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The effects of environmentally relevant doses of perchlorate (ClO(4)(-)) on Rana pipiens metamorphosis and development

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THE EFFECTS OF ENVIRONMENTALLY RELEVANT DOSES OF PERCHLORATE (ClO_4^-) ON *RANA PIPIENS* METAMORPHOSIS AND DEVELOPMENT

by

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ABSTRACT

The Effects of Environmentally Relevant Doses of Perchlorate (ClO_4^-) on *Rana pipiens* Metamorphosis and Development

by

Andrea N. Golli

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The purpose of this study was to assess the effects of environmentally relevant concentrations of perchlorate on the development and growth *of Rana pipiens*. Ammonium perchlorate is a rocket fuel oxidizer that is known to interfere with the function of the thyroid gland and some of the highest United States surface water concentration exists in the Las Vegas Wash, NV. Perchlorate not only blocks the body's ability to incorporate iodine into T_3 and T_4 hormones through the sodium iodine symporter system, but also depletes the thyroid glands' internal stores of iodine. Many of the steps that regulate metamorphosis in amphibians are also triggered by thyroid hormones therefore we investigated the ability of perchlorate to interfere with the development and metamorphosis of *Rana pipiens*. Aquaria containing concentrations of 40 ppb, 400 ppb, and 4000 ppb perchlorate with a 0 ppb perchlorate control as well as a magnesium control (since magnesium perchlorate was used) to evaluate the growth and development of *Rana pipiens*. Measuring snout to vent length, hindlimb length, tail

length and total body length weekly on 40 animals per tank allowed the growth of the tadpoles to be monitored throughout the study. Development of the tadpoles was determined by staging 20 animals per dose group according to the Taylor-Kollros Index. Metabolic rate for each dose group was assessed monthly by determining oxygen consumption; while deformities, as well as mortality, were tracked daily. The magnesium control completed metamorphosis during week 10, followed by the 40 ppb and 4000 ppb groups in week 21 with the control and 400 ppb group completing development during week 33. The magnesium control was larger (tail, snout to vent, total body, hindlimb) than all of the other groups during weeks 2-11. The weight adjusted oxygen consumption provided similar results with the magnesium and control groups consuming more oxygen than the other groups, while the 400 ppb group consistently consumed the least oxygen. Experimental problems significantly reduced the number of animals in 400 ppb, magnesium control and 4000 ppb groups thereby causing resource allocation problems. The magnesium control group developed faster than all other groups, which could be due to magnesium's up-regulation during glycolysis. The increased energy production could be accelerating metamorphosis, and hence attenuating perchlorate's inhibitory mechanism. Oxygen consumption appears to be a more sensitive biomarker, and is useful for detecting alterations prior to seeing adverse effects on growth and development. The concentrations of perchlorate used in this study did not delay development or metamorphosis in *Rana pipiens*.

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In the Las Vegas Wash Water Experiment, Lindy Horn and Shelly Maloney contributed to this research by hauling numerous buckets of water with a smile. They were the most dependable research assistants and always willing to help wherever they were needed. Once a second experiment was deemed necessary, there was overwhelming support from Sherri Powell, Jayson Brangan, Beth Domowicz, and Steve Oliveira. They completed everything from tadpole measuring to data entry. Sherri has taught me that life is to short to not go after your dreams, whatever they may be. Steve Weber and Jessica Larkin reviewed this manuscript in its entirety and provided valuable suggestions and comments.

There is no greater feeling than the joy of completing a research project, but I am also taking away invaluable research experience and friends that will last a lifetime.

CHAPTER 1

INTRODUCTION

The greater Las Vegas metropolitan area is a rapidly growing urban center in the southwestern United States that serves as home to approximately 1.2 million permanent residents (U.S. Census, 2000). The cities of Las Vegas, North Las Vegas and Henderson constitute the majority of the population in the Las Vegas Valley, which is surrounded by Mt. Charleston, Black Mountain, Sunrise Mountain and the Spring Mountains. The elevation of the basin ranges from approximately 3,000 feet at the base of the Spring Mountain range in the west to 1,500 feet in the east at the outflow of the valley (Las Vegas Wash Coordination Committee, 2003). The Las Vegas Valley is a 1600 square mile area in the basin and range province of the northern Mojave Desert, which is drained by the Las Vegas Wash (hereafter referred to as "the Wash") directly into Lake Mead (Las Vegas Wash Coordination Committee, 2003).

Several industrial facilities exist along the Wash and have released or spilled environmental contaminants into this area. Of particular concern is the release of ammonium perchlorate (NH_4ClO_4) (AP), an oxidizer used in the production of rocket fuel. This has resulted in concentrations of perchlorate (ClO_4^-) in the Wash water that far exceed the 18 parts per billion (ppb) coauthored recommended by the USEPA as a maximum safe threshold limit (USEPA, 2002).

Purpose of the Study

Perchlorate, in the mammalian system, is known to inhibit the uptake of iodide by the thyroid gland and to deplete the internal stores of thyroidal iodide (Wolff, 1998). This mechanism of action has been related to measurable adverse effects such as hypothyroidism, hypertrophy, and hyperplasia of thyroid follicular cells (Clark, 2000).

In amphibians, large doses of perchlorate salts ($KClO_4$, $Mg(ClO_4)_2$, $NaClO_4$) are known to block metamorphosis (Goleman, Urquidi, Anderson, Smith, Kendall, & Carr, 2002a). Many of the steps that regulate climax stages of metamorphosis in amphibians are controlled by the presence of the appropriate level of thyroid hormones at crucial times during development (Shi, 2000). Thus, high concentrations of these chemicals could alter normal development and metamorphosis. However, the effects of environmentally relevant concentrations of perchlorate on amphibian development in surface waters have not been accurately assessed. The purpose of this study was to evaluate environmentally relevant concentrations of perchlorate on normal development and metamorphosis of the Northern Leopard Frog (*Rana pipiens*).

Perchlorate in the southwestern United States may be playing a role in altering native amphibian development and subsequently altering amphibian populations. This research provides much needed insight on the developmental effect perchlorate may have on tadpoles.

CHAPTER 2

LITERATURE REVIEW

Ammonium perchlorate (AP) is manufactured as a solid propellant oxidizer and is therefore used in the production of rockets, missiles, and fireworks. Perchlorate salts are used as constituents of automotive airbag inflators, nuclear reactors and electronic tubes, additives in lubricating oils, electroplating and aluminum refining, in tanning and finishing leather, in analytical chemistry, and in the production of paints and enamels (Fisher, Todd, Mattie, Godfrey, Narayana & Yu, 2000). Perchlorates are also used as etching and engraving agents, as oxygen-generating devices for life-support systems in submarines, spaceships, bomb shelters and breathing apparatuses and in the manufacture of various esters (Burg, 1995). Of the perchlorate manufactured, 92% is used as an oxidizer and 7% is used as an explosive and the 1% is used in the other previously mentioned applicaitons (USEPA, 2002).

Large scale production and disposal of perchlorate salts began in the mid-1940s in the United States (USEPA, 2002), with production in the 1980s estimated at 20 to 30 million pounds per year. The United States Environmental Protection Agency (USEPA) has identified 40 states with perchlorate manufacturers or users; only Montana, South Dakota, Kentucky, Delaware, Connecticut, Rhode Island, New Hampshire, Vermont and Maine do not manufacture or use perchlorate (USEPA, 2000).

Ammonium perchlorate must periodically be replaced in rocket and missile supplies owing to its short shelf-life. The propellant containing AP is removed from rocket motors via high-pressure water washout, resulting in large amounts of waste aqueous solution containing AP (Wallace, Breen & Attaway, 1996).

In 1997, perchlorate–contaminated water supplies were found in the western United States in Nevada, Utah and California (Leising & Mace, 2001). These states are predominately affected owing to a large amount of perchlorate salts that were manufactured or used in the western United States (Gullick, Leachevallier & Barhorst, 2001).

Facilities in Henderson, Nevada, manufactured AP for the Department of Defense and the National Aeronautical and Space Administration from the early 1950s until the late 1990s (Leising & Mace, 2001). The perchlorate release that led to contaminated water occurred from perchlorate manufacturing by Kerr-McGee and Pepcon at the BMI (Black Mountain Industrial) Complex in Henderson (USEPA, 2000). Monitoring wells have identified a maximum groundwater concentration of 3,700,000 ppb, and a maximum surface water concentration of 120,000 ppb at Kerr-McGee (USEPA, 2000). At least one large plume from the BMI facilities drying beds has been characterized as leaking into the Wash. Monitoring programs within the Wash have suggested that in addition to the large plume entering east of Pabco Road, unconsolidated fine to coarse sediments of the Muddy Creek formation which comprise the bed of the Wash may act as a significant perchlorate reservoir and a subsurface conduit (Leising & Mace, 2001).

The Wash flow is defined as being unconfined to semiconfined (Leising & Mace, 2001). The flow is classified as unconfined if the water table exists under atmospheric pressure, as defined by levels in shallow wells (Bedient, Rifai, Newell,1997). An aquifer is classified as confined if it is overlain by a relatively impermeable unit such that the aquifer is under pressure and the pressure level rises above the confined unit (Bedient et al., 1997). The groundwater lies within alluvial deposits from the Quaternary period, which consists of silt, sand and pebble gravel that were deposited unconformably (Bales, 1987 and Leising & Mace, 2001). The lower Wash then cuts into an early to middle-Pleistocene gravel fill inset into the Tertiary Muddy Creek Formation (Glancy, 1986). Longwell, Pampeyan & Roberts (1965) defined this low-permeability layer to be composed of gypsiferous poorly sorted, fine-grained silty sandstone, siltstone and clay. Along much of the Wash, calcareous and gysiferous fine sandstone, siltstone and claystone of the Tertiary Thumb Formation are exposed (Leising & Mace, 2001). Limestone and siltstone of Horse Spring Formation also of Tertiary age overlie the Thumb Formation (Leising & Mace, 2001). Bell & Smith (1980) found NW and N trending normal faults in the vicinity of Three Kids Wash with a seep containing approximately 450 ppb perchlorate (Leising & Mace, 2001) is present within this fault zone.

This hydrogeology of the Las Vegas Wash watershed greatly impacts the movement of perchlorate from the BMI complex to Lake Mead. The unconsolidated gravel, sand and silt that unconformably overlie Quaternary and Tertiary deposits (Wash unit) along the entire Wash form a highly permeable layer. The surface water of the Wash and the

groundwater within the sediment exhibit dissimilar electrical conductivity and major ion composition (Leising & Mace, 2001). The Southern Nevada Water Authority (SNWA) found that groundwater and surface water contain significantly different perchlorate concentrations, presumably this seepage will continue until homeostasis or equilibrium is reached.

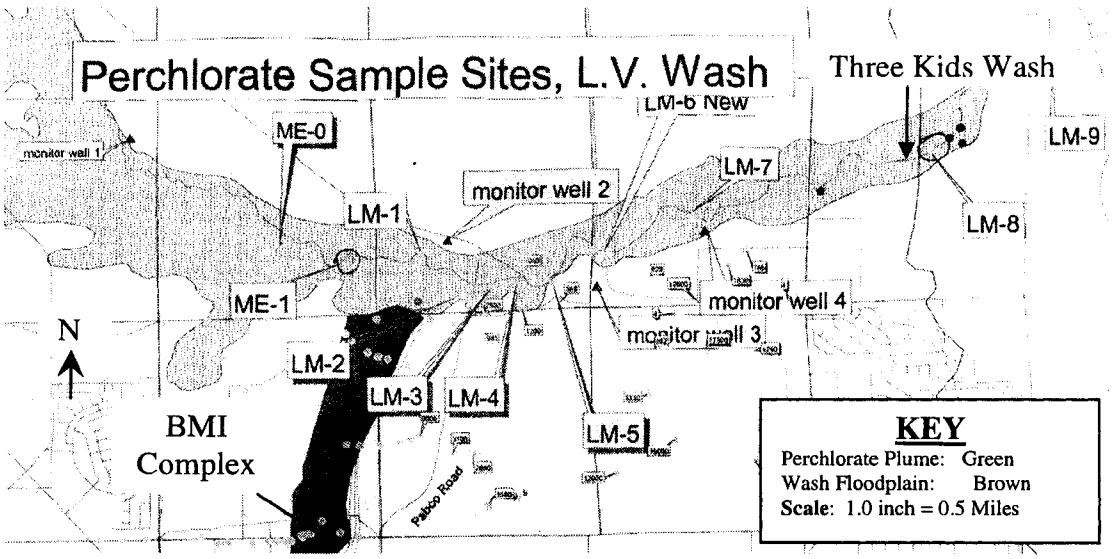


Figure 1 SNWA Perchlorate Sampling Sites

Adapted from Leising and Mace, 2001

More than one-half of the perchlorate entering the Wash can be attributed to the plume entering just east of Pabco Road (See Fig. 1) (Leising &Mace, 2001). Several smaller seeps along the Wash have been identified by SNWA, which account for the remaining perchlorate load that enters the Wash and subsequently Lake Mead. These seeps are within the unconsolidated Wash units, within gravel lenses of the Thumb Formation, and in Quaternary alluvial deposits form the banks of the Wash (Leising & Mace, 2001). These alluvial seeps emerge when the base elevation of the Wash drops

beneath the groundwater table, and cease once a new perchlorate equilibrium is reached with the surface water.

Below Pabco Road there are no significant surface or alluvial perchlorate sources. The SNWA believes that perchlorate enters the surface water from within the unconsolidated sediments that underlie the Wash (Leising & Mace, 2001). The following table lists the perchlorate concentrations observed in the Wash subsurface water from sampling wells or construction necessitated partial dewatering of the unconsolidated Wash units. (See Fig. 1 for locations).

Table 1 Subsurface Water Perchlorate Concentrations

Location	Concentration Range (ppb)
Source Slough	80,000-12,000
Pabco Road Dewatering	12,500-10,000
LM-6 Dewatering	3,500-1,600
Well LG-010 (near LM-8)	2,200-950
LM-8 Seeps	3,500-400

Adapted from Leising & Mace, 2001

These subsurface perchlorate seeps mix with the surface Wash water and lead to very scattered perchlorate concentrations along the Wash. The Nevada Department of Environmental Protection (NDEP) sampling indicates an average perchlorate concentration in the Wash to be approximately 1,000 ppb (Leising & Mace, 2001). The surface perchlorate concentration increases can be attributed to the water immediately beneath the unconsolidated sediment bed of the Wash encounters low-permeability bedrock that diverts the water upward into the Wash. Besides the large plume of perchlorate from the BMI complex north to the Wash, there are other significant seeps

further downstream in the Wash along the faults which impact the total perchlorate flux entering Lake Mead.

The Las Vegas Wash flows into Lake Mead, which is the drinking water source for 1.2 million people in the Las Vegas Valley (U.S. Census, 2000). Lake Mead was created by Hoover Dam, which impounds part of the Colorado River. The Colorado River is used as a drinking water source for more than 10 million people in southern California, and more than a million people in Arizona (USEPA, 2000). The concentrations of perchlorate in water supplies in California and Southern Nevada fluctuate between four and sixteen ppb (Li, Li, Gyrd, Deyhle, Sesser, Skeels & Lamms, 2000).

Chemical Forms and Properties

Perchlorate is an anion that is commonly associated with ammonium, magnesium, sodium, and potassium (Espenson, 2000). It's formula is characterized by a tetrahedral orientation of the four oxygen atoms around a central chlorine with a negative ionic value (Expenson, 2000). The oxygen atoms sterically block reductant molecules from direct attack at the chlorine. Another common form of perchlorate is perchloric acid ($HClO_4$), which is used for various laboratory techniques requiring a strong acid (USEPA, 2002).

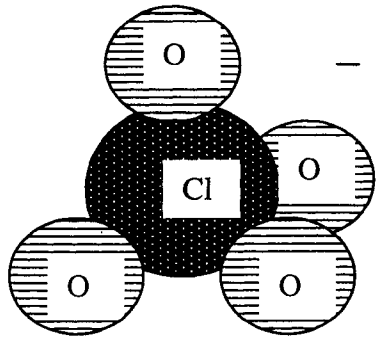


Figure 2 Structure of the Perchlorate Ion

Perchlorate salts and perchloric acid are readily soluble in water because the low charge density reduces their affinity for cations (Espenson, 2000). When in aqueous solution, perchlorate ions are inert and unreactive. Perchlorates are explosive only in combination with oxidizable components, such as in the presence of organic compounds or ammonium ions (Espenson, 2000). This means that when perchlorate is in the natural environment, it will dissolve in water and is not reactive. This stability results from the strong chlorine-oxygen bonds and the prerequisite that reduction must occur first through an oxygen atom abstraction rather than a direct involvement of the central chlorine atom (Urbansky & Schock, 1999). Thus, the reduction of the central chlorine atom from an oxidation state of +7 (perchlorate) to −1 (chloride ion) occurs exceptionally slowly (USEPA, 2002). The activation energy required for perchlorate reduction is so high that perchlorate cannot be expected to act as an oxidant within the human body because of the body's moderate temperatures and nearly neutral pH (USEPA, 2002). This supports in the contention that perchlorate is excreted virtually unchanged after absorption.

Biotransformation

Absorption of perchlorate through intact skin is unlikely due to its high ionic charge. Contact with moist mucus membrane results in absorption of inhaled perchlorate because of the high aqueous solubility (Gibbs, Ahmad, Crump, Houck, Leveille, Findleyk & Francis, 1998). However, exposure via inhalation of fumes or vapors is unlikely owing to perchlorate's low vapor pressure at room temperature (USEPA, 2002); however employees of AP facilities have been occupationally exposed through the respiratory and oral routes (Gibbs *et al.,* 1998).

The primary route of environmental perchlorate exposure for humans occurs through ingestion of drinking water (Gullick *et al.*, 2001). The high activation energy results in an insignificant amount of chemical reduction in the environment making perchlorate very mobile in aqueous systems and persistent for many years in ground and surface waters. Perchlorate salts readily dissolve in water and the resultant anion is easily absorbed from the gastrointestinal tract (Gibbs *et al.*, 1998). Limited adsorption, distribution, metabolism, and elimination studies were in existence prior to the strategy outlined in USEPA's draft of Perchlorate's Toxicological Review and Risk Assessment (USEPA, 2002). However, it is known that once inside of the body, perchlorate is not metabolized or biotransformed.

Once in the blood stream, only the perchlorate that is free, unbound, and available at the site of action is able to exhibit its effect. Perchlorate will travel through the blood stream until it accumulates selectively in the thyroid gland (Anabar, Guttmann & Lewitus, 1959)

Distribution

The water solubility of perchlorate suggests that it is readily absorbed by the gastrointestinal tract and is rapidly excreted primarily through the urine; excreted perchlorate is virtually unchanged structure and composition in humans (Burg, 1995; Anbar *et al.*, 1959). Perchlorate ions are not metabolized or involved in any chemical change in the body, they are primarily excreted as the parent compound in urine (USEPA, 2002). Peak blood levels after oral intake are reported at approximately three hours (Clark, 2000). Perchlorate is eliminated through the urine in a two phase process; the first phase removes 96% of the perchlorate with the half life being one to two hours (in rats) (USEPA, 2002). The second phase removed 4% in rats with the half life ranged from 72-80 hours (USEPA, 2002).

Once in the body perchlorate ions compete with iodide for the limited capacity of the thyroid gland (Anabar *et al.*, 1959; Wolff, 1998). In order to understand the toxic mechanism of perchlorate it is necessary to first consider the anatomy and standard function of the thyroid gland.

Thyroid Hormone System

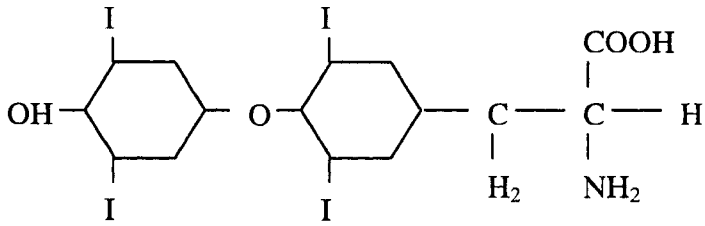
The adult thyroid weighs about approximately 25 grams and consists of two lateral lobes joined by a central isthmus lying over and around the trachea (Edwards, 1986). The functional components of the thyroid gland are the individual thyroid follicles (Hadley, 1998). Each thyroid follicle is spherical and lined with a monolayer of epithelial cells surrounding a central colloid-containing space (Edwards, 1986). The

follicular cells synthesize a protein, thyroglobulin (TG), which is released into the colloid space (Hadley, 1988). Thyroglobulin is an important substrate used in the synthesis of thyroid hormones (Hadley, 1988). An increased stimulation for thyroid hormones causes the follicular cells to expand to meet this demand and thereby reducing the colloid space. This indicates that the thyroid acts as a reservoir of thyroid hormones (Gard, 1998).

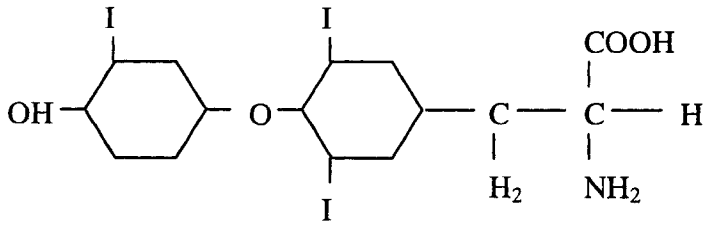
Inorganic iodide (I^-) from the human diet is trapped at the base of the thyroid follicular cells and is transported against an electrochemical gradient through an active pump mechanism across the follicular cells and into the colloid lumen (Hadley, 1988). Iodine in organic forms is converted mostly to iodide before adsorption (Cavalieri, 1997). Iodide is then oxidized by a peroxidase to an oxidized species of iodine, which is linked to tyrosyl groups of TG as monoiodotyrosine (MIT) and diiodotyrosine (DIT) (Hadley, 1988). A further coupling reaction occurs which results in the combination of two DIT molecules to form tetraiodothyronine (T_4, or thyroxine) or the combination of MIT with DIT to form triiodothyronine (T_3) (Gard, 1998) (See Figs. 3 & 4). T_4 is produced in quantities much greater than production of T_3, the more biologically active thyroid hormone.

These iodothyronines, still linked with TG, are then stored in the lumen of the follicle until release (Gard, 1998). T_3 and T_4 linked to TG are reabsorbed into follicular cells in colloid drops by endocytosis (Edwards, 1986). T_3 and T_4 are then separated from the TG by proteases contained in lysozomes (Edwards, 1986). Any uncoupled MIT and DIT are then deiodinated by a deiodinase and recycled for use within the cell (Hadley, 1988). The thyroid hormones, T_3 and T_4, are then released by exocytosis into circulation in the

bloodstream for distribution to the target tissue (Edwards, 1986). Thyroid hormones are insoluble in water, and therefore must be bound to plasma proteins in order to be transported (Gard, 1998). The function of the major thyroid hormone binding proteins in plasma is to maintain an equilibrium between extracellular and cellular hormone pools (Cavalieri, 1997).



