recently reviewed by Brown et al., (2004).

Perchlorate is an anion with similar partial specific volume to I⁻, and it competes with I⁻ for the sodium/Iodide symporter carrier thus inhibiting iodide uptake. Iodide deficiency causes a reduction in thyroid hormone synthesis and can lead to hypothyroidism. Hypothyroidism disrupts the feedback mechanism and causes hypersecretion of TSH by the pituitary gland (Capen, 2001). Hypersecretion of TSH causes hypertrophy and hyperplasia of the thyroid gland in mammals including humans (Paynter et al., 1988; Maiorana et al., 2003), rats (O'Connor et al., 2002) and rabbits (York et al., 2001a, b). Chronic hypersecretion of TSH may lead to profound goiter (Hard, 1998). In recent years, the toxicological effects of perchlorate on the thyroid gland have been reported by various authors (York et al., 2001a, b; Goleman et al., 2002a, b; Patiño et al., 2003). In Sprague-Dawley rats administered with ammonium perchlorate at 10 mg/kg/day, there was significant increase in thyroid weight and histopathology, consisting primarily of follicular cell hypertrophy with micro-follicle formation and colloid depletion within 14 to 90 days of exposure (Siglin et al., 2000). These changes were reversible after a recovery period of 30 days in the absence of perchlorate. Rats orally treated with 1% potassium perchlorate for more than 2 months showed a diffused homogenous hypertrophy and hyperplasia of follicular cells and decreased amount of colloid and increased vascularization (angiogenesis), but after 6 months of treatment,
neoplastic nodules appeared (Fernandez et al., 1991). The effects of perchlorate on thyroid function have also been examined in amphibians and fishes. In an amphibian, *Xenopus laevis*, ammonium perchlorate at 14 ppm reduced whole-body thyroxine content and caused significant hypertrophy of thyroid follicular epithelium (Goleman et al., 2002a). Potassium perchlorate (KClO₄) at 100 ppm caused a reduction in serum T₄ and T₃ levels by 62 and 72% respectively in sea lampreys (*Petromyzon marinus*) when compared to unexposed animals (Manzon and Youson, 1999). Observations by Patiño et al. (2003) reported that ammonium perchlorate induces hypertrophy, hyperplasia and angiogenesis in thyroid follicles of zebrafish. In eastern mosquitofish (*Gambusia holbrooki*), follicular epithelial cell height, hyperplasia, and hypertrophy increased with increasing perchlorate concentration, especially in fish exposed for 30 d, and these effects were statistically significantly different from controls at concentrations as low as 0.1 ppm (Bradford et al., 2005). Stonerollers (*Campostoma anomalum*) collected from a perchlorate-contaminated site in central Texas showed histopathological abnormalities including thyroid follicular hyperplasia, hypertrophy, and colloid depletion and these conditions correlated with the perchlorate concentrations in water, fish and periphyton (Theodorakis et al., 2006).

Effects of hypothyroidism and perchlorate on early development

It is well established that thyroid hormones are required for early-development of amphibians (Callery and Elinson, 2000), flounder (Inui and Miwa, 1985), sea bream, *Sparus sarba* (Nowell et al., 2001; Deane and Woo, 2003), rainbow trout (Jones et al.,
Thyroid hormones are also believed to have important roles in embryogenesis in fishes. High concentration of thyroid hormones, presumably of maternal origin, is carried in the eggs and, during embryogenesis, the concentration of T₄ and T₃ in the embryo decreases until endogenous production starts (Lam, 1994; Power, 2001). The larval development in zebrafish is rapid. Embryogenesis ends about 3 days post fertilization (dpf) and the newly hatched larvae begins to feed at about 5 dpf. In zebrafish, thyroid follicles are formed about 40 hours post fertilization (Roh and Concha, 2000). Zebrafish larvae start to concentrate radio-iodide for the first time at 3 dpf and this uptake increases throughout the larval and juvenile stages (Brown, 1997). Thyroid hormone deficiency-related problems in embryos or neonates might arise due to maternal hypothyroidism or direct exposure to thyroid disrupting compounds. Neonatal hypothyroidism due to maternal hypothyroidism has been observed in humans (Lazarus, 2002; Shaikh et al., 2003) and rats (Pickard et al., 2003). Thyroid dysfunction during pregnancy is associated with impaired neurological development in newborns babies (Lazarus, 2002a, b). Maternal hypothyroidism is harmful for the development of embryo and increases the risk of neuro-developmental deficits of the fetus of rats (Lavado-Autric et al., 2003). Maternal hypothyroidism has been associated with the retardation of fetal rat growth (Pickard et al., 2003) and brain development (Evans et al., 2002), and forebrain development in larvae of salamander, _Triturus cristatus_ (Gozzo and Castelvetri, 1988).

Environmentally relevant concentrations of ammonium perchlorate impair thyroid function and inhibit development and metamorphosis in _Xenopus laevis_ (Goleman et al., 2002 a). Treatment of zebrafish embryos with amiodarone (an antagonist of thyroid
hormone receptor) and methimazole (Thyoperoxidase inhibitor) leads to several deformities and growth retardation in larva (Liu and Chan, 2002). These developmental deformities in zebrafish were lethal and the larvae did not survive beyond 7 days after fertilization. However, developmental arrest caused by amiodarone-methimazole treatment in zebrafish larva was largely rescued by co-treatment with 10 nM T$_4$.

Zebrafish larva treated with potassium perchlorate showed stunted pectoral and pelvic fin growth and impaired pigmentation and, at highest concentration tested (500 ppm), perchlorate inhibited the radioiodide uptake by 90% (Brown, 1997). Exogenous T$_3$ treatment seems toxic to developing embryo at 10 nM (Liu and Chan, 2002) and to larvae at 5 nM (Brown, 1997). Developing (larval) fathead minnows, (Pimephales promelas) exposed to 1, 10, 100 ppm of ammonium perchlorate showed thyroid hypertrophy, hyperplasia, and depleted colloid in the thyroid follicles in all groups, affected growth in 10 and 100 ppm groups and elevated circulating T$_4$ in 100 ppm group compared to control group (Crane et al., 2005).

Sex differentiation during early stages of development is an important process in vertebrates. Fishes exhibit a wide diversity of sex determination patterns (Strüssmann and Nakamura, 2002). In addition to genetic sex determination, environmental factors like temperature, low pH, salinity, nutrition and exogenous estrogen and androgen levels are known to influence sex determination in fishes (Yamamoto, 1953; Nakamura and Takahashi, 1973; Schreck, 1974; Davis et al., 1995; Strüssmann and Patiño, 1995; Patiño et al., 1996; Kitano et al., 1999; Baroiller and D'Cotta, 2001; Devlin et al., 2001; Saillant et al., 2002; Strüssmann and Nakamura, 2002). The role of endogenous sex steroids is not
fully understood in fish. It has been hypothesized that male and female sex
differentiation in some teleosts are driven by androgen and estrogen hormones,
respectively (Afonso et al., 2001; Strüssmann and Nakamura, 2002). In addition to
steroids, several studies have indicated that thyroid hormones may also play important
roles in the gonadal differentiation in some vertebrates. For example, treatment of *X.
laevis* with thiourea, an antithyroid agent (iodination inhibitor), during larval
development resulted in the formation of all-female populations (Hayes, 1998), and
perchlorate skewed the sex ratio of developing *X. laevis* towards females at the time of
metamorphosis (Goleman et al., 2002a). However, Robertson and Kelley (1996) reported
that propylthiouracil, an antithyroid compound (coupling reaction inhibitor), did not
affect gonadal differentiation but it affected the development of male secondary sexual
characters in *X. laevis*. Therefore, the role of thyroid hormone on the early gonadal
development of aquatic vertebrates remains controversial. Moreover, the role of thyroid
hormones in fish gonadal sex differentiation has not been explored.

**Effects of thyroid hormone and perchlorate on reproduction**

The role of thyroid hormones in fish reproduction has been the subject of research
for many years. The results of a number of studies have suggested that thyroid hormones
are involved in the reproductive processes of some species (Brown et al., 1985). In
Sockeye salmon (*Oncorhynchus nerka*), T₃ and T₄ levels in blood were decreased
significantly in both sexes before spawning (Biddiscombe and Idler, 1983). Serum T₄
levels were highest both in females and males of Chum salmon (*O. keta*) in coastal sea
(before migration) and the levels decreased during the spawning migration (Ueda et al., 1984). In males and females of White sucker (*Catostomus commersoni*), the highest T₄ levels were found in pre-spawning fish and T₄ decreased significantly in spent fish (Stacey et al., 1984). The patterns of T₃ and T₄ in serum and oocytes of the tilapia (*Oreochromis mossambicus*) during oogenesis suggested that thyroid hormones in the oocytes increased throughout recrudescence as the oocytes increased in size, and T₃ appears to be incorporated (into the oocytes) selectively over T₄ (Weber et al., 1992). In wild striped bass, both male and female were in euthyroid state during the spawning season (Mylonas et al., 1997). In the latter study with striped bass, T₃ levels in the blood of females did not change during oocyte maturation but T₄ levels were reduced during the late phase of oocyte maturation. Thyroid hormones may also play an important role during follicular development and vitellogenesis in teleosts. For example, serum thyroid hormone levels in rainbow trout (*Salmo gairdneri*) were high during previtellogenesis but fell before spawning when sex steroids were at their peak, and then increased again following spawning (Cyr et al., 1988). The authors of this study (Cyr et al., 1988) suggested that T₃ enhances early ovarian development but that during energy-demanding process of vitellogenesis, T₃ formation is suppressed thereby curtailing somatic growth and favoring energy partition to the ova. Triiodothyronine (T₃) level was always greater than (T₄) and showed a consistent relationship with steroid hormone profiles.

Contrary to the conclusions of the studies just mentioned, the results of other studies are inconsistent with a possible role of thyroid hormones in the reproductive development of vertebrates. In a study of perchlorate exposure in rat, although the
function of the thyroid gland was affected at a low dose of exposure (3 mg/kg/day), reproductive performance was not affected even at the highest dose tested (30 mg/kg/day) (York et al., 2001b). Also, administration of exogenous thyroid hormone suppressed ovarian follicle growth in the frog, *Rana cyanophlyctis* (Saidapur and Kupwade, 1993). In cows, *Bos taurus*, ovarian function was not affected by hyper- or hypothyroid conditions (De-Moraes et al., 1998). In goldfish, *Carassius auratus*, plasma thyroid hormone levels caused decreased or no marked changes during gonadal development and the early spawning period (Sohn et al., 1999). In a recent study of the effects of perchlorate on the reproductive success of zebrafish, Patiño et al. (2003) found that environmental relevant concentrations of perchlorate (18 ppm) did not affect fecundity although thyroid histology was greatly affected and the number of eggs per unit volume was higher in the 18-ppm group relative to the control group.

**Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)**

Hexahydro-1,3,5-trinitro-1,3,5-triazine (CAS Reg. No. 121-82-4) commonly known as RDX (Royal Demolition eXplosive) is a cyclonite and like perchlorate it has been used primarily by the military. RDX was first synthesized by a German scientist named Henning and the intended use of this chemical was for medicinal purposes (http://www.chm.bris.ac.uk/webprojects2001/moorcraft/RDX test. htm). However, the explosive character of this chemical was not known until 1920. Only after 1940s, mass
production of RDX started and its application as a military explosive increased. RDX has a high degree of stability in storage and is considered the most powerful explosive and a main ingredient in plastic explosives for more than 60 years. Today, RDX is one of the most commonly used explosive in the world (Belden et al., 2005; Walsh and Jenkins, 1992; Talmage et al., 1999), is an ingredient in firework production, and is used for demolition of blocks (ATSDR, 1995). Occasional use of RDX as rodenticide has also been reported (Etnier, 1989).

RDX can be released into the environment directly and indirectly through manufacturing, loading, packing, utilization and demilitarization of antiquated munitions (Burrows et al., 1989; Walsh and Jenkins, 1992; Talmage et al., 1999). Contamination of soils, sediments, surface and ground waters with RDX has been reported in and around army ammunition plants in different parts of the world (Small and Rosenblatt, 1974; Aller, 1985; Walsh and Jenkins, 1992). The reported concentration of RDX in soil samples from various army sites in the United States ranges from 0.044 to 13,900 µg/g (Walsh and Jenkins, 1992). Sources of RDX in aquatic environments may include effluent release or leaching of RDX from contaminated soil into ground or surface waters (Sunahara et al., 1999). In ground water samples collected from the Bangor Annex ammunition processing waste disposal area (Washington, USA), the RDX concentration was up to 3.7 ppm. Concentrations of RDX in surface water at Brush Creek (Iowa, USA), near an army ammunition plant, ranged from 0.1 to 0.15 ppm (Small and Rosenblatt, 1974). In a stream near the Milan army ammunition plant (Tennessee, USA), concentrations of RDX as high as 109 ppm were reported (Ryon et al., 1984). RDX at
such high concentrations could be toxic to the terrestrial wildlife population as well as the aquatic biota.

Disposition of RDX

RDX can enter the body of a living organism through different routes of exposure. In human and laboratory animals, RDX can be slowly absorbed from the gastrointestinal tract after ingestion and from lungs after inhalation; however, absorption of RDX through skin has not been studied (US EPA, 1989). After absorption, RDX can be rapidly cleared from the plasma and distributed to the target tissues (US EPA, 1989). The highest concentrations are usually detected in kidneys, followed by brain, heart and liver. RDX is catalyzed by microsomal enzyme systems present in the liver and is excreted primarily through the urine and exhaled as carbon dioxide (Schneider et al., 1977). RDX can be sequentially metabolized (reduced) to different triazine products like, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (McCormick et al., 1981).

\[ \text{RDX} \quad \xrightarrow{\text{enzymes}} \quad \text{MNX} \quad \xrightarrow{\text{enzymes}} \quad \text{DNX} \quad \xrightarrow{\text{enzymes}} \quad \text{TNX} \]

*Figure b:* Anaerobic biodegradation of RDX (McCormick et al., 1981)
Toxicity of RDX

Cyclonitramines are generally considered toxic to living organisms (Talmage et al., 1999), and thus the presence of RDX in the natural environment could be detrimental to ecosystem health. The toxicity of RDX at acute and sub-acute levels has been relatively well characterized in mammals and birds following both oral and inhalation exposures (Cholakis et al., 1980; Levine et al., 1981a,b; Levine et al., 1983; Gogal et al., 1997). The acute toxicity of RDX including central nervous system toxicity ranging from convulsion to multiple seizures, nausea, vomiting and amnesia were observed in soldiers exposed to RDX (USEPA, 1988). The lethal dose (LD$_{50}$) in rats ($Rattus$ $norvegicus$) was determined to be approximately 200 mg/kg after single oral administration (Clayton and Clayton, 1982). In F344 rats, RDX at a dose of 300 or 600 mg/kg/day caused hyperactivity, tremors, convulsions and death (Levine et al., 1981a). However, information about the toxic effects of RDX in fishes is limited to a few species. The acute 96-h LC$_{50}$ of RDX for juvenile fathead minnow was estimated at 12.7 ppm (Burton et al., 1994) and for bluegill, at 6 mg L$^{-1}$ (Bentley et al., 1977).

Exposure to RDX in F344 rats at a dose of 40 mg/kg/day for 9 days caused several sublethal pathological conditions such as decreased weight gain and decreased food consumption, whereas no toxic effects were observed in B6C3C1 mice after a similar dosing treatment (Cholakis et al., 1980). In another study with mice, doses of RDX as high as of 320 mg/kg/day caused hyperactivity, increased liver weight accompanied by hepatocellular vacuolization (only in male mice), microgranulomas (in females), increased kidney weight, tubular nephrosis and death (Cholakis et al., 1980). In
a 13-week exposure study with F344 rats, subacute doses of RDX caused an increase in liver weight but no histological or biochemical alterations in liver were observed (Levine et al., 1981). However, in a 24-month study in F344 rats exposure to RDX at 40 mg/kg/day, severe toxicity was observed including mortality, hypoglycemia, weight loss, anemia with secondary splenic lesion, hepatotoxicity, cataracts and urogenital lesions (Levine et al., 1984a,b). In rhesus monkeys, the subacute toxicity of RDX includes central nervous system disorders, convulsion and frequent episodic vomiting (Litton 1974). In northern bobwhite (Colinus virginianus), dietary oral exposure to RDX at subacute doses for a period of 90-day caused a dose-dependent decrease in food consumption (Gogal et al., 2003). A terrestrial salamander, Plethodon cinereus exposed to 5000 mg RDX/kg in soil exhibited signs of neuromuscular impairments and significant weight loss, while it did not show any histological alterations in liver (Johnson et al., 2004).

Little information is available for fishes concerning the subchronic toxic effect of RDX at environmentally relevant concentrations. In fathead minnow (Pimephales promelas), larval survival and growth were affected at RDX concentrations ≥ 2.4 ppm after 28 days of exposure (Burton et al., 1994). In another study with the same species, larval size, but not survival, was affected at an RDX concentration of 5.8 ppm after a 30-day exposure (Bentley et al., 1977). Information regarding the bioaccumulation potential of RDX in fishes was not available until recently. This information is now available for channel catfish (Ictalurus punctatus; Belden et al., 2005) and sheepshead minnow (Cyprinodon variegates; Lotufo and Lydy, 2005).
The toxicity of RDX on reproduction has been studied only in a few species of invertebrates and vertebrates. In the earthworm (*Eisenia andrei*), RDX decreased fecundity at a concentration $\geq 46.7$ mg/kg in soil although RDX did not affect the growth of adult individuals at concentrations as high as 167 mg/kg in soil (Robidoux et al., 2002). RDX at a concentration of 209 mg/kg reduced juvenile production in the worm, *Enchtraeus albidus*, but did not affect juvenile production in *E. crypticus* up to the highest tested concentration of 658 mg/kg (Dodard et al., 2005). In a two-generation study with rats (*Rattus norvegicus*), RDX did not show any adverse toxic effects on reproduction at concentrations in the diet as high as 50 mg RDX/kg/day (Cholakis, 1980). Conversely, in northern bobwhite, dietary exposure to RDX caused a reduction in egg production (Gogal et al., 2003).

**Significance of the present study**

Perchlorate and RDX are highly energetic in nature and have been primarily used for military purposes. Much of the waste generated through the uses of explosive/energetic compounds finds their way into the aquatic ecosystem. Although the perchlorate ion and RDX are considered to be a potential health hazard to humans and wildlife population, knowledge of their toxicity to aquatic organisms especially in fish is limited. To our knowledge, there is no published information regarding the reproductive toxic effects of RDX in fishes and little information available for perchlorate. Ecotoxicological evaluations and risk assessments for perchlorate and RDX are a matter of concern for government agencies, researchers, and other organizations concerned with
environmental contamination and human health. Research is needed to develop a database useful for ecological risk assessment, formulation of regulations and the safe use and disposal of these chemicals. Therefore, this study was aimed at generating information regarding the ecotoxicological characteristics of these two chemicals, which could help towards better risk assessment and a safer environment. The specific objectives of this study are described below and their rationale has been described under each of the subsequent chapters. The model organism chosen for all of my experiments is zebrafish (Danio rerio), which is an important laboratory animal for aquatic toxicity studies.

Specific objectives

1. To develop and characterize biomarkers of perchlorate exposure in adult zebrafish (Chapter 1)

2. To determine the effects of perchlorate on gonadal development in larval zebrafish (Chapter 2)

3. To determine the effects of perchlorate on reproductive performance of adult zebrafish (Chapter 3)

4. To determine the acute toxic effects of RDX in larval zebrafish (Chapter 4)

5. To determine the subacute toxicity and bioaccumulation potential of RDX in zebrafish (Chapter 5)

6. To determine the effects of RDX on reproductive performance in adult zebrafish (Chapter 6)
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ABSTRACT

Perchlorate inhibits iodide uptake by thyroid follicles and lowers thyroid hormone production. Although several effects of perchlorate on the thyroid system have been reported, the utility of these pathologies as markers of environmental perchlorate exposures has not been adequately assessed. This study examined time-course and concentration-dependent effects of perchlorate on thyroid follicle hypertrophy, colloid depletion, and angiogenesis; alterations in whole-body thyroxine (T$_4$) levels; and somatic growth and condition factor of subadult and adult zebrafish. Changes in the intensity of the colloidal T$_4$ ring previously observed in zebrafish were also examined immunohistochemically. Three-month-old zebrafish were exposed to ammonium perchlorate at measured perchlorate concentrations of 0, 11, 90, 1131, and 11480 ppb for 12 weeks, and allowed to recover in clean water for 12 weeks. At two weeks of exposure, the lowest-observed-effective concentrations (LOECs) of perchlorate that induced angiogenesis and depressed the intensity of colloidal T$_4$ ring were 90 and 1131 ppb, respectively; other parameters were not affected (whole-body T$_4$ was not determined at this time). At 12 weeks of exposure, LOECs for colloid depletion, hypertrophy, angiogenesis, and colloidal T$_4$ ring were 11480, 1131, 90, and 11 ppb, respectively. All changes were reversible, but residual effects on angiogenesis and colloidal T$_4$ ring intensity were still present after 12 weeks of recovery (LOEC, 11480 ppb). Whole-body T$_4$ concentration, body growth (length and weight), and condition factor were not affected by

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perchlorate. The sensitivity and longevity of changes in colloidal $T_4$ ring intensity and angiogenesis suggest their usefulness as novel markers of perchlorate exposure. The 12-week LOEC for colloidal $T_4$ ring is the lowest reported of any perchlorate biomarker in aquatic vertebrates.

INTRODUCTION

Compounds containing oxyanions of chlorine, such as perchlorate, chlorate, or chlorine dioxide are of growing concern as environmental contaminants (Coates et al., 1999). Perchlorate has been used for more than 50 years in the manufacture of ammunitions, missiles, and match sticks and in industries such as leather processing, electroplating, aluminum refining, rubber manufacture, paints and enamel production (USEPA, 1999; [link](http://www.epa.gov/safewater/ccl/perchlorate/pdf/r9699fac.pdf)), and in fertilizers (Urbansky, 2002). Perchlorate salts (ammonium, sodium, potassium) are soluble in water and readily find their way to surface and ground waters (Urbansky, 2002; Smith et al., 2001; Tipton et al., 2003). Perchlorate also has been reported in the drinking water of many states around the country (USEPA, 2002). Perchlorate is relatively stable and persistent in the environment and some of its reported concentrations vary from 200 to 3,700 ppm in groundwater (Urbansky, 1998); 30 to 31 ppm in pond water on a former military base in Texas (Smith et al., 2001); and 4 to 16 ppb in Lake Mead (NV, USA) (USEPA, 1998).