3,3',5,5'-thyroxine (T_4)



3,3',5'-triiodothyronine (T_3)

Figure 3 Structures of T_3 and T_4 Hormones

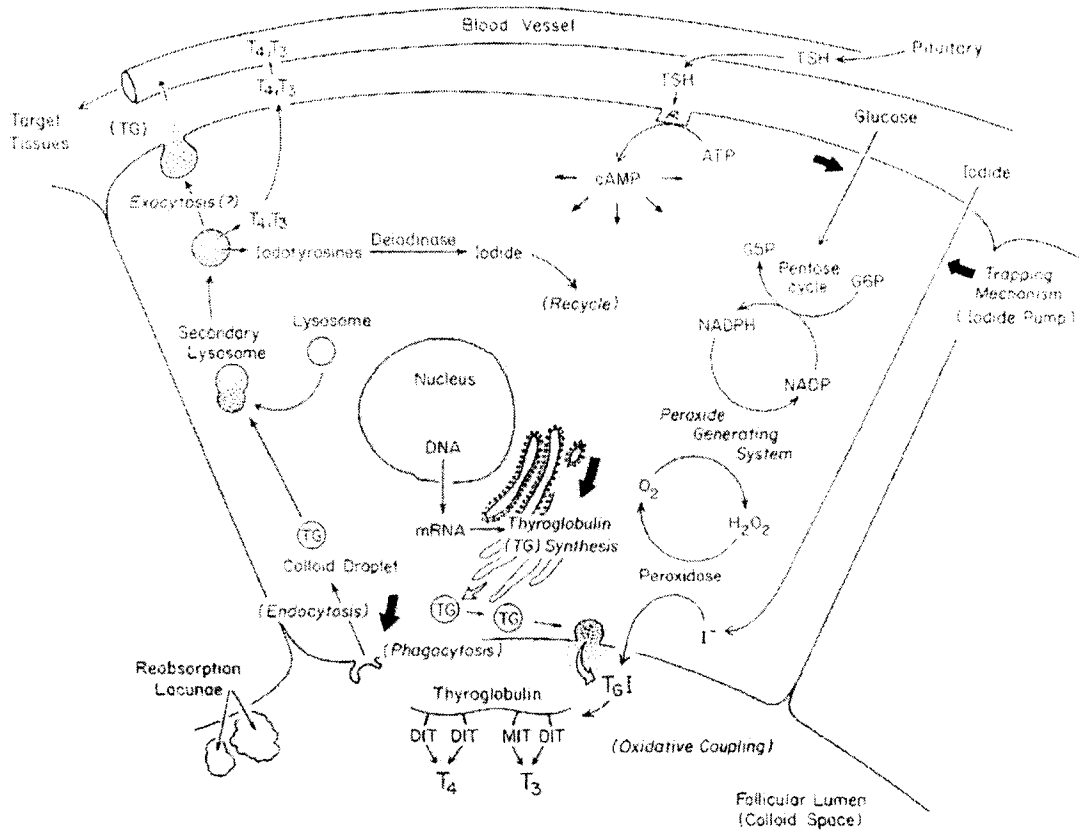


Figure 4 Thyroid Hormone Biosynthesis and Secretion

Reproduced with permission from Hadley, 1988

Hormonal Action

Chemically there are different classes of hormones. The first class, which T_3 and T_4 are members of, is predominately composed of amino acids, and are referred to as peptide or non-steroid hormones (Gard, 1998). The second class of hormones are steroid hormones which have a specific four-ringed structure called the cyclopentenoperhydrophenanthrene nucleus (Gard, 1998). Steroid hormones include estrogen and testosterone, which are able to pass through cellular membranes and thus are

able to interact with intracellular receptors (Gard, 1998). Steroid hormones are synthesized within elements of the smooth endoplasmic reticulum requiring a steroid-protein complex entering the nucleus and activating the synthesis of messenger ribonucleic acid (m-RNA) (Hadley, 1988). Ribonucleic acid (RNA) then leaves the nucleus and manufactures protein molecules that are then able to ellicit the hormonal action.

Non-steriod hormones are carried to the target by body fluid where they must bind to a receptor in the target cell membranes. The receptors are located on the membranes because non-steroid hormones cannot readily cross cellular membranes. They are able to interact with the receptor without the necessity of crossing the cell membrane nor entering the cell (Gard, 1998). Adenylate cyclase (AC) molecules are activated in target cells on non-steroid hormones and cause adenosine triphosphate (ATP) to be converted to adenosine 3′,5′-monophosphate (cAMP). cAMP then actives protein kinases, which are able to alter the key regulatory proteins by their phosphorylation (Gard, 1998). Kinases activate protein substrates to induce cellular changes. The second messenger system of the cAMP in non-steroid hormones allows for the effect of hormone-receptor complex to be greatly amplified. The first messenger is the hormone; however, because it is unable to cross the cellular membrane, it requires the use of a second messenger to influence processes within the cell (Gard, 1998). There is evidence that certain thyroid hormone-binding plasma proteins (i.e. apolipoproteins) may serve specific transport functions, although it generally is agreed that cellular uptake of thyroid hormones is a function of the unbound (free) form of the hormone (Cavalieri, 1997).

Thyroid hormones are unique non-steroid hormones because inorganic iodide is incorporated into their structures (Hadley, 1988). Specific binding proteins in the plasma and cell cytosol are needed to gain access to nuclear receptors because thyroid hormones are water-insoluble or lipophilic molecules (Hadley, 1988). The major thyroxine-binding proteins in human plasma are thyroxine-binding prealbumin (TBPA), thyroxine-binding globulin (TBG) and albumin (Hadley, 1988). The binding of the plasma protein as well as the lipid solubility and tissue affinity affect the volume of the thyroid hormones that are distributed throughout the body. Plasma protein binding allows the thyroid hormones to remain in the blood stream by making the complex polar with an increased molecular size, this results in the complex being unable to cross into organs via passive diffusion.

Even though thyroid hormones are non-steroid hormones the mechanism of action of T_3 is analogous to the model for steroid hormone action (Hadley, 1988). T_3 enters a target cell either by passive diffusion of by an unclarified carrier-mediated process. Once within the cytoplasm, T_3 interacts with a proteinaceous cytosol receptor and exists in equilibrium with its receptors (Hadley, 1988). T_3 is free to interact directly with chromatin; the cytosolic receptor may, however, provide a mechanism for concentrating the hormone within the thyroid hormone target cells. Thyroxine (T_4) is the primary circulating thyroid hormone.

Control of Thyroid Hormone Secretion

The synthesis and release of thyroid hormones is regulated by thyroid stimulating hormones (TSH) released from the anterior pituitary (Edwards, 1986). In response to TSH release, there is an immediate activation of follicular cell membrane receptors with

resulting activation and adenylate cyclase and cAMP production (Hadley, 1988). The release of TSH is in turn stimulated by thyrotropin releasing hormone (TRH) from the hypothalamus (Gard, 1998). The production of thyroid hormones is regulated by a classical negative feedback loop (Fig. 5). A decrease in T_3 and T_4 stimulates TSH release and increase of T_4 and T_3 suppresses TSH. This sequence of events leads to TSH often being used as a biomarker in research because its levels are often affected before a corresponding increase or decrease in T_4 and T_3 can be seen. Conversion of T_4 to T_3 may govern the secretion of TSH (Edwards, 1986). Normal circulating levels of TSH range from 0 to 6 mU/l in most commonly used radioimmunoassays (Edwards, 1986).

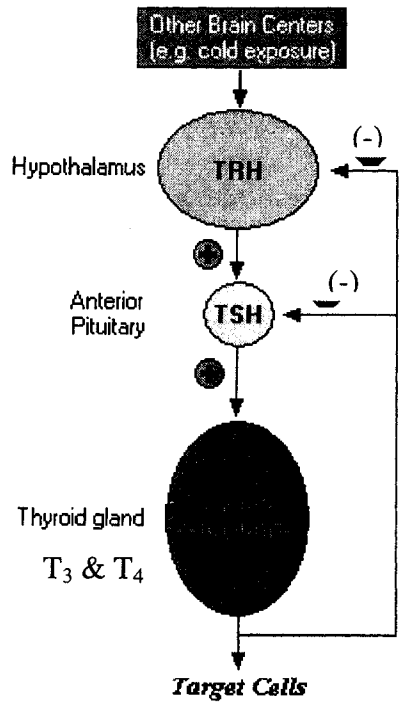


Figure 5 Negative Feedback of the Thyroid Gland

Reproduced with permission from Bowan 1999

Effects of Thyroid Hormones

The main effect of the thyroid hormones is to increase basal metabolic rate; this involves an increase in carbohydrate metabolism and an increase in the synthesis, mobilization, and degradation of lipids (Gard, 1998). The overall effects of T_4 and T_3 are to:

- Increase the basal metabolic rate
- Make more glucose available to meet the elevated metabolic demands
- Stimulate new protein synthesis, activation of lipoprotein lipase, and increase sensitivity of adipose tissue to lipolysis by other hormones
- Stimulate the heart rate, cardiac output, and blood flow
- Increase neutral transmission, cerebration, and neuronal development in young animals (Clark, 2000).

The main physiological difference between the two major thyroid hormones is that T_3 is thought to be the major physiologically active hormone regulating cellular activity (Hadley, 1988). T_3 is able to cross the blood brain barrier while T_4 cannot. However, T_4 is de-iodinated in order to form T_3. Type II iodothyronine deiodinase is responsible for the deiodination of T_4 into T_3 (Kamiya, Murakami, Araki, Hosoi, Ogiwara, Mizuma & Mori, 1999). This conversion is necessary to provide the brain with appropriate T_3 level during critical periods of development. The second type of deiodinase, Type I, is present in the thyroid gland, liver, and kidney, whereas Type II iodothyronine deiodinase activity is present in the brain, central nervous system and anterior pituitary (Kamiya *et al.*, 1999). Type I iodothyronine deiodinase is responsible for the deiodination of tyrosines which recycled for use within the cell (Hadley, 1988). Another difference between T_3 and T_4 is that only T_4 has the ability to exert a negative feedback on the hypothalamus, which is depicted in Fig. 5 (Hadley, 1988).

Mechanism of Perchlorate Action

Perchlorate affects the normal function of the thyroid gland by blocking iodide through the sodium/iodide symporter (NIS) pump and also by depleting stores of iodide (Anbar *et al.*, 1958 & Wolff, 1998). Iodide is needed for the normal production of thyroid hormones. The thyroid gland concentrates iodide against an electrochemical gradient by a carrier-mediated mechanism driven by adensoine triphosphate (ATP) (USEPA, 2002). The molecule responsible for this transport has been identified as the NIS. Active accumulation of iodide by the thyroid gland epithelium is a sodium dependent secondary active transport process mediate by the NIS. Two sodium ions are driven across the plasma membrane for every one iodide against the electrochemical gradient into the cell (O'Neill, Magnolato & Semenza, 1987). This means that the thyroid has a specialized ability to concentrate iodide selectively from surroundings where the concentrations are very low.

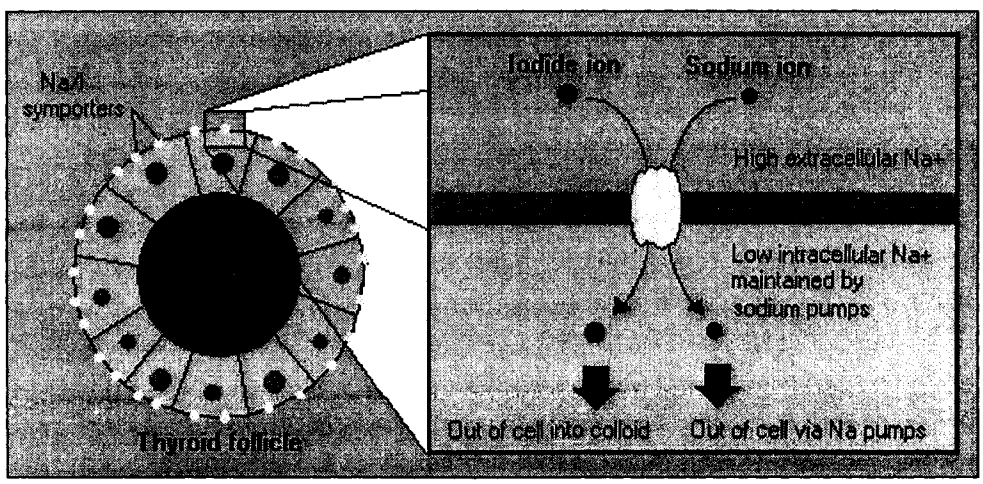


Figure 6 Sodium Iodide Symporter

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Anbar *et al.* (1959) found that perchlorate ions concentrate in the thyroid tissue and compete with iodide ions for the limited uptake capacity of the thyroid gland. The extent of perchlorate accumulation in the thyroid is comparable to that of iodide at high concentrations (Anbar *et al.,* 1959). This means that the thyroid accumulates perchlorate to the degree that it concentrates iodide. Chemically, perchlorate and iodide are exceptionally different. It is believed that the reason perchlorate and iodide behave similarly is because both ions are similar in size (Anbar *et al.*, 1959). Atterwill, Collins, Brown & Harland (1987) showed that after a 6-hour period following ingestion virtually all the accumulated iodide has been organified into thyroid hormones. This research is important in showing that perchlorate does not interfere with iodide organification. Thus, perchlorate appears to have no effect on the iodination process itself, but displaces iodide by competitive uptake at the NIS.

The conclusion that ion size was the determining factor in Anbar's research lead to further research of the role of ion size. Wyngaarden, Wright & Ways (1952) also found that monovalent anions like perchlorate are able to inhibit the collection and retention of the iodide ion in the thyroid gland. Wolff and Maurey (1962) confirmed the previous research that perchlorate in addition to other spherical halides and tetrahedral ions are concentrated by thyroid tissue as well as being competitors for iodine at the NIS.

The thyroid is thought to be three-compartment system, and much of the iodide in the intact thyroid is stored in the follicular lumen (Wolff, 1998). Perchlorate is concentrated in the lumen of the thyroid by a two-step electrochemically active process. It is actively transported across the basal membrane from the interstitial fluid into follicular cells, and

is further concentrated in passage across the apical membrane into the lumen (Chow & Woodbury, 1969). The thyroid of rats and guinea pigs concentrate perchlorate in serum ratios similar to standard iodide levels (Chow & Woodbury, 1970). All of the locations where perchlorate inhibition is exerted remain to be established owing to the complex anatomy of the thyroid follicle (Wolff, 1998).

Besides the interference of perchlorate with the NIS, the second mechanism of perchlorate is its ability to deplete endogenous stores of iodide (Anbar *et al.*, 1959). Bürgi, Benguerel, Knopp, Kohler & Studer (1973) used a very sensitive double isotope method to show that perchlorate leads to a small but significant increase of non-thyroxine iodine secretion which is owing to a discharge of endogenous iodide. It was previously thought that there were two separate iodide compartments because of various experiments with perchlorate. One argument for a two iodide compartments is the fact that perchlorate completely discharges transported iodine from the thyroid, while it had no such effect on endogenous iodide (Bürgi *et al.*, 1973). This observation of perchlorate's lack of effect on endogenous stores of iodide remained controversial and unconfirmed by other studies. Bürgi *et al.*(1973) concluded that the other investigators might have missed a small 26 μg/day increase in endogenous iodide due to less sensitive methods that were used.

Effects of Thyroid Dysfunction

Clearly, the research has shown that perchlorate both depletes the endogenous stores of iodine as well as competing with iodine at the NIS pump. When the body uptakes and

concentrates perchlorate into the thyroid gland instead of iodide, the synthesis of the thyroid hormones T_3 and T_4 decreases. The production of the thyroid hormones is regulated by a standard negative feedback loop. When perchlorate inhibits the uptake of iodine into the thyroid, this leads to an increase in serum TSH and TRH. TSH production continues until the thyroid hormone levels reach a sufficient level. Consequently, one of the health concerns associated with exposure to perchlorate is hypothyroidism, or an under-active thyroid. The classical symptoms of this disorder are decreased heart rate associated with decreased respiration rates and body temperatures (Gard, 1998). Weight gain is typical due to the decrease in the utilization of carbohydrates and reflexes are slowed due to decreased neuronal function (Gard, 1998). Edema is another symptom owing to an accumulation of skin proteins, polysaccharides and hyaluronic acid in the subcutaneous spaces (Gard, 1998).

The opposite dysfunction of hypothyroidism is termed hyperthyroidism, or an overactive thyroid, that produces excessive amounts of T_3 and T_4. The symptoms of hyperactivity include increased metabolism and increased heart activity (Hadley, 1988). Hyperactivity symptoms also include excessive sweating due to a combination of increased body temperature and increased activity of the sympathetic nervous system, agitation and anxiety, and an increased appetite (Gard, 1998). The most common cause of hyperthyroidism is Graves' disease, an autoimmune disease in which there is an antibody to TSH receptors on the thyroid gland causing constant stimulation of the thyroid hormones (Gard, 1998). Perchlorate has the ability to prevent iodide uptake into the thyroid gland and subsequently slow the formation of thyroid hormones, therefore it

has historically been used to treat Graves disease. Univalent inhibitors of iodide transport include thiocyanate and other monovalent cations (perchlorate, chlorate, periodate, etc.) (Hadley, 1988).

Potassium perchlorate was extensively used as an antithyroid drug in the late 1950s and early 1960s (Crooks & Wayne, 1960). Perchlorate's mechanism is unlike the other thiouracils and imidazole anithyroid drugs which prevent the iodination of tyrosine (Crooks & Wayne, 1960). Perchlorate was shown to be a superior antithyroid drug with less side effects when compared to methylthioracil and carbimazole in a 450 patient study (Crooks & Wayne, 1960). Following reports of toxicity (1g daily dose), in particular to bone marrow hypoplasia or aplastic anemia, potassium perchlorate fell into disfavor as a antithyroid drug (Barsilai & Sheinfeld, 1966). Gjemdal (1963) reported a case of fatal aplastic anemia at moderate doses (600 mg daily) of potassium perchlorate from a patient with previous signs of drug idiosyncrasy. Morgan & Trotter (1960) suggested that the adverse reactions to potassium perchorate is dose related and no more toxic than other thiourylene antithyroid drugs.

Martino, Mariotte, Aghini-lombardi, Lenziard, Morabito, Baschier, Pinchera, Breverman & Saffron (1986) researched short-term administration of perchlorate to amiodarone iodine-induced hypothyroidism. Amiodarone is a drug containing 37.2 mg iodine that is used for treatment of cardiac tachyarrhythmias, a rapid irregular heartbeat. The administration of potassium perchlorate to six patients lead to prompt restoration of euthyroidism (normal thyroid hormone levels), while the three untreated remained hypothyroid for 2-6 months (Martino *et al.*, 1986). "This anion reduces intrathyroidal

iodide content by competitively inhibiting thyroid iodide transport, so that sufficient iodide to inhibit thyroid hormones synthesis is no longer present within the thyroid follicular cells" (Martino *et al.*, 1986). Hyperthyroidism can complicate pregnancy so often the mothers are given antithyroid drugs as treatment.

Messer, Hauffa, Obricht, Benker, Kottulla & Reinwein (1990) found that the children of mothers who took such antithyroid drugs (carbimazole, thiamazole, or propylthiouracil) did not have any adverse effects when compared to a control group. The research examined thyroid size and function, as well as somatic and intellectual development (Messer *et al.*, 1990). Wenzel & Lente (1984) looked at three different thionamide drugs: methimazole, propylthiouracil and perchlorate on 69 patients with hyperthyroidism due to Graves' disease. After treatment, all three of the groups had high T_3 and T_4 hormone levels (Wenzel & Lente, 1984). Their work does not confirm the theory of either a general or an intrathyriodal immunosuppressive action of thionamide drugs, which was thought to be partly responsible for the beneficial effects of the drugs (Wenzel & Lente, 1984). All of this research confirms the iodide-trapping inhibition mechanism of perchlorate; otherwise, perchlorate would not have been used as a treatment of Graves' disease and thyrotoxicosis.

Goiter is an enlargement of the thyroid gland which produces an obvious swelling of the neck. Goiter may be a symptom of either an overactive or underactive thyroid gland (Gard, 1998). In the case of hypothyroidism, the pituitary gland will secrete increased amounts of TSH in an attempt to boost thyroid hormone synthesis and secretion. The thyroid then enlarges under the influence of TSH. In the case of hyperthyroidism, an

excessive production of either TRH or TSH will lead to an enlargement of the thyroid gland accompanied by excessive thyroid hormone secretion. A lack of iodine in the diet will cause the body to not be able to synthesize thyroid hormones and can cause goiter (Hadley, 1988).

Perchlorate has been shown to readily cross the placenta of the guinea pigs (*Cavia porcellus*) and may produce goiter in the fetus, whose thyroid appear to be more sensitive to this anion than that of the mother (Postel, 1957). If decreased thyroid activity occurs *in utero*, there is the risk of severe mental retardation owing to the failure of development of the central nervous system (Gard, 1998).

Patiño, Wainscott, Cruz-Li, Balakrishnan, McMurry, Blazer & Anduson (2003) focused their work on the reproductive performance of zebrafish (*Danio rerio*) reared in water containing ammonium perchlorate. Adult fish were raised for eight weeks in water containing 0 ppm, 18 ppm or 677 ppm perchlorate (Patiño *et al.*, 2003). The animals were then paired and spawned egg volume was accessed as an index of reproductive performance (Patiño *et al.*, 2003). Spawn volume was reduced within one week for the 677 ppm dose group and became negligible after four weeks (Patiño *et al.*, 2003). The 18 ppm dose group did not have significantly altered spawn volumes nor percentage of egg fertilization (Patiño *et al.*, 2003). Whole-body perchlorate levels were about one-hundredth of those of treatment water levels (Patiño *et al.*, 2003). At 677 ppm for four weeks, perchlorate lead to thyroid follicle cell hypertrophy and angiogenesis, whereas at 18 ppm for eight weeks, its effects were more prominent and included hypertrophy, angiogenesis, hyperplasia, and colloid depletion (Patiño *et al.*, 2003).

Biomarkers

The following section presents a summary of the current knowledge on perchlorate biomarkers from human exposure studies, mammalian research, and amphibian research. A biomarker is a biological response to a chemical that gives an indication of exposure, which is often more sensitive or easier to accomplish then to actually measure the chemical itself. The response can then be used to indicate harmful effects or to predict future harm to an organism.

Human Exposure Studies

Vulsma, Gons & DeVijlder (1989) reported that infants with severe congenital hypothyroidism receive substantial amounts of T_4 from their mother during late gestation. Twenty-five neonates with a complete inability to iodinate thyroid proteins were studied to determine whether there is a transfer of T_4 to the fetus (Vulsama *et al.*, 1989). The same serum levels were found in fifteen neonates with undeveloped thyroid as those neonates with a total organification defect, which suggests that these infants had T_4 that originated from their mother (Vulsama *et al.*, 1989).

Gibbs *et al.* (1998) monitored occupation AP airborne exposure for a eight hour shift that ranged from 0.2 to 436 μg/kg, with an average of 36 μg/kg. Working-lifetime cumulative doses in the high exposure group ranged from 8,000 to 80,000 μg/kg, with an average of 38,000 μg/kg. They concluded that these levels were two to three orders of magnitude less than doses historically prescribed in the treatment of Graves's disease. Further, they found no acute or chronic exposure-related effects on bone marrow, liver, or kidney function.

Lamm, Braverman, Li, Ricman, Pino, Howearth (1990) also monitored airborne occupational exposure to perchlorate in humans and found that no difference in measures of thyroid-function between the 1, 4, 11 and 34 mg per day groups. Thyroid function was assessed by measurements of serum TSH, T_4, T_3, free T_4 index, thyroid hormone binding ratio, thyroid peroxidase antibodies, and by clinical examinations (Lamm *et al.*, 1990). In another study, Lamm & Doemland (1999) reported that data from neonatal screening of the State Health Department of California and Nevada did not indicate an increased incidence of cogenital hypothyroidism between 1996 and 1997. Within the seven counties, 249 cases were identified, where 243 were expected, for an overall risk ratio of 1.0 (Lamm & Doemland, 1999).

Crump, Michaud, Tellez, Reyes, Gonzale, Montgomery, Crump, Lobo, Becerra, Gibbs (2000) studied 9,784 newborns and 162 school children in three cities in northern Chile with perchlorate water concentrations of 100-120 μg/L, 5-7 μg/L and non-detectable. No difference was found in TSH levels or goiter prevalence among school children after adjusting for age, sex, and urinary iodine (Crump *et al.*, 2000). Neonatal TSH levels were significantly lower in the cities with a perchlorate concentration (100-120 μg/L, 5-7 μg/L); this is opposite to the known pharmacological effect of perchlorate (Crump *et al.*, 2000). The findings of this study show that perchlorate in drinking water at concentrations as high as 100-120 ug/L do not suppress thyroid function in newborns or school-aged children (Crump *et al.*, 2000).

Li *et al.* (2000) performed another study examining whether exposure to perchlorate can affect neonatal blood thyroxine levels newborns in Las Vegas, Nevada and with a

control group in Reno, Nevada (2000). Perchlorate concentrations in Las Vegas drinking water supply during this research were non-detectable for eight months and ranged from nine to fifteen ppb for seven months. Reno drinking water perchlorate concentrates were below the four ppb detection limit (Li *et al.*, 2000). Of the more than 23,000 newborns in these two cities born between April of 1998 and June of 1999 no statistically significant differences were found in mean blood T_4 levels. There were no data on how many of the women studied consumed public water during their pregnancy (Li *et al.*, 2000).

Brechner, Parkhurst, Humble, Brown & Herman (2000) compared the TSH levels in newborns from Yuma, Arizona, whose drinking water comes completely from the Colorado River, to newborns in Flagstaff, Arizona where none of the drinking water is supplied from the Colorado. Brechner *et al.* (2000) found that TSH levels in Yuma were significantly higher than in Flagstaff. Adjustments of age in days at measurement and race/ethnicity were made (Brechner, *et al.*, 2000), however no adjustments where made for sex which is known to influence TSH levels (Goodman, 2001). They did not determine perchlorate concentrations in the test area; however, Goodman (2001) indicated that AZ drinking water contains approximately 6 ppb of perchlorate.

Li, Squartsoff & Lamm (2001) performed an analysis of the Medicaid database from Clark County, Nevada and Washoe County, Nevada to determine the prevalence of persons with thyroid diseases. Perchlorate has been detected in public drinking water in Clark County in the range of 5-24 ppb while Washoe County is non-detectable. There was no evidence in this comparison of an increased rate of thyroid disease (goiter,

nodule, thyrotoxicosis, congenital, hypothyroidism, thyroiditis, or thyroid cancer) associated with perchlorate exposure.

Greer, Goodman, Pleus & Greer (2002) determined that a perchlorate dose of 500 ppb in drinking water is necessary to suppress thyroid hormones. They determined that this dose was necessary to trigger an effect on iodide uptake by having 37 male and female adult human volunteers drink 400 ml of water laced with perchlorate doses ranging from 7-500 ppb. Subjects ingest ^{123}I before, during and fifteen days after they consumed perchlorate in order to measure iodine uptake. Based on their observations a NOAEL (no observable adverse effect level) was determined to be 7μg/ kg of body weight. Thus, the researches determined that perchlorate concentrations ranging from 180 to 220 ppb, "should be of no health concern in iodine sufficient populations."

Mammalian Research

Guinea pigs given 0, 8, 16 and 32 μg per kg of perchlorate in drinking water during the second or third week of pregnancy resulted in fetal goiter, but did not produce maternal goiter (Postel, 1957). These data provide evidence that perchlorate can cross the placenta. T_3 was ineffective when administered to the maternal guinea pigs in preventing fetal goiter (Postel, 1957). The placenta restricts the T_3's entry into the fetus, and it is rapidly degraded (Postel, 1957). Postel only reported an enlargement of the thyroid gland, the fetuses were not examined for other developmental effects.

Sprague-Dawley rats dosed with 100 mg/L of potassium perchlorate in their drinking water for 4-14 days experienced a decrease in T_3 and T_4 at parallel rates and TSH levels increased at the same time (Männistö, Ranta & Leppaluoto, 1979). The decrease to T_3

and T_4 levels depicts the classical feedback theory, which leads to a rise in serum TSH levels.

Mice (BALB/c strain) that drank 1.2% sodium perchlorate solution experienced strong hypothyroidism, hypertrophic and hyperplastic thyroid epithelial cells as pituitary thyrotropic cells (Pajer & Kališnik, 1991). The thyroid gland increased in total volume to 65 mm^3 in comparison to the control's 3 mm^3 (Pajer & Kališnik, 1991). The 72 female mice in the experiment were divided into six groups of twelve animals. Three groups drank 1.2 % sodium perchlorate solution awhile three control groups drank tap water. Eight or 32 weeks after the beginning of the experiment one perchlorate and one control group of animals were irradiated with each animal received a total dose of 4 Gy. The total volume increase of the pituitary distal parts after perchlorate treatment was 2.3 mm^3 in nonirradiated animals in comparison to 1.1 mm^3 in nonirradiated controls. Pajer & Kališnik concluded that the impairment of the iodine pump by perchlorate reduced the synthesis of the T_3 and T_4. A strong hypertrophy and enormous hyperplasia of the thyroid follicular and pituitary thyrotropic cells was accompanied by a high incidence of follicular cell carcinoma.

Siglin, Mattie, Dodd, Hildebrant & Baker (2000) performed a 90-day drinking water study on rats to determine the subchronic toxicity of AP. Perchlorate was administered to Sprague-Dawley rats for 14-90 days at levels of 0.01, 0.5, 0.2, 1.0 and 10 mg/kg/day. There was a 30 day non-treatment recovery period to evaluate the reversibly of any perchlorate induced effects at the 0.05, 1.0, and 10.0 mg/kg/day levels. The 10 mg/kg/day level experienced increased thyroid weights, increased follicular cell

hypertrophy with microfollicle formation and colloid depletion. These changes were reversible after the 30 day non-treatment recovery period. Statisically significant changes in TSH, T_3 and T_4 levels were observed at all dosage levels; however, no thyroid organ weight or histopathological effects were observed at dosage levels $\leq$ 1.0 mg/kg/day. Without any of these effects being seen in the lower dose groups, this research could not determine the toxicological significance of TSH and T_3 and T_4 levels in the dosage levels $\leq$ 1.0 mg/kg/day. No toxicologically meaningful differences were observed between the control and treated groups with respect to survival, clinical observations, body weights, food consumption, water consumption, ophthalmology, hematology, clinical chemistry, estrous cycling, sperm parameters, or bone marrow micronucleus formation. The study concluded that 1.0 mg/kg/day is regarded as the NOAEL for the study.

York, Brown, Girar & Dollarhide (2001a) reported that AP is not a reproductive toxicant in rats at doses as high as 30 mg/kg/day. Sprague-Dawley rats were given continuous access to AP in their drinking water at doses of 0, 0.3, 3.0, and 30.0 mg/kg/day. Perchlorate exposure caused statistically significant, dose-dependent changes in thyroid weight, histopathology, and hormone levels in all three generations of rats. In the F_1 adult rats, thyroid weights were significantly increased in all dose groups for female rats and in the 3.0 and 30.0 mg/kg/day does groups for male rats. Histopathologic changes in the thyroid consisted of hypertrophy and hyperplasia that increased in a dose-related manner in all three generations. Statistically significant changes in TSH, T_3 and T_4 levels occurred at doses higher than those resulting in changes in thyroid weight and histopathology. Perchlorate exposure did not seem to have an

effect on the thyroid hormones of either generation pups. In both P and F1 adult rats, there were no deaths, abortions, abnormal sperm parameters, abnormal fertility parameters or premature deliveries attributed to perchlorate exposures. The authors reported that based on their findings, 0.3 mg/kg/day was identified as the NOAEL for this study.

Another study by York, Brown, Girar & Dollarhide (2001b) investigated development toxicity of AP in New Zealand white rabbit (*Oryctolagus cuniculus*). The rabbits were given continual access to AP at does of 0, 0.3, 3.0, 30.0 and 100mg/kg/day synonymous with the previously mentioned study on gestation days six through 28. The rabbits were sacrificed on gestation day 29 and the fetuses were examined for developmental alterations. None of the dose groups had statistically significant litter characteristics that varied form historical laboratory ranges. However, there was an increased occurrence of thyroid follicular hypertrophy in animals treated with $\geq$ 10 mg/kg-day perchlorate and significantly decreased T_4 in doses treated with $\geq$ 30 mg/kg-day. This research concluded a maternal NOAEL to be 1.0 mg/kg-day for ammonium perchlorate and the developmental NOAEL was found to be 100 mg/kg-day for rabbits (York *et al.,* 2001b).

Thuett, Roots, Mitchell, Gentles, Anderson & Smith (2002) examined the effects of *in utero* and lactational exposure to AP on developing deer mice (*Peromyscus maniculatus*) and their data suggest the exposure at different concentrations may variably alter body weight and male heart weight during development. Breeding pairs were dosed continuously with 0, 1nM (117 ppt), 1 μM (117 ppb), or 1 mM (117 ppm) in their

drinking water until postnatal day (PND) 21 when the pups were sacrificed. Body weight in the 1 μM group was consistently lower than in the controls after PND 1. The 1 mM group had increased body weights however, the highest dose group did not vary from the control (Thuett *et al.*, 2002). Heart weights in male animals were decreased in the 1 μM and the 1mM dose groups (Thuett *et al.*, 2002). Heart weight decreased while body weight was increasing.

Mahle, Yu, Narayana, Mattie & Fisher (2003) found that female rat pups receiving perchlorate lactationally seem to be more sensitive than male pups. During this research pregnant rats were exposed to 1 mg/kg-day of perchlorate in their drinking water. Female pups receiving perchlorate had significantly lower levels of serum T_4 than control pups and parentally exposed pups, while serum T_4 levels in male pups were not affected by perchlorate. The study also found that *in utero* perchlorate exposure decreased serum T_4 levels in the fetus.

Amphibian Research

The research on amphibians is even more limited then the work that has been completed on humans and mammals. Miranda, Piscano & Casco (1996) examined the thyroid glands of *Bufo arenarum* larvae that were reared in 0.034% potassium perchlorate solution. They found that the thyroid gland volume and follicle cell height at months five and three were significantly larger in the treatment group then the control. Changes in the size and volume of follicular cells appeared to be mainly a function of the overstimulation of proteinpoietic apparatus as a result of a decrease in the thyroxine levels.

Goleman *et al.*, (2002a) performed two experiments on the African clawed frog (*Xenopus laevis)* exploring the effects of AP. The first experiment involved exposing eggs and larvae to varying environmentally relevant concentrations of AP for 70 days in order to examine the effects of metamorphosis (Goleman *et al.*, 2002a). The goal of the second experiment was to compare the recently developed USEPA Endocrine Disruptor Screening and Testing Committee Tier I screening test for frog metamorphosis with longer-term exposure scenarios (USEPA, 2003). In the second experiment larvae were raised in AP diluted FETAX (NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35mM, and Mg SO_4, 0.62 mM) to a target concentration of 14,040 ppb for 14 days. The developmental stage, snout to vent length, hindlimb length, tail length and height was measured on day 0 and at the end of exposure on day 14.

In the first Goleman *et al.* (2000a) experiment 50 fertilized eggs were raised in one of seven concentrations of AP diluted in FETAX solution: control, 1.41 ppm, 14.4 ppm, 133 ppm, 425 ppm, 5 ppb, 18 ppb, 147 ppb. Beginning on the day of hatch, hatching success, number showing bent tails, edema, abnormal swimming, percentage demonstrating forelimb emergence, morality and percentage metamorphosed were noted every day. Every five days, snout-vent length, hindlimb length, tail length, and developmental stage was determined from ten tadpoles per tank beginning sixteen days after hatching. The first experiment resulted in survival of larvae averaging 87-92% between the 5 ppb and 133 ppm groups but decreased to 6-7% in the 425 ppm group. The percentage of animals with bent tails, edema, and abnormal swimming increased in the 425 ppm dose group. This high dose group had reduced snout-vent length compared

to control at post-hatch days, 16, 21, 31, and 36. AP exposure reduced hindlimb growth in a concentration–dependent manner at post-hatch days 55, 60, 65, and 68. The lowest concentration capable of forelimb inhibition was found to be 5 ppb. AP also significantly reduced tail resorption in a concentration-related manner, with the lowest concentration capable of inhibiting resorption was 18 ppb. At concentrations above 147 ppb no animals completed tail resorption.

The second part of Goleman *et al.*'s (2002a) 14-day experiment resulted in mean tail lengths between the dose group and control being identical at the start and end of the experiment. However, AP did reduce the percentage of tadpoles completing resorption, but AP did not completely prevent the tail resorption at concentrations in the micrograms-per-liter range, well within the range of concentrations reported for AP leveled in contaminated surface waters.

Goleman, Carr & Anderson (2002b) performed similar research that focused on *Xenopus laevis* sex ratios. They raised embryos and larvae of *Xenopus laevis* in AP doses of 0 ppb, 38 ppb and 14 ppm mixed with FETAX solution for 70 days. There was also a 28-day recovery period after treatment in order to evaluate the reversibility of AP effect. The same developmental effects were measured as in the previous research every five days on 10 animals. This research involved T_4 being extracted from larvae and newly metamorphosed froglets as well as gonadal sex determination by visual inspection. The research resulted in no significant effects on mortality, hatching success, or developmental abnormalities (bent tails, edema, or abnormal swimming). Forelimb emergence, hindlimb development and tail resorption was inhibited by AP. The 14-ppm

dose group had no forelimb emergence during the 70 day treatment. The 14-ppm dose group had reduced whole-body T_4 levels, but both concentrations had significant hypertrophy or the thyroid follicular epithelium. Both concentrations also resulted in skewed sex ratios, with fewer males than females.

The experiment we performed was similar to the research performed in the Golemen studies except we used a completely aquatic species, did not use a FETAX solution, use magnesium perchlorate not AP and did not dose in the larval stage. The following hypotheses, reflect the same questions that Golemen *et al.* answered in their work.

> The survival rate of *Rana Pipiens* will be dose dependent with the highest survival rate among animals exposed to the lower doses of perchlorate.
>
> Deformaties in *Rana pipiens* will be dose related and will increase according to dose.

Sparling, Harvey & Nzengung (2003) exposed early larval *Hyla versicolor* obtained from constructed wetlands at Patuxent National Research Refuge, Laurel, Maryland, USA to perchlorate ranging from 2.2 to 50 ppm. In addition, three controls: 0 perchlorate, 0 perchlorate with 0.10 ppm iodide and 50 ppm perchlorate with 0.10 ppm iodide were tested. The authors found that mortality and growth was unaffected within all treatment groups. However, inhibition of hindlimb formation and metamorphosis was apparent in the latter stages of development. No tadpoles metamorphosed at 22.9 or 33.8 ppm perchlorate and only 1 metamorphosed at 50 ppm. There was no difference in the number of days to metamorphosis among treatments when only considering those tadpoles that entered the climax stage. However, there was a significant different in the number of days required to complete metamorphosis once a forelimb had emerged. They

did not find any evidence of inhibition in any of the controls, suggesting that a small amount of iodide can counter the effects of perchlorate.

Amphibian Life-cycle

Different taxonomic groups of amphibians undergo various stages or steps in their metamorphosis. For example, terrestrial species often develop under direct metamorphosis; meaning that the young hatch directly as adults (Etkins, 1968). Other species such as *Rana pipiens* and *Xenopus laevis* have the most complete aquatic larval period, which extends from laying eggs in the water to tadpole growth and the entire metamorphic process (Shi, 2000). This analysis of the amphibian life-cycle primarily focuses on the growth and development of *Rana pipiens*.

Taylor-Kollros Index

Taylor & Kollros (1946) developed twenty-five stages of development that *Rana pipiens* larvae undergo during metamorphosis. The term 'larval stage' is used to refer to an animal at any and all points throughout its larval period from the end of embryogenesis (hatching) to the start of metamorphosis (onset of tail resorption) (Rose, 1999). Taylor & Kollros (1946) based the larval stages on the appearance of new structures or readily detectable changes in structures. Pigmentation characteristics vary greatly between individuals; both in color and intensity as well the relative time of appearance. Therefore, pigmentary changes were not used as critical characteristics for staging. The following graph adapted from Taylor & Kollros (1946) records the length of the tadpoles (mm) against their age in days with the points on the graph indicating the

larval stages (See Fig. 7). It is important to note that various strains of tadpoles and different temperature and housing environments might lead to dissimilar growth rates and subsequently a very different rate of development.

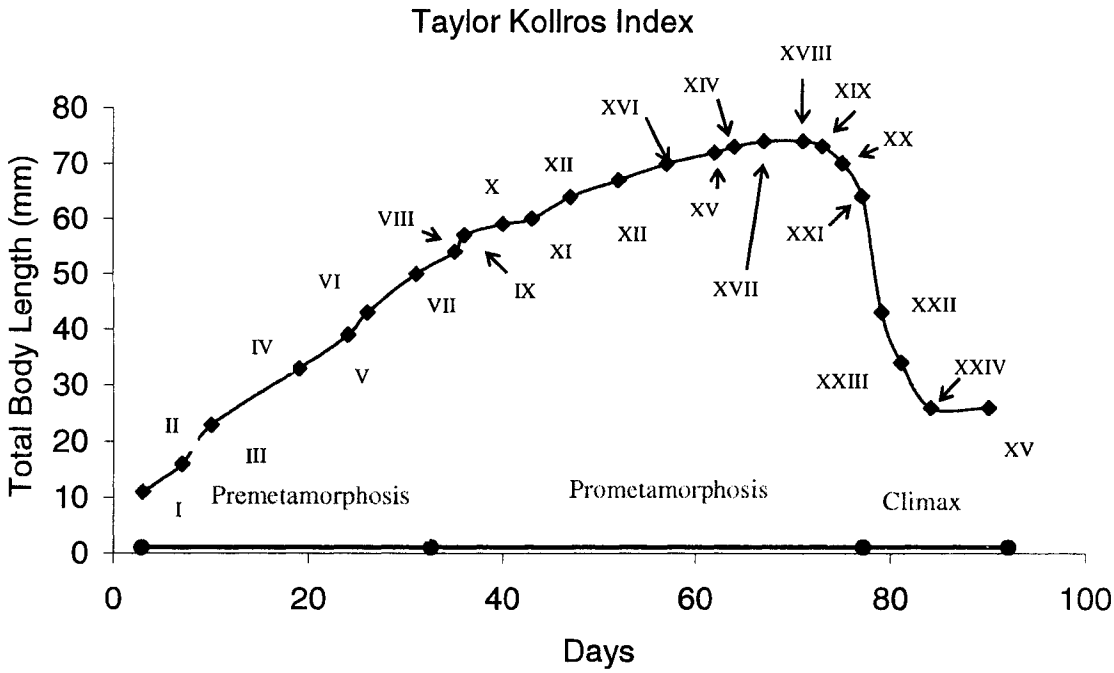


Figure 7 TKI Length (mm) and Average Age (days) at Each Stage

Adapted from Taylor & Kollros, 1946

During stages I to V the hindlimb is no more than a simple bud, therefore this group of stages are considered the "limb bud" stage. Between stages VI and X the bud is transformed to a paddle and then the paddle separates into several digits. These stages have been called the "paddle stages." The tadpole has reached 80% of its maximum length by the end of the paddle stages. The next group of stages (XI to VVII) has been termed the "foot" or "pre-metamorphic" stages as they involve more advanced development of the hindlimbs and precede the period of rapid transformation. The

"metamorphic" stages are initiated by the regression of the tail at stage XVIII and end at stage XXV when the resorption is complete and a fully developed juvenile frog emerges (Taylor, & Kollros, 1946). The Taylor-Kollros Index (TKI) was used in order to determine a rate of metamorphosis between the dose groups and lead to one of hypothesis that drove this research.

> *Rana pipiens* exposed to lower concentrations of perchlorate (4 and 40 ppb) will metamorphose at a faster rate when compared to those exposed to a higher concentration.

Premetamorphosis, Prometamorphosis and Metamorphic Climax

Etkins (1968) offers words of caution about the staging developed by Taylor & Kollros saying descriptively the TKI is highly useful, but it obscures the experimental analysis. Etkins (1968) warns that they do not recognize the differential acceleration of hindlimb growth as the initiation of metamorphosis but regard this process as beginning in what Etkins designates as prometamorphosis. Early leg growth (stages XII to XVIII) is dependent upon activation of the thyroid gland and is thus considered by Etkin (1968) to be a part of metamorphosis. Taylor and Kollros also do not differentiate a climax phase (Etkin, 1968).

Etkin (1968) therefore divides anuran metamorphosis into three specific periods: premetamorphosis, prometamorphosis, and metamorphic climax. Premetamorphosis is the period when embryogenesis and early growth and development take place in the absence of thyroid hormones (Shi, 2000). In *Rana pipiens*, the premetamorphic period lasts about seven weeks at conventional room temperatures (22 to 25° C) (Etkin, 1968).

The premetamorphosis larval growth period is a time of little external development; Taylor-Kollros define this period as stages I to XI.

During prometamorphosis, hindlimbs undergo differentiation of the toes and rapid and extensive growth of the hindlimbs (Shi, 2000) (TKI stages XI-XX). Prometamorphosis is characterized by rising concentrations of endogenous thyroid hormones (Shi, 2000). During the three-week period of prometamorphosis the hindlimbs of *R. pipiens* grow from 2 mm to about 20 mm while the total length only increases from about 55mm to 65 mm (Etkin, 1968). The emergence of the forelimbs, which usually take place within a few hours of each other, marks the end of prometamorphosis (Etkin, 1968). The tadpole has reached prometamorphosis when the hindlimb and snout to vent length ratio exceeds 0.2 and enters the metamorphic climax when the ratio approaches 1.0 (Etkins, 1968).

Thyroid hormones reach their highest levels during the metamorphic climax (Dodd & Dodd, 1976). Rapid morphological changes take place during this period. These changes include the loss of the horny beaks, the progressively widening of the mouth and most notably tail resorption (Shi, 2000). Regard, Taurog & Nakashima (1978) claimed that T_4 in *R. catesbeiana* tadpoles showed a 10-fold increase and T_3 increased 15-fold at climax when compared with premetamorphic levels during normal development. The completion of tail resorption marks the end of the metamorphic period (Shi, 2000). Total resorption of the gills, and a subsequent change to lung breathing, occurs around the same stages or slightly earlier than tail resorption (Shi, 2000). The research of Dodd & Dodd (1976) as well as Shi (2000) show that thyroid hormones are

needed for normal amphibian development, this led us to one of our hypothesis. The next hypothesis is based on the fact that thyroid hormones relate to metabolic rate of tadpoles, which can be measured as oxygen consumption (Warren, 1940).

> *Rana pipiens* exposed to higher concentrations (400 ppb, 4000 ppb) of perchlorate will have reduced morphometric characteristics (snout to vent, total body and hindlimb length) compared to those exposed to lower concentrations.
>
> Metabolic rate, as determined by oxygen consumption, will be reduced in *Rana pipiens* exposed to higher concentrations of perchlorate.

Hormone Regulation

Unlike the limbs and tails, virtually all other organs are present in both the tadpole and adult frog phases. However, these organs undergo extensive change during metamorphosis that is regulated by thyroid hormones. Amphibian development is controlled by hormone activity and tissue sensitivity (Rose, 1999). The removal of the thyroid gland prevents metamorphosis completely, and the return of the thyroid gland accelerates development (Hadley, 1988). All organs in amphibians are known to undergo thyroid hormone mediated changes (Rose, 1999). Allen (1927) concluded that "metamorphosis is due to the combined action of both the anterior lobe of the pituitary gland and the thyroid gland."

Low circulating levels of thyroid hormones in the amphibian larvae causes a slow maturation of hypothalamic neurosecretory center, resulting in greater thyroid hormone secretion (Hadley, 1988). This provides a positive feedback to the hypothalamus, thereby causing an increased maturation of the hypothalamus and an increase in TRH (Hadley, 1988). TRH causes an increase stimulation of TSH in the pituitary and subsequently an amplified production of T_3 and T_4 (Hadley, 1988). Hormonal pathways that affect

amphibian metamorphosis are illustrated in Fig. 8. Research by Yoshizato & Frienden (1975) suggests that in amphibians the maximum number of binding sites at the target organ, rather than the affinity constant, correlates with the biological activity of T_3 and T_4. Another pituitary hormone that has a role is metamorphosis is prolactin, which is a protein like TSH that is produced in the anterior lobe of the pituitary (Etkin, 1968). Prolactin appears to be under inhibitory rather than stimulatory control by the hypothalamus (Everett, 1966) (See Fig. 8). During premetamorphosis a large amount of prolactin is produced while there is substantial larval growth, but little organic differentiation (Fox, 1984). The antithyroid action of the prolactin diminishes as the hypothalamus is activated and the levels of TSH begin to rise during prometamorphosis and climax (Fox, 1984).

The function of prolactin in amphibians was initially considered a "juvenile" hormone, which inhibits metamorphosis (Shi, 2000). However the up-regulation of prolactin during metamorphosis suggests for an alternative function. One possible role for prolactin is to counteract high concentrations of thyroid hormones at the climax of metamorphosis to coordinate sequential transformations of different organs and tissues (Shi, 2000).

Environmental Factors

Besides thyroid hormones, environmental factors such as temperature, water availability, crowding, light, diet, and environmental iodine levels can regulate amphibian development (Dodd & Dodd, 1976). Some of these factors may inhibit or accelerate growth when present during metamorphosis. Higher temperature is an example of one of

the factors that can stimulate both tadpole growth and the rate of metamorphosis (Shi, 2000). Crowding, resource limitation and predation are factors that may inhibit growth when present during premetamorphic stages but can stimulate metamorphosis when present during prometamorphosis (Denver, 1988). Density-dependent effects on tadpole metamorphosis tend to be an inhibition of growth and development (Scott, 1994; Semlitsch & Caldwell, 1982).

Two Theories on the Controlling Mechanism

Etkin (1968) proposed an elaborate theory with the framework of the endocrine system to account for the mechanism controlling anuran metamorphosis. Fox (1984) summarized this broad theory as:

> During the premetamorphosis phase, when there is a large increase in larval body size but little hindlimb growth, the pituitary-thyroid axis is maintained at a steady low level of activity and the pituitary TSH cells are highly sensitive to negative thyroid hormonal feedback.

In this way, TSH cell secretion is minimal and the thyroid hormone level is thus so low it hardly differs from that of thyroidectomized larvae. Thyroid activity and substantial storage of hormones with thyroid follicles marks prometamorphosis (Fox, 1984). Maximum leg growth occurs in 10-20 ppb of T_4, while tail resorption at climax requires 200 ppb of T_4 (Fox, 1984). However, T_3 is two-to-five times as active as T_4 in causing tail recession (Yoshizato & Frienden, 1975).