Perchlorate has similar partial specific volume to iodide (Wyngaarden et al., 1952) and competes with it for its transporter, the sodium (Na)-iodide (I) symporter (NIS).
This symporter has greater affinity for perchlorate than iodide, and the trapping of iodide seems to be inhibited in the presence of perchlorate (Van Sande et al., 2003). Elemental iodide is required for the synthesis of thyroid hormones and iodide deficiency causes a reduction in thyroid hormone synthesis. Hypothyroidism disrupts the hypothalamic-pituitary-thyroid (HPT) feedback mechanism, which stimulates hypersecretion of thyroid stimulating hormone (TSH) by the pituitary gland (Capen, 2001). Hypersecretion of TSH causes hypertrophy and hyperplasia of the thyroid gland in mammals, including humans (Paynter et al., 1988; Maiorana et al., 2003), rats (O’Connor et al., 2002), and rabbits (York et al., 2001a, b). Chronic hypersecretion of TSH may lead to profound goiter, which is related to carcinogenesis (Hard, 1998). High levels of TSH also stimulate the vascularization (angiogenesis) of thyroid glands in humans (Fenton et al., 2001). Angiogenesis has been implicated in several pathological conditions such as inflammation (Koch et al., 1994; Brown et al., 1999) and cancer (Ferrara, 1995; Folkman, 1997). Ample information obtained with experimental animals indicates a relationship between disruption of HPT homeostasis and the development of thyroid neoplasms (Pasquini et al., 2003). Indeed, long-term reductions in circulating thyroid hormones and consequent increases in circulating TSH cause pathogenic angiogenesis and thyroid cancer (Cocks et al., 2000). In recent years, the toxicological effects of perchlorate have been examined in the thyroid gland or follicles of nonmammalian species (Goleman et al., 2002a,b; Patiño et al., 2003; McNabb et al., 2004). Patiño et al., (2003) showed that exposure to ammonium perchlorate (AP) can induce angiogenesis in thyroid follicles of zebrafish.
Thyroid gland histopathology and circulating levels of HPT hormones are often used as indicators of disorders of the thyroid gland in mammals (Maiorana et al., 2003; O’Connor et al., 2002; York et al., 2001a, b; Hard, 1998). Thyroid follicle cell hypertrophy is perhaps the most widely used histological marker of thyroid activity. Although hypertrophy has been used as indicator of thyroid pathology caused by perchlorate exposure (York et al., 2001a, Goleman et al., 2002a; Patiño et al., 2003; McNabb et al., 2004; Siglin et al., 2000) its sensitivity to low, environmentally relevant perchlorate concentrations has not been fully characterized. In some species, colloid depletion and alterations in thyroid hormone levels are considered to be relatively insensitive markers of perchlorate exposure (McNabb et al., 2004). Thyroidal angiogenesis caused by perchlorate has been described (Patiño et al., 2003; Fernandez Rodriguez et al., 1991) but not adequately assessed as a marker of exposure. An accumulation of thyroxine (T4) immunoreactivity along the periphery of the colloid, at the interphase between the follicular epithelium and the lumen, has been previously observed in teleost fishes using immunohistochemical methods (Raine et al., 2001; Wendl et al., 2002). However, the effects of perchlorate or other goitrogens on this colloidal T4 ring have not been examined. Further research is needed to characterize and validate biomarkers of perchlorate exposure that are capable of sensing its presence at low, environmentally relevant concentrations.

This study evaluated and compared current as well as potentially new biomarkers of perchlorate exposure using zebrafish as an experimental model. Seven different endpoints were examined: Thyroid follicle hypertrophy, angiogenesis, colloid depletion,
colloidal T₄ ring intensity, whole-body thyroxine concentration, body growth (weight and fork length), and condition factor. We exposed zebrafish for 12 weeks to different concentrations of perchlorate in water, and then allowed them to recover for 12 weeks to assess reversibility of effects. Samples were taken for analyses at various times during exposure and recovery. We determined and compared the lowest-observed-effective concentrations (LOECs) and the reversibility of the endpoints measured.

MATERIALS AND METHODS

Chemicals

Ammonium perchlorate (CAS 7790-98-9, Purity 99.999%) was purchased from Aldrich Chemical (Milwaukee, WI, USA). Stock solutions were prepared by mixing appropriate weight/volume of chemicals in zebrafish-water. For radioimmunoassay, L-[¹²⁵I]-T₄ (specific activity, 969 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). Primary T₄ antibody (rabbit anti-T₄ BSA serum) was purchased from ICN Biomedicals (Costa Mesa, CA, USA). This antibody has 100% reactivity towards L- and D-T₄ and 1.2% cross reactivity with L-triiodothyronine, and exhibits very low cross reactivity with mono- or di-iodo-L-tyrosine (<0.001%) at 50% displacement. Secondary antibody, anti-rabbit IgG developed in goat, and standard L-T₄ were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Animal husbandry

The use of animals in this study was reviewed and approved by the Texas Tech
University Animal Care and Use Committee (Lubbock, TX, USA). Two-month-old zebrafish (*Danio rerio*) were obtained from Aquatic Research Organisms (Hampton, NH, USA) and acclimatized for four weeks in zebrafish-water (180 mg of Sea Salt® per liter of deionized water) in 10-gallon glass aquaria. Each tank was fitted with two hand-made internal biofilters consisting of 250-ml glass beakers filled with glass wool and glass beads. The water flow inside the filter was maintained by airflow through a glass pipette.

All physico-chemical water parameters were maintained at optimal conditions for zebrafish (temperature, 26-28 °C; dissolved oxygen > 4 ppm; pH, 6.5-8; photoperiod, 14:10-h light:dark, unionized ammonia < 0.01 ppm). Fish were fed twice daily to satiation with commercial diet (Tetramin®, Tetra Sales, Blacksburg, VA, USA). Dissolved oxygen, temperature, conductivity, and salinity were measured daily using an YSI® model 85 meter (Yellow Springs, OH, USA), and total ammonia was measured with a HACH® spectrophotometer model DR/2000 (Loveland, CO, USA) at least once weekly. A small volume of water was siphoned daily from the bottom of the tank to remove debris, and one-half of the water volume was removed and replaced with clean water once weekly or as required to maintain water quality parameters.

**Experimental design and perchlorate exposure**

Zebrafish were exposed to nominal perchlorate concentrations of 0, 10, 100, 1000, and 10000 ppb. There were three replicate aquaria per treatment. Each experimental tank contained approximately 50 fish at the beginning of the exposure. Fish were exposed to
AP for 12 weeks and then were allowed to recover in the absence of perchlorate for an additional 12 weeks.

At the beginning of the exposure and at the time of each water exchange during the rearing period, the appropriate volume of AP stock solution was added to the aquaria to maintain the target perchlorate concentration. Water samples were collected for verification of actual perchlorate concentrations once every two weeks starting on the first day of exposure. At the end of the 12-week exposure period, the fish were carefully removed with a net from the exposure tanks, briefly rinsed in respective large beakers with aerated clean zebrafish water, placed in clean 10-gallon glass aquaria with fresh zebrafish water (without perchlorate), and reared for an additional 12 weeks. Water samples were collected during the recovery period at 1, 4, and 12 weeks.

**Fish sampling**

For thyroid histological observations and body growth and condition factor assessments, fish were collected at 2, 4, 8, and 12 weeks of exposure and at 4 and 12 weeks of recovery. At each sampling time, fish were allowed to swim (rinsed) in three consecutive baths of fresh zebrafish-water to remove external perchlorate (to minimize contamination of the laboratory), euthanized in MS-222 (1 g/L), and placed in Bouin’s fixative (LabChem, Pittsburgh, PA, USA) following an abdominal incision. Separate samples were also collected at 12 weeks of exposure and at 12 weeks of recovery for analysis of $T_4$ concentrations in whole-body extracts. For this purpose, fish were rinsed, euthanized, wrapped in aluminum foil, snap-frozen in liquid nitrogen, and stored at -80
°C until further analysis (Goleman et al., 2002a). Five fish from each replicate aquarium, irrespective of sex, were randomly collected at each sampling. The sex of each fish was later verified by histology (except those for hormone analysis, which were frozen).

**Perchlorate concentration in treatment water**

Perchlorate concentration was measured by ion chromatography in stock solutions and in water samples collected from experimental tanks (Anderson and Wu, 2002).

**Body growth**

To evaluate the effect of AP on growth of zebrafish, body weight and fork length (from tip of snout to the point where the caudal fin bifurcates) were measured at each time of sampling. Condition factor was also calculated according to the formula, 100000 x body weight (g)/length\(^3\) (mm\(^3\)). Since the growth patterns of adult male and female zebrafish are different, these measurements were analyzed separately for each sex.

**Histology**

Whole fish were kept in Bouin’s solution for 48 h at 4 °C and subsequently processed for histology according to procedures described for zebrafish by Patiño et al. (2003). The head of the fish was separated by incision from the trunk region, and each piece was used to prepare separate blocks of paraffin for thyroid histopathology and sex determination, respectively. Sections (6 μm) were processed and stained with hematoxylin and eosin.
For each fish, all thyroid histopathological analyses were conducted on the same cross-section of the head. The section was chosen according to its histological integrity and quality when viewing the first row of sections on the slide from left to right. Digital images of thyroid follicles were taken with an Olympus digital camera (DP10; Tokyo, Japan) attached to a compound microscope. Measurements were conducted digitally using Image-Pro® Express Software (Media Cybernetics, Silver Spring, MD, USA). The height of the thyroid follicle epithelium was used as index of hypertrophy. This height was measured at four predetermined positions (12, 3, 6, and 9 o’clock) in each of five follicles per fish. The average height was calculated for each follicle, and the average of the five follicles was determined for each fish. The number of blood vessels around the entire length of the follicular epithelium in these five follicles was also counted under the microscope, and the perimeter length of each follicle was measured digitally. The number of blood vessels was standardized to 100 μm of follicular perimeter and used as an index of angiogenesis for each follicle. The average of the five measurements was regarded as the fish value for angiogenesis. A semi-quantitative method was used to measure colloid depletion by assigning a score for each of the five follicles as follows: 0, no colloid depletion (no change relative to control); 1, up to one-third of colloid depleted; 2, up to two-thirds of colloid depleted; and 3, up to full colloid depletion. The average score for the five follicles was used as the fish value.

The procedures for processing and staining of the trunk regions were the same as for the head. The purpose of these preparations was to determine the sex of the fish by inspection of the gonads.
**Immunohistochemistry**

For this analysis, three fish per tank were randomly chosen from the five fish per tank sampled at 2 and 12 weeks of exposure and 12 weeks of recovery. Thyroxine immunohistochemistry for paraffin sections of thyroid follicles was performed as described previously for larval zebrafish (Wendl et al., 2002) with some modifications for the use of the VectaStain® Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Briefly, cross-sections of the head containing thyroid follicles were dewaxed, treated with 1% H₂O₂ to quench endogenous peroxidase activity, blocked with normal goat serum in phosphate-buffered saline containing 0.3% Tween (PBT), then rinsed and incubated with primary antibody diluted in PBT (1:8000 dilution of rabbit-anti T₄ BSA serum [ICN Biomedicals, Costa Mesa, CA, USA] in PBT) for 30 minutes. The sections were rinsed and incubated with a biotinylated secondary antibody containing horseradish peroxidase (in PBT) for 30 min, rinsed in phosphate-buffered saline and stained for 10 min using diaminobenzidine reagent. The slides were washed, dehydrated, cleared with xylene, and mounted with resin. No counter-stain was used.

Digital images of diaminobenzidin-stained thyroid follicles were taken and imported to the Image-Pro Express software. A typical control follicle has a ring of relatively strong T₄ signal along the periphery of the colloid (see Results). The optical density of the ring was measured digitally at eight predetermined positions (12, 1.5, 3, 4.5, 6, 7.5, 9, and 10.5 o’clock). Background staining was also measured at the same positions but immediately outside the follicle. The average optical densities of the ring and background staining were calculated from their eight respective values. Background
staining was subtracted from the ring staining to obtain a corrected value for colloidal T₄ ring intensity in each follicle. Two to six follicles per fish were examined and their values were averaged to calculate individual fish values.

**Thyroid hormone extraction and radioimmunoassay**

Thyroid hormone extraction was done according to Goleman et al. (2002a) with some modifications. The five fish taken from each tank replicate per sampling time were pooled for this procedure. The total weight of the pooled fish was recorded, the fish were broken into smaller fragments while still frozen, and then homogenized and sonicated. Approximately 1000 counts per minute of L-[^125]I]-T₄ in 50 µL of methanol (containing 1 mM propylthiouracyl) were added to estimate the recovery of endogenous hormone. Immediately before use, free iodine was removed from the radiotracer preparation using Sep-Pak® C₁₈ cartridges (Waters, Milford, MA, USA) according to Denver (Denver, 1993). Following a 30-min incubation, the homogenates were centrifuged and the supernatants were removed, mixed with two volumes of chloroform, and back-extracted into an aqueous phase with 2N NH₄OH. The back extraction was repeated two more times. The aqueous fractions were pooled and dried in a Jouan centrifugal evaporator overnight. The dried samples were resuspended in 1 ml of 2N NH₄OH, mixed with 2 ml chloroform, and centrifuged at 1500 g for 15 min at 4 °C. The aqueous phase was collected and purified by ion exchange chromatography as described by Morreale de Escobar et al. (1985). Fractions 4 through 9 were pooled, evaporated, reconstituted in radioimmunoassy buffer (300 µL), and the recovery of radiotracer was determined in an
aliquot using a Cobra 5005 gamma counter (Packard, Downers Grove, IL, USA). Under these conditions, the recoveries of radiotracer ranged from 32 to 63% (average 44%).

Thyroxine was measured in duplicate 50-μL aliquots of extract following the procedure of Mackenzie et al. (1978). Authentic T₄ standards were run in parallel to the samples and in duplicate for each concentration. The hormone content of the samples was determined using a four parameter logistic transformation of [¹²⁵I] T₄ displacement by the authentic standards. The values obtained for the two replicates per sample were averaged, and this average was corrected according to the estimated recovery for each sample. This assay procedure was validated for zebrafish whole-body extracts by confirming the direct proportionality of hormone dilution in serially diluted extracts, and by confirming full recovery of known concentrations of exogenously added authentic T₄ into the extracts.

Data analysis

All parametric analyses were conducted using the Statistica® software package (StatSoft, Tulsa, OH, USA), and nonparametric analysis with InStat® (GraphPad, San Diego, CA, USA), at the level of significance α = 0.05. The unit of replication for all treatments was the aquarium (fish tank), except for nonparametric analysis of colloidal depletion (see later description). Thus, fish values within a tank were averaged to obtain tank values, and the sample size per treatment per sampling time was three. However, because body size in zebrafish differs between sexes, the effects of AP on weight, fork length, and condition factor were analyzed separately for males and females. Namely, a male and a female tank value were determined for each aquarium prior to the analysis. A
preliminary parametric analysis of hypertrophy, angiogenesis, and colloid depletion (three-way analysis of variance [ANOVA]; factors: sex, perchlorate concentration, and sampling time) indicated no significant differences between sexes. Thus, in the definitive analyses, all male and female fish values were pooled within a tank to obtain overall tank values for hypertrophy, angiogenesis and colloid depletion. Analysis of colloidal T\textsubscript{4} ring intensity was also conducted on overall tank values irrespective of the sex of the fish.

A tiered approach was followed for the definitive data analyses. The first step was to conduct a parametric two-way ANOVA using perchlorate concentration (treatment) and sampling time as factors. If significant treatment or interaction effects were determined, the second step was to perform a one-way ANOVA for treatment at each sampling time. When appropriate, differences between treatment means at each sampling time were determined using Duncan’s multiple range tests. At each sampling time, ranks of colloid depletion were reanalyzed using the nonparametric tests, Kruskal-Wallis ANOVA by ranks and Dunn’s post-test. These nonparametric tests were conducted using individual fish values as unit of replication instead of tank values. For clarity, only the differences between each of the perchlorate treatments and the control group are reported.

RESULTS

Perchlorate concentration in water

The measured concentrations of perchlorate in tank water were close to the nominal concentrations. The mean perchlorate concentrations (± standard error, \(n = 7\) samples collected from each tank replicate) in the experimental tanks during exposure
were \(0 \pm 0, 11 \pm 0, 90 \pm 3, 1131 \pm 23,\) and \(11480 \pm 335\) ppb respectively for the 0, 10, 100, 1000, and 10000 ppb nominal concentrations. During the recovery period, perchlorate was detected only once, in the first water sample taken (one week after transfer to clean tanks) from one of the tank replicates of the 100 ppb-treatment group; the concentration in this tank was 24 ppb. There were no traces of perchlorate detected at any other time in this tank, or in any other tank during the recovery period. Although perchlorate levels during recovery were undetectable, for treatment identification purposes the results obtained during this period are associated with the original perchlorate concentrations.

**Effects of AP on general fish appearance, body growth and condition factor**

The AP-treated zebrafish did not show any behavioral signs of stress, and no treatment-related deaths occurred during the exposure or recovery period. One of the tank replicates for the 100-ppb (nominal) treatment group was lost due to a heater malfunction during the sixth week of exposure. Therefore, the number of tank replicates for this treatment was reduced to two for subsequent sampling times (8 weeks of exposure and later samplings).

The 3-month-old experimental fish were immature (juvenile stage) at the start of the exposures. The initial mean (± standard error) weight and fork length of the mixed-sex experimental population was 0.22 (± 0.01) g and 25.1 (± 0.34) mm (n = 20 fish). The fish had matured and showed signs of spawning behavior by the completion of the experiment 24 weeks later. Body weight, fork length, and condition factor of male and
female zebrafish were not affected by perchlorate exposure ($p > 0.05$), but there was an increase in their values during the experimental period (two-way ANOVA on each sex separately; $p < 0.05$). The overall weight, fork length and condition factor (mean of all groups ± standard error) taken at 2 weeks of exposure were 0.21 ± 0.01 g, 26.8 ± 0.3 mm and 1.06 ± 0.02 for males, and 0.26 ± 0.01 g, 27.5 ± 0.5 mm and 1.23 ± 0.04 for females. The same measurements taken at 12 weeks of exposure were 0.51 ± 0.01 g, 38.4 ± 0.2 mm and 1.23 ± 0.01 for males and 0.55 ± 0.02 g, 33.0 ± 0.3 mm and 1.40 ± 0.03 for females. At the end of the recovery period, the measurements were 0.70 ± 0.01 g, 38.4 ± 0.2 mm and 1.23 ± 0.01 for males and 0.84 ± 0.02 g, 38.2 ± 0.3 mm and 1.51 ± 0.02 for females.

**Effects of AP on thyroid histopathology**

Thyroid follicles from control fish are lined with a single layer of squamous or cuboidal epithelial cells and their lumens are filled with colloid (Figure 1.1A). Treatment with AP, especially at the higher perchlorate concentrations and longer exposure times, induced an increase in epithelial cell height (hypertrophy; Figure 1.1B, C), an increase in the number of blood vessels around (and perhaps within) the follicular layer (Figure 1B, C), and various degrees of colloidal depletion in individual follicles (Figure 1.1C).

Quantitative analysis indicated that exposure to AP caused concentration- ($p < 0.05$) and exposure length-related ($p < 0.05$) responses in the degree of thyroid follicle hypertrophy, angiogenesis, and colloid depletion (two-way ANOVA separately for each variable). At two weeks of exposure, the only variable for which significant treatment effects were noted was angiogenesis, and the lowest-observed-effective concentration
(LOEC) of measured perchlorate was 90 ppb (one-way ANOVA and Duncan’s multiple range test, \( p < 0.05 \)) (Figure 1.2). Hypertrophy became evident at four weeks of exposure, and the LOECs for angiogenesis and hypertrophy at this time were 1131 (one-way ANOVA and Duncan’s multiple range test, \( p < 0.05 \)) and 11480 ppb (one-way ANOVA and Duncan’s multiple range test, \( p < 0.05 \)), respectively (Figure 1.2). At eight weeks of exposure, the LOEC for hypertrophy remained at 11480 ppb (one-way ANOVA and Duncan’s multiple range test, \( p < 0.05 \)) and for angiogenesis was 90 ppb (one-way ANOVA and Duncan’s multiple range test, \( p < 0.05 \)) (Figure 1.2). At 12 weeks of exposure, the LOEC for hypertrophy had decreased to 1131 ppb (one-way ANOVA and Duncan’s multiple range test, \( p < 0.05 \)) and for angiogenesis remained at 90 ppb (one-way ANOVA and Duncan’s multiple range test, \( p < 0.05 \)) (Figure 1.2). At four weeks of recovery, the LOEC for hypertrophy remained at 1131 ppb (one-way ANOVA and Duncan’s multiple range test, \( p < 0.05 \)) and for angiogenesis had increased to 1131 ppb (one-way ANOVA and Duncan’s multiple range test, \( p < 0.05 \)) (Figure 1.3). At 12 weeks of recovery, hypertrophy was no longer detectable (one-way ANOVA, \( p > 0.05 \)) but angiogenesis was still observed with a LOEC of 11480 ppb (one-way ANOVA and Duncan’s multiple range test, \( p < 0.05 \)) (Figure 1.3). It should also be noted that the absolute values for hypertrophy and angiogenesis in response to AP exposure increased with time of exposure (Figure 1.2) and decreased with length of recovery (Figure 1.3). Signs of colloid depletion (mean score ± standard error) were first noticeable after eight weeks of exposure at perchlorate concentrations of 1131 ppb (0.04 ± 0.03) and 11480 ppb (0.27 ± 0.09), but only the latter was significantly different from the untreated
control (Kruskal-Wallis and Dunn’s post test, $p < 0.05$). The same dose-response pattern was observed at 12 weeks of exposure to 1131 ppb (0.17 ± 0.08) and 11480 ppb (1.19 ± 0.22), with only the highest concentration of perchlorate yielding results significantly different from control values (Kruskal-Wallis and Dunn’s post test, $p < 0.05$). During the recovery phase, thyroid follicles appeared to regain their lost colloid relatively quickly. Namely, although signs of colloid depletion were qualitatively noticed at four weeks of recovery in fish exposed to 1131 ppb perchlorate (0.12 ± 0.11), there was no significant difference from the control group (Kruskal-Wallis test, $p > 0.05$). No signs of colloid depletion were observed after 12 weeks of recovery in fish from the 1131 and 11480 ppb treatment groups, or in any of the fish exposed to 11 or 90 ppb perchlorate at any time during the experiment (all scores were zero; Figure 1.4).
Figure 1.1. Photomicrographs of sections of thyroid follicles (asterisks) of zebrafish reared in control water (A) or in water containing ammonium perchlorate-derived perchlorate at measured concentrations of 11480 ppb for 12 weeks (B-C). Note the squamous or cuboidal epithelial cell layer in control fish (A), and the cuboidal or tall-columnar shape and greater height (hypertrophy) of the cells in fish exposed to perchlorate (B-C). The follicles of perchlorate-exposed fish also developed higher numbers of blood vessels (angiogenesis) within the epithelial layer (B-C, arrows), and some follicles in these fish also showed signs of colloid depletion (C). Hematoxylin-eosin stain. Scale bar = 50 µm.
Figure 1.2. Changes in hypertrophy (□) and angiogenesis (■) of zebrafish thyroid follicles during exposure to ammonium perchlorate-derived perchlorate at measured concentrations of 0 to 11480 ppb from 2 to 12 weeks. Bars indicate mean values (+ standard error). Bars associated with an asterisk (*) are significantly different from the control (0 ppb) bar (one-way analysis of variance, Duncan’s multiple range test, \( p < 0.05 \)).
Figure 1.3. Changes in hypertrophy (□) and angiogenesis (■) of zebrafish thyroid follicles during recovery from a 12-week exposure to ammonium perchlorate-derived perchlorate at measured concentrations 0 to 11480 ppb. Measurements were taken at 4 and 12 weeks after removal of ammonium perchlorate from the tank water. The 12-week exposure values are the same as those shown in Figure 1; they are repeated here as baseline reference. Bars indicate mean values (+ standard error). Bars associated with an asterisk (*) are significantly different from the control (0 ppb) bar (one-way analysis of variance, Duncan’s multiple range test, p < 0.05).
Figure 1.4. Changes in colloidal depletion score in thyroid follicles during 8 and 12 weeks exposure and 4 weeks of recovery. Control fish showed no colloidal depletion at any point of exposure or recovery. During the exposure period (8 and 12 weeks) colloidal depletion in 11480 ppb was significantly affected ($p<0.05$, Kruskal-Wallis, Dunn’s post test). Asterisk (*) indicated a significantly different from the control (zero depletion score). No colloidal depletion was not detected (ND) at 4 and 12 weeks (not shown) of recovery period.
Effect of AP on colloidal T\textsubscript{4} ring intensity

Control follicles showed relatively strong immunostaining for T\textsubscript{4} along the periphery of their colloid (Figure 1.5A, B). Nonperipheral (luminal) colloid also reacted positively for T\textsubscript{4}. Negative-control follicles incubated with normal rabbit serum instead of primary anti-T\textsubscript{4} rabbit antiserum did not show immunostaining (data not shown). Exposure to perchlorate caused a decrease in the colloidal T\textsubscript{4} ring intensity (Figure 4C, D), and in extreme cases it became almost undetectable (Figure 1.5D).