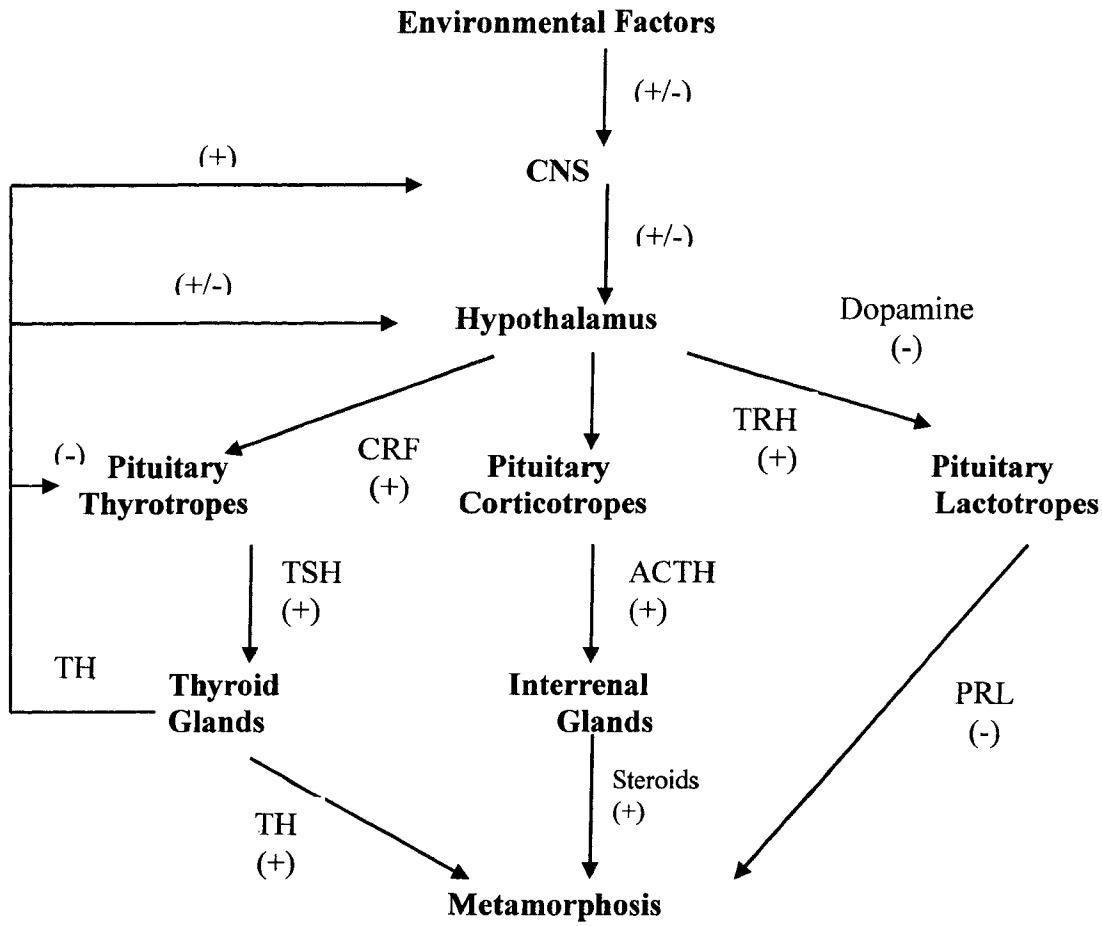


Figure 8 Amphibian Metamorphosis Hormonal Pathways

Adapted from Shi, 2000

Just before the onset of prometamorphosis, the TRH mechanism becomes sensitive to the initial level of circulatory thyroxine and a positive T_4 feedback occurs to induce prometamorphosis (Fox, 1984). The progressive increase in the production of TRH stimulates TSH, which in turn stimulates T_4 and T_3 secretion. Ultimately there is maximal activation of the pituitary-thyroid axis with the resulting climax corresponding to TKI stage XX (Fox, 1984). Etkin (1968) further postulated the TRH desensitizes the TSH cells of pituitary to the effects of negative feedback by a high circulatory level of

thyroid hormones (See Fig. 8). However, from the beginning of climax the hypothalamus ceases to be sensitive to positive feedback by circulatory T_4 and therefore TRH, TSH, T_4 and T_3 declines (Fox, 1984).

An alternative theory to Etkin's (1968) claim of relatively high and rising circulatory TH levels occurring during metamorphosis compared with that at the premetamorphic stage is proposed by Dodd and Dodd (1976). Their view is based on the peripheral tissues using TH continually and without moderation. This utilization increases during prometamorphosis and early climax stages, resulting in hormones being removed from circulation so quickly that their concentration levels rise only modestly (Fox, 1984). Dodd and Dodd (1976) found that TSH was present in prometamorphic *Xenopus laevis* tadpoles and rose to high levels at early metamorphic climax. There was a subsequent fall in TSH content in the pituitary at stage 61 (which marks the beginning of *Xenopus laevis* climax) followed by a rise to a peak value at stage 62 (development staging is from the work of Nieuwkoop and Faber (1956)). TSH levels dropped to below prometamorphic levels by the end of metamorphosis (Shi, 2000). Thus, high levels of TSH are present during metamorphosis when it is needed to stimulate T_4 release (Dodd and Dodd, 1976). The drop in TSH level in the pituitary at stage 61 coincides with the peak plasma T_4 (Shi, 2000). Thus, this drop is likely owing to increased secretion of TSH from the pituitary to the plasma to accelerate TH release, so that less TSH remains in the pituitary (Dodd and Dodd, 1976). However, no systematic quantification of TSH levels in the plasma is available (Shi, 2000).

There are no data to support a view of a positive feedback mechanism on hypothalamic TRH secretion, or that TRH desensitizes the pituitary TSH cells to a negative T_4 feedback (Fox, 1984). In mammals, the stimulation of the hypothalamus is through the secretion of TSH from the pituitary which is regulated by TRH (Shi, 2000) (See Fig. 8). TRH's presence in amphibians has been confirmed by numerous researchers; however, the role of TRH in metamorphosis has been controversial (Dodd & Dodd, 1976). Although experiments involving injecting TRH into adult frogs showed that TRH appears to increase TSH release, most experiments have failed to show a stimulation of TSH release or an acceleration of metamorphosis (Denver, 1988, 1996; Denver & Licht, 1989).

Etkin (1968) claims that any effective concentration of thyroxine can induce metamorphic changes if allowed to operate long enough, and that each tissue has a specific total requirement of TH needed to undergo metamorphic change. This is in contrast to Kollross' (1961) views that advocated that each tissue has a minimum hormone threshold level required for its response, and that different tissues have different thresholds that vary according to the temperature.

Etkin (1968) as well as Dodd and Dodd (1976) do agree on the fact the thyroid hormones regulate metamorphosis in tadpoles. Iodine is essential for synthesis of thyroid hormones therefore sufficient amounts of iodine must be present in the diet and/or water. Based on perchlorates' mechanism of action it can inhibit the synthesis of endogenous thyroid hormones but prevent iodine at the NIS pump and by depleting endogenous stores of iodine. This could result in a slowed or a complete block of metamorphosis of

anurans owing to their inability to uptake iodine and synthesize the necessary hormones to complete their development and metamorphosis. The concentration of perchlorate is thought to dictate whether there is complete block of metamorphosis or if the tadpoles will experience a decreased rate of development. Research also suggests that once the animal is removed from the iodine deficient environment (perchlorate laden water) they are able to recover and reach metamorphic climax (Goleman et al., 2002).

Declining Amphibian Populations

One of the reasons our research investigated the effects that perchlorate is having on amphibian populations is because local amphibian populations have been declining in southern Nevada. Bradford (2002) investigated amphibian populations in the eastern Mojave desert that surrounds Las Vegas, NV. In upland sites where springs exclusively feed wetland habitat, five amphibians were found. Seventy three percent of the 128 upland sites contained the red-spotted toad, which was the most abundant species followed by the Pacific treefrog (*Pseudacris regilla*), Woodhouse toad (*Bufo woodhousii*), introduced American bullfrog (*Rana catesbeiani*), and the relict leopard frog (*Rana onca*). Amphibian species have changed greatly at lowland sites due to sparsely distributed wetlands habitats being altered or eliminated by human activities. The most noticeable changes are the nearly complete replacement of native leopard frogs by the introduced bullfrog, and the complete replacement of the Arizona toad by the Woodhouse's toad (Bradford, 2002).

Reaser (2000) performed a study that examined size, mass, sex ratios and age structure of the Columbia spotted frog (*Rana luteiventris*) at seven sites in the Toiyabe Range in central Nevada. Knowledge of demographic patterns of this amphibian is essential in identifying stress agents in the environment, setting management priorities to protect critical sources habitat and monitoring recovery efforts. Reaser (2000) concluded that localized factors are influencing recruitment and mortality rates. "The introduction of exotic trout and cattle are likely the two most important anthropogenic factors limiting the distribution and persistence of *R. luteiventris* in the Toiyabe Range" (Reaser, 2000).

Matthews, Knapp and Pope (2002) agree with the problems that non-native trout are causing in the Sierra Nevadas. Their research suggests the introduction of trout has led not only to the decline of amphibians but also to the decline of garter snakes. The presence of amphibians is a prerequisite for garter snake persistence in high-elevation portions of the Sierra Nevada and that the introduction of trout into an ecosystem can have serious effects, not just on their prey but also on other predators in the ecosystem. Knapp and Matthews (2000) point to several introduced species of trout owing to the decline of the mountain yellow-legged frog (*Rana muscosa*) in California's Sierra Nevada. The research included surveys of more than 1700 sites in two adjacent and historically fishless protected areas that differed primarily in the distribution of introduced fish.

A study on the Snake Range of Nevada found 8 *Rana pipiens* out of the 406 total amphibians identified, all of which were in pinyon-juniper wetland habitat (Setser, Meik & Mulcahy, 2002). Amphibians are often throught to have a metapopulation structure,

which may render them vulnerable to habitat fragmentation. Research on *Bufo punctatus* in the Mojave Desert did not support this classical metapopulation model (Bradford, Neale, Nash, Sada, & Jaeger, 2003). *B. punctatus* occurs primarily in populations within mountain ranges that are isolated from patchy populations in other ranges. The influence of local environmental characteristics on patch occupancy demonstrates the importance of including patch quality metrics in test of predictions for patch occupancy based on metampopulations theory (Bradford *et al.*, 2003).

Besides amphibian declines in the desert southwest amphibians are declining in numerous geographical locations throughout the world. In most cases, the cause or causes are unknown, but are assumed to result from anthropocentric alterations in the environment. For example pesticide and other toxicants, increased UV radiation resulting from atmospheric ozone depletion has been correlated with mortality of amphibian eggs, introduced nonnative predators, acid rain and habitat destruction, disturbance and fragmentation have all been suggested as potential causes of amphibian declines (Carey & Bryant, 1995). Bette (1999) implicates not only agricultural chemicals but also disease and climate change as important factors in amphibian populations. Amphibian decline has been getting widespread attention due to the rapid declines from apparently pristine remotes regions. Interior Secretary Bruce Babbitt drastically increased the budget in 1999 for amphibian research and was quoted, " my fellow Cabinet members joined me in responding to an increasing environmental threat showing up in unexplained declines, deformities, and even disappearances of frogs, toads, and salamanders, species that have been on Earth for 350 million years" (Bette, 1999).

Ankley, Diamond, Tietge, Holcombe, Jensen, Defoe & Peterson (2002) assessed the potential role of solar ultraviolet radiation in causing limb malformations in *Rana pipiens*. Full sunlight caused approximately 50% mortality of frogs during early larval development and there was a dose-dependent induction of hindlimb malformations in frogs. Besides the sole effect that UV radiation might have on amphibians there was research done of on the link between pathogen outbreaks and UV-B exposure. Kiesecker, Blaustein & Belden (2001) report that pathogen outbreaks in amphibian populations in the western USA are linked to climate-induced changes in UV-B exposure. Climate-induced reductions in water depth at oviposition sites have caused high mortality of embryos by increasing their exposure to UV-B exposure and, consequently, their vulnerability to *Saprolegnia ferax* infection.

Sullivan and Spence (2003) results indicate that environmentally realistic concentrations of atrazine exert a negative impact on amphibian metamorphosis. This research also looked at the mixture of atrazine with nitrate and even if the mixtures of agricultural chemicals are at sublethal concentrations they may exert negative and not necessarily consistent mixture effects. Another common herbicide, acetochlor, was investigated and found to accelerate T_3 induced forelimb emergence and increase mRNA expression of thyroid hormone ß receptors in Ranid tadpoles (Crump, Werry, Veldoen, Van Aggelen & Helbing, 2002). Pesticide drift in California's Central Valley was researched using a geographical information system and compared to eight known declining amphibian populations in adjacent Sierra Nevada by Davidson, Shaffer & Jennings (2001). Besides examining pesticide drift, habitat destruction, ultraviolet

radiation and climate change where also investigated. In four species, a strong positive association between amphibian declines and the amount of upwind agricultural land use existed. This suggests that pesticides may be important factor in amphibian declines. For two other species declines were strongly associated with their habitat-destruction hypothesis.

CHAPTER 3

MATERIALS AND METHODS

The hypotheses that motivated this research are restated as follows:

1. *Rana pipiens* exposed to higher concentrations (400 ppb, 4000 ppb) of perchlorate will have reduced morphometric characteristics (snout to vent, total body and hindlimb length) compared to those exposed to lower concentrations.

2. *Rana pipiens* exposed to lower concentrations of perchlorate (4 and 40 ppb) will metamorphose at a faster rate when compared to those exposed to a higher concentration.

3. Metabolic rate, as determined by oxygen consumption, will be reduced in *Rana pipiens* exposed to higher concentrations of perchlorate.

4. The survival rate of *Rana pipiens* will be dose dependent, with the highest survival rate among animals exposed to the lower doses of perchlorate.

5. Deformaties in *Rana pipiens* will be dose related and will increase according to dose.

To address these hypotheses the research was conducted in two parts. An initial study, *Effects of Perchlorate on Development and Metamorphosis in Northern Leopard Frogs: Las Vegas Wash Water,* was conducted June 24, 2002 until October 21, 2002. This was followed by a secondary study *Effects of Perchlorate on Development and Metamorphosis in Northern Leopard Frogs: Laboratory Mixed Perchlorate Water* conducted March 10, 2003 until October 27, 2003 . There were slight modifications that were made to the second study that utilized the information from the preliminary study;

however, the methods are discussed separately in order to avoid confusion.

Preliminary Study: Las Vegas Wash Water

Animals

A total of 215 Northern Leopard Frog tadpoles (*Rana pipiens*) equivalent to stages 0-1 according to the TKI were purchased from Trans-Mississippi Biological Supply, St. Paul, Minnesota. Northern Leopard Frog tadpoles were separated into four dose groups of fifty-two each, and 7 animals were sacrificed for chemical analysis prior to dosing. Animals were held in 20 gallon aquaria containing 22 ± 2 degree Celsius water which was changed weekly (for protocol, refer to Appendix A), aerated and subject to a 12 hour light: dark cycle. The University of Nevada, Las Vegas (UNLV) has a certified American Association for Laboratory Animal Care (AALAC) facility and the appropriate animal use and care protocols (UNLV Protocol No. R993-0502-166) were submitted and approved prior to the beginning of this project. The preliminary Las Vegas Wash water experiment began when the tadpoles arrived on June 30, 2002 and ended on October 21, 2002 when the vast majority of the tadpoles had undergone metamorphosis. Tadpoles were fed powdered frog brittle (NASCO, Ft. Atkinson, WI, USA) three times a week. A sample of frog brittle was removed at the beginning and end of the project, frozen and shipped to Clayton Laboratories (Novi, MI) for iodine and perchlorate analysis.

The animals were sacrificed when metamorphosis was near completion (Taylor Kollros stages 22-24). The animals were euthanized by immersion in 3- aminobenzioc acid ethyl ester (MS-222 1g/L in distilled water). The animals were kept until

metamorphosis was complete or they died. Tail length, snout to vent length, right and left hindlimb length, right and left forelimb length and weight were taken after the animals were euthansized. Days to complete metamorphosis were noted in order to address the second hypothesis. The fourth hypothesis was answered by checking the tadpoles daily for mortality.

Water Collection and Water Analysis

Water samples were collected once every two weeks from the Las Vegas Wash at monitoring site LM-8 (N 36' 36'35.3", W 114' 14'.28.3") (Fig. 1) and Overton Beach (N 36' 26' 39.3", W 114' 20' 52.8") at Lake Mead (control location). The LM-8 site is known to have concentrations of approximately 439 ± 198 ppb of perchlorate (Joseph Leising, Personal Communication), while the Overton site is below the detection limit of 4 ppb and thus assumed to be at or very near to zero. Following water collection, a 1000 ml sample of water was shipped to Clayton Laboratories packed in a cooler of ice, where the exact concentration of perchlorate and iodide in the water was determined. After receiving the results of the initial water tests, the concentrations of perchlorate in each Las Vegas Wash sample were diluted with Overton Beach water to approximate 0, 4, 40, and 400 μg/L. Once the respective tanks were diluted, a 1000 ml sample of water was obtained and sent to Clayton Laboratories to determine exact concentrations of perchlorate and iodide in each tank (started 18 June 2002). In order to accurately assess the potential environmental impacts of perchlorate on development and metamorphosis of *Rana pipiens*, environmental concentrations of perchlorate were allowed to fluctuate within one standard deviation (439 ± 198 ppb) of the target range. The protocol was

designed to mimic the environmental conditions and natural fluctuations that occur in the Las Vegas Wash. Thus, the two lower dose ranges were also allowed to fluctuate with the perchlorate fluctuations that occur in the Wash; however, these were directly proportional to the concentration of the highest dose.

Water was changed weekly in accordance the project protocol. The water was allowed to acclimate to room temperatures for a minimum of twelve hours before the tanks were changed. Any additional water collected but not immediately used, was place into capped, 5-gallon buckets and stored at 4° C until needed. No water was held longer than two weeks, or allowed to stay in a tank for more than one week.

The water used for the control animals was acquired from Overton Beach at Lake Mead, well upstream from the inputs of the Las Vegas Wash, using the same methods described above. The Overton water was used as a control and also used to dilute the Las Vegas Wash samples to achieve the desired concentrations listed above.

Five gallon buckets were filled from Overton Beach water and labeled "O" for use in the control tank and in dilution of the low perchlorate doses. Five gallon buckets were filled from Las Vegas Wash site LM-8 and labeled "W," and used as the high dose.

From June 20 to July 1, 2002, an Orion Research 811 meter was used to determine pH, an Orion Research 810 meter was used to determine dissolved oxygen, and an Orion Research 115 meter was used for conductivity and temperature. From July 2, 2002 to the end of both experiments, conductivity, pH, temperature, and percent dissolved oxygen were calculated daily using YSI model 63 and YSI model 85 digital meter for each tank (for protocol, refer to Appendix A).

Oxygen Analyzer

Oxygen consumption was performed in order to determine the metabolic rate of tadpoles by monitoring their total oxygen consumption, which addresses the third hypothesis. Total oxygen consumption provides an accurate measure of metabolic rate, which is related to thyroid hormones (Warren, 1940). Once a month, 10 tadpoles from each dose group were tested. Two chambers were set up with 5 animals from the same dose group in each chamber for month 1, 2, 3, and 4 however, in month 5 the analysis was performed with only 2 animals per chamber owing to the fact most of the tadpoles had already undergone metamorphosis. The Sable System's model ReadOx-4 was used for all measurements and recorded with DATACAN software, also from Sable Systems.

The first step in preparing the oxygen analyzer was to assemble the electrodes by covering the electrodes with 0.001" or 0.0009" thick polyethylene membrane and placing the electrode into de-ionized water for calibration. Sodium dithionite is used to zero the equipment by removing all of the oxygen from the de-ionized water that sodium dithionite is dissolved in. The channels were then spanned back to 150 mm Hg because this takes into account 720 mm Hg at Las Vegas's elevation and that air consists of 20% oxygen or approximately 150 mm Hg. The animals were then placed in the chamber filled with water from the appropriate dose group and the electrode was connected. The acquisition program was set for 30 minutes to obtain the data points.

Oxygen consumption (VO_2) data were calculated from the slope of the regression line fit to data points showing the decline in PO_2 over time. The ambient air temperature and barometer pressure remained relatively constant around 23 degrees Celsius and 0.95 mb.

Using a table of oxygen solubility in fresh water at 23 degrees Celsius, the dissolved oxygen is 8.56 mg/L. The conversion factor of 0.7 was used because we needed to convert from mg/L to ml/L. The elevation in Las Vegas changes the standard of 1 atm to 0.95 atm, which is used in the following formula to obtain the saturation point of oxygen.

$$(8.56 \text{ mg } O_2/\text{ L } H_2O) \text{ X } (0.7 \text{ ml } O_2/\text{ L } H_2O) \text{ X } (0.95 \text{ mb}) = 5.69 \text{ ml } O_2/\text{L}$$

The chamber volume remained constant at 0.5 L and the PO_2 at sea level is 159 but was altitude corrected to 151. The following calculation was used calculate VO_2/min.

$$\frac{(\text{Sat}[O_2] \text{ X Chamber Volume})}{PO_2} \text{ X (Slope) X (1000)} = VO_2/\text{minute}$$

This number was then multiplied by 60 minutes to determine the VO_2/hour which was multiplied by the mass of the animals in the chamber to get the final computation of VO_2/hour or the milliliters of dissolved oxygen per gram hours.

Developmental Assessments

Once per month, an assessment of metamorphosis was completed on the tadpoles according to normal stages of *Rana pipiens* development as defined by Taylor, and Kollros, (1946). Ten tadpoles from each tank were examined once a month and were euthanized by immersion in 3- aminobenzioc acid ethyl ester (MS-222 1g/L in distilled water). After they were euthanized, the tadpoles were placed under a microscope and the TKI was used to determine the development stage of each animal. In order to compare the frequency of the number of animals at each stage within the different dose groups, the stages were combined to perform a log-likelihood ratio or G-statistic for independence. A G-statistic uses frequency data comparing the expected (control dose group) to that of the observed data from the other dose groups. In order to perform this statistical analysis, the

assumption was made that the tadpoles were chosen in a random manner and that there was independence of these observations (Kinnear & Gray, 2000). Unlike any of the other statistical tests performed, a G-statistic does not require normal distributed but it does require symmetry of distribution (Kinnear & Gray, 2000). The G-statistic was used rather than a Chi-Square because of the small frequencies per cell (less than 5) (Kinnear & Gray, 2000). The G-statistic was manually calculated according to Zar (1999). Significance was noted if $p < 0.05$.

The second developmental assessment were the weekly measurements where twenty tadpoles were measured from each dose group each week. Each tadpole's tail length, body length, snout to vent length, total body length and hindlimb length (if applicable) were determined. The tadpoles were measured using an electronic caliper. The first hypothesis states that *R. pipiens* exposed to high concentrations of perchlorate will have reduced body size characteristics. This hypothesis was tested by performing an ANOVA with a Tukey's post hoc on the weekly measurements. This analysis was performed in SPSS (Version 11.5 for Windows). There was an overall significance between the dose groups when $p < 0.05$ on the overall ANOVA, and significance between the dose groups was noted in the Tukey's post hoc when $p < 0.05$.

In order to comply with the assumptions of an ANOVA, normality was first assessed using the Shapiro-Wilk statistic (calculated in SPSS); this was used because the sample size was less than 200. The data were considered normal if $p > 0.05$. However, ANOVA is robust with respect to the assumption of the underlying populations' normality and the validity of the analysis is affected only slightly by even considerable deviations from

normality (Zar, 1999). The two other assumptions that must be made are independence and homoscedasticity (homogeneity of variances). Homoscedasticity was checked by a visual inspection of the stem and leaf plots of the data. If these data do not meet the assumptions, non-parametric tests such as Kruskal-Wallis and Mann-Whitney were performed.

Weekly, all of the tadpoles were monitored for one of the three types of deformatities, including: edema, bent tail, and abnormal swimming as described by Golemen *et al.* (2002). These deformities were tracked in order to address the fifth hypothesis. Monitoring for these deformities will indicate if the deformities are dose related and if the frequency increases according to dose.

Secondary Study: Laboratory Mixed Perchlorate Water

Owing to the development of a parasite (See Fig. 9) during the fifth week of the preliminary study involving the use of Las Vegas Wash water, we determined a second study was necessary. (See results for a further discussion of the parasite). Slight modifications in the procedure were made in order to improve the experimental design; the differences from the pilot study are discussed below.

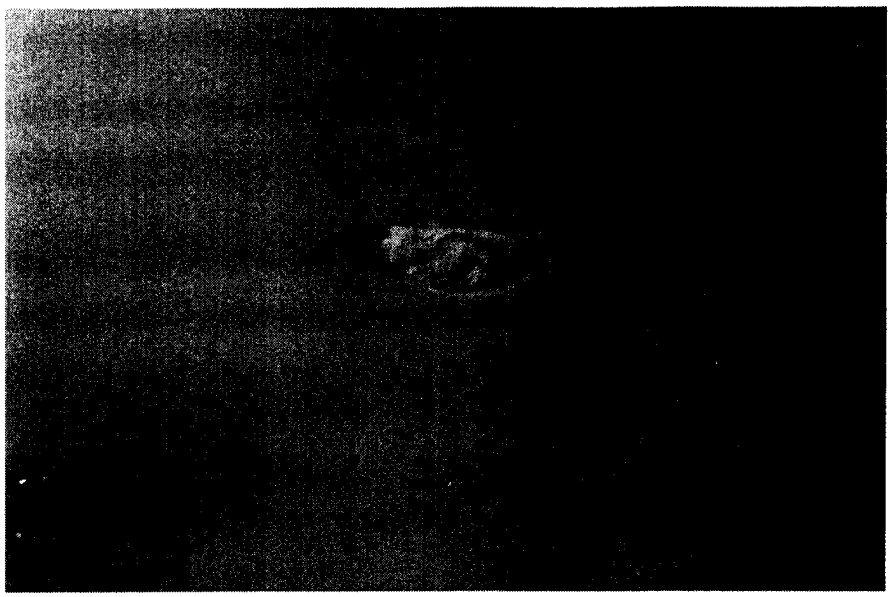


Figure 9 *Dactylogyrus vastator* as Identified on the 4, 400 ppb Groups in the Preliminary Study Using Las Vegas Wash Water.

Animals

The second experiment, *Effects of Perchlorate on Development and Metamorphosis in Northern Leopard Frogs: Laboratory Mixed Perchlorate Water*, began on March 10, 2003 with 114 *Rana pipiens* in the magnesium control tank and 115 animals in the control, 40 ppb, 400 ppb and 4,000 ppb dose groups. The *Rana pipiens* for this study were also obtained from Trans-Mississippi as in the preliminary Las Vegas Wash experiment

Water Collection and Water Analysis

During the second experiment we eliminated the Las Vegas Wash water and instead mixed de-iodized water with magnesium perchlorate (Fisher Chemical, reagent grade anhydrous, CAS 10034-81-8, Lot No. 935896) in the laboratory. We included one higher dose group and eliminated the lowest dose groups; resulting in 40 ppb, 400 ppb, and 4000

ppb dose groups. Two control groups were included, a standard control group and a magnesium control group. De-ionized water had to be used instead of tap water because the local tap water contains 4 ppb of perchlorate. Sodium chloride, (VWR Scientific Products, reagent grade crystals, CAS 7647-14-5), in the amount of 4.3 grams/ 80 Liters, was added to all of the dose groups in order to replace some ions that the de-ionized water removed. The magnesium in the magnesium control tank was added in the form of magnesium chloride (J.T. Baker Chemical Co., CAS 7786-30-3, Lot No. 45130) to the same proportion of magnesium available in the 4000 ppb dose group (30 mg or 0.375 ppm of magnesium). Water samples were obtained at the beginning of the experiment to confirm that the dose groups were as expected.

Water analysis (pH, conductivity, temperature, and dissolved oxygen) was completed on each tank prior to weekly tank change and post tank change. The same equipment was used to perform these measurements as in the preliminary study used.

Oxygen Analyzer

The same procedure was used to perform the oxygen consumption analysis. We selected the "best" run from the 3 or 4 trials that were performed on each tank from each month. The selection ideology was to pick the set of data points with the best r^2, or coefficient of determination value. The data set that met this condition had data points that best conformed to a straight line with few, or no, dips in oxygen consumed. These declines typically occurred when the tadpoles came in contact with the electrode in the chamber. The slope of the line created from these data points was then used to determine which doses consumed more or less oxygen. However, the slope was not considered

during the selection of which data was to be considered the "best" only the coefficient of determination (r^2) was used. If more than one repetition produced the same r^2, the first run at that value was chosen. These chosen data points were then used (approximately 640 per run) in order to compare the slopes to the other groups. We used an F-statistic in order to test for significant difference among slopes of the multiple regression lines. Significance indicated where F is < 0.05. If there was an overall significance, a comparison between the groups was performed to determine where the difference between the dose groups was occurring. If the post hoc did not identify any significance, a comparison of the elevations was then completed (Zar, 1999).

The weight of the animals being tested in the chamber directly relates to the amount of oxygen that they will consume. The VO_2/gram hours takes into account solubility of the water and the animal weight. In order to perform a comparison from our "best" run we adjusted the data points based on the total weight of the animals (typically 5 per chamber). If the adjusted data were determined in SPSS not to be normal by the Shapiro-Wilks method, every effort was made to transform the data. Once the data were weight adjusted, we ran the same F statistic to determine significance between the slopes.

Developmental Assessments

The preliminary Las Vegas Wash water experiment used 10 animals per month to perform the TKI, during the second round we used 20 animals in order to have a larger sample size. The G-statistic was manually calculated as before (Zar, 1999). Significance was noted if $p < 0.05$.

Sample size was also increased for weekly measurements, by measuring 40 animals per tank each week to obtain the developmental landmarks (snout to vent, hindlimb length, tail length, and total body length). As before, deformities of edema, abnormal swimming, or bent tail were noted. Measurements of both the right and left hindlimb were taken to check for asymmetrical development in the tadpoles. Performing a paired t-test in SPSS allowed us to complete this analysis, significance was noted when $p < 0.05$. If the normality assumption were not met for the t-test a Wilcoxon signed ranks test were performed to assess the symmetrical development of the hindlimbs.

A second ANOVA was completed in SPSS comparing the developmental landmarks on the tadpoles at the day of metamorphosis. Performing this analysis allowed us to compare the final measurements on the tadpoles when they were scarified at TKI stage 22-24.

CHAPTER 4

RESULTS

Preliminary Study: Las Vegas Wash Water

During the fifth week, July 22, 2002, we noticed that all of the animals in the 400 ppb had developed a parasite, and the group died during week 5 of the experiment. The parasite was later identified as *Dactylogyrus vastator*. The low dose group (4 ppb) also experienced numerous deaths starting on September 5, these deaths were later attributed to the presense the same parasite. The control and 40 ppb groups were unaffected by the parasite and completed their development and were sacrificed on October 21, 2002.

The TKI stages were only different during the first month of the experiment with the control group being larger than 4 ppb and 400 ppb (See Fig. 7, Appendix A). The 40 ppb group's hindlimb length was significantly larger than the 40 ppb group during week 11. During weeks 15 and 16, the 40 ppb group had significantly longer hindlimbs than the control group. The snout to vent length remained fairly consistent between the dose groups throughout the experiment. However, there was a significant difference in the snout to vent length between groups during week one of the experiment, as the control group was significantly larger at the start of the study. Major differences were noted during week 1 of the experiment between the control group and 4 ppb group and 400 ppb group snout to vent length. During week three, significant differences in the snout to

vent length were noted between the 4 ppb and 400 ppb group when compared to the 40 ppb group. The total body lengths of the tadpoles were similar to the snout to vent results, with a steady consistent gain between dose groups. There were statistical differences in the total body length during the first three weeks of the study, these differences were noted between the control group and 4 ppb group and 400 ppb group during the first week as well as during the second week (Appendix A, Fig. 13-16). Week three also presented differences in the total body length with the control group being larger than the 4 ppb group. The tadpole tail length was consistent with the previous developmental trends seen in the hindlimb, snout to vent and total body measurements, as there was only a significant difference in the tail length during the first three weeks of the study. All effects seen in the snout to vent, total body length and tail length were attenuated by the end of the third week.

Secondary Study: Laboratory Mixed Perchlorate Water

Water chemistry measurements of dissolved oxygen, pH, temperature, and conductivity were taken twice a week, one before and one following weekly water changes. Water chemistry weeks do not necessarily correspond with the morphometric characteristic weeks that are discussed in detail hereafter. The water chemistry data were not normally distributed therefore we used a Kruskal-Wallis nonparametric statistic to analyze ranks in the water chemistry measurements between the tanks. This analysis was performed in order to assure that the water quality was not a variable in the experiment. No statistical significance in any of the variables (pH, temperature,

conductivity, dissolved oxygen) was found between any of the groups with an overall U=7.552 and p=0.109. See Appendix E for graphs of weekly water quality analysis.

Survival

The second study involving laboratory mixed perchlorate concentrations began on March 10, 2003 and ended on October 27, 2003. Table 2 illustrates the number of tadpoles that were alive each week. Numbers had to be adjusted for a few weeks due to difficulties encountered while counting such small animals. During the first week of the experiment, malfunctioning water filters caused serve deaths in the magnesium, 40 ppb, and the 4000 ppb tanks. After the problem was identified, all of the tanks were switched to bubble stones to alleviate this problem. During week 8, 20 animals from the control, 400 ppb and 4000 ppb group were scarified for tissue analysis and the number of animals were similar in range (see Table 4).

Table 2 and 3 also show that the magnesium group completed metamorphosis in week 10 while 40 ppb and 4000 ppb group completed metamorphosis during the 21st week. The control and the 400 ppb group completed metamorphosis the slowest. Table 3 (b) shows weekly and cumulative percents of tadpoles that completed metamorphosis. The percentage was calculated by subtracting out unnatural mortality from sacrifices and the filter problems discussed above. The magnesium control group had the highest percent of successful metamorphosis completion at 89%. The next two groups, 40 ppb and 4000 ppb, to complete metamorphosis had a success of 73% and 40%, respectively. The last two groups to finish metamorphosis, control and 4000 ppb, had 31% and 50% completion, respectively.

Table 5 (a-e) contains the three deformities that were tracked during the experiment, bent tail, edema and abnormal swimming. Only a weekly deformity percent could be calculated because we did not track individual animals, thus we had no way of knowing if a particular deformity was a new case or a carry over from the previous week. The "% Deformities" column is a total percent of deformities, and was calculated by adding together the number of animals in each of the three categories of deformities.

Table 2 Weekly Number of Rana pipiens Known Alive Following Perchlorate Exposure

Week	Mg Control	Control	40 ppb	400 ppb	4000 ppb
1	114	115	115	115	115
2	62	102	40	111	85
3	18	102	40	*111**	*80**
4	18	*91**	*40**	106	80
5	18	91	*40**	104	77
6	17	83	40	101	69
7	15	79	39	90	67
8	13	59	*39**	62	40
9	9	*57**	36	55	40
10	8	57	26	52	35
11		53	20	47	31
12		44	15	44	29
13		*44**	13	41	24
14		39	11	38	21
15		39	11	35	19
16		38	11	*35^*	*19^*
17		37	9	40	11
18		37	9	39	11
19		34	7	35	8
20		33	4	32	6
21		23	2	25	4
22		18		22	
23		18		22	
24		16		22	
25		13		19	
26		12		18	
27		12		17	
28		11		13	
29		10		12	
30		7		11	
31		6		11	
32		5		7	
33		5		7	

*Adjusted numbers based on pre and post week numbers
^Five tadpoles from 400ppb were mistakenly put into 400ppb

Table 3 Number of Rana pipiens Successfully Completing Metamorphosis in Each Perchlorate Dose Group per Week

(a) Week of Completion

Week	Mg Control	Control	40 ppb	400 ppb	4000 ppb
1					
2					
3					
4					
5					
6	1	0	0	0	0
7	1	0	0	0	0
8	2	0	2	0	0
9	4	0	0	0	0
10	1	0	4	2	3
11	7	0	5	2	2
12		2	5	2	3
13		1	2	1	1
14		3	2	1	3
15		0	0	3	1
16		1	1	0	0
17		0	1	3	1
18		1	2	1	2
19		1	1	6	2
20		3	1	2	2
21		4	2	4	1
22		0	0	0	0
23		1		0	
24		0		2	
25		2		0	
26		0		1	
27		0		1	
28		1		3	
29		1		0	
30		3		0	
31		0		1	
32		1		3	
33		0		0	
34		2		1	

Table 3 Number of *Rana pipiens* Successfully Completing Metamorphosis in Each Perchlorate Dose Group per Week

(b) Weekly and Cumulative Percentages

	Weekly	Cumulative	Weekly	Cumulative	Weekly	Cumulative	Weekly	Cumulative	Weekly	Cumulative
Week	Mg Control		Control		40 ppb		400 ppb		4000 ppb	
6	5.6	5.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7	5.6	11.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8	11.1	22.2	0.0	0.0	5.3	0.1	0.0	0.0	0.0	0.0
9	22.2	44.4	0.0	0.0	0.0	5.3	0.0	0.0	0.0	0.0
10	5.6	50.0	0.0	0.0	10.5	15.8	2.6	0.0	5.8	0.1
11	38.9	88.9	0.0	0.0	13.2	28.9	2.6	5.3	3.8	9.6
12			2.5	0.0	13.2	42.1	2.6	7.9	5.8	15.4
13			1.3	3.8	5.3	47.4	1.3	9.2	1.9	17.3
14			3.8	7.6	5.3	52.6	1.3	10.5	5.8	23.1
15			0.0	7.6	0.0	52.6	3.9	14.5	1.9	25.0
16			1.3	8.9	2.6	55.3	0.0	14.5	0.0	25.0
17			0.0	8.9	2.6	57.9	3.9	18.4	1.9	26.9
18			1.3	10.1	5.3	63.2	1.3	19.7	3.8	30.8
19			1.3	11.4	2.6	65.8	7.9	27.6	3.8	34.6
20			3.8	15.2	2.6	68.4	2.6	30.3	3.8	38.5
21			5.1	20.3	5.3	73.7	5.3	35.5	1.9	40.4
22			0.0	20.3	0.0	73.7	0.0	35.5	0.0	40.4
23			1.3	21.5			0.0	35.5		
24			0.0	21.5			2.6	38.2		
25			2.5	24.1			0.0	38.2		
26			0.0	24.1			1.3	39.5		
27			0.0	24.1			1.3	40.8		
28			1.3	25.3			3.9	44.7		
29			1.3	26.6			0.0	44.7		
30			3.8	30.4			0.0	44.7		
31			0.0	30.4			1.3	46.1		
32			1.3	31.6			3.9	50.0		
33			0.0	31.6			0.0	50.0		

Table 4 Weekly Number of *Rana pipiens* Found Dead in Tanks

Week	Mg Control	Control	40 ppb	400 ppb	4000 ppb
1	0	1	0	0	0
2	(52#)	0	3 (30#)	0	1
3	(44#)	(13#)	(42#)	(13#)	(39#)
4	0	0	0	0	0
5	0	0	0	0	0
6	0	0	0	0	0
7	0	0	0	0	0
8	0	(20*)	1	(20*)	(20*)
9	0	0	0	0	0
10	0	0	0	0	0
11		0	1	0	2
12		0	0	0	0
13		0	0	0	0
14		0	0	0	0
15		0	0	0	0
16		0	0	0	0
17		0	0	0	0
18		0	0	0	0
19		0	0	0	0
20		1	1	0	0
21		1	0	2	0
22		0	(2^)	0	(4^)
23		0		0	
24		0		0	
25		0		0	
26		0		0	
27		0		0	
28		0		1	
29		0		1	
30		0		0	
31		0		0	
32		0		0	
33		0		0	

* 20 tadpoles from the Control, 400 ppb and 4000 ppb tanks were sacrificed; # Mortaility related to filter malfunction; ^ Final tank sacrifice

Table 5 Weekly Cumulative Deformities in *Rana pipiens* Exposed to Perchlorate
(a) Control Group

Week	Bent Tail	Edema	Abnormal Swimming	# of Tadpoles	% Deformities
1	0	0	0	115	0
2	0	0	0	102	0
3	0	0	0	102	0
4	1	0	0	91	1
5	1	0	0	91	1
6	1	0	1	83	2
7	1	0	0	79	1
8	2	0	0	59	3
9	1	0	0	57	2
10	3	0	1	57	7
11	1	0	0	53	2
12	6	5	4	44	34
13	14	5	1	44	45
14	11	12	2	39	64
15	8	2	1	39	28
16	10	8	4	38	58
17	10	8	4	37	59
18	4	3	2	37	24
19	1	3	2	34	18
20	1	7	2	33	30
21	1	1	0	23	9
22	1	1	0	18	11
23	0	6	0	18	33
24	0	3	1	16	25
25	1	1	1	13	23
26	1	2	0	12	25
27	0	3	0	12	25
28	0	2	1	11	27
29	0	2	0	10	20
30	0	2	0	7	29
31	0	1	0	6	17
32	0	1	0	5	20
33	0	1	0	5	20

Table 5 Weekly Cumulative Deformities in *Rana pipiens* Exposed to Perchlorate

(b) Mg Deformaties

Week	Bent Tail	Edema	Abnormal Swimming	# of Tadpoles	% Deformity
1	0	0	0	114	0
2	0	0	0	62	0
3	0	0	1	18	6
4	0	0	0	18	0
5	0	0	0	18	0
6	0	0	1	17	6
7	1	0	1	15	13
8	1	0	1	13	15
9	0	0	0	9	0
10	0	1	0	8	13

Table 5 Weekly Cumulative Deformities in *Rana pipiens* Exposed to Perchlorate

(c) 40 ppb Group

Week	Bent Tail	Edema	Abnormal Swimming	# of Tadpoles	% Deformities
1	0	0	0	115	0
2	0	0	1	40	3
3	0	0	0	40	0
4	0	0	0	40	0
5	1	0	0	40	3
6	2	0	0	40	5
7	0	0	0	39	0
8	0	1	1	39	5
9	1	0	1	36	6
10	1	0	0	26	4
11	2	2	0	20	20
12	2	2	3	15	47
13	2	0	0	13	15
14	2	2	1	11	45
15	1	1	0	11	18
16	2	2	1	11	45
17	2	2	1	9	56
18	0	2	1	9	33
19	0	1	1	7	29
20	0	0	0	4	0
21	0	0	0	2	0

Table 5 Weekly Cumulative Deformities in *Rana pipiens* Exposed to Perchlorate

(d) 400 ppb Group

Week	Bent Tail	Edema	Abnormal Swimming	# of Tadpoles	% Deformities
1	0	0	0	115	0
2	0	0	1	111	1
3	0	0	0	111	0
4	0	0	0	106	0
5	1	0	0	104	1
6	2	0	0	101	2
7	0	0	0	90	0
8	0	1	1	62	3
9	1	0	1	55	4
10	1	0	0	52	2
11	2	2	0	47	9
12	2	2	3	44	16
13	2	0	0	41	5
14	2	2	1	38	13
15	1	1	0	35	6
16	2	2	1	35	14
17	2	2	1	40	13
18	0	2	1	39	8
19	0	1	1	35	6
20	0	0	0	32	0
21	0	0	0	25	0
22	1	1	0	22	9
23	0	6	0	22	27
24	0	3	1	22	18
25	1	1	1	19	16
26	1	2	0	18	17
27	0	3	0	17	18
28	0	2	1	13	23
29	0	2	0	12	17
30	0	2	0	11	18
31	0	1	0	11	9
32	0	1	0	7	14
33	0	1	0	7	14

Table 5 Weekly Cumulative Deformities in *Rana pipiens* Exposed to Perchlorate

(e) 4000 ppb Group

Week	Bent Tail	Edema	Abnormal Swimming	# of Tadpoles	% Deformities
1	0	0	0	115	0
2	0	0	0	85	0
3	0	0	0	80	0
4	0	0	0	80	0
5	0	0	0	77	0
6	4	0	2	69	9
7	1	0	2	67	4
8	0	0	0	40	0
9	0	0	0	40	0
10	1	0	0	35	3
11	2	0	1	31	10
12	4	5	3	29	41
13	1	2	0	24	13
14	3	11	1	21	71
15	8	10	1	19	100
16	5	6	0	19	58
17	5	6	0	11	100
18	0	2	0	11	18
19	0	3	0	8	38
20	0	2	0	6	33
21	0	3	0	4	75

Oxygen Analyzer

The preliminary study involving Las Vegas Wash water allowed for modifications to be made to the oxygen analyzer during the laboratory mixed water experiment. One of the problems we identified was temperature fluctuations and accurate electrode performance is temperature dependent. In order to address this problem a water bath was used during each month's experiment in order to decrease temperature fluctuations

which often occur in climate-controlled buildings. A smaller stir bar was also used during the secondary study involving laboratory mixed perchlorate concentrations because sick or weak tadpoles can get caught in the swirling vortex of the oxygen consumption chamber thereby causing inaccurate oxygen consumption numbers.

The oxygen consumption we performed was routine metabolic rate. Routine metabolic rate is different from standard metabolic rate which takes place at a specific temperature and measures minimum activity. Routine metabolic rate has an additional source of error due to spontaneous activity that is recorded. The following table provides the slope equations and the volume of oxygen consumed for one repetition for each dose group each month. The Methods and Materials section describes the actual equations that were used for the VO_2 calculations as well as the method used to select one of the repetitions.

Table 6 Volume of Oxygen Consumed (grams per hour) for *Rana pipiens* Following Exposure to Perchlorate

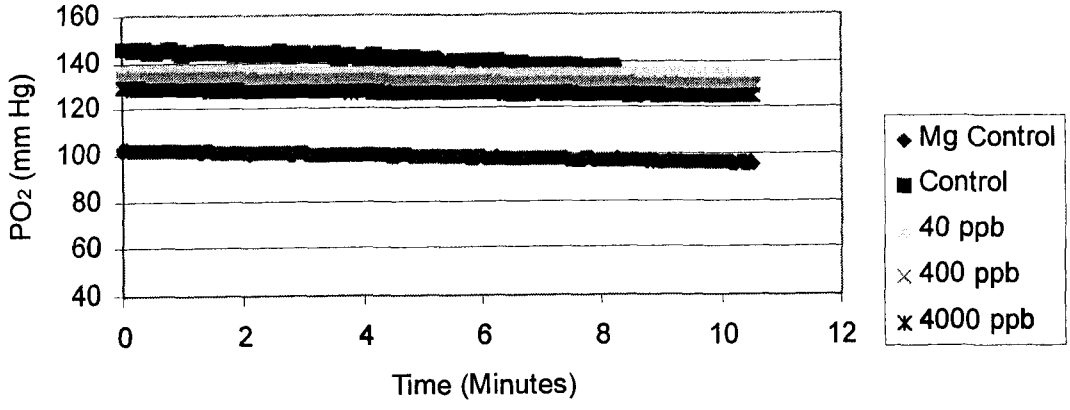
Month	Concentration	Slope Equation	R^2	Mass (g)	VO_2 (g/hr)
0	Mg	Y= -.00611x+1.02	0.99	2.59	2.67
	Control	Y= -.0055x+0.99	0.99	1.27	4.9
	40 ppb	Y= -.00236x+1.36	0.98	1.73	1.54
	400 ppb	Y= -.00422x+1.34	0.99	1.79	2.67
	4000 ppb	Y= -.0038x+1.28	0.97	1.78	2.41
1	Mg	Y=-.0270x+1.08	0.99	11.1	2.75
	Control	Y=-.0072x+1.18	0.97	3.07	2.64
	40 ppb	Y=-.0103x+1.07	0.98	8.01	1.45
	400 ppb	Y=-.0045x+0.81	0.83	4.49	1.13
	4000 ppb	Y=-.0061x+0.92	0.98	4.5	1.52
2	Mg	Y=-.018x+0.64	0.99	14.27	1.82
	Control	Y=-.014x+1.13	0.93	6.69	2.37
	40 ppb	Y=-.015x+1.07	0.99	11.04	1.54
	400 ppb	Y=-.0054x+1.12	0.94	4.71	1.3
	4000 ppb	Y=-.0069x+0.86	0.95	6.82	1.14
3	Control	Y=-.0086x+1.17	0.89	7.2	1.35
	40 ppb	Y=-.011x+0.883	0.98	11.43	1.09
	400 ppb	Y=-.004x+1.09	0.81	6.12	0.74
	4000 ppb	Y=-.0086x+0.95	0.96	10.57	0.92
4	Control	Y=-.013x+1.11	0.90	11.97	1.23
	40 ppb	Y=-.0295x+1.20	0.99	18.41	1.81
	400 ppb	Y=-.00771x+1.16	0.78	11.61	0.75
	4000 ppb	Y=-.0074x+0.85	0.89	12.74	0.62
5	Control	Y=-0.0149x+1.12	0.99	8.7	1.94
	400 ppb	Y=-0.00740x+1.04	0.97	10.9	0.77

In Month 0 the magnesium control group had a steeper slope and therefore consumed more oxygen than all of the other tanks. The control group also consumed more oxygen than the 4000 ppb group.

Once again during Month 1 the accelerated development of the magnesium control group resulted in the tadpoles consuming more oxygen than the control and 40 ppb group. The control and 40 ppb group consumed more oxygen than the 400 ppb and 4000 ppb groups. This is consistent with our hypothesis of the lower dose groups consuming

more oxygen than the higher dose groups. The results from the second month show that the magnesium group consumed more oxygen than all of the dose groups just like the results from Month 0. The control, 40 ppb and 400 ppb groups all consumed more oxygen than the 4000 ppb group. The magnesium control group is no longer a part of the analysis during Month 3 because all of the animals have completed metamorphosis. The control and 4000 ppb group both consumed more oxygen than the 400 ppb dose group during the third month. During the third month the 40 ppb dose group also consumed more oxygen than the control group. The results of Month 4 show the control and 40 ppb consumed more oxygen than the 4000 ppb and the 40 ppb also consumed more oxygen than the 400 ppb group. This month is opposite from Month 3 with the 400 ppb consuming more oxygen than 4000 ppb. The final two dose groups, control and 400 ppb, have no significant difference between their slopes during month 5.