At two weeks of exposure, 1131 and 11480 ppb of perchlorate inhibited the optical density of the colloidal T\textsubscript{4} ring (one-way ANOVA, and Duncan’s multiple range test, \(p < 0.05\)) (Figure 1.5). After 12 weeks of exposure, the intensity of the colloidal T\textsubscript{4} ring was suppressed in all perchlorate-treated groups, and the LOEC was 11 ppb (one-way ANOVA, and Duncan’s multiple range test, \(p < 0.05\)) (Figure 5). After 12 weeks of recovery, the effect of perchlorate exposure was still present in fish that had been exposed to the highest concentration (11480 ppb; one-way ANOVA and Duncan’s multiple range test, \(p < 0.05\)) (Figure 1.6).

We also measured the optical density of the luminal colloid in samples taken at 12 weeks of exposure. The staining intensity of luminal colloid was inhibited in the presence of perchlorate, but this inhibition was very minor (LOEC, 11480 ppb; data not shown) compared with the inhibition observed in colloidal T\textsubscript{4} ring intensity (LOEC, 11 ppb) (Figure 1.5).
Figure 1.5. Photomicrographs of sections of thyroid follicles (asterisks) immunostained for T4. Follicles from control fish showed a relatively strong signal (dark brown color) along the periphery of their colloid, at the interphase with the epithelium, giving the appearance of a colloidal T4 ring (A-B, arrows). The ring signal declined in intensity (C, arrow) or even disappeared (D, arrow) in follicles from fish exposed to perchlorate (11480 ppb in the photomicrographs shown). The luminal colloid also showed positive signal (light brown color). Scale bar = 25 µm.
Figure 1.6. Changes in colloidal T4 ring intensity (optical density) of zebrafish thyroid follicles at 2 and 12 weeks of exposure to ammonium perchlorate-derived perchlorate at measured concentrations 0 to 11480 ppb for 12 weeks, and after 12 weeks of recovery. Bars indicate mean values (+ standard error). Bars associated with an asterisk (*) are significantly different from the control (0 ppb) bar (one-way analysis of variance, Duncan’s multiple range test, $p < 0.05$).
Figure 1.7. Effect of perchlorate exposure on whole-body thyroxine (T4) levels. Either the length of exposure or the treatment had any effect on the T4 concentration (p>0.05, two-way ANOVA).
Effect of AP on thyroid hormone levels

No significant effect of AP exposure on whole-body T\textsubscript{4} concentrations (ng/g body wt) was observed at any time during this experiment (two-way ANOVA; factors: treatment and sampling time; \( p > 0.05 \)). The mean (± SE) concentration of T\textsubscript{4} (grouped by sampling time) was 1.37 ± 0.09 ng/g at 12 weeks of exposure and 1.73 ± 0.15 ng/g at 12 weeks of recovery.

DISCUSSION

This study evaluated seven different biological endpoints as possible markers of exposure to environmentally relevant concentrations of perchlorate in zebrafish: Thyroid follicle angiogenesis, thyroid follicle hypertrophy, thyroid follicle colloid depletion, intensity of colloidal T\textsubscript{4} ring, whole-body T\textsubscript{4} concentration, body growth (weight and length), and condition factor. The results obtained indicated that colloidal T\textsubscript{4} ring intensity is the most sensitive of these markers when measured after relatively prolonged exposures to perchlorate (12 weeks). Structural (histological) indices of thyroid activity were, as a group, the second most sensitive markers of exposure to AP-derived perchlorate. Somatic growth and condition factor as well as whole-body thyroxine concentration were not affected by perchlorate under the present experimental conditions. Thus, these latter traits are not useful as makers of perchlorate exposure in subadult-to-adult zebrafish.

The LOECs for changes in the intensity of colloidal T\textsubscript{4} ring were 1131 ppb at two weeks of exposure and 11 ppb at 12 weeks. The 12-week LOEC for this parameter (11
ppb) is one order of magnitude lower than the next most sensitive marker examined in this study (angiogenesis; see later discussion), and several-fold lower than the lowest concentrations of perchlorate for which effects have been previously reported in other aquatic vertebrates (e.g., 59 ppb for hypertrophy in *Xenopus* tadpoles (Goleman et al., 2002a)). The intensity of the colloidal T₄ ring was still reduced after 12 weeks of recovery in fish exposed to the highest concentration of perchlorate tested in this study (11480 ppb). Thus, changes in the intensity of the colloidal T₄ ring occurred relatively rapidly upon the onset of the exposure, responded with high sensitivity after prolonged exposure periods, and “stored” information for relatively long periods of time following termination of the exposure.

Uptake of iodide into the thyroid follicle cell is facilitated by NIS present at basolateral membrane (De La Vieja et al., 2000), and apical efflux into the follicular lumen is probably facilitated by pendrin, a transport protein in the apical surface of the cell (Royaux et al., 2000). In the rat thyroid follicle, radioactive iodide seems to accumulate at the apical surface of the epithelium shortly after administration of the radiotracer, thus forming a ring around the periphery of the colloid (Wollman and EKholm, 1981; Ofverholm and Ericson, 1984). The location and appearance of this iodide ring is similar to the colloidal T₄ ring observed in zebrafish ((Wendl et al., 2002); present study), medaka, and rainbow trout (Raine et al., 2001) thyroid follicles. Thyroperoxidase, an integral membrane protein of thyroid follicle cells, catalyzes iodination and coupling, two major process involved in T₄ synthesis (Nilsson, 2001). Therefore, the colloidal T₄ ring of fish thyroid follicles may reflect the relative accumulation of T₄ at its site of
synthesis. As free T₄ is soluble in water and alcohol, the immunohistochemical technique used in the present study most likely detects only the thyroglobulin-bound T₄ present in the colloid. The thyroid follicles of teleost fishes seem to produce predominantly T₄ and little triiodothyronine (Raine et al., 2001).

The occurrence of angiogenesis, hypertrophy, and colloid depletion in thyroid follicles of zebrafish exposed to AP has been previously reported (Patiño et al., 2003). However, the present study is the first to methodically examine the relative sensitivities of these histological changes as markers of perchlorate exposure in zebrafish, and the first to examine angiogenesis as marker of perchlorate exposure in any species. Angiogenesis was the most sensitive of the histological markers examined either on temporal or perchlorate-concentration scales. Namely, angiogenesis appeared at two weeks, well before hypertrophy and colloid depletion, and also responded to water perchlorate concentrations (90 ppb) that are at least one order of magnitude lower than those affecting the other parameters measured. Curiously, the LOEC of perchlorate for angiogenesis did not change with exposure time, essentially remaining at 90 ppb from 2 to 12 weeks of exposure. The absolute number of blood vessels per unit length of follicular perimeter, however, generally increased with time of exposure to perchlorate. On a temporal scale, hypertrophy was second to angiogenesis in order of sensitivity, becoming evident for the first time at four weeks but only at the highest concentration of perchlorate tested (11480 ppb). Colloid depletion was first detected at eight weeks of exposure, also at 11480 ppb. The LOEC for colloid depletion remained at 11480 ppb even after 12 weeks of exposure, whereas LOEC for hypertrophy declined from 11480.
ppb to 1131 ppb from 8 to 12 weeks. Although these histological alterations were reversible after the removal of AP from tank water, significantly higher levels of thyroid follicle vascularization were still observed at 12 weeks of recovery in fish exposed to the highest concentration of perchlorate (11480 ppb) compared to the control fish. Thus, angiogenesis is also the most persistent of the histological biomarkers examined in this study, as persistent as changes in the intensity of the colloidal T4 ring (see earlier discussion). Unlike the findings of Patiño et al. (2003), hyperplasia was not observed in the present study. This difference in results may be due to the higher concentrations of perchlorate (18000 ppb) used by Patiño et al. (2003), or to differences in sensitivity to perchlorate between the fish populations used by the two studies. Like the findings of Patiño et al. (2003), no sex-linked effects of AP were noted on the thyroid histology of fish from the present study.

The effects of perchlorate on thyroid histology also have been examined in amphibians and other vertebrates. In tadpoles of *Xenopus laevis*, water concentrations of AP-derived perchlorate as low as 59 ppb caused hypertrophy of thyroid follicles after 10 weeks of exposure (Goleman et al., 2002a). This finding with larval amphibians contrasts with the results of the present study with subadult-to-adult zebrafish, where the lowest concentration of perchlorate that induced thyroid follicle hypertrophy after 12 weeks of exposure was 1131 ppb. In rats, oral administration of AP at 10 mg/kg/day caused significant increases in thyroid gland weight, follicular cell hypertrophy, micro-follicle formation, and colloid depletion within 14 to 90 days of exposure (Siglin et al., 2000). Similar to the present observations with zebrafish, perchlorate-induced changes in thyroid
Increased vascularization (angiogenesis) of the thyroid gland has been reported in rats orally treated with 1% potassium perchlorate for two months (Fernandez Rodriguez et al., 1991). Comparing the phenomenon of angiogenesis between zebrafish and rats is complicated because of differences in their thyroid structure. Thyroid follicles of zebrafish and other teleost fishes are not encapsulated in a discrete gland like they are in most other vertebrate taxa, but are found dispersed among the branchial arterioles of the lower throat region. The increased vascularization observed in the present study seemed to occur inside the follicles within the follicular epithelium, but Patiño et al. (2003) also reported a general increase in the vascularization of the extrafollicular space around the thyroid follicles of zebrafish exposed to AP. In the rat thyroid gland, perchlorate-induced vascularization seems to occur in the extrafollicular compartment (Fernandez Rodriguez et al., 1991). Regardless of the microanatomical location of the angiogenic response to perchlorate (electron microscopy may be needed to clarify the location in zebrafish), the increased vascularization can be regarded as a physiologically adaptive mechanism to compensate for the disruption in thyroid hormone production. Namely, an increase in the number of blood vessels in or around thyroid follicles may increase the supply of nutrients (including iodide) to the follicles, and also enhance the removal and distribution of thyroid hormones stored in their lumen in order to maintain thyroid hormone homeostasis.

Given the various effects of perchlorate exposure on the histology and $T_4$-
immunological traits of the thyroid, its lack of effects on whole-body T\textsubscript{4} levels may seem unexpected. However, previous studies of changes in HPT hormone levels in blood or whole-body have yielded inconsistent results. For example, exposure to a nominal concentration of approximately 100 ppm caused a reduction in serum T\textsubscript{4} and triiodothyronine levels in sea lamprey (\textit{Petromyzon marinus}) larvae after several weeks of treatment (Manzon and Youson, 1997). Perchlorate at 14140 ppb, but not 59 ppb, caused a slight reduction in whole-body T\textsubscript{4} concentrations of \textit{Xenopus} tadpoles after 70 days of exposure (Goleman et al., 2002a). Decreased production of T\textsubscript{4}, but not triiodothyronine or TSH, was observed in rabbits after about 3 weeks of oral administration of AP at \( \geq 30 \text{ mg/kg/day} \) (York et al., 2001a). Conversely, total T\textsubscript{4} concentrations were significantly increased in deer mice after prolonged oral administration of AP at 1 nM and 1 \( \mu \text{M} \), whereas triiodothyronine levels were unaffected (Thuett et al., 2002). The results of a recent study by McNabb et al. (2004) with bobwhite quail indicated that thyroidal T\textsubscript{4} content is a more reliable marker of decreased thyroid function than is the circulating level of T\textsubscript{4}. The reasons circulating levels of thyroid hormone are generally unreliable as markers of thyroid function may include the presence of an efficient feedback mechanism to restore hormone production/secretion levels, and the relatively large reserves of thyroid hormone contained in the follicular colloid. Namely, even under conditions of complete or near-complete cessation of thyroid hormone production, it may take relatively long periods of time before changes in circulating hormone levels are detected. In fact, colloid depletion scores and changes in the intensity of luminal colloid T\textsubscript{4} immunoreactivity were among the least sensitive
markers of perchlorate exposure in the present study with zebrafish.

Exposure to AP had no effect on the growth and condition factor of zebrafish. The fish were immature at the onset of the exposures but by the end of the experiment, 24 weeks later, they had grown in size and condition factor and had also matured irrespective of treatment as judged by displays of spawning behavior. The time-dependent increase in condition factor of both males and females is likely due to the development of their gonads. Indeed, cursory inspection of gonadal sections prepared to determine the sex of the fish confirmed that they had reached reproductive condition during the course of the study. It appears, therefore, that the general health and pubertal development of zebrafish are not affected by exposure to environmentally relevant concentrations of AP-derived perchlorate. Patiño et al. (2003), who exposed adult (mature) zebrafish to AP-derived perchlorate at 18000 ppb for eight weeks, also concluded that the general behavior and reproductive performance of zebrafish are unaffected by perchlorate exposure despite marked alterations in thyroid histology. Similar results were obtained in a study with pregnant rabbits given AP orally, where fetal growth was unaffected at maternal dosages as high as 100 mg/kg/day (York et al., 2001a). In *X. laevis*, however, AP in the ppb range inhibited the growth and metamorphosis of young larvae (Goleman et al., 2002b). Although perchlorate at very high concentrations (~ 500 ppm) can affect the development of zebrafish embryos and larvae (Brown, 1997; Elsalini et al., 2003), the effects of environmentally relevant concentrations of perchlorate on larval fishes are presently unknown. More information is needed to determine if the growth and development of amphibians (larval or adults) are
more susceptible to perchlorate exposure than those of fishes.

In conclusion, the results of this study indicated that the intensity of the colloidal T\textsubscript{4} ring and thyroid follicle angiogenesis are the most sensitive biomarkers of perchlorate exposure presently available for zebrafish. These two biomarkers responded at environmentally relevant levels of perchlorate that are one to two orders of magnitude lower than the levels detected by standard biomarkers such as hypertrophy and colloid depletion. After prolonged periods of exposure, the intensity of the colloidal T\textsubscript{4} ring was particularly sensitive, responding to the presence of perchlorate in water at levels as low as 11 ppb. Further, the longevity of these new biomarkers following removal of perchlorate from water was much greater than that of the standard biomarkers. Thus, they also may serve to provide a more reliable historical record of past exposures. Finally, it should be noted that the thyroidal biomarkers examined in this study (new as well as standard) not only mark exposure to perchlorate but also thyroidal effects of perchlorate. Namely, they indicate effects of perchlorate on the thyroidal condition of the exposed animals. Thus, the finding that thyroid condition (colloidal T\textsubscript{4} ring) is affected by perchlorate at concentrations of 11 ppb may be of relevance to assist in the resolution of current disputes about safe levels of perchlorate in the environment. It remains to be determined if the new biomarkers of goitrogen exposure characterized in the present study are applicable to fishes other than zebrafish and to nonpiscine vertebrates.
REFERENCES


CHAPTER - 2

EFFECTS OF PERCHLORATE AND THYROXINE ON SEX RATIOS IN ZEBRAFISH

ABSTRACT

Perchlorate inhibits thyroid hormone synthesis by preventing iodide uptake into the thyroid epithelial cells. Disruption of thyroid function is known to impair metamorphosis in teleost fishes and anuran amphibians, and to affect sex ratios in anurans. However, the effect of thyroid disruption on gonadal sex differentiation and sex ratios in teleosts is unknown. The objective of this study was to determine the effect of perchlorate, via thyroid disruption, on sex ratios in zebrafish. Three days postfertilization (dpf), zebrafish were exposed to untreated water or water containing perchlorate at 100 ppm or 250 ppm for a period of 30 days. Rescue treatments consisted of a combination of perchlorate and exogenous T₄ (10 nM). Thyroid histology was assessed at the end of the treatment period (33 dpf), and gonadal histology and sex ratios were determined in fish that were allowed an additional 10-day period of growth in untreated water. Perchlorate alone did not affect survival of zebrafish. In the presence of T₄, larval survival at 43 dpf was reduced compared to untreated fish but, compared to the corresponding perchlorate concentration, exogenous T₄ caused increased mortality only at a perchlorate concentration of 100 ppm. Perchlorate alone or in the presence of T₄ suppressed body length at 43 dpf. As expected, exposure to perchlorate caused thyroid hypertrophy and colloidal depletion but these effects were blocked in the presence of exogenous T₄. Sex
ratios showed a perchlorate concentration-dependent trend to change in favor of females: 50.5, 58 and 65% females in the control and the 100- and 250-ppm perchlorate treatments, respectively. Co-treatment with T₄ not only reversed the effect of perchlorate but also overcompensated by skewing the sex ratio towards males: from 40% males to 72% males at 100-ppm perchlorate, and 34% males to 76% males at 250-ppm perchlorate. Co-treatment with T₄ also accelerated the process of spermatogenesis in the males. The gonads of a small percentage of fish were classified as ovotestes regardless of treatment. The changes in sex ratios were not associated with rates of larval survival or growth. Thus, we conclude that thyroid hormones are necessary for the maintenance of balanced sex ratios in zebrafish populations.

INTRODUCTION

Thyroid hormone (TH) is required for metamorphosis of amphibians (Callery and Elinso, 2000) and of teleosts such as flounder, Paralichthys olivaceus (Inui and Miwa, 1985). Larval or juvenile development of teleosts such as sea bream, Sparus aurata (Nowell et al., 2001) and salmonids (Sullivan et al., 1987; Lema and Nevitt, 2004; Jones et al., 2002) also requires the presence of TH. Therefore, the presence in the environment of contaminants that alter the thyroid endocrine system could potentially affect larval and juvenile development of natural fish and amphibian populations. Perchlorate (ClO₄⁻) is an environmental contaminant that is well known for its goitrogenic effects. Perchlorate inhibits iodide uptake into the thyroid follicles and consequently also inhibits TH synthesis (Wolff, 1998). Environmentally relevant concentrations of perchlorate have
been shown to impair thyroid function and to inhibit the larval or juvenile development of several aquatic species including the African clawed frog, *Xenopus laevis* (Goleman et al., 2002) and fathead minnow, *Pimephales promelas* (Crane et al., 2005).

Embryogenesis in zebrafish ends about 3 days postfertilization (dpf) (Westerfield, 2000) and larval development is relatively rapid compared to amphibians. Thyroid follicles in zebrafish begin to form about 40 hours post fertilization (Rohr and Concha, 2000), and iodide uptake is seen for the first time at 3 days after fertilization (at hatching) and increases during larval and juvenile development (Brown, 1997). These observations indicate that the requirement for endogenous production of TH does not begin until after hatching. A role for endogenous TH in the development of pectoral and pelvic fins (Brown, 1997) and immune function (Lam et al., 2005) during the period of metamorphosis has been proposed for zebrafish. However, little is known for zebrafish or any other teleost concerning additional possible functions of TH during larval development.

The results of some studies have suggested a role for TH during gonadal sex differentiation in anurans. For example, treatment of *X. laevis* with a goitrogenic compound (thiourea) during larval development resulted in the formation of all-female populations (Hayes, 1998). Also, exposure to perchlorate during metamorphosis significantly altered the sex ratio of developing *X. laevis* towards females (Goleman et al., 2002). The basic pattern of gonadal sex differentiation has been established for zebrafish. In this species, all individuals first develop ovary-like gonads regardless of genotypic sex and, subsequently, approximately half the individuals undergo regression
of the ovarian tissue and develop testes (Takahashi, 1977). This condition has been termed juvenile hermaphroditism. The onset of gonadal sex differentiation occurs between 21 and 23 dph, and most oocytes disappear from testes about 30 dph (Takahashi, 1977; Uchida et al., 2002). Like in other teleost fishes (Strüssmann and Nakamura, 2002), sex steroids seem to be involved in the process of gonadal sex differentiation in zebrafish (Uchida et al., 2004). However, the possible role of TH during gonadal sex differentiation has not been examined in zebrafish or in any other teleost.

The objective of this study was to determine the role of TH during the development of the gonads and the establishment of sex ratios in zebrafish. We used the TH synthesis inhibitor, perchlorate, alone or in combination with exogenous TH to manipulate the status of the thyroid system and examine its association with gonadal sex differentiation. The information obtained may useful for a better understanding not only of the biology of gonadal sex determination and differentiation in teleosts, but also of the possible ecotoxicological effects of environmental goitrogens.

MATERIALS AND METHODS

Chemicals and preparation of treatment solutions

Sodium perchlorate (CAS 7790-98-9, Purity 99.99%) was purchased from Aldrich Chemical (Milwaukee, WI, USA). A stock solution of 150,000 ppm perchlorate concentration was prepared by mixing the appropriate amount of sodium perchlorate in “zebrafish-water” (25 g of R/O Right®/100 L of reverse osmosis water). Working solutions of perchlorate (100 and 250 ppm) were prepared by mixing the appropriate
volume of stock solution with 30 L zebrafish-water in two separate aquaria. These aquaria were fitted with heaters to maintain the temperature at approximately 28 °C. In addition, T₄ “rescue” treatments were prepared for each of the perchlorate treatments. In the rescue treatments, T₄ at a final concentration of 10 nM was added to each of the tanks containing perchlorate using a T₄ stock solution of 5 mM (prepared in DMSO; final DMSO concentration in treatment water, 0.002%).

General embryo handling and maintenance

Zebrafish larvae were obtained from a colony of wild-type broodstock maintained in our laboratory at Texas Tech University (Lubbock, TX, USA). Embryos were collected from breeding chambers 2 h after lights-on (artificial dawn), cleaned with zebrafish water, and groups of 100 embryos were placed 1-L beakers containing 600 ml of zebrafish water. Methylene blue (Aquatrol, Inc. Anaheim, CA, USA) was added at 4 drops/30 L to prevent fungal growth. All beakers were placed in a water bath set at 28 °C. After 9 h, unfertilized (opaque) eggs were removed from each beaker. Approximately 500 ml of water were removed daily from each beaker and replaced with fresh zebrafish water preheated to 28 °C and dead embryos, if any, were removed from the beakers. Unhatched embryos were removed at 3 dpf (72 hours after fertilization), at which time the number of viable larvae per beaker was standardized to 90 individuals. The exposure to treatment solutions was started at 3 dpf in the beakers by replacing zebrafish water with the appropriate treatment media. The animal protocol for this study was reviewed
and approved by the Institutional Animal Care and Use Committee of Texas Tech University (Lubbock, TX, USA).

Experimental design and exposures

There were five treatment groups: control, perchlorate at 100 ppm, perchlorate at 100 ppm with T₄ at 10 nM, perchlorate at 250 ppm, and perchlorate at 250 ppm with T₄ at 10 nM. The highest concentration of perchlorate (250 ppm) was chosen because of its previously shown inhibitory effects on thyroid function in zebrafish (Elsalini and Rohr, 2003). Similarly, T₄ at 10 nM was used in the rescue treatments because of the effectiveness of this concentration in reversing the inhibitory effects of hypothyroid conditions in zebrafish larvae (Liu and Chan, 2002). Each treatment was conducted in triplicated 1-L beakers containing 90 larvae (3 dpf) each in 600 mL of treatment solution. Beakers representing one full set of replicates were placed in each of three water baths set at a temperature of 28 °C. Every day in the morning, dead larvae (if any) were counted and removed from each beaker, and approximately 80% of the water was replaced with fresh treatment water. Feeding was started at 5 dpf using powdered dry food (Tetramin®) twice daily. Each beaker was cleaned twice following feeding to remove uneaten food and debris. The temperature of the water bath was monitored daily, and the beakers were covered with a lid to help maintain a homogenous temperature. After 12 dpf, one of the two daily meals was replaced with newly hatched Artemia nauplii.

At 24 dpf, juveniles from each treatment beaker were transferred into individual 5-gallon aquaria fitted with heaters and hand-made biofilters (glass beakers containing
glass wool with water circulation driven by airflow). Exposure to the respective treatment solutions continued in the aquaria until 33 dpf. Water pH and temperature were monitored daily, and unionized ammonia was determined at least once weekly. Fifty percent of the treatment solution was exchanged every other day. At 33 dpf, exposures were terminated by transferring the fish to aquaria containing untreated zebrafish water. At this time, 12 fish from each treatment (4 fish per replicate) were euthanized, measured for fork-length (length from snout to the point of bifurcation of the caudal fin) and observed for gross morphological abnormalities, and fixed with Bouin’s fixative for thyroidal histology. All remaining fish were euthanized at 43 dpf, measured for fork-length and observed for gross morphological abnormalities, and fixed with Bouin’s solution for gonadal histology.