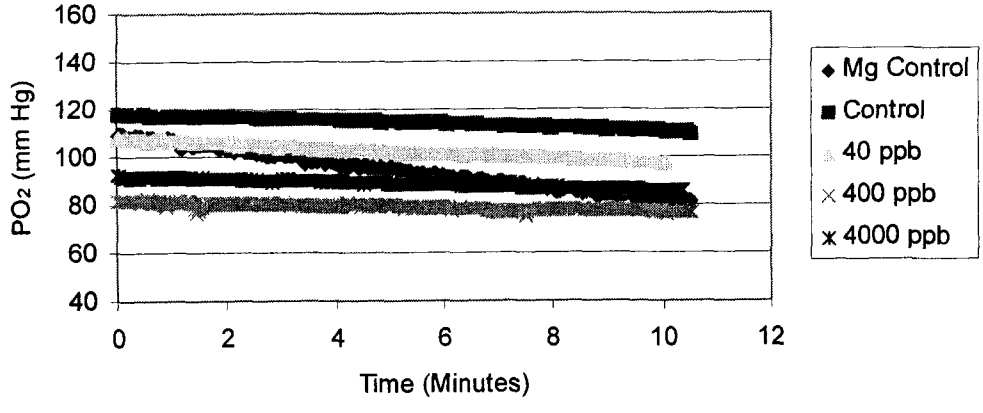
(a) Month 0



Month 0 Slope Equations:
Mg y = -0.0061x + 1.02, Control y = -0.0055x + 0.99, 40 ppb y = -0.0024x + 1.36, 400 ppb y = -0.0422x + 1.34, 4000 ppb y = -0.0038x + 1.28

Overall significance $p<0.001$. Multiple comparison; Mg v. Control, 40 ppb, 400 ppb, 4000 ppb; Control v. 4000 ppb

(b) Month 1



Month 1 Slope Equations
Mg y = -0.027x + 1.08, Control y = -0.0073x + 1.18, 40ppb y = -0.0103x + 1.07, 400 ppb y = -0.0045x + 0.81, 4000ppb y = -0.0061x + 0.92

Overall significance $p<0.001$. Multiple comparison; Mg v. Control, 40 ppb; Control v. 400ppb, 4000ppb; 40 ppb v. 400 ppb, 4000ppb.

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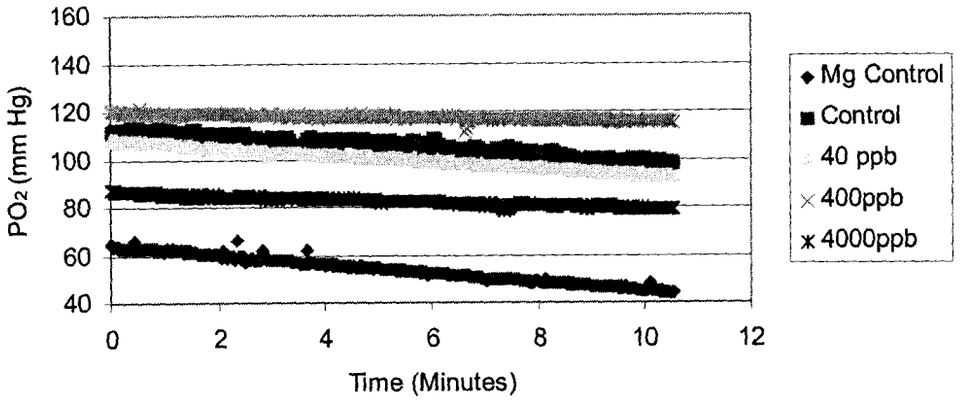
Figure 10 Oxygen Consumption Rate in *Rana pipiens* Exposed to Perchlorate

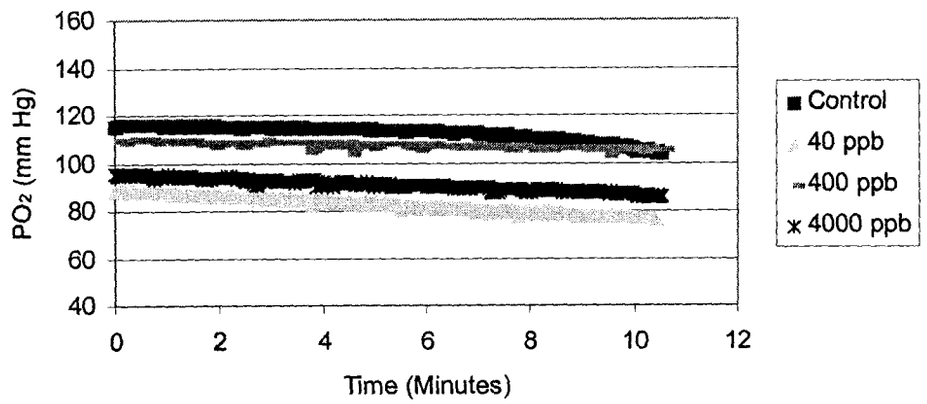
(c) Month 2



Month 2 Slope Equations
Mg y = -0.0180x + 0.64, Control y = -0.0140x + 0.13, 40 ppb y = -0.015x + 1.07, 400 ppb -0.0054x + 1.12, 4000 ppb: -0.0069x + 0.86

Overall significance $p<0.001$. Multiple Comparison; Mg v. Control, 40 ppb, 400 ppb, 4000ppb; Control v. 4000 ppb; 40 ppb v. 4000ppb; 400 ppb v. 4000 ppb

(d) Month 3

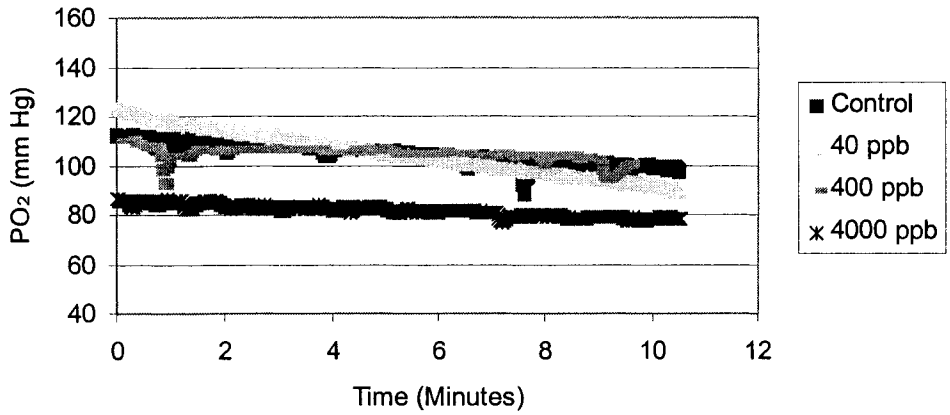


Month 3 Slope Equations
Control y = -0.0086x + 1.17, 40 ppb y = -0.011 x + 1.11, 400 ppb y = -0.0040x + 1.09, 4000ppb y = -0.0086x + 0.95

Overall significance $p<0.001$. Multiple Comparison; Control v. 40 ppb, 4000ppb; 400 ppb v. 4000 ppb

Figure 10 Oxygen Consumption Rate in *Rana pipiens* Exposed to Perchlorate

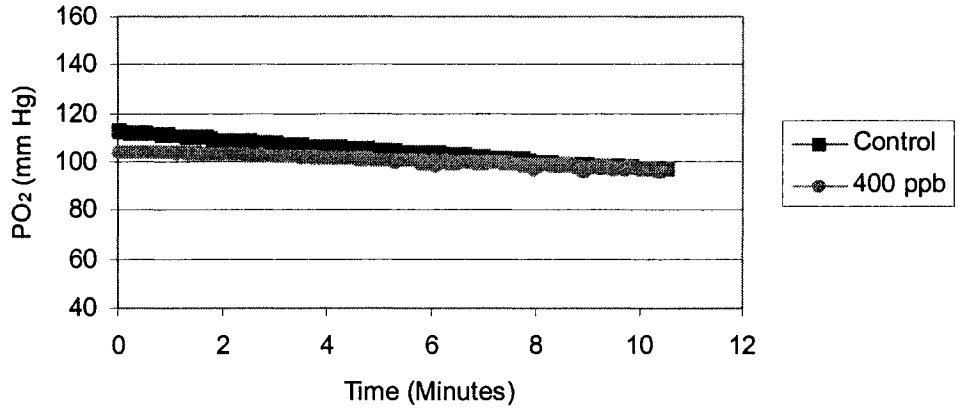
(e) Month 4



Month 4 Slope Equations
Control y = -0.0130x + 1.11, 40 ppb y = -0.0295x + 1.20, 400 ppb y = -0.0771x + 1.16, 4000 ppb y = -0.0074x + 0.85

Overall significance $p<0.001$. Multiple Comparison; Control v. 4000 ppb; 40 ppb v. 400 ppb, 4000 ppb; 400 ppb v. 4000 ppb

(f) Month 5



Month 5 Slope Equations
Control y = -0.0149x + 1.12, 400 ppb y = -0.0074x + 1.04

Figure 10 Oxygen Consumption Rate in *Rana pipiens* Exposed to Perchlorate

Oxygen Consumption is dependent on the weight of the animals therefore we adjusted the slope equations for the weight of the tadpoles in each group. The weight adjusted consumption produced results relatively similar to the non-weight adjusted. The Month 0 weight adjusted slope comparison resulted in the magnesium control group consuming more oxygen than all of the other groups just like the non-weight adjusted Month 0. The weight adjusted control group also consumed more oxygen than all of the other groups.

Month 1 weight adjusted produced weight adjusted equations similar results to Month 0 with the magnesium and the control group consuming more oxygen than all of the other groups. This trend is similar to the non-weight adjusted just more pronounced by the being significant against more groups. Both control groups consumed more oxygen than the perchlorate dose groups, which is consistent with our hypothesis. The weight adjusted 4000 ppb tank consumed more oxygen than the 40 ppb and 400 ppb groups and the 40 ppb group consumed more oxygen than the 400 ppb group. These results are different from the non-weight adjusted slope comparison.

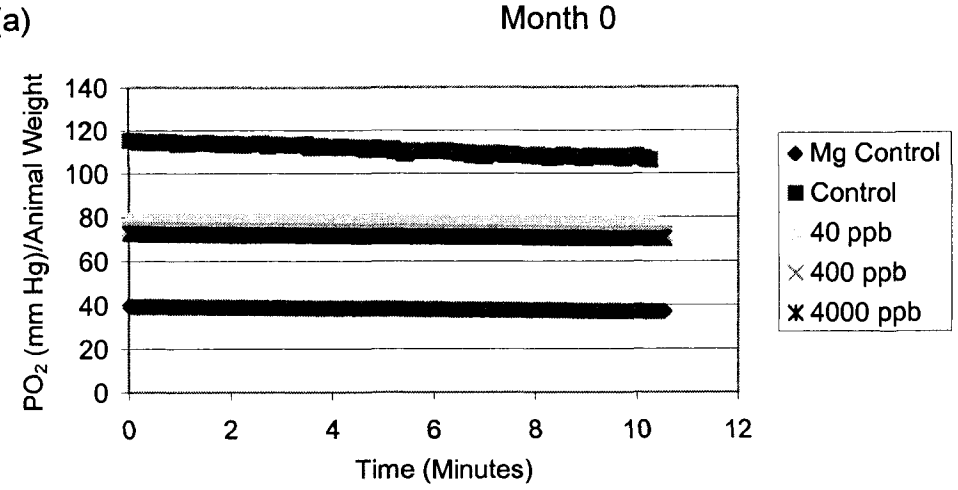
Identical to the previous two weight adjusted months, the magnesium and control group are statistically different from all of the other dose groups with the magnesium control group consuming more than the control group during the second month. The 40 ppb group produced a steeper slope than the 400 ppb and 4000ppb group while the 4000 ppb group consumed more oxygen when compared to the 400 ppb. This is different from the non-weight adjusted where the 40 ppb and 400 ppb groups consumed more oxygen then the 4000 ppb group.

Since the magnesium group completed metamorphosis by Month 3, only the control group is significant versus the 40 ppb and 4000 ppb groups. These results were the same as the non-weight adjusted slope comparison. The 40 ppb weight adjusted group consumed more oxygen than the 400 ppb group. This result was not seen in the non-weight adjusted where a difference was noted between the 400 ppb and 4000 ppb group.

The Month 4 weight adjusted slope comparison show that the control is once again significantly different than all of the other dose groups. In the non-weight adjusted control group a different only noted to the 4000 ppb group. The 40 ppb group consumed more oxygen than the 400 ppb and 4000ppb group and the 400 ppb group consumed more than the 4000 ppb group. These results were the same as the non-weight adjusted slope comparison results.

The weight adjusted slope comparison ended in the final month with the control group consuming more oxygen than the 400 ppb group, which is consistent with our hypothesis. The non-weight adjusted Month 5 did not identify a statistical different between these groups.

(a)

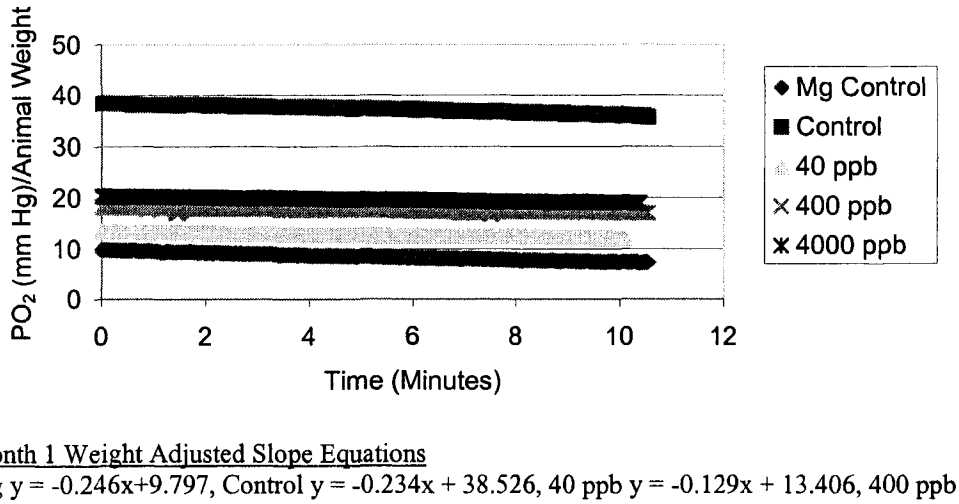


Month 0 Weight Adjusted Slope Equations

Mg y = -0.236x + 39.48, Control y = -0.833x + 115.13, 40 ppb y = -0.1356x + 78.574
400 ppb y = -0.236x + 74.614, 4000 ppb y = -0.217x + 72.385

Overall significance p<0.001. Multiple Comparison; Mg v. Control, 40 ppb, 400 ppb, 4000ppb; Control v. 40 ppb, 400 ppb, 4000ppb.

(b)

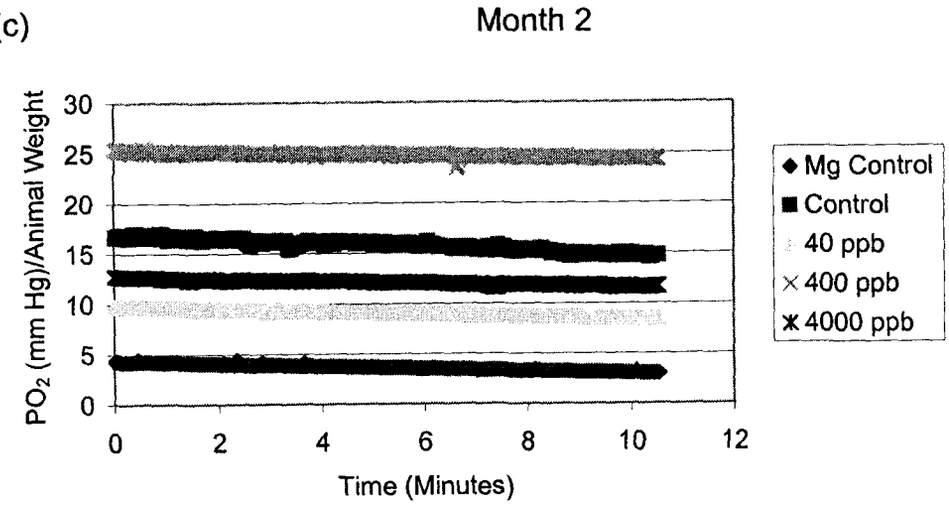


Month 1 Weight Adjusted Slope Equations
Mg y = -0.246x+9.797, Control y = -0.234x + 38.526, 40 ppb y = -0.129x + 13.406, 400 ppb y = -0.1001x + 18.126, 4000 ppb y = -0.135x + 20.421

Overall significance p <0.001. Multiple Comparison; Mg v. Control, 40 ppb, 400 ppb, 4000ppb; Control v. 40 ppb, 400 ppb, 4000 ppb; 40 ppb v. 400 ppb; 4000 ppb v. 40 ppb, 400 ppb.

Figure 11 Oxygen Consumption Rate of Weight Adjusted *Rana pipiens* Exposed to Perchlorate

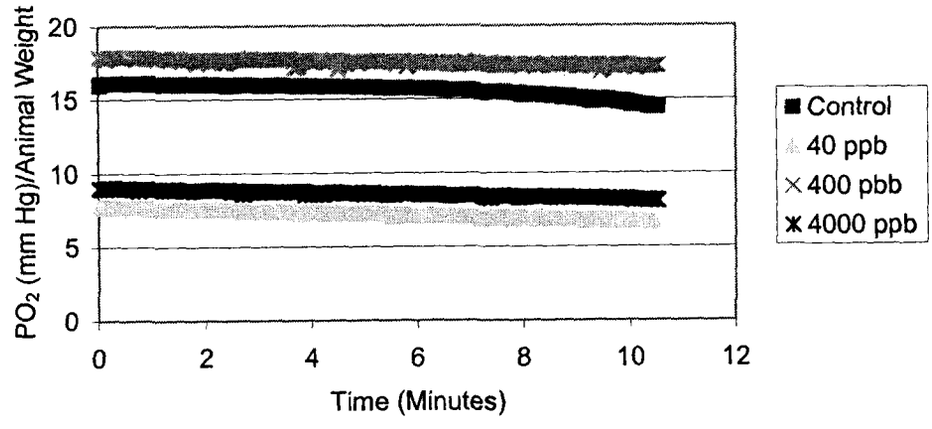
(c)



Month 2 Weight Adjusted Slope Equations
Mg y = -0.132x + 9.76, Control y = -0.207x + 16.90, 40 ppb y = -0.133x + 9.76, 400 ppb y = -0.083x + 25.251, 4000 ppb: y = -0.101x + 12.635

Overall significance p<0.001. Multiple Comparison; Mg v. Control, 40 ppb, 400 ppb, 4000ppb; Control v. 40 ppb, 400 ppb, 4000 ppb; 40 ppb v. 400 ppb, 4000 ppb; 4000 ppb v. 400 ppb.

(d)

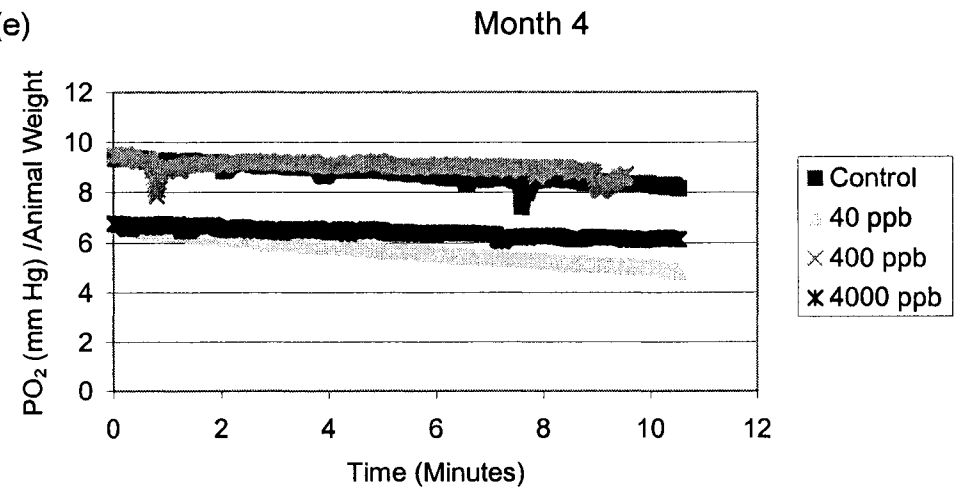


Month 3 Weight Adjusted Slope Equations
Control y = -0.136x + 16.344, 40 ppb y = -0.100x + 7.725, 400 ppb y = -0.065x + 17.871
4000 ppb y = -0.082x + 9.022

Overall Significance p<0.001. Multiple Comparison; Control v. 40 ppb, 4000 ppb; 40 ppb v. 400 ppb.

Figure 11 Oxygen Consumption Rate of Weight Adjusted *Rana pipiens* Exposed to Perchlorate

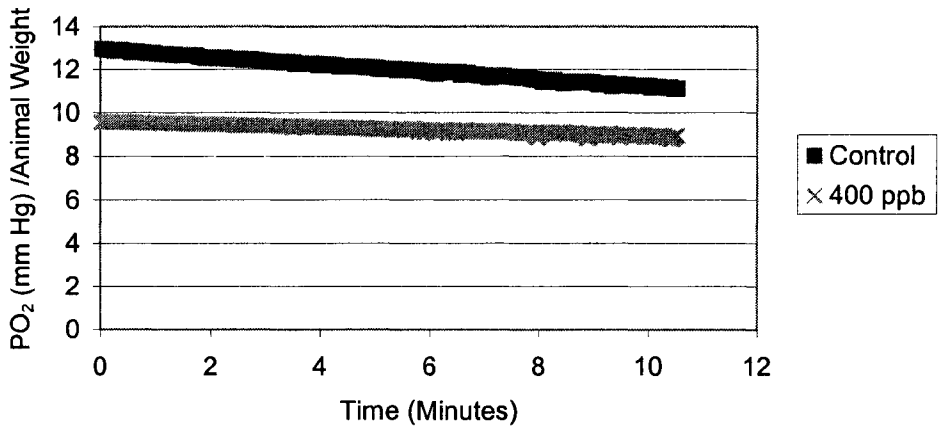
(e)



Month 4 Weight Adjusted Slope Equations
Control y = -0.107x + 9.33, 40 ppb y = -0.1604x + 6.53, 400 ppb y = -0.051x + 9.27
4000 ppb y = -0.056x + 6.71

Overall significance $p<0.001$. Multiple Comparison; Control v. 40 ppb, 400 ppb, 4000 ppb, 40 ppb v. 400 ppb, 4000 ppb; 400 ppb v. 4000 ppb.

(f)



Month 5 Weight Adjusted Slope Equations

Control y = -0.172x + 12.913, 400 ppb y = -0.0679x + 9.604

Overall significance $p<0.001$. Multiple Comparison; Control v. 400 ppb

Figure 11 Oxygen Consumption Rate of Weight Adjusted *Rana pipiens* Exposed to Perchlorate

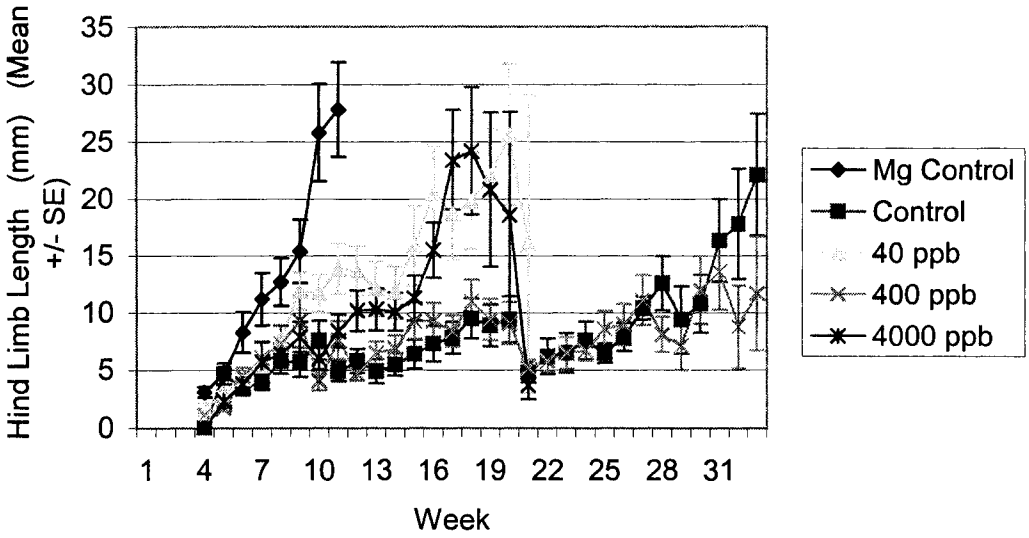
Developmental Assessments

The three tests that were used to assess the development of the tadpoles were the morphometric measurements, asymmetrical limb development and the TKI. The morphometric measurements and the asymmetrical limb development were performed using both a temporal and stage based approach. The temporally based method involves measuring animals weekly therefore, animals can be at different developmental stages. The stage-based approach involved measurements taken during TKI stages 23-25, when the animals were sacrificed prior to completing metamorphosis. The decision was made to include a stage based approach because tadpoles are known development asymmetrically, but can even out when metamorphosis is complete.

Temporally Based

Snout to vent, total body, tail, and hindlimb length were measured weekly in 40 animals per tank and the data are presented in Fig. 12 a-d. Significance values were not added to the Fig. 12 graphs because they became too busy but they are listed in the subsequent tables (Table 7 overall and Table 8 post-hoc). A post-hoc analysis was only performed on the weeks that had an overall significance. The sample size for the temporally based morphometric characteristics (hindlimb, snout to vent, total body and tail length) is available in Appendix B.

(a) Hind Limb Length



(b) Snout to Vent Length

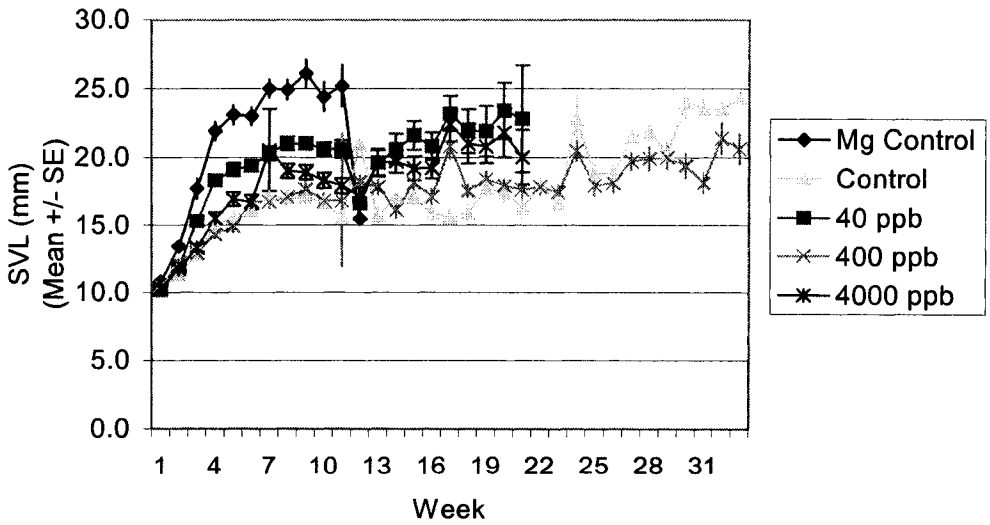
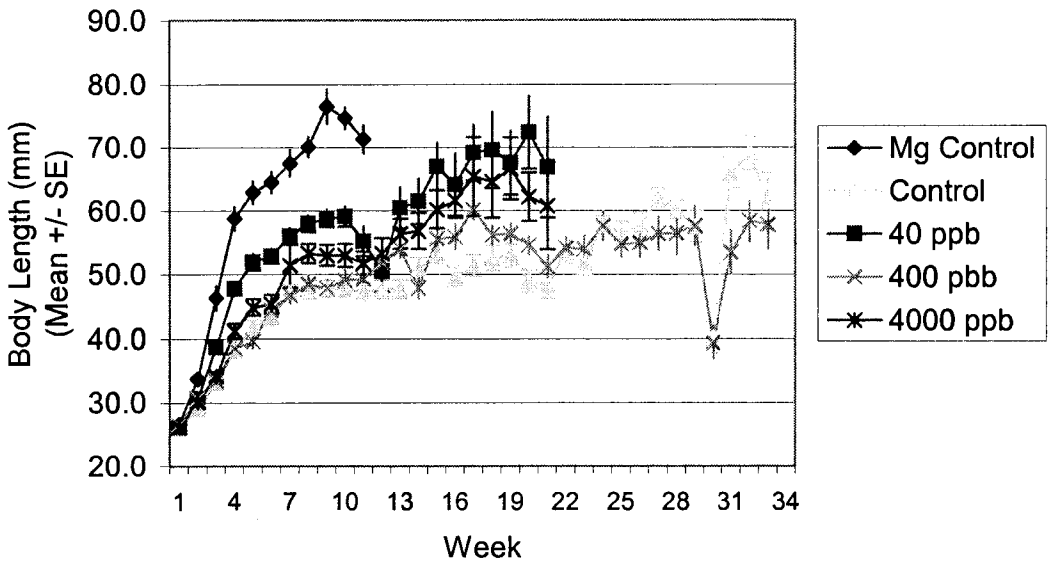


Figure 12 Temporally Based Morphometric Characteristic of *Rana pipiens* Exposed to Perchlorate

(c) Total Body Length



(d) Tail Length

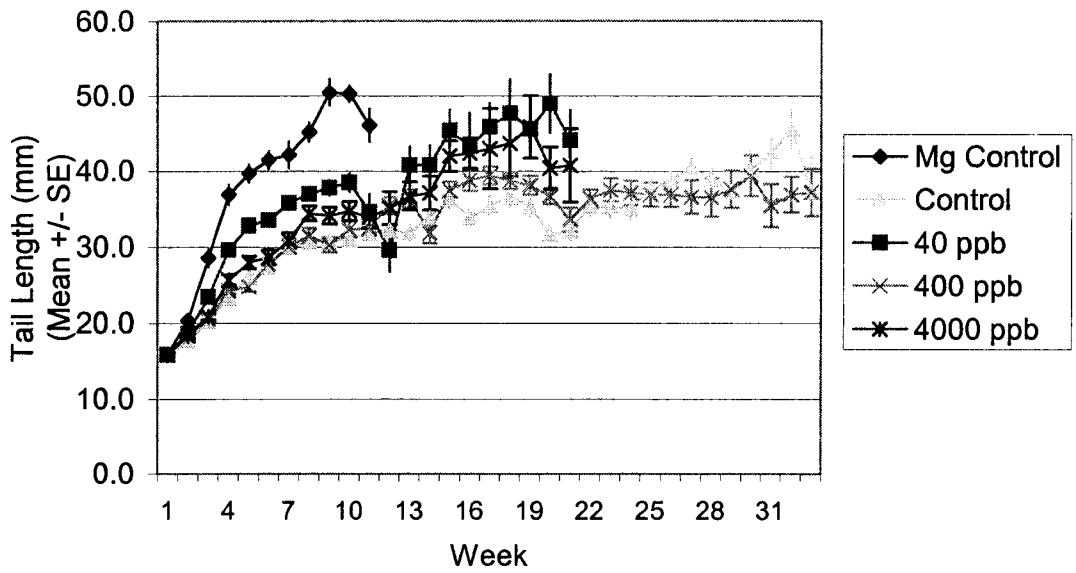


Figure 12 Temporally Based Morphometric Characteristic of *Rana pipiens* Exposed to Perchlorate

The experiment was initated with all aniamls being equal with respect to morphometric characteristics. Beginning on week 2 there are differences in the tail, snout to vent, total body and right hindlimb length and this trend was consistant through week 21 (Table 7a). The tail and total body length had an overall significance in weeks 27, 28, and 31.

The magnesium control group had a larger body size compared to all other groups (Table 8). The magnesium control group was larger in snout to vent, tail, and total body length than in all other groups in weeks 2-11, at which time all of the animals had completed metamorphosis. The right hindlimb lengths were not as consistantly larger.

Starting in week 3, the 40 ppb group was also physically larger with respect to the control, 400 ppb and the 4000 ppb group in the snout to vent, tail and total body length (Table 10a). These results were consistent the first 6 weeks. After this time the 40 ppb group was larger than the other groups, but it was not as consistent as the first 6 weeks. The 40 ppb group was larger than the control group.

The 4000 ppb group was larger than the control group starting in week 11 snout to vent length. This continued through week 20 in one or all of the morphometric measurements. The 4000 ppb group then completed metamorphosis in week 21.

The control group and the 400 ppb group ended the experiment with the control group having larger animals than the 400 ppb dose group. This trend was first seen in week 17 and continued through the end of the experiment, differences were not consistantly significant every week, nor were the measurements (snout to vent, tail, total body, or right hind limb).

Table 7a Weekly Morphometric Characteristics in *Rana pipiens* Exposed to Perchlorate

Week		F	p
1	Tail	0.039	0.997
	SVL	1.004	0.407
	Total	0.296	0.880
	RtHind	N/A	N/A
2	Tail	5.4	0.000*
	SVL	7.83	0.000*
	Total	6.93	0.000*
	RtHind	N/A	N/A
3	Tail	7.57	0.000*
	SVL	10.38	0.000*
	Total	10.09	0.000*
	RtHind	N/A	N/A
4	Tail	36.33	0.000*
	SVL	32.84	0.000*
	Total	36.99	0.000*
	RtHind	0.762	0.575
5	Tail	37.59	0.000*
	SVL	30.51	0.000*
	Total	36.85	0.000*
	RtHind	3.753	0.008*
6	Tail	28.54	0.000*
	SVL	23.23	0.000*
	Total	28.394	0.000*
	RtHind	3.43	0.013*
7	Tail	17.49	0.000*
	SVL	3.006	0.020*
	Total	10.9	0.000*
	RtHind	3.194	0.018*
8	Tail	18.57	0.000*
	SVL	23.44	0.000*
	Total	21.81	0.000*
	RtHind	3.28	0.015*
9	Tail	23.81	0.000*
	SVL	18.94	0.000*
	Total	25.19	0.000*
	RtHind	3.31	0.014*
10	Tail	16.75	0.000*
	SVL	14.94	0.000*
	Total	17.22	0.000*
	RtHind	18.56	0.000*
11	Tail	3.84	0.005*
	SVL	18.34	0.000*
	Total	8.38	0.000*
	RtHind	13.12	0.000*
12	Tail	2.36	0.074
	SVL	11.55	0.000*
	Total	1.83	0.146
	RtHind	7.67	0.000*
13	Tail	5.63	0.001*
	SVL	6.73	0.000*
	Total	7.3	0.000*
	RtHind	4.93	0.003*
14	Tail	4.13	0.008*
	SVL	7.81	0.000*
	Total	5.76	0.001*
	RtHind	3.43	0.021*
15	Tail	5.86	0.001*
	SVL	5.7	0.001*
	Total	5.5	0.002*
	RtHind	3.47	0.020*
16	Tail	5.86	0.001*
	SVL	8.28	0.000*
	Total	7.04	0.000*
	RtHind	6.57	0.001*

SVL (Snout to Vent Length); RtHind (Right hind limb length)

* Statistically significant ($p<0.05$) by ANOVA with Tukey's post-hoc

Table 7b Weekly Morphometric Characteristics in *Rana pipiens* Exposed to Perchlorate

Week		F	p
17	Tail	4.1	0.009*
	SVL	12.86	0.000*
	Total	8.52	0.000*
	RtHind	9.72	0.000*
18	Tail	4.12	0.009*
	SVL	8.1	0.000*
	Total	5.54	0.002*
	RtHind	4.75	0.005*
19	Tail	5.49	0.002*
	SVL	6.07	0.001*
	Total	3.89	0.012*
	RtHind	4.51	0.006*
20	Tail	11.03	0.000*
	SVL	5.65	0.002*
	Total	10.16	0.000*
	RtHind	2.96	0.039*
21	Tail	2.97	0.041*
	SVL	3.52	0.022*
	Total	3.67	0.018*
	RtHind	3.84	0.015*
22^	Tail	U=170.00	0.446
	SVL	U=192.5	0.881
	Total	U=171.00	0.463
	RtHind	U=16.0.00	0.587
23^	Tail	U=15.0.5	0.196
	SVL	U=138.0	0.103
	Total	U=140.5	0.118
	RtHind	U=140.5	0.248
24^	Tail	U=160.5	0.132
	SVL	U=145.5	0.830
	Total	U=122.0	0.320
	RtHind	U=96.00	0.228

^ Nonparametric Mann-Whitney

* Statistically significant ($p<0.05$) by ANOVA with Tukey's post-hoc

Week		F	p
25	Tail	2.71	0.110
	SVL	0.22	0.646
	Total	1.78	0.192
	RtHind	8.49	0.007*
26	Tail	0.292	0.593
	SVL	1.78	0.193
	Total	0.95	0.359
	RtHind	2.59	0.119
27	Tail	11.03	0.003*
	SVL	7.51	0.011
	Total	11.66	0.002*
	RtHind	1.69	0.205
28	Tail	8.53	0.008*
	SVL	2.2	0.152
	Total	11.83	0.002*
	RtHind	1.98	0.173
29	Tail	2.92	0.103
	SVL	0.04	0.834
	Total	2.55	1.260
	RtHind	0.67	0.430
30	Tail	2.84	0.111
	SVL	0.02	0.879
	Total	2.84	0.111
	RtHind	3.57	0.077
31	Tail	9.57	0.007*
	SVL	3.85	0.069
	Total	9.42	0.008*
	RtHind	2.52	0.154
32	Tail	0.065	0.803
	SVL	9.045	0.013*
	Total	1.986	0.189
	RtHind	0.06	0.812
33	Tail	0.785	0.397
	SVL	7.902	0.018*
	Total	0.011	0.917
	RtHind	0.189	0.673

Table 8a Weekly ANOVA Morphometric Characteristic Post-hoc Significance by Perchlorate Tank Concentrations

Week		Mg v. Conrol	Mg v. 40 ppb	Mg v. 400 ppb	Mg. v. 4000 ppb	40 ppb v. Control	40 ppb v. 400 ppb	40 ppb v. 4000 ppb	4000 ppb v. 400 ppb	Control v. 400 ppb	4000 ppb v. Control
1	SVL	0.370	0.644	0.924	0.582	0.992	0.981	1.000	0.966	0.859	0.997
	Tail	0.999	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.996	0.999
	Total	0.856	0.965	0.998	0.960	0.997	0.996	1.000	0.995	0.957	0.998
	RtHind	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2	SVL	0.000*	0.002*	0.027*	0.000*	0.608	0.937	0.987	0.702	0.181	0.887
	Tail	0.000*	0.022*	0.012*	0.0128*	0.414	0.996	0.979	0.996	0.647	0.771
	Total	0.000*	0.010*	0.014*	0.001*	0.432	1.000	0.978	0.962	0.374	0.790
	RtHind	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
3	SVL	0.000*	0.002*	0.000*	0.000*	0.000*	0.000*	0.003*	0.937	1.000	0.865
	Tail	0.000*	0.000*	0.000*	0.000*	0.000*	0.004*	0.010*	0.999	0.995	0.967
	Total	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.003*	0.988	0.997	0.921
	RtHind	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
4	SVL	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.291	0.968	0.987
	Tail	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.719	0.988	0.302
	Total	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.512	1.000	0.310
	RtHind	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Table 8b Weekly ANOVA Morphometric Characteristic Post-hoc Significance by Perchlorate Tank Concentrations

Week		Mg v. Conrol	Mg v. 40 ppb	Mg v. 400 ppb	Mg. v. 4000 ppb	40 ppb v. Control	40 ppb v. 400 ppb	40 ppb v. 4000 ppb	4000 ppb v. 400 ppb	Control v. 400 ppb	4000 ppb v. Control
5	SVL	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.006*	0.029*	0.879	0.263
	Tail	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.031*	0.748	0.421
	Total	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.001*	0.024*	0.785	0.328
	RtHind	1.000	0.247	0.029*	0.238	0.247	0.696	0.978	0.991	0.029*	0.238
6	SVL	0.000*	0.000*	0.000*	0.000*	0.000*	0.001*	0.001*	1.000	0.884	0.880
	Tail	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.001*	0.954	0.994	0.798
	Total	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.001*	0.989	0.973	0.811
	RtHind	0.051	0.030*	0.291	0.038*	0.987	1.000	0.997	0.995	1.000	0.997
7	SVL	0.039*	0.428	0.025*	0.467	0.596	0.471	1.000	0.408	1.000	0.530
	Tail	0.000*	0.003*	0.000*	0.000*	0.001*	0.001*	0.005*	0.975	1.000	0.970
	Total	0.000*	0.016*	0.000*	0.000*	0.015*	0.010*	0.458	0.467	1.000	0.467
	RtHind	0.028*	0.027*	0.317	0.090	0.964	0.999	1.000	1.000	0.945	0.953
8	SVL	0.000*	0.001*	0.000*	0.000*	0.000*	0.000*	0.040*	0.052	1.000	0.053
	Tail	0.000*	0.000*	0.000*	0.000*	0.000*	0.001*	0.287	0.199	0.981	0.055
	Total	0.000*	0.000*	0.000*	0.000*	0.000*	0.000	0.131	0.105	0.996	0.040
	RtHind	0.210	0.067	0.149	0.150	0.852	0.999	0.924	0.994	0.971	0.998
9	SVL	0.000*	0.001*	0.000*	0.000*	0.000*	0.000*	0.048*	0.397	0.912	0.048
	Tail	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.106	0.077	0.995	0.114
	Total	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.062	0.103	1.000	0.054
	RtHind	0.025*	0.760	0.336	0.092	0.079	0.828	0.307	0.978	0.676	0.894

Table 8c Weekly ANOVA Morphometric Characteristic Post-hoc Significance by Perchlorate Tank Concentrations

Week		Mg v. Conrol	Mg v. 40 ppb	Mg v. 400 ppb	Mg. v. 4000 ppb	40 ppb v. Control	40 ppb v. 400 ppb	40 ppb v. 4000 ppb	4000 ppb v. 400 ppb	Control v. 400 ppb	4000 ppb v. Control
10	SVL	0.000*	0.036*	0.000*	0.000*	0.000*	0.000*	0.053	0.261	1.000	0.157
	Tail	0.000*	0.000*	0.000*	0.000*	0.000*	0.005*	0.196	0.545	0.903	0.087
	Total	0.000*	0.001*	0.000*	0.000*	0.000*	0.001*	0.110	0.408	0.967	0.086
	RtHind	0.000*	0.000*	0.000*	0.000*	0.302	0.001*	0.019*	0.814	0.446	0.930
11	SVL	0.000*	0.033*	0.000*	0.000*	0.000*	0.000	0.015	0.599	0.354	0.010*
	Tail	0.002*	0.037*	0.005*	0.018*	0.000*	0.000*	0.015*	0.942	0.996	0.771
	Total	0.000*	0.011*	0.000*	0.001*	0.013*	0.141	0.696	0.814	0.897	0.267
	RtHind	0.000*	0.003*	0.000*	0.000*	0.000*	0.069	0.115	0.997	0.677	0.352
12	SVL	n/a	n/a	n/a	n/a	0.000*	0.000*	0.066	0.261	0.509	0.009*
	Tail	n/a	n/a	n/a	n/a	0.000	0.108	0.176	0.999	0.359	0.525
	Total	n/a	n/a	n/a	n/a	0.793	0.968	0.834	0.961	0.300	0.156
	RtHind	n/a	n/a	n/a	n/a	0.001*	0.000*	0.386	0.031	0.921	0.093
13	SVL	n/a	n/a	n/a	n/a	0.010*	0.424	1.000	0.243	0.087	0.001*
	Tail	n/a	n/a	n/a	n/a	0.002*	0.258	0.409	0.997	0.045*	0.066
	Total	n/a	n/a	n/a	n/a	0.001*	0.209	0.651	0.818	0.024*	0.006*
	RtHind	n/a	n/a	n/a	n/a	0.012*	0.095	0.880	0.236	0.763	0.025*
14	SVL	n/a	n/a	n/a	n/a	0.021*	0.002	0.920	0.001*	0.661	0.029*
	Tail	n/a	n/a	n/a	n/a	0.084	0.011*	0.628	0.101	0.667	0.520
	Total	n/a	n/a	n/a	n/a	0.036*	0.02*	0.689	0.020	0.923	0.221
	RtHind	n/a	n/a	n/a	n/a	0.044*	0.140	0.917	0.323	0.832	0.105

Table 8d Weekly ANOVA Morphometric Characteristc Post-hoc Significance ($p<0.05$) by Perchlorate Tank Concentrations

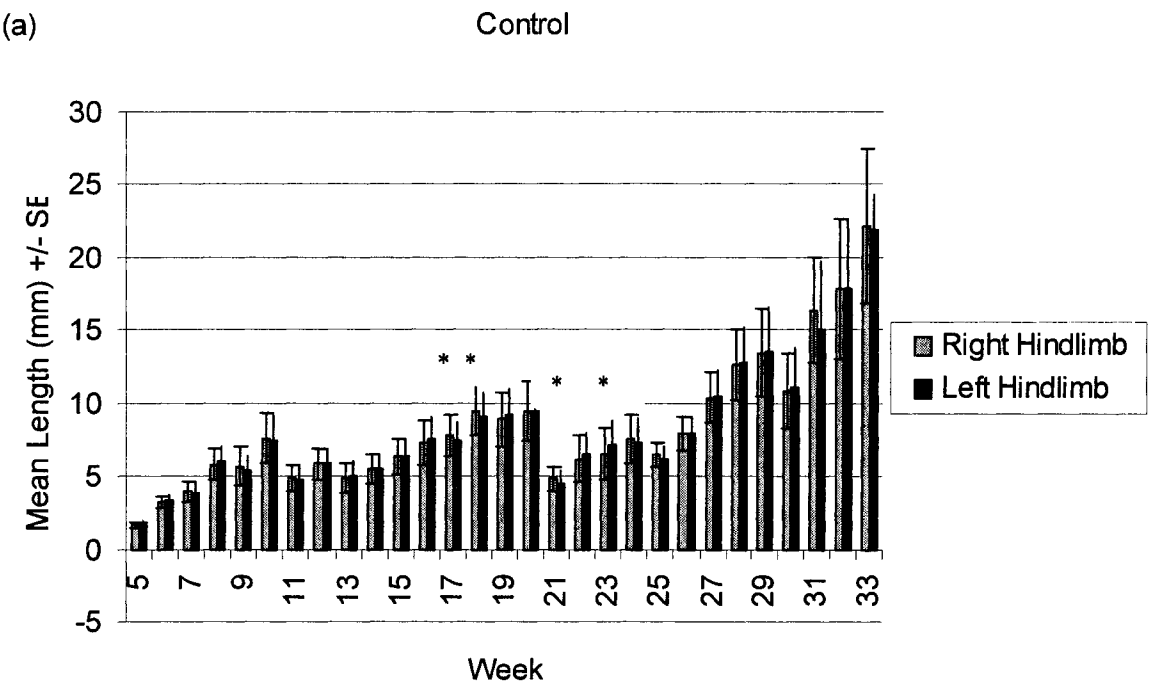
Week		40 ppb v. Control	40 ppb v. 400 ppb	40 ppb v. 4000 ppb	4000 ppb v. 400 ppb	Control v. 400 ppb	4000 ppb v. Control
15	SVL	0.001*	0.017*	0.239	0.681	0.521	0.120
	Tail	0.004*	0.017*	0.041	0.641	0.905	0.041*
	Total	0.002*	0.013*	0.335	0.013	0.794	0.099
	RtHind	0.016*	0.508	0.213	0.508	0.540	0.213
16	SVL	0.000*	0.008*	0.581	0.119	0.461	0.004*
	Tail	0.012*	0.463	0.004	0.995	0.081	0.004*
	Total	0.002*	0.172	0.944	0.944	0.110	0.002*
	RtHind	0.001*	0.008	0.559	0.173	0.831	0.035*
17	SVL	0.000*	0.497	0.975	0.975	0.000*	0.000*
	Tail	0.017*	0.222	0.892	0.658	0.279	0.097
	Total	0.001*	0.177	0.890	0.582	0.007	0.004*
	RtHind	0.019*	0.022*	0.714	0.000*	0.994	0.000*
18	SVL	0.001*	0.023*	0.958	0.958	0.287	0.002*
	Tail	0.014*	0.065	0.796	0.419	0.726	0.137
	Total	0.005*	0.037	0.841	0.231	0.558	0.039*
	RtHind	0.147	0.246	0.874	0.019*	0.949	0.009*
19	SVL	0.025*	0.079	0.927	0.300	0.862	0.122
	Tail	0.019*	0.132	1.000	0.084	0.528	0.009*
	Total	0.008*	0.065	0.999	0.075	0.554	0.009*
	RtHind	0.054	0.063	0.998	0.055	0.998	0.046*
20	SVL	0.01*	0.023*	0.895	0.895	0.925	0.03*
	Tail	0.000*	0.004*	0.183	0.588	0.013*	0.018*
	Total	0.000*	0.003*	0.323	0.561	0.075	0.010*
	RtHind	0.089	0.999	0.824	0.299	0.323	0.261
21	SVL	0.049*	0.153	0.780	0.501	0.546	0.164
	Tail	0.128	0.218	0.951	0.278	0.886	0.139
	Total	0.060	0.139	0.889	0.501	0.546	0.164
	RtHind	0.010*	0.013*	0.017*	0.926	0.991	0.968
25^	SVL	n/a	n/a	n/a	n/a	0.646	n/a
	Tail	n/a	n/a	n/a	n/a	0.110	n/a
	Total	n/a	n/a	n/a	n/a	0.192	n/a
	RtHind	n/a	n/a	n/a	n/a	0.007*	n/a
26^	SVL	n/a	n/a	n/a	n/a	0.193	n/a
	Tail	n/a	n/a	n/a	n/a	0.593	n/a
	Total	n/a	n/a	n/a	n/a	0.339	n/a
	RtHind	n/a	n/a	n/a	n/a	0.119	n/a

Table 8e Weekly ANOVA Morphometric Characteristic Post-hoc Significance ($p<0.05$) by Perchlorate Tank Concentrations

Week		40 ppb v. Control	40 ppb v. 400 ppb	40 ppb v. 4000 ppb	4000 ppb v. 400 ppb	Control v. 400 ppb	4000 ppb v. Control
27^	SVL	n/a	n/a	n/a	n/a	0.011*	n/a
	Tail	n/a	n/a	n/a	n/a	0.003*	n/a
	Total	n/a	n/a	n/a	n/a	0.002*	n/a
	RtHind	n/a	n/a	n/a	n/a	0.205	n/a
28^	SVL	n/a	n/a	n/a	n/a	0.152	n/a
	Tail	n/a	n/a	n/a	n/a	0.008*	n/a
	Total	n/a	n/a	n/a	n/a	0.002*	n/a
	RtHind	n/a	n/a	n/a	n/a	0.173	n/a
29^	SVL	n/a	n/a	n/a	n/a	0.834	n/a
	Tail	n/a	n/a	n/a	n/a	0.103	n/a
	Total	n/a	n/a	n/a	n/a	0.126	n/a
	RtHind	n/a	n/a	n/a	n/a	0.423	n/a
30^	SVL	n/a	n/a	n/a	n/a	0.879	n/a
	Tail	n/a	n/a	n/a	n/a	0.111	n/a
	Total	n/a	n/a	n/a	n/a	0.111	n/a
	RtHind	n/a	n/a	n/a	n/a	0.077	n/a
31^	SVL	n/a	n/a	n/a	n/a	0.069	n/a
	Tail	n/a	n/a	n/a	n/a	0.007*	n/a
	Total	n/a	n/a	n/a	n/a	0.008*	n/a
	RtHind	n/a	n/a	n/a	n/a	0.008	n/a
32^	SVL	n/a	n/a	n/a	n/a	0.013*	n/a
	Tail	n/a	n/a	n/a	n/a	0.803	n/a
	Total	n/a	n/a	n/a	n/a	0.189	n/a
	RtHind	n/a	n/a	n/a	n/a	0.812	n/a
33^	SVL	n/a	n/a	n/a	n/a	0.018*	n/a
	Tail	n/a	n/a	n/a	n/a	0.397	n/a
	Total	n/a	n/a	n/a	n/a	0.917	n/a
	RtHind	n/a	n/a	n/a	n/a	0.973	n/a

^ Weks 25-33 a t-test was performed on the two remaining groups (Control and 400 ppb).