**Histology**

Procedures for thyroid and gonadal histology have been previously described (Patiño et al., 1996; Patiño et al., 2003; Mukhi et al., 2005). Slides containing paraffin sections (7 μm) were stained with hematoxylin and eosin and observed under a compound microscope. Thyroid morphometric measurements (hypertrophy and colloid depletion) were measured as described elsewhere (Mukhi et al., 2005) using image-analysis software (Image-Pro® Express, Media Cybernetics, Silver Spring, MD, USA). With few exceptions, sagittal sections of the gonad were used for observations. The gonadal sex of each fish was confirmed after observing serial sections through both entire gonads under the microscope. Female fish were identified by the presence of
previtellogenic oocytes, and male fish were confirmed by the presence of spermatogonial cells or onset of spermatogenesis. Some fish contained both female and male-like germ cells in their gonads and were classified as ovotestes.

**Data Analysis**

The vast majority of the natural and treatment-related mortality in this study occurred before 23 dpf, when fish samples for various analyses began to be taken from the tanks. Thus, to estimate survival rates, fish sampled at and after 23 dpf were included among the survivors. Survival (percent) data were transformed to their arcsine value before analysis; and fork-length (mentioned as ‘length’ henceforth) and thyroid hypertrophy (epithelial cell height) were transformed to their logarithmic value. Following transformation, these data were subjected to parametric analysis using 1-way nested-analysis of variance (ANOVA; tanks nested into treatments). If significant effects of treatment were found, mean separations were conducted using Tukey’s honestly significant differences (HSD) test. Colloidal depletion scores were analyzed by Kruskal-Wallis nonparametric ANOVA followed by built-in multiple comparisons for rank means. Chi-square contingency tables were used to compare sex ratios. Specifically, Chi-square for trends was used to compare the sex ratio of the control and the two concentrations of perchlorate; and two-tailed Chi-square was used to compare the sex ratios of each rescue treatment against its corresponding perchlorate concentration. In addition, assuming that ovotestes are undifferentiated gonads in transition to becoming testes (Takahashi, 1977), we added the ovotestes to the male population and repeated the
analyses. All results are represented as mean ± standard error of the mean (SEM). All data analyses were conducted using the Statistica® Data Miner software package (StatSoft, Tulsa, OH, USA) at the level of significance of $\alpha = 0.05$.

RESULTS

Effects on survival, growth and general appearance

The highest overall survival was observed in the control group, which showed an average survival of 70.7 ± 3.3% (Figure 2.1). Perchlorate alone did not affect the survival of larvae at either of the concentrations, whereas in the presence of exogenous T$_4$, larval survival was reduced beyond what was observed in control. However, the T$_4$ co-treatment caused higher mortality than perchlorate only in the 100-ppm treatment series (nested ANOVA and Tukey’s HSD, $p<0.05$). At a perchlorate concentration of 250 ppm, addition of 10 nM T$_4$ did not cause additional mortality ($p>0.05$).

At end of the exposure period (33 dpf), perchlorate alone did not affect body length (nested ANOVA, Tukey’s HSD, $p>0.05$; Figure 2.2A). Lower body lengths compared to control fish were observed in both T$_4$ co-treatment groups, whereas compared to the corresponding perchlorate treatments, co-treatment with T$_4$ affected body length only in the 250 ppm perchlorate series ($p<0.05$). Interestingly, 10 days after the termination of the exposure the effects on larval length were more prominent (Figure 2.2B). The length of juveniles in all the exposure groups was significantly lower than the control group, and T$_4$ co-treatment led to significantly lower lengths at both perchlorate concentrations (nested-ANOVA, Tukey’s HSD, $p<0.05$).
Qualitatively, the most obvious abnormalities observed in 33-dpf larvae were a reduced development of body pigmentation in the perchlorate treatment groups, and a silvery coloration in the T₄ co-treatment groups. Also, T₄-treated fish had an extended lower jaw compared to fish in the other groups. At 43 dpf, 10 days after termination of treatments, the effects of perchlorate on body pigmentation had partially returned to “normal” compared to control fish, but some fish treated with T₄ still showed elongated jaws as well as other abnormalities such as kinked tails.
Figure 2.1. Effect of perchlorate (ClO$_4$) and thyroxine (T4) co-treatment on zebrafish survival over the treatment period (3-43 dpf). Survival rate (%) was not affected by any of the perchlorate treatments. Survival was lower in both of the T4 co-treatment groups compared to control group (1-way ANOVA and Tukey’s HSD, p<0.05); however, compared to perchlorate, T4 co-treatment yielded lower survival only in fish from the 100-ppm perchlorate group (1-way ANOVA and Tukey’s HSD, p<0.05).
Figure 2.2. Effect of perchlorate (ClO4) and thyroxine (T4) co-treatment on the fork-length of juvenile zebrafish at 33 dpf (panel A) and 43 dpf (panel B). At 33 dpf, fish length was not affected by any of the perchlorate treatments, but lower growth compared to control was observed in fish from the T4 co-treatments (nested-ANOVA and Tukey’s HSD, p<0.05). At 43 dpf, fish from all treatment groups had shorter lengths than control fish, and fish from both T4 co-treatments fish had shorter lengths than their respective perchlorate-only group (nested-ANOVA and Tukey’s HSD, p<0.05).
Effects on thyroidal histology

At the completion of the exposure period (33 dpf), thyroid follicles from control fish were lined with a single layer of squamous epithelial cells and their lumens were typically filled with colloid (Figure 2.3A). Treatment with either concentration of perchlorate increased epithelial cell height (nested ANOVA and Tukey’s HSD, p<0.05) and induced colloid depletion (Kruskal-Wallis and multiple comparisons for rank means, p<0.05) compared to the control group (Figure 2.3B and Figure 2.4A,B). Co-treatment with T₄ blocked the effects of perchlorate on epithelial cell height (nested ANOVA and Tukey’s HSD, p<0.05) and colloid depletion (Kruskal-Wallis and multiple comparisons for rank means, p<0.05); namely no differences in these parameters were observed between control fish and fish treated with perchlorate (at either concentration) and exogenous T₄ (Figure 2.3B and Figure 2.4A,B). However, the size of thyroid follicles was clearly smaller in the T₄ co-treatment groups compared to the control group (Figure 2.3C).
Figure 2.3. Photomicrograph of thyroid follicles of zebrafish reared in control water (asterisk; panel A), water containing 250 ppm perchlorate (panel B), or water with perchlorate (250 ppm) and thyroxine (10 nM) co-treatment (panel C) for a period of 33 dpf. Note the squamous or cuboidal epithelial cell layer in control as well as thyroxine co-treatment fish (arrow; panel A and C), and the cuboidal or tall-columnar shape and greater height (hypertrophy) of the cells in fish exposed to perchlorate alone (panel B). Exposure to perchlorate alone caused severe colloid depletion (panel B), but such effect was not observed in fish form the other groups (see panel A and C). Thyroxine co-treatment resulted in relatively small follicles compared to follicles from control fish. Scale bar = 10 μm.
Figure 2.4. Quantitative evaluation of thyroid morphometry at 33 dpf. Perchlorate (ClO₄) alone significantly increased the epithelial cell height (hypertrophy), but this effect was blocked in the presence of thyroxine (T₄, 10 nM; nested-ANOVA and Tukey’s HSD, p<0.05; panel A). A semi-quantitative measurement of colloid depletion indicated that perchlorate alone at both the concentration caused a severe colloid depletion compared to control and but of the rescue treatment in presence of 10 nM T₄ had significantly lower colloid depletion compared to their respective perchlorate-alone treatments (Kruskal-Wallis test, p<0.05; panel B).
Effects on gonadal development and sex ratio

The gonads of most male and female fish had clearly differentiated by 43 dpf based on their histological appearance. Ovaries from control females contained numerous previtellogenic oocytes (Figure 2.5A), and testes from control males were filled with spermatogonial germ cell nests (Figure 2.5B). Regardless of treatment, the gonads of a small percentage of the fish could not be classified into either sex because of the presence of a few previtellogenic oocytes embedded within spermatogonial germ cells; these gonads were classified as ovotestes (Figure 2.5C). Ovotestes often contained degenerating bodies (Figure 2.5C). None of the females observed contained ovaries beyond the previtellogenic stage of development and spermatogenesis was not observed in the testes of males form the control and perchlorate treatments. However, 13% and 19% of the males treated with exogenous T₄ in the 100-ppm and 250-ppm perchlorate groups, respectively, contained testes in various stages of spermatogenesis (Figure 2.5D).

Compared to the control group (50.5% female), the sex ratio in both perchlorate treatments showed a concentration-dependent trend to change in favor of females (100 ppm perchlorate, 58% female and 40% male; 250 ppm perchlorate, 65% female and 34% male) (Chi-square for trends, p=0.0216; Figure 2.6). Compared to their corresponding perchlorate treatment, co-treatment with T₄ significantly skewed the sex ratio towards male (100-ppm perchlorate + T₄, 24% female and 72% male; 250-ppm perchlorate + T₄, 21% female and 76% male) (Chi-square, p<0.0001, Figure 2.6). The results of Chi-square for trends (p=0.0089) and Chi square for sex ratios (p<0.0001) were the same when ovotestes were added to the testes count.
Figure 2.5. Photomicrographs of ovary (panel A), testis (panel B) and ovotestis (panel D). Ovaries were identified by the presence of previtellogenic oocytes (PO) and testes by the presence of spermatogonia (SG). Some gonads were classified as ovotestes because of the presence of both male- and female-like germ cells; degenerating bodies were often observed in these gonads (arrows, panel E). None of the germ cells in the ovaries or testes of control fish had began meiosis, but the testes of a number of males from the thyroxine co-treatment group were at an advanced stage of spermatogenesis (panel C). Scale bar = 20 μm.
Figure 2.6. Effect of perchlorate (ClO₄) and thyroxine (T₄) co-treatment on sex ratios. Treatment with perchlorate significantly skewed the ratio towards females compared to the control group (p=0.0216, Chi-square for trend). In the presence of 10 nM T₄, the sex ratio was significantly skewed towards male compared to the respective perchlorate treatment groups (p<0.0001, Chi-square test). A small incidence of ovotestis was observed irrespective of treatment. The total number of fish (n) were 134, 135, 80, 89 and 86 for the control, 100-ppm perchlorate, 250-ppm perchlorate, 100-ppm perchlorate + T₄ and 250-ppm perchlorate + T₄, respectively.
DISCUSSION

The period of exposure in the present study was from 3 to 33 dpf in order to bracket the onset of endogenous TH production (3 dpf; Brown, 1997; Wendel et al., 2002) and the period of gonadal sex differentiation (Takahashi, 1977; Uchida et al., 2002) in zebrafish. Perchlorate was used to block endogenous TH production and co-treatment with T4 to assess the reversibility of the TH-dependent effects of perchlorate. The results obtained suggested that endogenous TH is necessary to achieve normal (1:1) sex ratios in zebrafish populations.

Perchlorate inhibits TH synthesis by suppressing uptake of iodide via the sodium/iodide symporter, and consequently disrupts negative feedback mechanisms that regulate the activity of the thyroid endocrine system (Wolf, 1998; Capen, 2001). It is well established for mammals that thyroid follicle cell height correlates positively with the level of stimulation by thyroid-stimulating hormone (TSH) under conditions of altered TH production (Paynter et al., 1988; Capen, 2001). The results of the present and previous studies with teleost fishes are consistent with this knowledge. Namely, hypertrophy of the thyroid follicle epithelium following perchlorate stimulation has been reported in adult (Patiño et al., 2003; Mukhi et al., 2005) and juvenile (present study) zebrafish, mosquitofish, Gambusia holbrooki (Bradford et al., 2005), and fathead minnow (Crane et al., 2005). In addition, co-treatment with exogenous T4 in the present study blocked the perchlorate-dependent increase in thyrocyte height. Complete inhibition of the synthetic ability of thyroid follicles may not have occurred even at the highest concentration of perchlorate used (250 ppm), because some of the follicles in the
perchlorate-treated fish contained colloid although at a much reduced level. However, consistent with observations of hypertrophy, perchlorate-induced reductions in follicular colloid content were blocked by co-treatment with exogenous T₄. These observations suggest that perchlorate treatment disrupted TH synthesis and negative feedback mechanisms, thus causing higher levels of TSH production; and that co-treatment with T₄ restored negative feedback mechanisms and lowered TSH production. The size (diameter) of thyroid follicles was not quantified in the present study, but treatment with exogenous T₄ clearly inhibited the growth of thyroid follicles relative to control values. By contrast, the size of thyroid follicles in the perchlorate-treated fish did not seem to have been affected to the same degree although many were devoid of colloid and thus showed a “collapsed” appearance. These observations suggest that adequate levels of TSH, which presumably are reduced in T₄-treated fish, are necessary for the normal growth of zebrafish thyroid follicles. Overall, histological observations of thyroid status at the end of the exposure period confirmed the hypothyroid effects of perchlorate and suggested the induction of hyperthyroid conditions by co-treatment with exogenous T₄.

Control fish had a cumulative survival rate of 70% during the experimental period covered by this study (3-43 dpf). Similar rates of survival for comparable rearing periods have been reported in other studies of zebrafish (Fenske et al., 2005). Exposure to perchlorate alone did not affect zebrafish survival compared to control values; again, similar observations were obtained with fathead minnow exposed to 100-ppm ammonium perchlorate for 28 dpf (Crane et al., 2005). In the present study, however, co-treatment with 10-nM T₄ (in the presence of perchlorate) resulted in lower survival rates compared
to control values, although the difference between a given T₄ co-treatment and its respective perchlorate reference was significant only at the perchlorate concentration of 100 ppm. The cause of increased larval mortality in the presence of 10-nM T₄ is not clear. Liu and Chan (2002) did not observe lethally toxic effects of 10 nM T₄ in larval zebrafish, although their exposure period was only 7 days (0-7 dpf). Lam et al. (2005) exposed zebrafish to T₄ at 30 nM for 28 days beginning at 5 dpf, but they did not report the survival rates of their experimental fish.

In fathead minnow, exposure to ammonium perchlorate at 100 ppm from 0 to 28 dpf caused a reduction in body length (Crane et al., 2005). Also, treatment of 5-dpf zebrafish larvae with the TH synthesis inhibitor, methimazole, reduced the length of the fish when measured after 14 and 28 days of continuous exposure (Lam et al., 2005). In the present study, perchlorate at 100 or 250 ppm did not significantly affect the length of zebrafish when measured at the completion of the exposure period (3-33 dpf), but there was a trend for mean values to be smaller compared to the control fish. However, 10 days after removal of perchlorate (at 43 dpf), fish length was significantly reduced in perchlorate-treated fish compared to control values. Thus, perchlorate-dependent effects on growth seemed to be enhanced with age (or growth) and thus may be irreversible. Curiously, co-treatment with T₄ at 10 nM enhanced the negative effects of perchlorate on growth. This finding is inconsistent with those of a previous study with zebrafish, which reported a reversal of the inhibitory effects of methimazole on larval growth by co-treatment with T₄ at 30 nM (Lam et al., 2005). It is difficult to explain the differences between the present and previous (Lam et al., 2005) studies in regards to the effect of
exogenous T₄ on larval growth. Observations on survival and growth in the present study suggest the occurrence of toxic effects of T₄ at 10 nM, and a previous study with larval zebrafish reported toxicity of T₄ at 30 nM even after short-term exposures (Liu and Chan, 2002). Larval fish are generally sensitive to husbandry conditions, and it is possible that unknown differences in rearing protocols are responsible for the differences between studies (Lam et al., 2005; and present study). However, consistent with the findings of Lam et al. (2005), the present study showed that treatment with exogenous T₄ caused an abnormal increase in the length of the lower jaw.

The role of TH in gonadal sex differentiation has been previously examined in amphibians. Treatment of X. laevis with thiourea (inhibitor of iodination) during larval development resulted in the formation of all-female populations (Hayes, 1998). Similarly, exposure Xenopus tadpoles to perchlorate skewed the sex ratio towards females (Goleman et al., 2002). Conversely, Robertson and Kelley (1996) reported that the antithyroid compound, propylthiouracil (inhibitor of the coupling reaction), did not affect gonadal sex differentiation in X. laevis but blocked the development of secondary male sexual characters. The reason for the difference in results concerning the effects of goitrogens in primary (gonadal) sex differentiation between Robertson and Kelley’s (1996) study and the later reports is unclear. In teleosts, the effect of TH on gonadal development had not been examined prior to the present study. This study found that treatment with perchlorate during the larval-to-juvenile transition in zebrafish, which encompasses the normal period of gonadal sex differentiation (Takahashi, 1977; Uchida et al., 2002), significantly skews the sex ratio of the experimental population toward
females. In addition, co-treatment with exogenous T₄ not only blocked the effect of perchlorate, but also overcompensated by skewing the sex ratios toward males and also by accelerating the onset of spermatogenesis in some of the males. [Precocious onset of spermatogenesis has been also observed in immature male carp treated with exogenous T₄ (Timmermans et al., 1997).] Co-treatment with T₄ caused higher mortality compared to control values, thus allowing the possibility of confounding effects of sex-linked differential mortality; however, such scenario is not supported by the present results. Namely, at a perchlorate concentration of 250 ppm, the proportion of females in the experimental populations changed from 65% in the perchlorate treatment to 24% in the T₄ co-treatment without a significant change in mortality rates. Overall, the present observations strongly suggest that hypo- and hyper-thyroid conditions during the period of gonadal sex differentiation have feminizing and masculinizing consequences, respectively, on the sex ratio of zebrafish populations. These results with zebrafish are consistent with some of the previous observations made with anuran amphibians (Hayes, 1998; Goleman et al., 2002).

The mechanisms of regulation of gonadal sex by TH are uncertain for zebrafish. Zebrafish follow the “undifferentiated” pattern of gonadal sex differentiation, in which the gonads of all individuals begin development as ovaries and subsequently the ovarian elements in the gonads of genetic males are eliminated (by apoptosis) as testicular development is initiated (Takahashi, 1977; Uchida et al., 2002). Like in many other teleosts (Strüssmann and Nakamura, 2002), sex steroids seem to regulate or modulate gonadal sex differentiation in zebrafish. Uchida et al. (2004) showed that experimental
inhibition of aromatase activity caused germ cell apoptosis and sex-reversal of genetic female zebrafish into males. This observation suggests that the absence of estrogen, or an increased concentration of androgen due to their lack of conversion into estrogen, is sufficient for testicular differentiation in zebrafish. Thus, it may be postulated that hypothyroid conditions in zebrafish lead to increased aromatase activity and ovarian differentiation, whereas hyperthyroid conditions result in the reduced activity of this enzyme and testicular formation. In fact, there is evidence available to support this hypothesis. For example, aromatase activity and estradiol content were enhanced in Sertoli cells of hypothyroid rats, whereas treatment with TH lowered aromatase activity and estradiol content (Panno et al., 1994) and down regulated estrogen receptors (Panno et al., 1996). Also, TH positively regulates androgen receptor content in the rat Sertoli cell (Panno et al., 1996; Arambepola et al., 1998), and treatment with TH increases the expression of the androgen receptor gene in the testis of lizards (Cardone et al., 2000).

In summary and conclusion, exposure to perchlorate inhibited thyroid function and skewed the sex ratio towards females; whereas co-treatment with exogenous T_4 caused hyperthyroid conditions and skewed the sex ratio towards males. These observations suggest an important role for TH in the process of gonadal sex differentiation and the maintenance of a balanced sex ratio in zebrafish. The implications of these findings from an environmental point of view are uncertain. Although the concentrations of perchlorate used in this study are within the levels reported in some contaminated aquatic habitats (Smith et al., 2001), they are at the high end of the observed range. However, the results of this study indicate that studies of endocrine and
reproductive disruption caused by xenobiotics, particularly those with estrogenic or androgenic activities, should also consider the involvement of the thyroid system.

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ABSTRACT

Perchlorate is an environmental contaminant that inhibits thyroid hormone (TH) synthesis and thus can potentially affect TH-dependent functions. A role of TH in the regulation of teleost reproduction has been proposed but is not fully understood. The objectives of this study were to determine the effects of long-term perchlorate exposure on (1) thyroid status and reproductive performance of adult zebrafish, and (2) viability and development of F1 embryos and early larvae. Mixed-sex populations of adult zebrafish (*Danio rerio*) were exposed to 0, 10, and 100 ppm nominal concentrations of perchlorate for 10 weeks. Females (8 fish per tank replicate, 3 replicates per treatment) and males (single pool of 12 fish per treatment) were separated and further exposed to their respective treatments for 6 additional weeks. Every week during this period (Week 11 to 16), the 8 females from each tank replicate were paired within spawning containers with 4 males from the same respective treatment, and packed-egg volume (PEV) was measured 2.5 h after onset of spawning (the first weekly spawn was a trial and was not included in the analyses). Other than PEV, endpoints of interest could not be measured at each spawning event because of treatment-dependent declines in egg production. Thus, each of these endpoints was measured once during the period between Week 14 and 16 and included fertilization rate, fertilized egg diameter, hatching rate, length and
craniofacial development of early larvae, TH content of embryos (embryo pools, 3.5 h after onset of spawning) and of exposed mothers (two pooled individuals per replicate collected at the end of the treatment period; 16 weeks). Exposure to both concentrations of perchlorate inhibited T4 concentrations in mothers and embryos relative to control values, but T3 concentrations were unchanged. Perchlorate at both concentrations also caused a marked reduction in PEV especially towards the end of the exposure period, but fertilization and embryo hatching rates were unaffected. Egg diameter and standard length of 4-day-old larvae were slightly increased by perchlorate treatment. Although head depth of 4-day-old larvae was unaffected by perchlorate, mean heights of the jaw-forming cartilage complexes, Meckel’s and ceratohyal, were slightly decreased in broods from the perchlorate-treated parents. In conclusion, long-term exposure of adult zebrafish to perchlorate not only disrupts thyroid homeostasis and inhibits fecundity, but also alters the early development of their F1 progeny.

INTRODUCTION

Perchlorate has been used for many years in the manufacture of ammunitions, as an oxidant in fuel for missiles and rockets, and in industries such as leather processing, electro-plating, aluminum-refining, and rubber manufacturing (US EPA, 1998; Logan, 2001). Salts of perchlorate are highly soluble in water and are often are found in surface and ground waters near their sites of manufacture, use or disposal (Urbansky, 2002; Tipton et al., 2003). Perchlorate has a similar partial specific volume to iodide, with which it competes for the sodium-iodide symporter (NIS) (Van Sande et al., 2003).
Perchlorate-dependent iodide deficiency results in a reduction of thyroid hormone (TH) synthesis and can lead to hypothyroidism (Capen, 2001). The toxicological effects of perchlorate on vertebrate thyroid gland/follicles have been reported by various authors (e.g., York et al., 2001; Goleman et al., 2002a, b; Thuett et al., 2002; Patiño et al., 2003; McNabb et al., 2004; Bradford et al., 2005; Crane et al., 2005; Mukhi et al., 2005; Liu et al., 2006).

The results of a number of studies have indicated that TH may be involved in the reproductive development of some teleosts (Cyr et al., 1988; Cyr and Eales, 1988; Weber et al., 1992; Van der Geyten et al., 2001). For example, in a study with rainbow trout, inhibition of the transformation of T\textsubscript{4} into T\textsubscript{3} by ipodate caused hypothyroidism in females and depressed their gonadosomatic index (GSI); whereas moderate hyperthyroidism induced by exogenous T\textsubscript{3} treatment led to higher GSI. However, the results of other studies seem to be inconsistent with a positive role of TH in teleost reproduction. For example, in a study with zebrafish, Patiño et al. (2003) found that environmental relevant concentrations of perchlorate (18 ppm) did not affect female fecundity (volume of eggs laid) although thyroid histology was greatly affected after a period of exposure of 8 weeks. In addition, a recent study with zebrafish reported that exposure to the thyroid disrupting chemical, propylthiouracil, caused an increase in egg number despite the development of hypothyroid conditions (Van der Ven et al., 2006). Also, in non-teleost vertebrates previously examined, exposure to perchlorate at environmentally relevant levels does not seem to cause major impairments in reproductive performance (York et al., 2001, 2005; Gentles et al., 2005).
The apparent inconsistency of previous studies indicates that additional research is necessary to clarify and understand the role of TH in, and the effects of thyroid endocrine disruptors on, teleost reproduction. Therefore, the primary objective of the present study was to determine the effects of long-term exposure to perchlorate on the reproductive performance of zebrafish. In view of recent information suggesting that the levels of TH in zebrafish are resilient to perchlorate exposure (Mukhi et al., 2005), the length of the exposure period in this study was 16 weeks. This exposure period is twice the length of the exposure period (8 weeks) used in a previous study with zebrafish that yielded negative results of perchlorate on reproduction (Patiño et al., 2003). Thyroid hormones are maternally transferred into teleost eggs, and it has been suggested that TH are necessary for embryonic development (Power et al., 2001). Thus, a secondary objective of this study was to determine the consequences of maternal exposures to perchlorate on parameters of egg quality and early larval development.

MATERIALS AND METHODS

Animal husbandry

Four-month-old, wildtype zebrafish were obtained from a local vendor (Lubbock, TX, USA) and allowed to acclimatize to our laboratory conditions. Animal husbandry procedures for this study were as described by Mukhi et al. (2005). Briefly, each aquarium was filled with 30 L of zebrafish-water (25 g of R/O Right®/100 liter of reverse-osmosis water) and fitted with two hand-made internal biofilters. A water current through the filter was maintained by airflow via a glass pipette. The tanks were treated
with Stresszyme® (Aquatic pharmaceuticals, Pittsburgh, PA, USA) weekly to facilitate mineralization of nitrogenous waste. The range of observed water quality parameters were pH 6.0-8.0, temperature 27-29 °C, and 12h/12h light/dark cycle. If pH fell below 6.5, an appropriate volume of 5 M NaOH solution was added to the affected aquarium to bring it within the desired range of 6.5-8.0. Fish were fed twice daily to satiation with adult frozen Artemia and/or Tetramin® flakes (Tetra Sales, Blacksburg, VA, USA). Leftover food and fecal material were removed by siphoning every evening. Temperature and pH were measured daily and dissolved oxygen, specific conductivity, salinity, unionized ammonia and nitrate was measured at least once weekly. Half of the water volume (15 L) was removed and replaced with clean system-water twice weekly. The protocol for the use of animals in this study was reviewed and approved by the Texas Tech University Animal Care and Use Committee (Lubbock, TX, USA).

Perchlorate exposure prior to spawning

A static-renewal exposure procedure was applied as previously described (Patiño et al., 2003; Mukhi et al., 2005). Each aquarium (see above) contained 35 fish from a mixed-sex population. The nominal perchlorate concentrations chosen for this study were 0 (control), 10 and 100 ppm. A 12-week exposure to perchlorate at 10 ppm did not induce hypothyroidism in zebrafish (Mukhi et al., 2005). Therefore, the exposure period in the present study was increased up to 16 weeks. The first 10 weeks of exposure was conducted in 9 treatment tanks (3 treatments X 3 replicates). At the end of this period, 3 females per replicate were sampled for thyroid histological analysis to determine the
effectiveness of perchlorate treatment. For this purpose, the fish were euthanized in 1 mg/L tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Inc., Redmond, WA, USA), fixed in Bouin’s solution at 4 °C for 48 h, rinsed in tap water, and stored in 70% ethanol until processed. Histological procedures and thyroid follicle analyses were as described by Patiño et al. (2003).

**Spawning procedures and sample collections**

After the initial 10 weeks of exposure, females and males were separated into a new set of tanks and the exposures were continued until 16 weeks. Each female tank contained 8 fish and each male tank, 12 fish. Female tanks were set in triplicate for each treatment. Only one male tank per treatment was prepared. Three groups of 4 males were used to breed with the 3 groups of females per treatment.

A summary of the spawning schedule is presented in figure 3.1. The combined wet-weight of the 8 female fish from each tank was taken in the evening before spawning by placing them in a pre-zeroed, 1-L beaker with water immediately before their transfer to spawning chambers. The spawning procedure utilized spawning chambers with false bottoms to collect eggs as described earlier (Patiño et al., 2003). The following morning at 2.5 h after lights-on (artificial dawn), fish were removed from the spawning chambers and returned to their original tanks. The spawn was collected from each chamber and fecal matter and other debris were removed using glass pipettes and by rinsing 4 times with fresh zebrafish water. Packed-egg volume (PEV) was determined by volume displacement in a graduated 5-ml glass cylinder. The first spawning was conducted at
Week 11 of exposure to synchronize breeding, but no data was collected from this spawning event. The fish were then spawned once weekly for five additional weeks. Packed-egg volume was measured for every spawning (Week 12 to Week 16) and analyzed as weekly PEV through time per treatment or as cumulative PEV per treatment (see Data Analysis).

Additional measurements were taken from Week 14 to Week 16. However, because of limited PEV in the perchlorate-treated groups especially towards the end of the exposure period, we were not able to take all measurements at each spawning event. Briefly, fertilization and hatching rates were measured at Week 14, and the embryonic contents of T₄ and T₃ were determined at Week 14 and 15, respectively. For the latter, samples were snap-frozen in liquid nitrogen and stored at -80 °C until analyzed. Standard length and craniofacial development of 4-dpf-larvae were determined at Week 15. For this purpose, embryos were fixed in 4% paraformaldehyde at 4 °C overnight and stored in 70% ethanol until further processed. Egg diameters were measured at Week 16. Finally, 2 female fish per replicate were randomly collected one day after the last spawning for analysis of whole-body T₄ and T₃ content. For this purpose, fish were snap-frozen in liquid nitrogen and stored at -80 °C until further processing. Larvae and adults were euthanized in a lethal concentration of MS-222 (1 g/L) prior to processing. Embryo and larval rearing were conducted in untreated zebrafish water.
Figure 3.1. Schematic representation of the spawning schedule. Male and female fish were reared together and exposed to perchlorate (0, 10 and 100 ppm) for 10 weeks. They were separated by sex at Week 10 and the exposure continued until Week 16. A trial spawning was conducted at Week 11, and definitive spawning occurred from Week 12 to 16 (see text for details).
Thyroid hormone extraction and analysis

Thyroid hormones, T₃ and T₄, were extracted from 2 pooled females per tank replicate collected at Week 16; T₃ was extracted from 0.7-2.8 g of PEV per tank replicate collected at Week 14; and T₄ was extracted from 0.4-2.2 g of PEV per tank replicate collected at Week 15. Extraction procedures were as previously described for whole body of zebrafish (Mukhi et al., 2005). Samples were homogenized using a Polytron® homogenizer (Glen Mills Inc., Clifton, NJ, USA) in ice-cold methanol [volume in ml = tissue weight (g) x 4] containing 1 mM propylthiouracyl and then sonicated. Approximately 1000 counts per minute of [¹²⁵I]-T₃ or [¹²⁵I]-T₄ in 50 µL of methanol (containing 1 mM propylthiouracyl) were then added to each sample to monitor the recovery of endogenous hormone. Following a 30-min incubation, homogenates were centrifuged and the supernatants were removed, mixed with two volumes of chloroform and back-extracted into an aqueous phase with 2N ammonium hydroxide. The back extraction was repeated two more times. The aqueous fractions were pooled and dried in a Jouan® centrifugal evaporator overnight at 30 °C. The dried samples were reconstituted in 300 µL of barbital buffer (barbital 15.47 g/L, EDTA 0.5 g/L, bovine gamma globulin 1 g/L, thimerosal 1 g/L; pH 8.6) prior to the assay, and the recovery of radiotracer was determined in an aliquot using a Cobra 5005 gamma counter (Packard, Downers Grove, IL, USA). Under these conditions, the average extraction recovery efficiencies for T₃ and T₄ were 64% and 67% for the embryos and 71% and 62% for whole-body homogenates.

Thyroid hormones were measured in duplicate 50-µL aliquots of extract
following the procedure of Mackenzie et al. (1978). Authentic T$_3$ or T$_4$ standards were used for assay calibration and run in duplicate for each concentration. The hormone content of the samples was determined using a four parameter logistic transformation of [$^{125}$I] T$_3$ or [$^{125}$I] T$_4$ displacement by the authentic standards. The sample values obtained were corrected for the estimated recovery of each sample. This assay procedure was validated for both embryo and whole-body homogenates by confirming the direct proportionality of hormone dilution in serially diluted extracts (Pearson $r = 0.97-0.99$; $p<0.05$), and by confirming the recovery of known concentrations of exogenously added authentic T$_3$ and T$_4$ into the extracts (all within 86-93% of expected values).

F1 embryo-larval measurements

Fertilization rates were estimated at approximately 9 h after lights-on as the number (percent) of viable embryos relative to the total number of eggs in a sub-sample of approximately 100 eggs per replicate; and hatching rates were determined as the number (percent) of the viable embryos that hatched by 3 days post fertilization (dpf; 72 h). Egg diameter was measured in 40-50 eggs per replicate tank using an ocular micrometer attached to a stereomicroscope and calibrated against an external (stage) micrometer. As the zebrafish egg is almost spherical, the diameter was measured randomly on any part of the egg.

Standard length and craniofacial development was examined in 15 viable embryos per replicate reared through hatching until 4 dpf, at which time they were sampled for analysis. Gross developmental deformities were also recorded. Craniofacial development
was assessed only in embryos derived from the control and 100-ppm perchlorate groups. Parameters of craniofacial development examined included head depth at the middle of the eye and height of the Meckel’s and ceratohyal cartilage complexes (see below). These measurements were also made with an ocular micrometer. Four dpf was chosen for larval analysis because a previous report indicated that craniofacial development of zebrafish is sensitive to TH during the embryo-to-larva transition (3-5 dpf; Liu and Chan, 2002).

Whole-mount cartilage staining with Alcian Blue was conducted to facilitate cartilage analysis according to procedures described by Liu and Chan (2002). Briefly, the larvae preserved in 70% ethanol were dehydrated with series of graded concentrations of ethanol and stained overnight with a 0.1% solution of Alcian Blue dissolved in 80% ethanol/20% glacial acetic acid. The samples were washed with acidic ethanol (80% ethanol/20% glacial acetic acid, then 95% ethanol/5% glacial acetic acid), rehydrated to phosphate buffer saline (PBS), and rinsed twice with PBS. The samples were then consecutively treated with 50 mg trypsin per ml of a saturated sodium tetraborate solution at 4 °C overnight, and with 3% H₂O₂ for 5 minutes. The stained samples were washed several times with PBS and preserved in 50% glycerol (in PBS). The Meckel’s and ceratohyal cartilages each form a U- or V-shaped structure that can be approximated to an isosceles triangle (Figure 3.2). The height (h) of each structure was estimated by measuring the side (s) and the base (b) of the triangle under a stereoscope and applying the Pythagorean formula, \( h = \sqrt{s^2 - b^2/4} \).
Figure 3.2. The height of Meckel’s ($h_1$) and ceratohyal ($h_2$) cartilage complexes (dotted double arrow) were measured in this study according to the Pythagorean formula, $h = \sqrt{s^2 - b^2/4}$. 
Data analysis

All statistical analyses were done with the Statistica® Data Miner software package (StatSoft, Tulsa, OK, USA). Parameters such as wet-weight of females, packed-egg volume, TH levels in embryo and whole-body pool extracts, hatching rate and fertilization rate are measures of tank performance. Thus, in all these cases, sample size per treatment for statistical analyses is the number of tank replicates (n = 3). Treatment-related effects were assessed by 2-way analysis of variance (2-way ANOVA; e.g., for weekly female wet-weight or weekly PEV), 1-way ANOVA, or repeated-measure ANOVA as appropriate. Measurements taken from individuals such as egg diameter, larval length, and cartilage height were analyzed by nested ANOVA. Raw data were transformed to logarithmic or arcsine values as appropriate. Separation of means was done using Tukey’s honestly significant differences (HSD) test. Unless otherwise mentioned, the results are shown as mean ± standard error of the mean (SEM). Analyses were performed at the level of significance of \( \alpha = 0.05 \).

RESULTS

Effect of perchlorate on maternal thyroid histology

A qualitative assessment of the thyroid follicles in female zebrafish at the time of separation of females into spawning tanks (Week 10) indicated a mild colloid depletion in fish exposed to perchlorate at 10 ppm (Figure 3.3B), and mild to severe depletion at 100 ppm (Figure 3.3C). Females from both treatment groups exhibited hypertrophic thyroid follicle cells. In addition, some females exposed to perchlorate at 100 ppm
contained hyperplastic follicles (Figure 3.3D). Control fish had thyroid follicles full of colloid that contained non-hypertrophic epithelial cells (Figure 3.3A).

**Effects of perchlorate on maternal and embryonic thyroid hormone levels**

Measurements made at Week 16 indicated that whole-body T$_4$ content, but not T$_3$ content, was greatly reduced in perchlorate-treated females compared to control females (1-way ANOVA, Tukey’s HSD, p<0.05; Figure 3.4A,B). Although there was a trend for whole-body T$_3$ levels to be lower in perchlorate-treated fish (Figure 3.4A), this trend was not statistically significant (p>0.05).

Embryonic T$_3$ and T$_4$ contents were measured at Week 14 and 15, respectively. Embryonic T$_3$ content was not affected by maternal exposure to perchlorate (1-way ANOVA, p>0.05; Figure 3.5A). However, embryonic T$_4$ content was decreased in both the 10- and 100-ppm groups compared to the control treatment (1-way ANOVA, Tukey’s HSD, p<0.05; Figure 3.5B).
Figure 3.3. Photomicrographs of thyroid sections of female zebrafish after 10 weeks of treatment. Control thyroid follicles were filled with colloid and had non-hypertrophic epithelial cells (A). Exposure to perchlorate caused mild colloid depletion at 10 ppm (B) and mild to severe colloid depletion at 100 ppm (C). Hypertrophic epithelial cell were observed at both perchlorate concentrations. In some fish, exposure to perchlorate at 100 ppm also caused hyperplasia (panel D). Bar = 20 µm (A, B, C) or 40 µm (D).
Figure 3.4. Effects of perchlorate exposure on thyroid hormone levels in whole-body extracts of female zebrafish at the end of the treatment (16 weeks). Triiodothyronine (T₃) concentration was not affected by perchlorate treatment (1-way ANOVA, p>0.05; panel A), whereas thyroxine (T₄) was significantly reduced at both perchlorate concentrations compared to control values (1-way ANOVA, Tukey’s HSD, p<0.05; panel B).
Figure 3.5. Effects of maternal exposure to perchlorate on embryonic thyroid hormone content 3.5 h after fertilization. At Week 14, maternal exposure to perchlorate did not affect the triiodothyronine (T3) levels in the control group (1-way ANOVA, p>0.05; panel A). At Week 15, the levels of thyroxine (T4) in embryos from mothers exposed to perchlorate at 10 or 100 ppm were lower than control values (1-way ANOVA, Tukey’s HSD, p<0.05; panel B).
Effect of perchlorate on maternal wet-weight

Two-way ANOVA (treatment X length of exposure) indicated that both treatment and length of exposure had effect on the wet-weight of females (p<0.05). At Week 12 of exposure, the mean weight of females prior to spawning was significantly lower in the 100-ppm group compared to the control and 10-ppm groups (1-way ANOVA, Tukey’s HSD, p<0.05). From Week 13 to Week 16, the mean weight of females in the 10 and 100-ppm treatment groups were significantly lower than control values (1-way ANOVA, p<0.05), but there was no significance difference between the 10 and 100-ppm treatment groups (p>0.05; Figure 3.6).

Effect of perchlorate on packed-egg volume production

Two-way ANOVA (treatment X length of exposure) indicated that treatment, length of exposure and their interaction all had significant effects on PEV (p<0.05). One-way ANOVA conducted at each spawning event indicated that treatment effects were significant (p<0.05) at 12, 13, 15, and 16 weeks of exposure but not at 14 weeks. The inhibitory effect of perchlorate on PEV was evident earlier in the 10-ppm exposure group (Week 12, 13, 15 and 16) than in 100-ppm exposure group (Week 15 and 16) (1-way ANOVA, Tukey’s HSD; Figure 3.7A). The cumulative PEV in the 10- and 100-ppm treatment groups were significantly lower than the control cumulative PEV (repeated-measure ANOVA, Tukey’s HSD, p<0.05; Figure 3.7B).
Figure 3.6. Effect of perchlorate on female wet-weight. The group weight of the 8 females per replicate was measured the day before spawning immediately before transfer to spawning chambers. The weight of control females remained constant throughout the spawning period, but the weight of females exposed to perchlorate at 10 and 100 ppm was significantly reduced (1-Way ANOVA, Tukey’s HSD, p<0.05 for each spawning event). Asterisks indicate significant difference from control females.
Figure 3.7. Effects of perchlorate on weekly packed-egg volume (panel A) and cumulative packed-egg volume (panel B). Perchlorate at 10 ppm inhibited weekly egg production starting at Week 12 of exposure; and at 100 ppm, packed-egg volume was reduced compared to control values starting at Week 15 (1-Way ANOVA, Tukey’s HSD, p<0.05). Cumulative packed-egg volume was significantly reduced by exposure to perchlorate at 10 and 100 ppm perchlorate (repeated-measure ANOVA, Tukey’s HSD, p<0.05). Asterisks indicate significant difference from control (panel A), and columns associated with common letters are not significantly different (panel B)
Effects of parental exposure to perchlorate on F1 embryo-larval development

Measurements of fertilization and hatching rates at Week 14 indicated that these parameters of gamete and embryo quality were not affected by parental exposure to perchlorate at 10 or 100 ppm (1-way ANOVA, p>0.05; Table 1). However, mean egg diameter at Week 16 was slightly larger in spawns from both perchlorate treatments compared to control values (nested ANOVA, Tukey’s HSD, p<0.05; Table 1).

Observations conducted at Week 15 indicated that parental exposures to perchlorate did not cause obvious developmental deformities in F1 progeny (data not shown). However, larval length was slightly but significantly increased in progeny from the 100 ppm-treated parents compared to progeny from the control parents (Table 2). Also, analysis of jaw morphometry indicated that the height of Meckel’s and ceratohyal cartilage complexes were slightly but significantly reduced compared to control larvae (Table 2). Cartilage measurements could not be normalized for larval length because these measurements were not recorded for the same individual fish (larvae from each spawn were processed in groups for cartilage staining). However, because the mean standard length in the 100-ppm larvae was longer than in control larvae, cartilage measurements normalized for larval length would only amplify the difference observed between treatments.
Table 1. Effects of parental exposure to perchlorate on selected egg and embryo traits

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fertilization Rate (%)</th>
<th>Hatching Rate (%)</th>
<th>Fertilized egg Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 WK</td>
<td>14 WK</td>
<td>16 WK</td>
</tr>
<tr>
<td>Control</td>
<td>86±4</td>
<td>93±4</td>
<td>1.23±0.01</td>
</tr>
<tr>
<td>10 ppm</td>
<td>89±4.1</td>
<td>96±3</td>
<td>1.26±0.03*</td>
</tr>
<tr>
<td>100 ppm</td>
<td>75±9.2</td>
<td>86±7.3</td>
<td>1.26±0.03*</td>
</tr>
</tbody>
</table>

Asterisk (*) indicates significant difference from control (1-way ANOVA and Tukey HSD, P<0.05)
Table 2. Effect a 15-week maternal exposure to perchlorate (100 ppm) on early larval development

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Standard Length (mm)</th>
<th>Head Depth (mm)</th>
<th>Height of Ceratohyal (µm)</th>
<th>Height of Meckel’s (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.21±0.04</td>
<td>0.47±0.02</td>
<td>201.0±3.4</td>
<td>113.9±2.2</td>
</tr>
<tr>
<td>Perchlorate</td>
<td>3.28±0.03*</td>
<td>0.48±0.0</td>
<td>189.8±7*</td>
<td>104.4±5.3*</td>
</tr>
</tbody>
</table>

Asterisk (*) indicates significant difference from control (nested-ANOVA, P<0.05)
DISCUSSION

Perchlorate competitively inhibits iodide uptake into the thyroid follicles (Capen, 2001; Wolff, 1998), and at environmentally relevant concentrations it impairs thyroid histology of several aquatic vertebrates including the African clawed frog, *Xenopus laevis* (Goleman et al., 2002a), fathead minnow, *Pimephales promelas* (Crane et al., 2005) and zebrafish (Patiño et al., 2003; Mukhi et al., 2005; Van der Ven et al., 2006; present study). In zebrafish, perchlorate-induced alterations of thyroid follicles include hypertrophy, hyperplasia, colloidal depletion, angiogenesis and suppression of the immunocytochemistry-based colloidal T₄ ring (Patiño et al., 2003; Mukhi et al., 2005). Several of these conditions were qualitatively evident in the female fish of the present study exposed to perchlorate at 10 and 100 ppm, and their TH levels were also affected. These observations confirm the effectiveness of perchlorate as disruptor of thyroid function in zebrafish. In addition, the results of the present study showed that long-term exposure to perchlorate can also disrupt reproductive function.

A previous study with adult zebrafish found that exposure to perchlorate at 10 ppm for 12 weeks had no effect on whole-body T₄ concentration; however, the same study suggested that longer exposure periods may be necessary to alter TH status in this species (Mukhi et al., 2005). Indeed, the results of the present study showed that exposure to perchlorate at 10 or 100 ppm for 16 weeks caused a decline in whole-body T₄ concentrations. Other studies with various vertebrate species have yielded inconsistent results regarding the effects of perchlorate on T₄ concentrations. For example, exposure to perchlorate caused a decline in whole-body T₄ concentration of African clawed frog
(Goleman et al., 2002a) and mosquitofish, *Gambusia holbrooki* (Bradford et al., 2005), and in plasma T₄ of rabbits (York et al., 2001); whereas perchlorate treatment increased T₄ concentration in whole-body of fathead minnow (Crane et al., 2005) and in plasma of deer mice, *Peromyscus maniculatus* (Thuett et al., 2002). Curiously, T₃ concentrations seem to be more resistant to change during exposure to perchlorate. For example, exposure to perchlorate did not affect T₃ levels in rabbits (plasma; York et al., 2001) and zebrafish (whole-body; present study) despite a decline in T₄ levels in both cases. Although whole-body T₃ concentrations in the present study showed a trend to decrease in perchlorate-treated fish, this trend was not statistically significant. The resilience of T₃ homeostasis was also recently demonstrated in a study of rats (*Rattus norvegicus*) fed iodine-deficient diets, in which reductions in the T₃ level of various tissue compartments occurred only after the corresponding T₄ level had declined by 75-95% (Pedraza et al., 2006). In the present study, T₄ levels in female fish exposed for 16 weeks to perchlorate at 100 ppm were reduced by 95% compared values recorded in control fish. Thus, it seems likely that zebrafish treated with perchlorate were borderline hypothyroid in the present study and that longer exposure periods may have led to clear reductions in T₃ levels.

The thyroid gland (or thyroid follicles) is the only endocrine organ of vertebrates that can store considerable amounts of hormone. Because T₄ is the major hormone synthesized and stored in the thyroid follicles of teleosts, it is not surprising that exposure to compounds that inhibit T₄ synthesis (e.g., perchlorate) take relatively long periods of time to significantly suppress T₄ levels. Triiodothyronine is biologically more active than
T₄, and in teleosts it is synthesized by peripheral tissues via outer ring deiodination of T₄ (Brown et al., 2004). Treatment of tilapia (Oreochromis mossambicus) with methimazole, also a TH synthesis inhibitor, induces the activities of type I and type II deiodinases (which enhance T₃ production from T₄) and suppresses the activity of type III deiodinase (which metabolizes T₃ and T₄ to inactive forms) in the liver (Van der Geyten et al., 2001). These changes in deiodinase activities are believed to help maintain T₃ homeostasis under conditions of reduced but still adequate T₄ supply (Van der Geyten et al., 2001). Thus, increased conversion rates of T₄ to T₃ under near hypothyroid conditions may explain why T₃ levels are more resistant to change than T₄ levels following exposure to goitrogens, as shown by the present and previous (York et al., 2001; Pedraza et al., 2006) studies.

In an earlier study with zebrafish, PEV was not affected after exposure to ammonium perchlorate at 18 ppm for 8 weeks although TH levels were not measured (Patiño et al., 2003). Mukhi et al. (2005) subsequently showed little change in TH levels (whole-body) of zebrafish exposed to perchlorate at 10 ppm for up to 12 weeks. In the present study, sodium perchlorate at 10 and 100 ppm generally had an inhibitory effect on PEV after an exposure period of 12-16 weeks (inhibition was consistent at both perchlorate concentrations during the last two weeks of exposure), and whole-body T₄ levels were also greatly reduced when measured at 16 weeks. The effects of perchlorate on the weight of female fish generally matched the effects on PEV; most likely, female fish weight is a direct reflection of the mass of eggs they produced prior to spawning. Taken together, the present and previous (Patiño et al., 2003; Mukhi et al., 2005)
observations indicate that exposure to perchlorate impairs egg production in zebrafish only if TH levels are disrupted. These observations and conclusion are consistent with a role of TH in the maintenance of zebrafish reproductive condition.

There is correlative as well as experimental evidence suggesting that TH is also necessary for successful reproduction in other teleosts (Cyr et al., 1988; Cyr and Eales, 1988; Weber et al., 1992; Van der Geyten et al., 2001). In a notable study with rainbow trout (Cyr and Eales, 1988), hypothyroidism induced by treatment with ipodate (inhibitor of outer ring deiodination) resulted in depressed gonadosomatic index (GSI) of female fish, whereas moderate hyperthyroidism induced via dietary T\textsubscript{3} supplementation resulted in increased GSI levels. These and other observations have led to the proposal that TH is necessary for the regulation of gonadal growth (recrudescence) in teleosts (Cyr and Eales, 1996). Although GSI was not measured in the present study, the lower body weights and PEV values suggests a depressed GSI in perchlorate-treated fish.

However, there is also disagreement over the effects of goitrogens, and consequently the specific role of TH, on teleost reproduction. For example, in a recent study with zebrafish, a 3-week exposure to propylthiouracil (like perchlorate, an inhibitor of TH synthesis) caused plasma T\textsubscript{4} levels to be depressed at 10 and 100 ppm and plasma T\textsubscript{3} levels at 100 ppm, and reportedly also caused an increase in cumulative egg production in females spawned twice weekly over the exposure period (Van der Ven et al., 2006). However, the control fish in this recent study (Van der Ven et al., 2006) spawned an average of only 2.3 times out of 6 spawning attempts, an observation which may raise questions about the condition of some of the fish at the start of the exposure. In
the present study, each spawning replicate (n = 3) consisted of 8 females and 4 males (total females per treatment, 24), and reproductive cycles were synchronized by a trial spawn prior to the initiation of data collection; whereas in the recent study (Van der Ven et al., 2006), each replicate (n = 3) consisted of 2 females and 1 male (total females per treatment, 6). The use of small numbers of fish in the latter study may increase the risk of biased results due to random differences in initial fish condition. In another study with eastern mosquitofish (Gambusia holbrooki), a livebearer, exposure to sodium perchlorate at 100 ppm reportedly also had a stimulatory effect on fecundity (embryo numbers corrected for body length) at the end of an 8-week exposure period (Park et al., 2006). However, eastern mosquitofish are multiple spawners under natural field conditions (Perez-Bote and Lopez, 2005) and, under laboratory conditions, the brood production cycle of the closely related Gambusia affinis is approximately 21 days (Koya et al., 1998). Thus, the fecundity estimates in the eastern mosquitofish study (Park et al., 2006) should be interpreted with caution because the frequencies of spawning as well as the total number of embryos produced during the 8-week exposure period are unknown.

Exposure to perchlorate affected the TH content of early embryos in a pattern similar to that observed in female fish. Namely, T₄ levels were decreased and T₃ levels were unchanged in extracts of embryos derived from mothers exposed to perchlorate at 10 or 100 ppm for 14-15 weeks. Because TH concentrations in whole embryos were determined shortly following fertilization (3.5 h), the measured hormone levels are most likely a direct reflection of maternally inherited concentrations with little contribution from T₄ or T₃ metabolism by the embryos. Maternally derived TH concentrations decline
during development of the teleost embryo prior to the onset of endogenous hormone production (e.g., Power et al., 2001) and, in zebrafish, endogenous TH production does not begin until after hatching (Wendl et al., 2002). Thus, embryos from perchlorate-treated zebrafish mothers, which contained suppressed reserves of T₄ at the beginning of their development, may have become hypothyroid prior to the completion of embryogenesis. This situation may explain the alterations in craniofacial development observed in 4-dpf larvae derived from perchlorate-treated mothers. Namely, the mean lengths of the Meckel’s cartilage (which forms the lower jaw) and ceratohyal cartilage (which supports the lower jaw) complexes were slightly but significantly smaller in F1 larvae derived from parents exposed to perchlorate (100 ppm) compared to control larvae. This observation is consistent with the results of a study with zebrafish embryos and early larvae treated with TH synthesis inhibitor (methimazole) and T₃ antagonist (amiodarone), where the length of the lower jaw in 5-dpf larvae was shortened by the inhibitor/antagonist treatment and restored by co-treatment with exogenous T₄ (Liu and Chan, 2002). Thus, the earlier study with zebrafish (Liu and Chan, 2002) concluded that TH is necessary for proper development of the lower jaw. Some differences in results also exist between the studies. For example, whereas larval head depth was not affected by maternal perchlorate treatment in the present study, embryo-larval hypothyroidism caused a reduced in head depth in the previous study (Liu and Chan, 2002). Differences in methodology must be taken into consideration when assessing the similarities and differences between the results of Liu and Chan (2002) and the present study (maternal versus embryo-larval treatment, respectively). However, taken together, the present and
previous (Liu and Chan, 2002) studies with zebrafish provide evidence for a role of maternal TH in the early development of the lower jaw.

Although PEV was inhibited by maternal exposure to perchlorate in the present study (see preceding discussion), egg fertilization and hatching rates were unaffected. These observations are consistent with those of a study with medaka, where fertilization and hatching of hypothyroid F1 eggs and embryos derived from thiourea-treated hypothyroid mothers were similar to those seen in control individuals (Tagawa and Hirano, 1991; this study with medaka did not examine effects on fecundity). Other studies with zebrafish have consistently observed no effects of maternal goitrogen exposure on egg fertilization or hatching rates (Patiño et al., 2003; Van der Ven et al.; 2006). Curiously, the diameter of fertilized eggs and the length of 4-day-old (post fertilization) larvae were slightly stimulated by maternal exposure to perchlorate in the present study. The reason for these observations is uncertain, but it could be the result of a physiologically regulated trade-off between fecundity and egg size. In mosquitofish, exposure to perchlorate also caused a slight increase in the mass of individual embryos (Park et al., 2006).

In conclusion, long-term exposure of zebrafish to perchlorate affects not only the integrity of their thyroid endocrine system but also their reproductive performance and the early development of their F1 progeny. Therefore, perchlorate can be classified not only as a thyroid endocrine disruptor but also as a potential reproductive disruptor.
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ABSTRACT

Hexahydro-1,3,5-trinitro-1,3,5-triazine, a cyclonitramine commonly known as RDX, is used in the production of military munitions. Contamination of soil, sediment, and ground and surface waters with RDX has been reported in different places around the world. Acute and subacute toxicities of RDX have been relatively well documented in terrestrial vertebrates, but among aquatic vertebrates the information available is limited. The objective of this study was to characterize the acute toxicity of RDX to larval zebrafish. Mortality (LC50) and incidence of vertebral column deformities (EC50) were two of the end points measured in this study. The 96-h LC50 was estimated at 22.98 and 25.64 ppm in two different tests. The estimated no-observed-effective-concentration (NOEC) values of RDX on lethality were 13.27 ± 0.05 and 15.32 ± 0.30 ppm; and the lowest-observed-effective-concentration (LOEC) values were 16.52 ± 0.05 and 19.09 ± 0.23 ppm in these two tests, respectively. The 96-h EC50 for vertebral deformities on survivors from one of the acute lethality tests was estimated at 20.84 ppm, with NOEC and LOEC of 9.75 ± 0.34 and 12.84 ± 0.34 ppm, respectively. Behavioral aberrations were also noted in this acute toxicity study, including the occurrence of whirling movement and lethargic behavior. The acute effects of RDX on survival, incidence of deformities, and behavior of larval zebrafish occurred at the high end of the most
frequently reported concentrations of RDX in aquatic environments. The chronic effects of RDX in aquatic vertebrates need to be determined for an adequate assessment of the ecological risk of environmental RDX.

INTRODUCTION

Hexahydro-1,3,5-trinitro-1,3,5-triazine, a cyclonitramine commonly known as RDX (royal demolition explosive), is a high energetic explosive compound used mainly for the production of munitions (Walsh and Jenkins, 1992; Talmage et al., 1999) as well as firework production and demolition of blocks (ATSDR, 1995). Occasional use of RDX as rodenticide has also been reported (Etnier, 1989).

Royal Demolition Explosive can be released into the environment directly and indirectly through manufacturing, loading, packing, utilization and demilitarization of antiquated munitions (Burrows et al., 1989; Walsh and Jenkins, 1992; Talmage et al., 1999). Contamination of soils, sediments, surface and ground waters with RDX has been reported in and around army ammunition plants in the USA and elsewhere (Small and Rosenblatt, 1974; Aller, 1985; Walsh and Jenkins, 1992). The reported concentration of RDX in soil samples from various army sites in the USA ranges from 0.044 to 13,900 µg g⁻¹ (Walsh and Jenkins, 1992). Sources of RDX in aquatic environments may include effluent release or leaching of RDX from contaminated soil into ground or surface waters (Sunahara et al., 1999). In ground water samples collected from the Bangor Annex ammunition processing waste disposal area (WA, USA), the RDX concentration was up to 3.7 ppm. Concentration of RDX in surface water at Brush Creek (IA, USA), near an
army ammunition plant, ranged from 0.1 to 0.15 ppm (Small and Rosenblatt, 1974). In a stream near the Milan army ammunition plant (TN, USA), concentrations of RDX as high as 109 ppm were reported (Ryon et al., 1984).

Cyclonitramines are generally considered to be toxic to living organisms (Talmage et al., 1999), and thus the presence of RDX in the natural environment could be detrimental to the health of the ecosystem. Toxic effects of RDX have been well documented in various laboratory animals including mammals and birds (Hart, 1977; Cholakis et al., 1980; Levine et al., 1981a,b; Levine et al., 1983; Gogal et al., 1997). Based upon microbial toxicity tests, RDX has been also classified as toxic to aquatic vertebrates and invertebrates (Drzyzga et al., 1995). However, information about the toxic effects of RDX in fishes is limited to a few species. The acute 96-h LC50 of RDX for juvenile fathead minnow was estimated at 12.7 ppm (Burton et al., 1994) and for bluegill, at 6 ppm (Bentley et al., 1977). Information about the sublethal effects of RDX is unavailable for fishes.

Much of the waste generated through the use of explosive compounds finds its way into the aquatic ecosystem. Therefore, additional information on the toxic effects of RDX is necessary for an adequate assessment of the ecological risk of RDX in the aquatic environment. The objectives of the present study were to evaluate the acute effect of RDX on survival and developmental deformities of larval zebrafish (Danio rerio).
MATERIALS AND METHODS

Chemical and Safety Procedures

Hexahydro-1,3,5-trinitro-1,3,5-triazine (CAS Reg. No. 121-82-4) was obtained from Accurate Energetics (McEwen, TN, USA). The chemical was 99% pure and supplied in desensitized form, containing 20% water by volume. Standard solutions for RDX at a concentration of 1000 ppm in acetonitrile were obtained from Supelco (Bellefonte, PA, USA). Acetonitrile was obtained from Fisher Scientific (Pittsburg, PA, USA). Stock solutions for the exposures were prepared by mixing the appropriate weight of RDX in zebrafish-water (180 mg of Sea Salt™/L) at 28°C under constant stirring for 24 h. Working solutions were prepared by mixing the appropriate volumes of stock solution and zebrafish-water. The RDX concentration in stock solutions was determined to be stable for at least one week after preparation. Therefore, the stock solution was made just prior to the start of the 96-h acute toxicity study. The final concentration of RDX in the stock solution, the working solution, and water samples from exposure beakers were analyzed by high performance liquid chromatography (HPLC) (see chemical analysis section below for additional detail).

RDX was stored in a specially designed bunker on campus and handled with utmost care to prevent spark or shock which could trigger explosions. The parent compound and its metabolites are considered possible carcinogens (USEPA, 1994). Therefore, personnel involved in the study wore protective gloves and goggles. All solvents retained from water solutions, extractions or from solvent reduction procedures were collected and discarded according to standard safety procedures.
Chemical Analysis

Water samples were analyzed for actual RDX concentrations by HPLC. To construct the standard curve, standards (20 - 10,000 ppb) were prepared from the standard stock solution (1000 ppm) in 50:50 acetonitrile: water (zebrafish-water). At least 9 standards were prepared for calibration. Each analysis sequence began with injections of at least three continuing calibration standards within the calibration curve range. Single continuing calibration standards were run with every 10-15 samples in the sequence, and responses were averaged into the existing standard curve. If the instrument response of the continuing calibration standards had changed by 15%, then a new standard curve was developed using all calibration standards. Before injection, water samples were diluted with 50% acetonitrile and filtered through a 0.2 µm PTFE syringe filter (Fisher Scientific, NH, USA) into the autosampler vials.

A Hewlett-Packard 1100 HPLC instrument was used with a UV detector set at a wavelength of 254 nm. Separation were performed with a Supelco C18 column (4.6 x 25 mm, 5-µm packing, Supelco, Bellefonte, PA, USA) using a mobile phase of 50% acetonitrile to 50% water. The solvent flow rate was 1 ml/min, and the injection volume was 15 µl. Each run took 7 min. Peak areas were obtained using a HP 3390A integrator.

Fish breeding and larval rearing

Protocols for the use of animals in this study were reviewed and approved by the Texas Tech University Animal Care and Use Committee (Lubbock, TX, USA). Larvae for the acute toxicity study were obtained from a colony of adult zebrafish maintained on
These colony fish were purchased from Aquatic Research Organisms (Hampton, New Hampshire, USA) and reared in 200-L aquaria with external biofilters. Water quality parameters were maintained at optimal for zebrafish (pH 6.5 - 8.0; 28 ± 2°C; 14:10 light:dark cycle; unionized NH3 <0.01 ppm). Male and female broodstock zebrafish were maintained separately and fed adult brine shrimp twice daily to satiation. Male and female fish were placed together for breeding in the evening according to methods described by Patiño et al., (2003), and eggs were collected 3 h after lights-on the following morning. Eggs were washed several times with zebrafish-water to remove fecal matter and other debris. Eggs were then counted into a 1-L beaker (200 eggs per beaker) containing 400 ml of zebrafish-water (temperature 27-28°C). Beakers were maintained at 27-28°C in a water bath, and other parameters were kept optimal for zebrafish as described above, except that the light:dark cycle was 12:12 h. Unfertilized (opaque) eggs were removed from the beakers 12 h after spawning. Fifty percent of the water was exchanged with fresh zebrafish-water (preheated to 27-28°C) everyday after removing any dead eggs or egg shells from the hatched fish. Most eggs hatched 3-4 days after fertilization. Unhatched eggs were removed and discarded on day 4 after fertilization to maintain homogeneity in the age-since-hatching among larvae. Five-day-old larvae were used for acute toxicity tests.

**Range-finding test**

The 96-h acute toxicity of RDX was determined according to guidelines provided by the American Society for Testing and Materials (ASTM 1997). Five-day-old larvae
were assigned to seven RDX treatments and one control. The nominal concentration of RDX in the highest treatment was designed to be 40,000 ppb and the lowest treatment group was 0.04 ppb. Each treatment concentration was 10 fold higher than the previous one (except the 0.04 ppb-group). The RDX concentration in treatment beakers was verified by HPLC measurement. As the detection limit of RDX in test solution was 20 ppb, RDX could be detected only in the highest four treatment concentrations. The mortality data generated from this preliminary range-finding test was utilized in designing the definitive tests.

Definitive Test 1

A randomized complete block design was implemented with 40 experimental units (250-mL exposure beakers), eight treatment levels, and five replicates per treatment. Within each of the water baths, each beaker was randomly assigned one of the eight treatments. Each beaker contained 100 mL of zebrafish water and 15 larvae. The larvae were acclimatized to the beaker for 24 h before starting the exposure. To start the exposure, approximately 95% of water was removed from each beaker and replaced with treatment water containing the appropriate RDX concentrations. In subsequent days, approximately 95% of water volume was replaced daily with freshly prepared treatment solutions. Water quality parameters (pH, temperature, unionized ammonia, and dissolved oxygen) were measured daily. The temperature of the water bath was used as indicator of the temperature of the water in the beakers. Dissolved oxygen was measured with a YSI 85™ meter (YSI Inc., Yellow Springs, OH, USA) and pH was measured with a pH Tester
(Oakton Instruments, IL, USA) before the daily water exchanges. Water samples were collected daily from each beaker before the water exchange for determination of actual RDX concentrations and unionized ammonia content. Dead larvae were counted and removed daily before exchanging the water. At the end of the 96-h exposure period, the total number of live fish was determined in each beaker. The fish were then euthanized with 1 g/L of MS-222 (Sigma, St. Louis, MO, USA) and waste water was disposed in an appropriate waste container.

Definitive Test 2

The design for this test was similar to that of the preceding Definitive Test 1. Results from the preceding tests indicated that the variability among the measured RDX concentration for each replicate within a treatment was narrow and close to the measured RDX concentration in the working solutions. Thus, RDX concentrations for this test were not measured in each beaker but instead were measured only in the working solutions that were used as source for beaker water. All fish handling and monitoring procedures for this test were as described earlier.

From the preliminary observations of the Definitive Test 1, it was determined that RDX caused vertebral column deformities in the developing larvae. These observations prompted us to determine the incidence of vertebral column deformities in Definitive Test 2. Mortality rates in second test were also recorded and used for calculation of LC 50 for this definitive test. Data collected on vertebral deformities were used to determine the EC50. Behavioral effects were also noted as gross observations. Larvae were observed
for gross behavioral abnormalities at 12 hour intervals, and at the end of the 96-h exposure all surviving fish were euthanized in MS-222 (Sigma, St.Louis, MO, USA) and preserved in 10% neutral buffered formalin (Sigma, St.Louis, MO, USA). The overall incidences of vertebral column deformities (Brown and Nunez, 1998; Silverstone and Hammell, 2002) were determined on the survivors. For this purpose, all larvae per beaker were observed under a stereo microscope to determine the percentage of those showing vertebral deformities.

Data Analyses

Data were subjected to probit analysis to calculate LC50 and EC50 with 95% confidence limits (CL) using SPSS (SPSS Inc., Chicago, IL, USA) after logarithmic transformation of RDX concentrations. For the Definitive Test 1, the RDX concentrations measured for each individual beaker over the 96 h period (24 h interval) were averaged to obtain the mean for each beaker, and the average of the five replicate beakers was used to represent the concentration of each treatment. For the Definitive Test 2, the concentrations of the working solutions prepared daily for each treatment group were averaged over the 96-h to obtain the respective treatment concentrations. Percent values for mortality and deformities were transformed by arcsine of square-root before application of one-way analysis of variance (ANOVA, \(\alpha= 0.05\)); and Duncan’s multiple range test was used (\(\alpha= 0.05\)) to determine the Lowest-Observed-Effect Concentration (LOEC) and the No-Observed-Effect-Concentration (NOEC) for lethality and vertebral deformities. These statistical analyses were conducted using the Statistica® software.
package (StatSoft, Tulsa, OK, USA). A linear regression analysis was done to assess correlation between the percent vertebral deformities and the water borne RDX concentration. Behavioral observations were qualitatively assessed.

RESULTS

Chemical analysis

The HPLC procedure yielded an excellent linearity with a correlation coefficient of 1.00 over the range of RDX standard concentrations. The retention time for RDX was approximately 5.4 min. RDX is not highly soluble in water and its highest solubility in the zebrafish water was estimated to be 42 ppm at 28°C. The measured concentration of RDX (mean ± SE, \( n = 5 \)) in the treatment groups of the first test (Definitive test 1) were 0 ± 0, 10.44 ± 0.20, 13.27 ± 0.05, 16.52 ± 0.05, 20.56 ± 0.06, 25.93 ± 0.11, 32.08 ± 0.03 and 39.88 ± 0.09 ppm; and in Definitive test 2 were 0 ± 0, 9.75 ± 0.34, 12.84 ± 0.34, 15.32 ± 0.30, 19.09 ± 0.23, 23.98 ± 0.31, 29.89 ± 0.29 and 37.12 ± 0.31 ppm.

Acute toxicity of RDX

Concentration-dependent mortality was observed during the course of the 96-h acute toxicity tests (Figure 4.1). No mortality was observed at any RDX concentration within the first 24 h of exposure in both definitive tests. The first mortality occurred after 48 h of exposure in both definitive tests, and the incidence at the highest RDX concentration after 96 h reached 100 percent in Definitive Test 1 and 83 percent in the Definitive Test 2. No mortality occurred in the control beakers. The 96-h LC50 was
estimated to be 22.98 ppm (CL 21.45- 24.64 ppm) and 25.64 ppm (CL 17.06-38.89 ppm) for the Definitive Test 1 and Definitive Test 2 respectively. The NOEC values on lethality of RDX were 13.27 ± 0.05 and 15.32 ± 0.30 mg L⁻¹ and LOEC values were 16.52 ± 0.05 and 19.09 ± 0.23 ppm, in Definitive Test 1 and Definitive Test 2 respectively (p < 0.05).

Vertebral column deformities

There was a concentration-dependent increase in the overall incidence of vertebral column deformities at the completion of the 96-h exposure to RDX. Kyphosis (Silverstone and Hammell, 2002) was the most frequently observed deformity (Figure 4.2). A strong positive correlation (r= 0.80) was observed between the RDX concentration and percent of vertebral deformities. The 96-h EC50 was estimated to be 20.84 ppm (Figure 4.1), with 95% confidence limit of 18.73-23.19 ppm. The NOEC and LOEC of RDX for vertebral deformities were 9.75 ± 0.34 and 12.84 ± 0.34 ppm, respectively (p < 0.05, Figure 4.3).
Figure 4.1. Acute toxic effect of waterborne RDX on larval zebrafish. Survival (mean ± standard error) was determined after 96 h of exposure to various concentrations of RDX in two separate definitive tests. Note that the measured RDX concentrations were slightly different between Definitive test 1 (●) and Definitive test 2 (○). For each test, the 96-h LC50 and EC50 were estimated by probit analysis from the percent survival after 96 h of exposure.
Figure 4.2. Vertebral column deformities caused by waterborne RDX in larval zebrafish. Control larvae (A) had a straight longitudinal axis running from the head through the trunk to the caudal fin (dashed line). RDX caused different types of vertebral deformities in exposed larvae (B), the most common being kyphosis - a downward curvature of the longitudinal axis (dashed line) in the region over the abdominal cavity.
Figure 4.3. Concentration-dependent effect of waterborne RDX on vertebral deformities (mean ± standard error) in larval zebrafish. The data shown was collected during the Definitive Test 2 (see text), and includes all types of deformities. There was a significant effect of waterborne RDX concentration on overall vertebral deformities ($p < 0.05$). The treatments that were significantly different from control are indicated by an asterisk (*, $\alpha = 0.05$). The NOEC and LOEC of RDX for vertebral deformities were 9.75 ± 0.34 and 12.84 ± 0.34 ppm, respectively ($p < 0.05$).
Behavioral observations

Experimental units were observed daily and the general behavior of the fish was noted. There were no grossly abnormal behaviors observed within the first 12 h of exposure in any of the treatment groups. At 24 h of exposure, erratic (whirling) movement was conspicuous at RDX concentrations of $\geq 19.09$ ppm. After 72 h, most larvae at RDX concentrations of $\geq 23.98$ ppm had lost their ability to react when chased with a probe and had become lethargic, lying on the bottom of the beaker with only intermittent periods of swimming activity. There were no obvious behavioral alterations in fish from the control beakers or the three lowest treatment groups ($\leq 15.32$ ppm) during the exposure.

DISCUSSION

Although the toxicity of RDX has been relatively well studied in mammals (Hart, 1977; Levine et al., 1981a,b), the information for aquatic organisms is limited to few species. The acute toxicity of RDX has been determined in fathead minnows, bluegill, rainbow trout and channel catfish (Burton et al., 1994; Bentley et al., 1977). Other than the study of the Burton et al., (1994) with fathead minnow, the acute toxicity in most other studies was estimated from the nominal concentrations of RDX either in test water or in DMSO. In the present study with zebrafish we used the measured concentration of RDX to evaluate the LC50, NOEC and LOEC of this compound in larval zebrafish. In fathead minnow, Bentley et al., (1977) reported that the 96-h LC50 of RDX (nominal concentrations) is $> 100$ ppm for embryos, 43 ppm for 1-h post hatched larvae, 3.8 ppm
for 7-day-old larvae, 16 ppm for 30-day-old juveniles, and 11 ppm for 60-day-old fish. Burton et al., (1994) reported that the 96-h LC50 (measured concentration) for 15-17-day-old fathead minnow was 12.7 ppm. Similarly, the 96-h LC50 (nominal concentrations) for juvenile bluegill was 6.0-6.4 ppm; for fingerling rainbow trout was 6.4 ppm; and for juvenile catfish was 4.1-13 ppm (Bentley et al., 1977). In the present study, the 96-h LC50 for 5-day-old larval zebrafish was estimated at 22.98 ppm (95% confidence limit of 21.45-24.64 ppm). Thus, the acute toxicity of RDX in zebrafish is similar to those reported in other fishes at various stages of development.

It is noteworthy that RDX seems to be more toxic to aquatic vertebrates than aquatic invertebrates (Etnier, 1986). In one study, RDX concentrations at the solubility limit of the compound under specific test conditions were found to be not toxic to daphnia (*Ceriodaphnia dubia*), hydra (*Hydra littoralis*), and midge (*Paratanytarsus parthenogeneticus*) (Peters et al., 1994). Similarly, RDX at concentrations >100 ppm (nominal concentration) was not toxic to the water flea (*Daphnia magna*), scud (*Asellus militaris*) and the midge (*Chironomous tentans*) (Bentley et al., 1977). It is unclear why there is a relatively large difference in RDX toxicity levels between aquatic vertebrates and invertebrates, but it may be attributed to physiological differences between the taxa or to differences in the respective mechanism of RDX toxicity.

Although RDX is not highly soluble in water, acute toxic effects of this compound in aquatic vertebrates are observed within its solubility range. The origin of RDX in water bodies is mainly wastewater discharges from army ammunition plants or run off from contaminated sites (Sunahara et al., 1999). Concentrations of RDX in water
have been found to be as high as 109 ppm (Ryon et al., 1984), and at this concentration
RDX has been demonstrated to be well above the acute toxic level to aquatic vertebrate
species (Bentley et al., 1977; Burton et al., 1994; present study). The toxicity of RDX
may also depend on the stage of development of organisms. In fishes, larvae are
generally considered to be the more susceptible to contaminant exposure than the adults
(Wedemeyer, 1996). Thus, larval toxicity data may be a better indicator of contaminant
effects in fishes for the purpose of ecological risk assessment.

Vertebral deformities also showed a concentration-dependent pattern with an
estimated EC50 value of 20.84 ppm. The NOEC and LOEC of RDX for vertebral
deformities were 9.75 ± 0.34 and 12.84 ± 0.34 ppm, respectively, which are slightly
lower than the NOEC and LOEC for lethality (13.27 ± 0.05 ppm and 16.52 ± 0.05 ppm,
respectively). These values must be considered with some caution, however, since they
are based on measurements taken on fish that survived the exposures; and are biased
toward the most tolerant individuals of the population. (It is difficult to determine
deformities on dead fish because of postmortem artifacts.) However, there was a strong
correlation between RDX concentration and the incidence of vertebral deformities.
Spinal curvatures have been previously observed in fathead minnow after chronic
exposure to RDX, although concentration-dependent effects were not established
statistically (Burton et al., 1994). In feral fishes, vertebral column deformities have been
reported in individuals inhabiting places contaminated with heavy metals, bleached kraft
mill effluent, and chlorinated benzoquinones (Bengtsson, 1975; Bengtsson, 1979 and
Halver et al., 1969). In zebrafish, spinal curvatures have been associated with exposure
to organophosphate, organochlorine, and carbamate insecticides (Bengtsson 1975; Couch et al., 1977; Incardona et al., 2004) and also with old-age and muscular abnormalities (Gerhard et al., 2002). The mechanism of the vertebral deformities caused by RDX is unknown for fishes. RDX has mutagenic effects in mice and has been classified as a possible human carcinogen (class C) (USEPA, 1994).

Exposure to RDX in mammals causes a variety of central nervous system disorders (Levine et al., 1981a,b), including abnormal behavior that correlates positively with dosage of RDX exposure (MacPhail et al., 1985). Acute toxic effects of RDX have been reported in humans upon ingestion of C-4, which consists mostly of RDX (Stone et al., 1969). The symptoms of acute RDX exposure in this report concerning humans (Stone et al., 1969) included generalized convulsions and unconsciousness, muscular twitching, hyperactive reflexes, headache, temporary amnesia, disorientation and asthenia. In the present study, acute toxic effects RDX on behavior of larval zebrafish included erratic swimming patterns (whirling movement) and, after several days, lethargic behavior. Little information is presently available on the effects of RDX on the behavior of aquatic vertebrates.

The information obtained in this study may be of use in efforts to assess the ecological risk of RDX in the aquatic environment. In this regard, further research is needed to determine the sublethal effect of RDX on the general health, growth, and reproduction of fishes. In addition, the toxicity of RDX metabolites should also be examined.
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ABSTRACT

A previous study with larval zebrafish showed that the 96-h no observable effect concentration (NOEC) for RDX is approximately 13-15 ppm. The objectives of the present study were to determine the chronic effects of RDX at concentrations below the larval NOEC on survival, somatic condition (body length and weight) and gonadal condition (histology) of adult zebrafish, and to determine the bioconcentration factor for RDX. For this purpose, mixed-sex populations of 3-month-old, adult zebrafish were exposed in static-renewal systems to measured RDX concentrations of 0, 1 or 9.6 ppm for up to 12 weeks followed by a 15-day rearing period in untreated water. RDX caused considerable mortality at 9.6 ppm, with most deaths occurring within the first 8 weeks of exposure. No treatment-related mortalities occurred in the other groups. Somatic measurements of fish taken at 4, 8 and 12 weeks indicated that RDX at 9.6 ppm caused lower body weights at 4 and 8 weeks of exposure and at 1 ppm, lower body weight at 4 weeks; whereas fish lengths were not affected at any time during the exposure. Histological observations of the gonads did not reveal any treatment-related abnormalities. In addition, the estimated sperm content of the testis did not vary among treatments, and a number of females from all treatment groups showed evidence of spawning activity (appearance of postovulatory follicles). RDX was detected in whole-
body extracts of fish exposed to waterborne RDX at 4, 8, and 12 weeks of exposure. The bioconcentration factor for RDX was influenced by time of exposure but not by water RDX concentration; the combined values (for the 1- and 9.6-ppm treatments) were 1.01 ± 0.13, 0.91 ± 0.06 and 2.23 ± 0.04 at 4, 8 weeks and 12 weeks, respectively. RDX was not detected in fish collected after the 15-day recovery period. In conclusion, chronic exposure to RDX is lethally toxic to adult zebrafish at concentrations that are below the acute larval NOEC. Although no evidence of gross impacts on gonadal condition was noted at any of the concentrations tested, functional studies are needed to properly address potential RDX effects on reproduction. The bioconcentration factor for RDX in zebrafish is low but it increased slightly with time of exposure.

INTRODUCTION

Environmental contamination with explosives and their transformation products has been reported in the USA and around the world (Small and Rosenblatt, 1974; Aller, 1985; Walsh and Jenkins, 1992). One of the most widely used explosives by the military is hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Reddy et al., 1999). Contamination of soil, ground and surface waters with RDX results from the production and use of RDX and from the disposal of antiquated munitions near the production and disposal facilities, respectively (Burrows et al., 1989; Walsh and Jenkins, 1992; Talmage et al., 1999). Surface water concentrations of RDX as high as 109 ppm have been reported near some munitions plants (Ryon et al., 1984). RDX is persistent in the environment and under anaerobic conditions can be sequentially metabolized (reduced) to different triazine
products such as hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-
dinitroso-5-nitro-1,3,5-triazine (DNX), hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)
(McCormick et al., 1981). Contamination of water bodies with RDX and its metabolites
may adversely affect aquatic biota (Talmage et al., 1999).

The toxicity of RDX after acute and chronic exposures is relatively well
documented for mammals (Hart, 1977; Cholakis et al., 1980; Levine et al., 1981a, b),
birds (Gogal et al., 2003) and amphibians (Johnson et al., 1999; Sparling et al., 2000;
Johnson et al., 2004). For instance, RDX decreased feed consumption and reduced weight
gain in rats (*Rattus norvegicus*) but not in mice (*Mus musculus*) at any doses ranging
between 10 to 40 mg/kg/day (Cholakis et al., 1980). In F344 rats, RDX at a dose of 100
mg/kg/day caused weight gain and increased liver weight (Levine et al., 1981a). Dietary
RDX at levels exceeding 10.6 mg/kg/day decreased feed consumption and egg
production in northern bobwhite (*Colinus virginianus*; Gogal et al., 2003). At soil
concentrations of 5000 mg/kg, RDX caused neuromuscular disorders and significant
weight loss in terrestrial salamanders (*Plethodon cinereus*; Johnson et al., 2004).

The acute toxicity of RDX has been characterized in several teleosts. In larval
zebrafish (*Danio rerio*), for example, the 96-h no observable effect concentration
(NOEC) for RDX was approximately 13-15 ppm and the 96-h LC50 was 23-26 ppm
(Mukhi et al., 2005b). However, little information is available for teleosts concerning the
chronic toxic effects of RDX. In fathead minnow (*Pimephales promelas*), larval survival
and growth were affected at RDX concentrations ≥ 2.4 ppm after 28 days of exposure
(Burton et al., 1994). In another study with the same species, larval size, but not survival,
was affected at an RDX concentration of 5.8 ppm after a 30-day exposure (Bentley et al., 1977). Information regarding the bioconcentration potential of RDX in fishes was not available until recently. This information is now available for channel catfish (*Ictalurus punctatus*; Belden et al., 2005) and sheepshead minnow (*Cyprinodon variegatus*; Lotufo and Lydy, 2005).

It is evident that the information currently available is inadequate for assessing the ecological risks of exposure to RDX in aquatic environments. Therefore, the objectives of the present study were to determine the chronic effects of RDX on survival, somatic condition (body length and weight), and gonadal condition of adult zebrafish, and to determine the bioconcentration factor for RDX. The concentrations tested are below the 96-h NOEC value for zebrafish (Mukhi et al., 2005b).

**MATERIALS AND METHODS**

**Chemical and Safety Procedures**

Hexahydro-1,3,5-trinitro-1,3,5-triazine (CAS Reg. No. 121-82-4) was obtained from Accurate Energetics (McEwen, TN, USA). The chemical was 99% pure and supplied in desensitized form, containing 20% water by volume. Standard solutions for RDX and MNX were obtained from Supelco (Bellefonte, PA, USA). RDX was stored in a specially designed bunker on campus and handled with care. RDX was transported from the bunker to the study facility in the form of slurry.
Animals

Two-and-one-half-month-old, wildtype zebrafish (*Danio rerio*) were obtained from a local vendor and acclimatized to our laboratory for about 2 weeks prior to experimentation. Animal husbandry procedures for this study were as described by Mukhi et al. (2005a). Briefly, each treatment unit (tank) consisted of a 10-gallon aquarium filled with 30 L of zebrafish-water (180 mg of Sea Salt® per 1 L of deionized water) and fitted with two hand-made, glass-based internal biofilters driven by airflow. Water quality (pH, temperature, dissolved oxygen, specific conductivity, and salinity) was measured daily and ammonia-N was measured at least twice weekly. Water quality parameters were maintained at recommended levels for zebrafish (pH 6.5-8.0, 26-28.5 ºC, 12/12 light/dark cycle; Mukhi et al., 2005a). Fish were fed until satiation either with adult frozen *Artemia* or Tetramin® flakes (Tetra Sales, Blacksburg, VA, USA) twice daily (morning and afternoon). On every evening, leftover food and fecal material were removed by siphoning. Protocols for the use of animals were reviewed and approved by the Texas Tech University Animal Care and Use Committee (Lubbock TX, USA).

Experimental design

Three concentrations (nominal) of RDX were chosen for this study: 0 (control), 1 and 10 ppm. Fifty-five zebrafish were randomly distributed to each of 9 experimental tanks (3 replicates per treatment). A static-renewal exposure method was followed. Treatment solutions were prepared in 9 separate tanks (10-gallon aquaria) situated on a rack above the corresponding exposure tanks. The required amount of test material (RDX
slurry) was poured into each of the overhead tanks at least 24 h before water exchange. These tanks were aerated to create water circulation and help dissolve RDX, and heated to the appropriate temperature before adding to the exposure tanks. Every week, either 50 percent of treatment water twice, or 100 percent of treatment water once, was renewed with fresh treatment water. On the day of water renewal, the appropriate volume of water was removed by siphoning and disposed appropriately, and replaced with appropriate volume of fresh exposure water by gravity flow. For 100 percent water exchanges, fish were simply transferred to new 10-gallon tanks. The volume of water exchange was decided depending upon measurements of water quality such as unionized ammonia, turbidity or algae infestation. For verification of actual RDX exposure concentration, water samples (2-3 ml) were collected from the exposure tanks before and after each water exchange. Feeding behavior (general activity) was observed and recorded qualitatively once weekly during the morning feeding throughout the exposure period. Mortality, if any, was recorded and dead fish were removed and disposed appropriately.

Fish were sampled at 4, 8 and 12 weeks of exposure. Ten fish from each replicate were collected at 4 and 8 weeks of exposure for histological and tissue RDX content analysis. The same numbers of fish were collected at 12 weeks from the control and 1-ppm replicates, but only 3-5 fish per replicate could be sampled at this time for the 9.6-ppm group due to high treatment-related mortality. The latter were used for RDX content analysis. All fish were euthanized with 1 g/L tricaine methane sulfonate solution prior to processing. Body weight and fork-length were recorded for all fish sampled.
RDX elimination

At the completion of the 12-week exposure period, 4-6 fish per tank were rinsed in zebrafish water several times and transferred to new respective tanks. Fish were held in these tanks for a total of 15 days. Water samples were collected during the recovery period to check for the presence of RDX in water. One water exchange was conducted at the end of the first week. After 15 days, fish were euthanized and snap-frozen in liquid nitrogen for RDX analysis.

Chemical analysis in treatment water and stock solution

Water samples were analyzed for actual RDX concentrations by high performance liquid chromatography (HPLC) according to Mukhi et al. (2005b). Our original study design did not include measurement of RDX metabolites in the treatment solution. However, since MNX was detected in the tissue extract (see Results section), we decided to assess the possible contamination of our RDX stock with RDX metabolites. For this purpose, liquid chromatography and mass spectrometry (LC-MS) were used.

RDX and MNX extraction and analysis

A preliminary analysis showed that RDX and MNX were present in whole-body extracts from RDX-exposed fish. Other RDX metabolites such as TNX and DNX were not observed. The procedure used for extraction, cleanup and analysis of RDX and its metabolites in fish tissue was according to Pan et al. (2005) with some modifications. Briefly, 3-5 fish per treatment replicate were pooled and dehydrated by grinding with 8-
10 g of Na₂SO₄ (which also served as dispersing agent). To estimate the percent recovery for RDX and MNX, extracts from untreated fish were spiked with standard solutions. Ground tissue samples were loaded into 22-ml cells and extracted with 20 ml of acetonitrile using a Dionex ASE 200 extractor (Salt Lake, UT, USA). Extracts were evaporated to 1-2 ml using a rotary evaporator and subsequently cleaned using Florisil® cartridges (Supelco, Bellefonte, PA, USA). Extracts were evaporated under nitrogen gas and concentrated to a final volume of 1 ml. The samples were analyzed by gas chromatography with an electron-capture-detector (HP 6890 series GC-ECD Agilent, Palo Alto, CA, USA). Each analysis sequence began with injections of at least three calibration standards that spanned the needed calibration range. The limits of detection of the instrument were 17 and 21 ppb for RDX and MNX, respectively. The presence of RDX and MNX in tissue extracts was confirmed by LC-MS. The extraction recovery efficiencies for RDX and MNX were 97 ± 9 and 88 ± 7 %, respectively; and the sample values obtained were corrected for the estimated recovery.

**Gonad histology**

Gonadal histology was conducted to assess reproductive effects. For this purpose, following euthanasia and an abdominal incision, whole fish were placed in Bouin’s fixative for 48 h and were subsequently processed for histological observations using standard techniques. Seven-micron sections taken within the anterior one-third region of the gonad were stained with hematoxylin and eosin and observed under a compound microscope. Stages of germ cell development present in the ovary or testis were recorded.
for each fish. Specific stages recorded for ovarian follicles were pre-vitellogenic (perinucleolar), vitellogenic, post-ovulatory and atretic. Spermatogonia, spermatocytes, spermatids and sperm were recorded for the testis. In addition, the percent area of testicular cross-sections occupied by sperm was determined using digital images taken with an Olympus digital camera (DP70) attached to a compound microscope and Image-Pro® Express software (Media Cybernetics, Silver Spring, MD, USA). Gonadal preparations were also inspected for the presence of standard histopathological features (Blazer, 2002).

Data analysis

As there was no substantial difference in the RDX concentration (ppm) before and after water exchange in each tank, the average of all measurements taken each week were calculated and reported. For somatic and reproductive condition parameters, individual fish values within each aquarium were averaged to obtain tank values. RDX bioconcentration was measured in pools of 3-5 fish per aquarium. Thus, in all cases, sample size per treatment for statistical analyses is the number of tank replicates (n = 3). The bioconcentration factor for RDX was calculated by dividing the tissue RDX concentration by the weekly average water RDX concentration at 4, 8 and 12 weeks of exposure, respectively.

Unless otherwise noted, the effects of waterborne RDX (concentration and exposure time) on somatic condition and reproductive condition and on RDX bioconcentration were initially assessed by two-way analysis of variance (ANOVA). The
effects of different concentrations of RDX at each exposure period were then analyzed with one-way ANOVA followed by Duncan’s multiple range test (Statistica®, StatSoft, Tulsa, OK, USA). These analyses were performed at the level of significance of $\alpha = 0.05$. Behavioral observations were qualitatively documented and reported.

Because (live) fish were sampled for analysis at the end of each 4-week exposure interval, cumulative mortality could not be calculated. Thus, mortality is expressed as percent dead fish during each 4-week interval with respect to the number of fish present in each replicate at the beginning of each interval.

RESULTS

Exposure media

The average, measured concentrations of RDX in treatment tanks were close to the nominal concentrations: 0, 1 and 9.6 ppm for the target (nominal) concentrations of 0, 1 and 10 ppm, respectively (Figure 5.1). These observations indicate that, under the conditions of the present study, RDX concentrations were stable throughout the exposure period. RDX was not detected in any of the tanks during the recovery period of the study. MNX was not measured in water from the treatment tanks during the exposures, but it was measured in stock solution. The LC-MS analysis indicated that a 10-ppm RDX stock solution contained approximately 10 ppb of MNX.
Effect of RDX on survival and somatic condition

Mortality was observed only at the highest concentration of RDX (9.6 ppm). Most mortality, 33 percent, occurred within first 4 weeks of exposure; and 24 percent occurred during the subsequent 4-week period. The mortality rate in the last 4-week exposure period was only 5 percent (Figure 5.2). There was no mortality in the 1-ppm group and only one death occurred in the control group.

There was no growth in the control fish in the period between 4 and 12 weeks of exposure (one-way ANOVA using data from control fish; p < 0.05). (The weight and length of fish at the start of the experiment was not recorded) Fish length was not affected by RDX at any time during the exposure period (Figure 5.3A; p > 0.05). Body weight was affected at 4 weeks of exposure to RDX at both 1 and 9.6 ppm relative to the control fish (Figure 5.3B; p < 0.05); and at 8 weeks it was affected only at 9.6 ppm (Figure 5.3B). No effects of RDX on body weight were observed at 12 weeks of exposure (Figure 5.3A,B; p > 0.05). Weight and length of the fish sampled at the end of the 2-week period in clean water did not vary among treatment groups (p > 0.05; data not shown).

Behavioral observations were not quantified in this study. However, it was clear that feeding behavior was affected during the first several weeks of exposure to RDX at 9.6 ppm. Fish from this treatment showed relatively lethargic behavior during feeding compared to fish in the control and 1 ppm-treatment. These differences in behavior eventually disappeared towards end of the exposure (8-12 weeks), when fish in the 9.6-ppm aquaria displayed behaviors similar to those of fish from the other groups.
Figure 5.1. Measured RDX concentration in treatment water during the 12-week period of exposure. The values shown are the mean (± SEM) of the weekly average of 2-4 measurements for each tank replicate (n = 3). See text for details of weekly measurements.
Figure 5.2. Mortality of fish exposed to RDX. Treatment-related mortality was observed only in the 9.6-ppm exposure group, and most deaths in this group occurred within the first 8 weeks of exposure. The values shown (mean ± SEM, n = 3) were independently estimated for each 4-week interval (see text).
Figure 5.3. Effects of RDX on fork length (panel A) and weight (panel B) of zebrafish at 4, 8, and 12 weeks of exposure. Each bar represents the mean value (± SEM, n =3). Only those values that were significantly different from the control value are indicated by an asterisk (*, $\alpha < 0.05$). See text for details of statistical analysis.
RDX and MNX bioconcentration and elimination

RDX was present in whole-body extracts of zebrafish exposed to waterborne RDX but not in fish from the control tanks (Figure 5.4A). Although the concentration of RDX in fish was influenced by water RDX concentration and length of exposure (2-way ANOVA; p < 0.05), its BCF was affected only by length of exposure. The combined BCF values (for the 1- and 9.6-ppm treatments) were 1.01 ± 0.13, 0.91 ± 0.06 and 2.23 ± 0.04 at 4, 8 weeks and 12 weeks, respectively (Figure 5.4B). RDX was not detected in whole-body extracts of zebrafish after the 15-day recovery period (data not shown).

MNX was also detected in tissue extracts from RDX-exposed fish but not from control fish. The whole-body concentration of MNX was influenced by the concentration of RDX in water (Figure 5.4C). The BCF of MNX could not be determined since MNX concentrations in tank water were not measured.
Figure 5.4. RDX bioaccumulation (panel A) and bioconcentration factor (BCF) (panel B), and MNX bioaccumulation (panel C) in whole-body of zebrafish at 4, 8 and 12 weeks of exposure. Each bar represents the mean value (± SEM, n=3). Only those values that were significantly different from the control value are indicated by an asterisk (*, α < 0.05). RDX and MNX were not detected (ND) in control fish. See text for details of statistical analysis.
Effect of RDX on gonadal histology

Male and female fish examined at the completion of the 4, 8 weeks of exposure (in all groups) and at 12 weeks (in control and 1-ppm group) contained germ cells at all stages of development (fish from the 9.6-ppm group were unavailable for histology at 12 weeks). Notably, post-ovulatory follicles were observed in several females from all treatment groups at 4 and 8 weeks of exposure and in control and 1-ppm treatment group at 12 weeks. Also, the percent testicular section area occupied by sperm did not vary among treatments at 4, 8 and 12 weeks period. No gross histopathological features were noted in any of the preparations examined.

DISCUSSION

Exposure of young adult zebrafish to a measured concentration of RDX of 9.6 ppm caused significant mortality within the first 8 weeks of exposure, but no mortality was observed at exposure concentrations of 0 or 1 ppm. Similar observations were previously reported for fathead minnow. For example, Burton et al. (1994) observed significant mortality after a 4-week exposure of fathead minnow juveniles to RDX at a concentration of 9.83 ppm but not at 1.35 ppm. In addition, Bentley et al. (1997) reported that survival of fathead minnow juveniles was not affected at RDX concentrations below 5.8 ppm following a 30-day exposure. In a recent study with juvenile channel catfish, Belden et al. (2005) reported no incidence of mortality after a 7-day exposure to RDX at 2 ppm. In the present study with zebrafish, the mortality rate observed at an RDX exposure concentration of 9.6 ppm decreased with length of exposure and became
relatively small after 8 weeks. The high incidence of mortality after exposure of adult zebrafish to RDX at 9.6 ppm was unexpected because this concentration is below the 96-h NOEC for lethality (13-15 ppm) in larval zebrafish (Mukhi et al., 2005b). It appears, therefore, that the results of acute toxicity testing with larval zebrafish (Mukhi et al., 2005b) are of little value to predict the chronic effects of RDX on the survival of adult individuals (present study).

Histological observations of the gonads did not indicate the incidence of gross effects of RDX on gonadal condition during the 12-week period of exposure. Namely, the estimated sperm content of the testes did not differ among fish from the various treatments; and several of the ovaries inspected, irrespective of treatment, contained postovulatory follicles thus indicating the incidence of spawning. Behavioral observations taken once weekly at feeding time (morning) suggested a reduction in spawning activity at an RDX concentration of 9.6 ppm (males chasing females; data not shown), but these observations were not quantified. Follow-up functional studies (e.g., reproductive output and gamete quality) will be necessary to adequately assess the chronic effects of RDX on zebrafish reproduction.

Qualitative observations of feeding behavior indicated that fish exposed to RDX at 9.6 ppm were less aggressive at seeking food than fish from the other treatments, but these effects lasted only until about 8 weeks after the initiation of the exposure. Although the present study was not designed to measure actual feeding rates, it is reasonable to suggest that the abnormal feeding behavior observed was associated with suppressed feeding rates. In fact, abnormal behavior and decreased feed consumption rates has been
previously reported during exposure to RDX in northern bobwhite (Gogal et al., 2003), salamander (Johnson et al., 2004), and rat (Cholakis et al., 1980). In addition, exposure to RDX at 9.6 ppm in the present study also suppressed body weight at 4 and 8 weeks but not at 12 weeks of exposure, when the effects on feeding behavior had also disappeared. Thus, the suppression in body weight may have been at least partly due to a parallel suppression in food consumption induced by RDX at 9.6 ppm. Significant weight loss has been previously reported for a number of vertebrate species exposed to RDX (Brown, 1975; Cholakis et al., 1980; MacPhail et al., 1985; Johnson et al., 2004), including the fathead minnow (Burton et al., 1994). Curiously, RDX at 1 ppm also affected body weight in the present study with zebrafish at 4 weeks of exposure without an apparent effect on feeding behavior. This observation suggests that exposure to RDX at 1 ppm has subtle somatic effects that are not related to gross behavioral alterations.

The BCF for RDX was influenced by time of exposure but not by water RDX concentration. The combined BCF values (for the 1- and 9.6- ppm treatments) increased from ≤1 at 4 and 8 weeks to >2 at 12 weeks. The low BCF values for RDX in zebrafish are consistent with its low octanol-water partitioning coefficient (log $K_{ow} = 0.9$; Monteil-Rivera et al., 2004). Short-term measurements (< 7 days) of RDX bioconcentration were recently conducted in channel catfish (Belden et al., 2005) and sheepshead minnow (Lotufo and Lydy 2005) with BCF values reported at 2 and 1.7, respectively. Interestingly, the BCF for RDX in zebrafish remained close to one within the first 8 weeks of exposure, but this value doubled by 12 weeks (present study). The reason for this time-dependent change in BCF is unknown.
Whole-body extracts of zebrafish exposed to RDX had detectable concentrations of MNX. The source of fish tissue MNX cannot be clearly established because our RDX stock contained MNX at a 1000-fold lower relative concentration. However, to explain the level of tissue MNX observed in the present study as the result of stock contamination, one would have to assume a BCF value for MNX of about 30 based on the relative concentrations of the two compounds in the stock solution (an unlikely possibility). Other possibilities include microbial or fish tissue biotransformation of RDX into MNX. Biotransformation of RDX into MNX has been reported by plant tissue (Van Aken et al., 2004) and bacteria (Clostridium bifermentans; Zhao et al., 2003) but, to our knowledge, not by animal tissues.

In conclusion, chronic exposure to RDX is lethally toxic to adult zebrafish at concentrations that are below the acute larval NOEC. Although no evidence of gross impacts on gonadal condition was noted at any of the RDX concentrations tested, functional studies are needed to properly address the potential reproductive effects of RDX. The bioconcentration factor for RDX in zebrafish is low but it increased slightly with time of exposure.

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CHAPTER - 6

REPRODUCTIVE EFFECTS OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE IN ZEBRAFISH

ABSTRACT

The objective of this study was to examine the effect of RDX on the reproductive performance of zebrafish. Adult males and females were exposed to control water or two measured concentrations of RDX, 0.5 and 3.2 ppm, for a period of 6 weeks. Male and female fish were exposed separately. Aquaria containing 8 females each were the unit of replication, and there were 3 replicates per treatment. Every two weeks after the onset of exposure, the 8 females in each tank were paired with 4 similarly-treated males to induce spawning. Spawn (packed-egg) volume, egg fertilization rate and embryo hatching rate were determined. Mean PEV was increased relative to the control value after exposure to the low concentration of RDX (0.5 ppm) at 2 weeks, but not at 4 or 6 weeks. This observation suggests the occurrence of a temporary stimulatory response to RDX at low concentrations. No significant effects of RDX on PEV were noted in fish exposed to RDX at 3.2 ppm. Egg fertilization and embryo hatching rates were not affected by RDX at any concentration during the exposure period. Overall, the results of the present study with zebrafish do not suggest the occurrence of deleterious effects on reproductive performance or egg quality of exposure to RDX at sublethal concentrations. However, this study did not include measurements of F1 larval or juvenile health.
INTRODUCTION

Environmental contamination with military explosive compounds and their byproducts has been reported in several parts of the world. These explosives or their metabolic products are considered toxic and could act as ecological stressors if released into the environment in significant amounts. One such explosive compound is hexahydro-1,3,5-trinitro-1,3,5-triazine, a cyclonitramine commonly known as RDX (Royal Demolition Explosive). RDX often ends up in soil, sediment, ground and surface waters through its use as an explosive, wastewater release from production facilities, and disposal of antiquated munitions. In the United States, environmental contamination with RDX has been reported in 16 Superfund sites across the nation (ASTDR, 1995).

Cyclonitramines are generally considered toxic to living organisms (Talmage et al., 1999), and thus the presence of RDX in the natural environment at high concentrations could be harmful to the health of aquatic ecosystems. Toxic effects of RDX have been documented in various laboratory animals including mammals (Hart, 1977; Cholakis et al., 1980; Levine et al., 1981a,b; Levine et al., 1983), birds, (Gogal et al., 2003) and fishes (Bentley et al., 1977, Burton et al., 1994, Belden et al., 2005, Lotufo and Lydy, 2005, Mukhi et al., 2005b). We have recently characterized lethal and sublethal effects of RDX in zebrafish. In an acute toxicity study with larval zebrafish, the 96-h LC50 for RDX was found to be 23 ppm (Mukhi et al., 2005b). In a chronic exposure study, RDX at 9.6 ppm caused high mortality rates, but an examination of the gonads of surviving fish did not suggest the occurrence of reproductive effects (Mukhi and Patiño,
in preparation). However, the latter study suggested that functional (performance) studies are necessary to understand the effects of RDX on zebrafish reproduction.

The impact of environmental contaminants on reproduction is of concern because disturbances in the ability to reproduce could affect the long-term viability of animal populations. Information about the toxicity of RDX on reproduction is limited for vertebrates and no patterns of effects have yet emerged. For example, dietary exposure to RDX (10.6 mg/kg/day) reduced egg production in northern bobwhite (Colinus virginianus) (Gogal et al., 2003). Conversely, in a two-generation study with rats (Rattus norvegicus), RDX did not show any adverse toxic effects on reproduction at concentrations in the diet as high as 50 mg/kg/day (Cholakis, 1980). To our knowledge, there are no studies available that address the effects of RDX exposure on the reproductive performance of fishes. Therefore, the objective of this study was to determine the effects of RDX on the reproductive output of a model aquatic organism, the zebrafish.

The nominal concentrations of RDX used in this study (0-5 ppm) are within the range of concentrations previously reported in contaminated aquatic habitats (e.g., Ryon et al., 1984; Small and Rosenblatt, 1974). The highest nominal concentration (5 ppm) was chosen to limit mortality of exposed fish which, in a previous study, occurred at a measured concentration of 9.6 ppm (Mukhi and Patiño, in preparation). The interest of this study is the reproductive effect of RDX at sublethal concentrations.
MATERIALS AND METHODS

Chemicals, safety procedures, and chemical analysis

Hexahydro-1,3,5-trinitro-1,3,5-triazine (CAS Reg. No. 121-82-4) was obtained from Accurate Energetics (McEwen, TN, USA). The chemical was 99% pure and supplied in desensitized form, containing 20% water by volume. Standard solutions for RDX were obtained from Supelco (Bellefonte, PA, USA). RDX was stored in a specially designed bunker on campus and handled with care. RDX was transported from the bunker to the study facility in the desensitized form. Water samples from the treatment tanks were analyzed for actual RDX concentrations by high performance liquid chromatography (Mukhi et al., 2005a).

Experimental animal and standard rearing conditions

Four-month-old, adult wildtype zebrafish (*Danio rerio*) were obtained from a local vendor (Lubbock, TX, USA) and allowed to acclimatize to laboratory conditions for a period of 3 weeks before the spawning trials began. Male and female zebrafish were reared separately. Eight females were randomly distributed into each of nine 10-gallon aquaria, and 12 males into each of three 10-gallon aquaria. Animal husbandry procedures for this study were as described by Mukhi et al. (2005a). Briefly, each aquarium was filled with 30 L of zebrafish water (25 g of R/O Right®/100 L of reverse-osmosis water) and fitted with two hand-made, internal glass biofilters driven by air flow. Stress Zyme® (Aquarium Pharmaceuticals, Pittsburgh, PA, USA) was added at weekly intervals to maintain adequate levels of denitrifying bacteria in the biofilters. Water quality
parameters were maintained at recommended levels for zebrafish (pH 6.5-8.0, 26-28.5 °C, 12h/12h light/dark cycle). If water pH in the aquaria fell below 6.5, the appropriate volume of a 5 M NaOH solution was added bring it back within the optimal range. Fish were fed adult frozen *Artemia* and Tetramin® flakes (Tetra Sales, Blacksburg, VA, USA) twice daily to satiation. Every evening, leftover food and fecal material were removed by siphoning. Temperature and pH were measured daily and dissolved oxygen, specific conductivity, salinity, unionized ammonia and nitrate were measured at least once weekly. Fifty percent of the water volume (15 L) was replaced with fresh zebrafish water twice weekly. Due to anticipated difficulty in identifying the males from the females after spawning, males were tagged with Visible Implant Elastomer at the base of the dorsal fin according to the manufacturer’s protocol (Northwest Marine Technology, Shaw Island, WA, USA). Males were allowed to recover for at least 2 weeks after tagging before the start of spawning. The experimental animal protocols for this study were reviewed and approved by the Texas Tech University Animal Care and Use Committee (Lubbock, TX, USA).

**RDX exposure**

Two nominal concentrations of RDX (0.5 and 5 ppm) were used in addition to a control treatment. Because the purpose of this study was to examine the sublethal effects of RDX on reproduction, the higher nominal concentration (5 ppm) was deliberately chosen to be below the concentration that in a previous study caused significant mortality in adult zebrafish (9.6 ppm; Mukhi and Patiño, in preparation).
Glass aquaria housing the female fish were considered the unit of replication for this study, and each treatment was conducted in triplicate for females (8 fish per tank). Males for each treatment were maintained together in a single tank (12 fish per tank). Therefore, this study included a total of 9 female tanks and 3 male tanks. A static renewal exposure to RDX procedure was followed as described earlier (Mukhi and Patiño, in preparation). Briefly, 50 percent of tank water was replaced twice weekly with preheated-aerated-fresh treatment water. Because RDX values under the present aquarium conditions and experimental protocols are stable (Mukhi and Patiño, in preparation), water samples (2-3 ml) for verification of actual RDX concentrations were collected from each tank only once a week before the second water exchange. Fish feeding and swimming behaviors were qualitatively observed daily at the time of feeding, and any sign of abnormal behavior was recorded.

**Spawning procedures**

The spawning procedure used was described by Patiño et al. (2003). Briefly, spawning chambers were made of plastic shoe boxes (30 cm long X 15 cm wide X 5 cm deep) coated with silicon, and consisted of an upper chamber for holding the fish and a lower chamber for collecting the eggs. Holes were drilled on the side walls of the upper chamber to allow air exchange to occur, and its bottom was replaced with a silicon-coated plastic mesh to allow the eggs to fall into the lower chamber. The day before spawning, fish were fed only the morning meal and a single spawning container was placed in each female fish tank. The 8 females in each aquarium were combined with
four males from the same RDX-treatment tank in the spawning chamber. The group weight of all female fish in each replicate was taken by placing them in a pre-zeroed, 1-L beaker containing zebrafish water just prior to their transfer to the spawning chambers. The following morning, fish were removed from the spawning chambers and returned to their tanks approximately 2.5 h after lights-on (artificial dawn). Fecal matter and other debris were removed from the egg slurry using pipettes and rinsing 4 times with fresh zebrafish water. Packed-egg volume (PEV) was determined by volume displacement in a graduated 5-ml glass cylinder.

The first spawning trial was conducted one week before the initiation of RDX treatment. The purpose of this trial was to confirm spawning readiness and to synchronize reproductive cycles; only fish weight data obtained during the procedure for this spawning was included in the analysis. The fish were then spawned once every 2 weeks after the initiation of RDX exposure. Therefore the PEV measured at 2, 4, 6 weeks after initiation of exposure. One female was lost from one of the (nominal) 5 ppm-treatment tanks following the first spawning; therefore, this replicate was subsequently eliminated from the study.

Rates of fertilization and hatching were also calculated. For this purpose, approximately 100 eggs from each treatment replicate were incubated in 500-ml beakers containing 300 ml of zebrafish water at 28 °C. Six to 8 h after artificial dawn, unfertilized eggs became white (opaque) and were removed from the incubation beaker and counted. The fertilized, translucent eggs were also counted and kept in the same beaker until hatching. Fifty percent of the water in the beakers was replaced with preheated (28 °C),
fresh zebrafish water daily. Three days after spawning, the number of unhatched eggs was determined in each beaker. Percent hatching was calculated from the total fertilized eggs present in the beaker.

Data analysis

The weight of female fish was measured as group-weight (8 fish), and PEV and fertilization and hatching rates were measured in each tank replicate. Thus, in all cases, sample size per treatment for statistical analyses was the number of tank replicates (n = 3). Fertilization rate and hatching rate were subjected to arcsine transformation before analysis. Unless otherwise noted, the effects of waterborne RDX on fish weight, spawn volume, fertilization rate and hatching rate were initially assessed by two-way analysis of variance (two-way ANOVA, concentration X exposure time). If significant factor or interaction effects were found, treatment effects at each exposure time were examined by one-way ANOVA followed by Duncan’s multiple range tests (Statistica®, StatSoft, Tulsa, OK, USA). These analyses were performed at the level of significance of $\alpha = 0.05$. Behavioral observations were qualitatively documented and assessed.

RESULTS

Measured RDX concentration in experimental tanks

The average measured concentrations of RDX in the experimental tanks were $0 \pm 0$, $0.5 \pm 0.1$ and $3.2 \pm 0.2$ ppm in control, 0.5 and 5 ppm nominal concentration groups, respectively.
General effects of RDX

Two-way ANOVA (treatment X length of exposure) indicated that only treatment had an effect on the weight of females (p<0.05); whereas length of exposure and treatment X length of exposure had no significant effects. Mean weight of females did not differ one week prior to RDX exposure (p>0.05) and RDX did not affect weight at 2 and 6 weeks after the onset of exposure (1-way ANOVA, p). However, mean weights differed at 4 weeks of exposure (p<0.05, 1-way ANOVA), at which time the weight of females in the 0.5-ppm treatment group was significantly higher (p<0.05) than the 3.2-ppm group (Figure 6.1). A trend for female weight to be also higher than in control fish at 2 weeks of exposure to RDX at 0.5 ppm was not statistically significant.

Fish aggregation behavior (feeding activity) was recorded qualitatively at the time of feeding every day during the period of exposure. In the control and 0.5 ppm-treatment groups, feeding activity did not seem to change at any point during the exposure. However, feeding activity was reduced within one day of exposure in females from the 3.2 ppm-treatment group, and by the end of the first week of exposure in the males. However, this effect on feeding behavior was temporary and only lasted until the end of the second week of exposure.

Effect of RDX on packed-egg volume

Two-way ANOVA (treatment X length of exposure) indicated that treatment (p<0.05) but not length of exposure or the interaction between the treatment and length of exposure (p>0.05), had significant effects on PEV. One-way ANOVA for treatments at
each spawning event indicated that the treatment effect was significant ($p<0.05$) only at 2 weeks of exposure. At this spawning event, RDX at 0.5 ppm stimulated an increased in PEV ($p<0.05$, Duncan’s multiple range test) compared to the control and 3.2-ppm exposure groups (Figure 6.2). Packed-egg volume in the 0.5-ppm treatment group subsequently decreased from its high value at 2 weeks, although at 4 weeks it was still numerically (but not statistically) higher than control values. Packed-egg volume seemed to decrease in the 3.2-ppm group relative to controls, but this decrease was not statistically significant.

The cumulative PEV was also analyzed. For this purpose, all 3 spawn volumes for each treatment replicate were added during the 6-week exposure. Results of one-way ANOVA indicated that treatment had an effect on the cumulative PEV ($p<0.05$); RDX at 0.5 ppm caused a higher cumulative egg volume relative to RDX at 3.2 ppm, with a trend to be also higher than control values (Figure 6.3). This analysis yielded results generally consistent with those of the biweekly analysis (Figure 6.2); namely, an overall increased in spawn volume at an RDX concentration of 0.5 ppm was confirmed. However, only the biweekly spawn analysis is able to establish treatment differences at specific times of exposure. Short-lasting or temporary effects (e.g., those of 0.5-ppm RDX at 2 weeks) can be obscured in the cumulative spawn volume analysis.
Figure 6.1. Effect of RDX on the wet-weight of females. Treatment but not length of exposure had an effect on the weight of the females (2-way ANOVA). At 4 weeks of exposure, the weight of females in 0.5 ppm treatment group was significantly higher than in the 3.2 ppm-treatment group (p<0.05)
Figure 6.2. Effect of RDX on biweekly packed-egg volume. RDX stimulated packed-egg volume in the 0.5 ppm treatment group at 2 weeks of exposure (1-way ANOVA and Duncan’s multiple range test; p<0.05). Although RDX reduced the egg production (almost by half) in the 3.2 ppm-group compared to the control, the effect was not significant (p>0.05). The stimulatory effect of RDX at 3.5 ppm was no longer statistically significant at 4 weeks of exposure and was numerically similar to control values by 6 weeks. For any given week, bars associated with common letters are not significantly different.
Figure 6.3. Effect of RDX on cumulative packed-egg volume. Cumulative packed-egg volume in the 0.5-ppm treatment was significantly higher than in the 3.2-ppm group (1-way ANOVA and Duncan’s multiple range test; p<0.05). The control value was intermediate between the 0.5- and 3.2-ppm values. For any given week, bars associated with common letters are not significantly different.
Figure 6.4. Effect of RDX on fertilization and hatching rates. Parental exposure to RDX had no significant effect on the fertilization rate and hatching rate of the embryos, although a slight increase in fertilization rate occurred through time (not shown on graph) (2-way ANOVA; p>0.05).
Effect of RDX on egg fertilization and embryo hatching rate

Values for percent fertilization and percent hatching were subjected to an arcsine transformation to achieve homogeneity of variances. Two-way ANOVA (treatment X length of exposure) showed no treatment or interaction effects (p>0.05) either on fertilization rate or hatching rate, but length of exposure had an effect on fertilization rate with a slight time-dependent increase being apparent in all treatment groups (p<0.05, Figure 6.4).

DISCUSSION

Information on the effect of RDX on reproductive performance is limited to few species. Among vertebrates, dietary exposure to RDX (10.6 mg/kg/day) inhibited food intake, reduced weight gain, and suppressed egg production in northern bobwhite (Gogal et al., 2003). However, RDX showed no adverse reproductive effects in rats exposed to RDX in the diet at daily doses of 50 mg of RDX/kg (Cholakis, 1980). In the present study, no deleterious effects of RDX were observed on egg production (spawn volume) in zebrafish at exposure concentrations of 0.5 or 3.2 ppm. On the contrary, biweekly analysis of packed egg volume showed that RDX at 0.5 ppm treatment significantly increased at 2 weeks of exposure, with a trend for higher volume also seen at 4 weeks. This observation suggests that RDX at 0.5 ppm had a stimulatory effect on egg production during the early period of the exposure. This stimulatory effect on packed-spawn volume was short lived and was not observed at the higher concentration of RDX tested. An analysis of the cumulative PEV produced during the exposure period also
indicated an overall increase in egg production at an RDX concentration of 0.5 ppm, but this analytical approach was not as sensitive as the biweekly analysis to detect short-lived effects. The body weight of female fish exposed to RDX at 0.5 ppm also showed a similar pattern for higher values at 2 to 4 weeks of exposure, with the values being significantly different from those of control fish at 4 weeks. This pattern of RDX effects on pre-spawn female body weight and its similarity with the pattern of effects on (biweekly) PEV are consistent with the conclusion that RDX at 0.5 ppm caused an increase in egg production. The biological relevance of the temporary increase in egg production after exposure to RDX at 0.5 ppm is uncertain at the present time. Stimulatory effects of certain contaminants at low concentrations on the reproductive performance of fishes have been previously reported; this phenomenon has been termed “hormesis” (Calabrese and Blain, 2005). However, the results of the present study are insufficient to determine if the effect of RDX at 0.5 ppm reflects a hormetic response.

Maternal exposure to RDX did not affect egg fertilization and embryo hatching rates. These observations suggest that RDX at water concentrations that do not cause significant mortality of exposed parental fish (up to 3.2 ppm) also does not cause deleterious effects on F1 egg quality. We are unaware of any similar information available for other vertebrate species with respect to RDX exposure. In deer mice (*Peromyscus maniculatus*), exposure to the RDX metabolite, hexahydro-1,3,5-trinitroso-1,3,5-triazine (HMX), caused postpartum mortality and decreased weight from birth to weaning (Smith et al., 2006).

Overall, the results of the present study do not suggest the occurrence of
deleterious effects of sublethal concentrations of RDX (up to 3.2 ppm) on zebrafish reproductive performance. However, the results of this study can not rule out the possibility of impairments in F1 larval or juvenile health caused by parental exposures to RDX.

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