The second temporal analysis was performed using a t-test to determine if there was asymmetric develop of the hindlimbs occuring within each dose group. The data did not have a normal distribution during weeks 22-24, therefore a Wilcoxon nonparametric analysis was performed. This analysis only examined differences within dose groups, not among the dose groups, therefore each dose group is represented in a separate graph. The sample size is located in Appendix C.

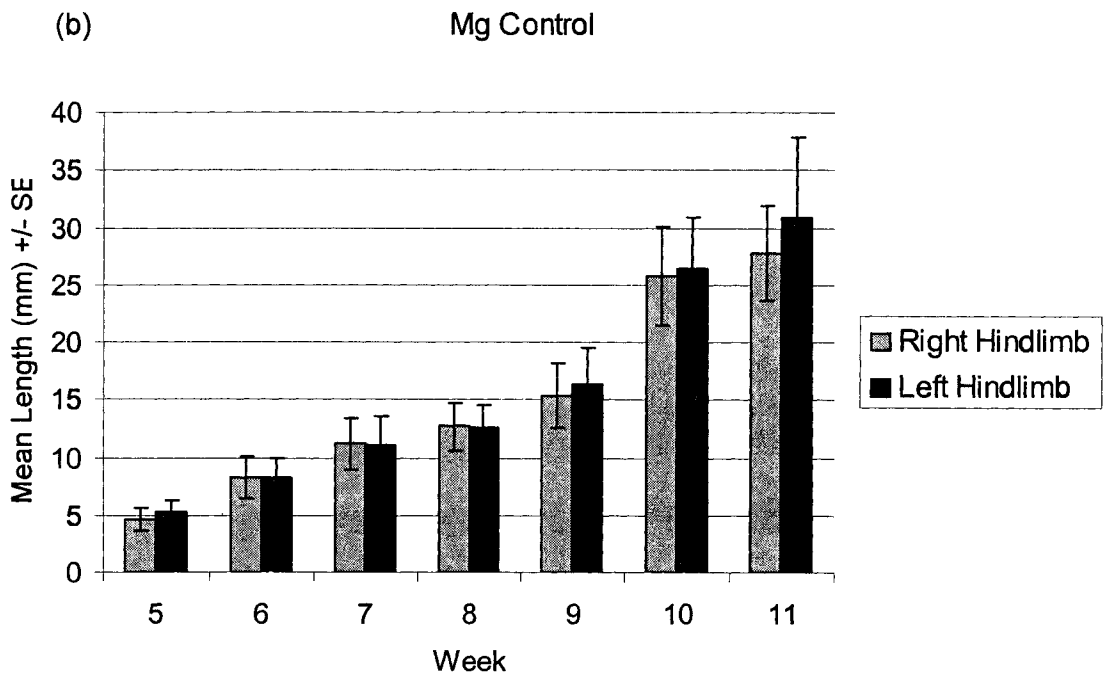


Control Significance: Week 17 (t=2.16; p=0.04); Week 18 (t=2.34; p=0.028); Week 21 (t=2.57; p=0.018); Week 23 (Z=-2.13, p=0.03)

Figure 13 Temporally Based Hindlimb Symmetry of *Rana pipiens* Following Exposure to Perchlorate

The control group had asymmetrical hindlimb development during various weeks in the middle of the *R. pipiens* metamorphosis period (Fig. 13a). The magnesium control

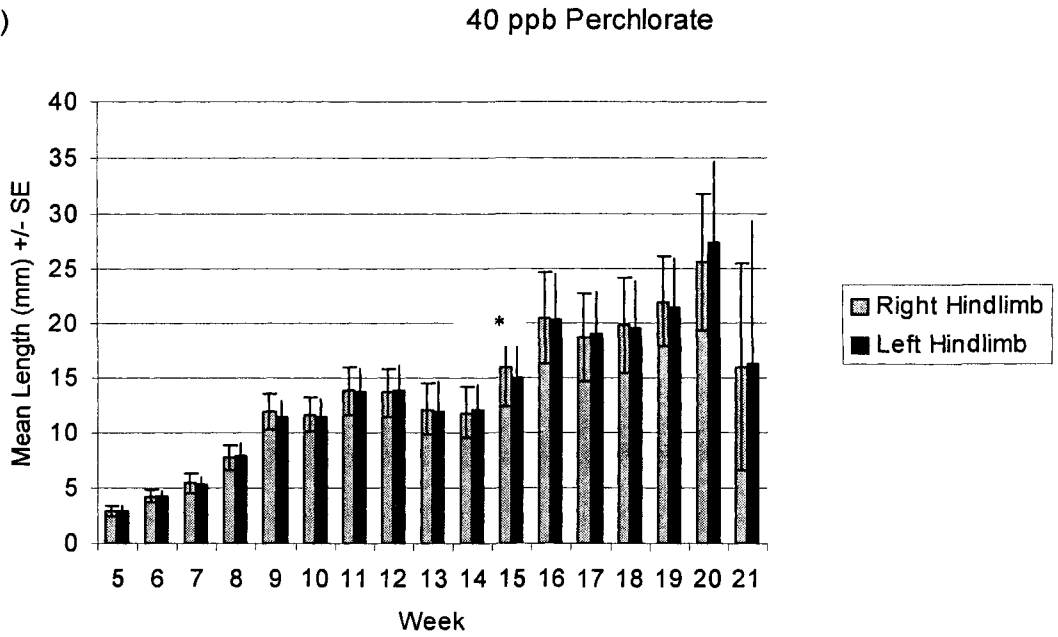
group did not exhibit any hind limb asymmetrical development (Fig. 13b). The 40 ppb, 400 ppb and 4000 ppb dose group experienced asymmetrical development, however no obvious trends could be drawn from this analysis.



Mg Control Significance: None

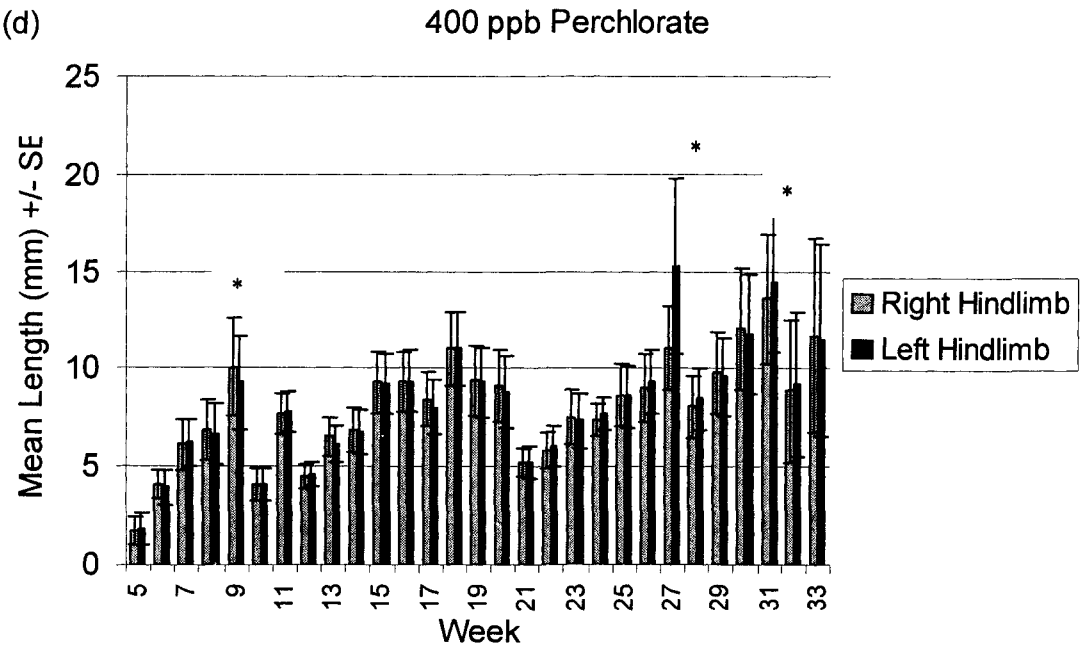
Figure 13 Temporally Based Hindlimb Symmetry of *Rana pipiens* Following Exposure to Perchlorate

(c)



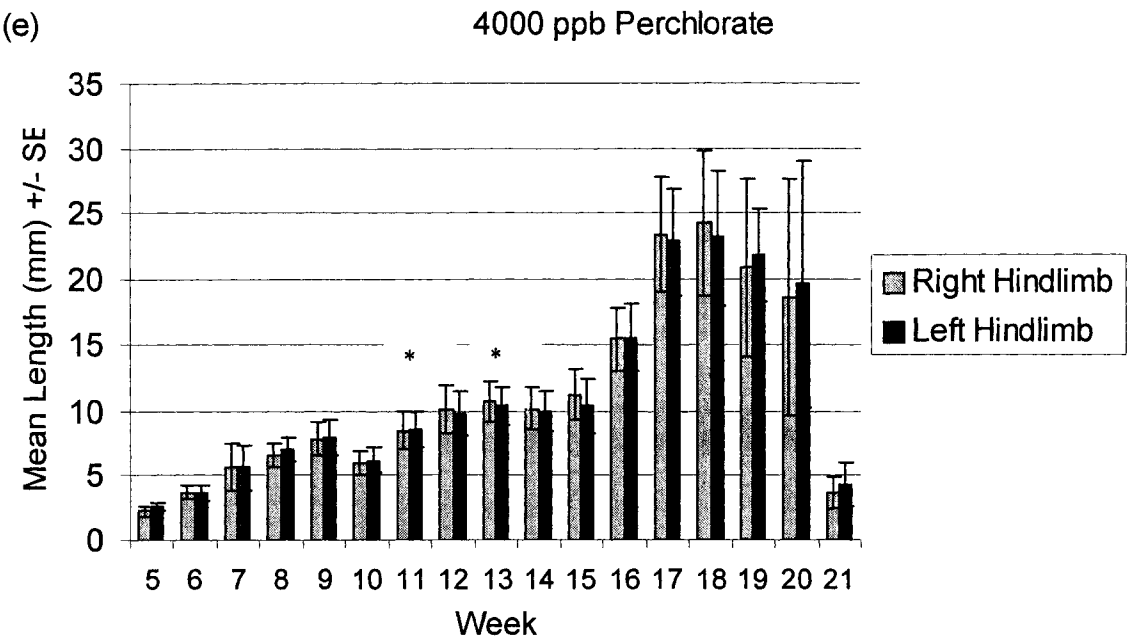
Week 28 (t=-3.171; p=0.008); Week 32 (t=-3.032; p=0.023)

(d)



400 ppb: Week 9 (t=2.791; p=0.016); Week 28 (t=-3.171; p=0.008); Week 32 (t=-3.032; p=0.023)

Figure 13 Temporally Based Hindlimb Symmetry of *Rana pipiens* Following Exposure to Perchlorate



4000 ppb Significance: Week 11 (t=-3.26; p=0.003); Week 13 (t=2.5; p=0.018)

Figure 13 Temporally Based Hindlimb Symmetry of *Rana pipiens* Following Exposure to Perchlorate

Stage-Based

Owing to the fact that tadpoles can develop at varying rates, we felt it was necessary to compare the morphometric characteristics on a stage-based analysis. Completing the comparison in this fashion will account for differences that may have occurred during development but that are not present when tadpoles complete metamorphosis. The stage-based analysis used measurements at approximately the same stage of TKI development (stages 22-23). Following stage based analyses we only noted an difference in the right forelimb length (See Fig. 14), but the post-hoc did not show any significance.

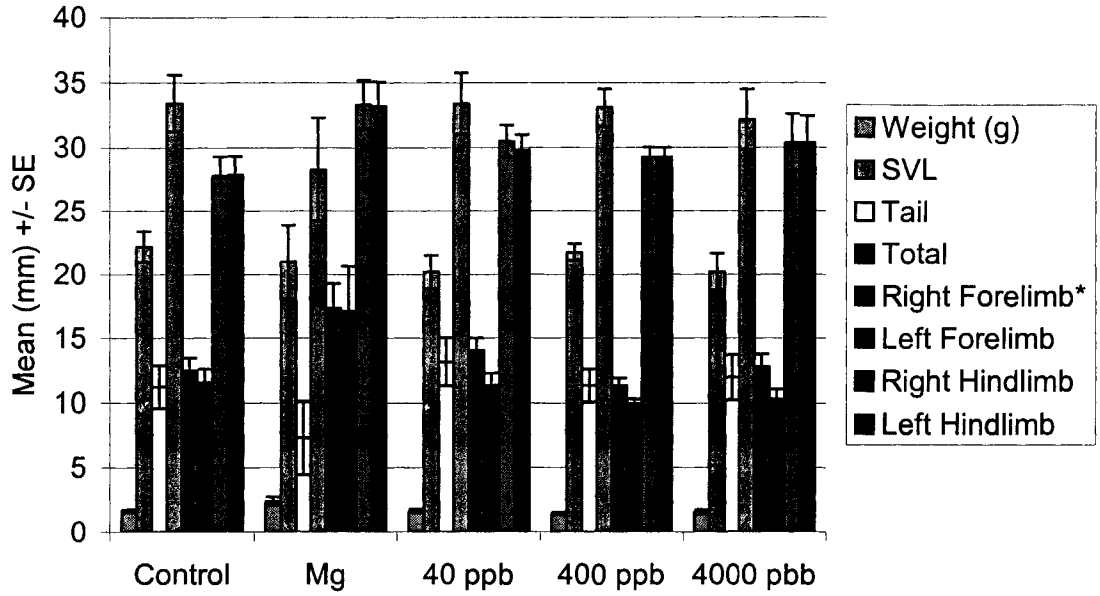


Figure 14 Stage-Based Morphometric Characteristics of Rana pipiens Exposed to Perchlorate

* Statistically significant ($p<0.05$) by nonparametric Kruskal Wallis analysis with Nemenyi post-hoc

The forelimb asymmetrical develop was not performed on a temporal basis because there was not a large enough sample size during the weekly measurements. Once a tadpole develops its forelimbs, it is only a week later that it is sacrificed because it completed metamorphosis. No significance was noted in the forelimb nor hindlimb stage based symmetry within each dose group (Fig. 16).

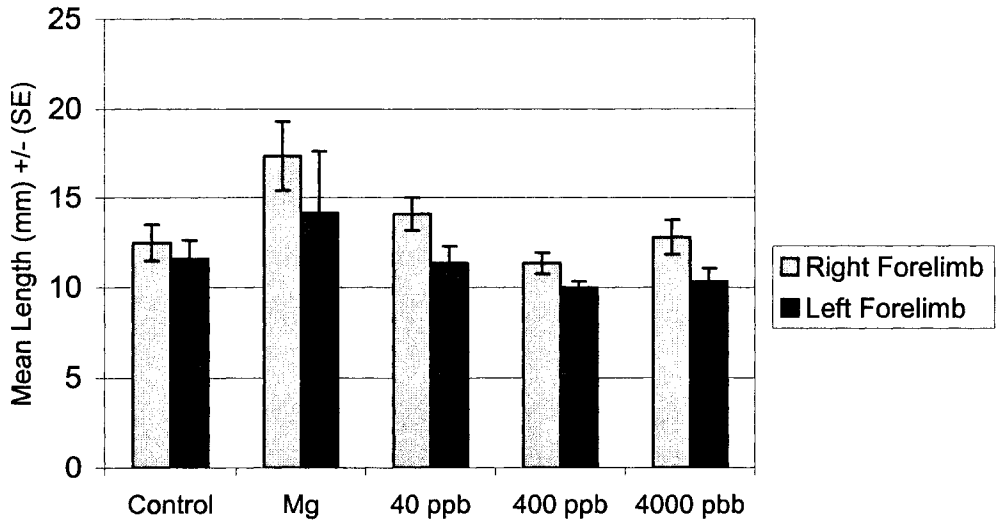


Figure 15 Stage-Based Forelimb Asymmetrical Development of *Rana pipiens* Following Exposure to Perchlorate

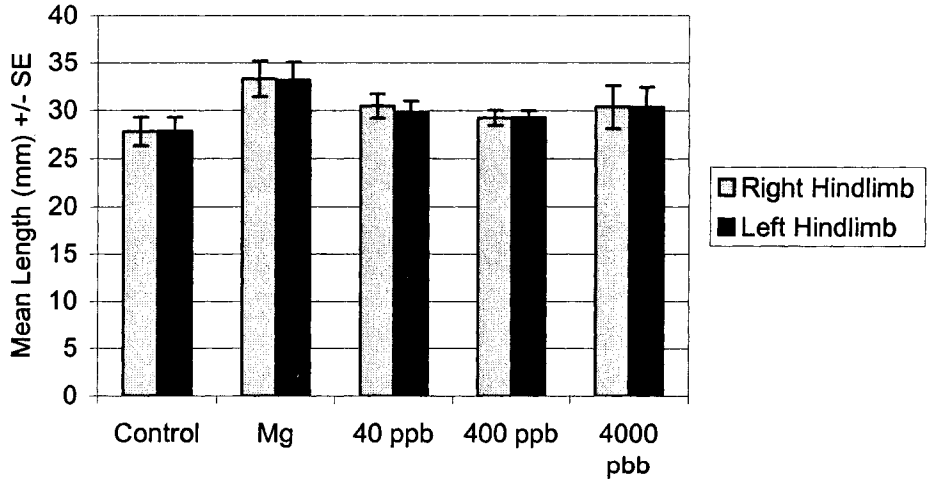
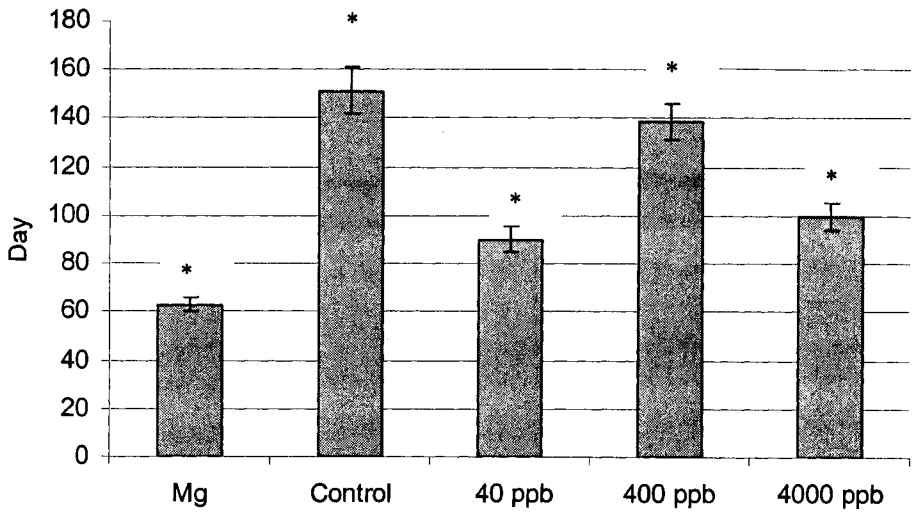


Figure 16 Stage-Based Hindlimb Assymetrical Development of Rana pipiens Following Exposure to Perchlorate

TKI

The TKI analyzes the metamorphosis and development of the tadpoles, which is different than the morophometric characteristics that are an analysis of the physical size of the animals. Fig. 17 shows the number of animals that completed metamorphosis each week in each of the dose groups. The magnesium control group completed metamorphosis the fastest; followed by the 40 ppb and 4000ppb with the last groups being the control and 400 ppb. The 40 ppb group completed metamorphosis the second fastest followed by the 4000 ppb group. Table 9 provides the month by month frequencies of animals in the corresponding group of TKI stages.



*Overall Significance F=21.133, p=0.000. Mg v Control, p=0.000; Mg v 400 ppb, p=0.000; Mg v 4000 ppb, p=0.035; 40 ppb v Control, p=0.000; 40 ppb v 400 ppb, p=0.000; 4000 ppb v Control, p=0.000; 4000 ppb v 400 ppb, p=0.002.

Figure 17 Day *Rana pipiens* Exposed to Perchlorate Completed Metamorphosis

Table 9 Monthly TKI of *Rana pipiens* Exposed to Perchlorate

(a) Month 0

TKI Stage	0	1	2+	
40 ppb	1 (1)	12 (14)	7 (5)	$0.950 \leq p \geq 0.975$
400 ppb	1(1)	13 (14)	6 (5)	$0.975 \leq p \geq 0.990$
4000ppb	2 (1)	13 (14)	5 (5)	$p \leq 0.999$
Mg^	1 (1)	11 (14)	8 (5)	

(b) Month 1

TKI Stage	1-3	4-6	7-9+	
40ppb	1 (7)	6 (12)	13 (1)	$p \geq 0.001$*
400ppb	10 (7)	8 (12)	2 (1)	$0.50 \leq p \geq 0.75$
4000ppb	9 (7)	9 (12)	2 (1)	$0.75 \leq p \geq 0.50$
Mg^	0 (7)	1 (12)	16 (1)	

(c) Month 2

TKI Stage	1-6	7-9	10-12	13-18	
40ppb	1 (13)	3 (2)	9 (3)	7 (2)	$0.01 \leq p \geq 0.005$*
400ppb	11 (13)	4 (2)	2 (3)	3 (2)	$0.90 \leq p \geq 0.75$
4000ppb	9 (13)	2 (2)	5 (3)	4 (2)	$0.75 \leq p \geq 0.50$
Mg^	0 (13)	0 (2)	1 (3)	11 (2)	

(d) Month 3

TKI Stage	1-9	10-12	13-15	16-18	19-23	
40ppb	1 (4)	3 (9)	4 (4)	2 (1)	5 (2)	$0.90 \leq p \geq 0.75$
400ppb	5 (4)	8 (9)	3 (4)	3 (1)	1(2)	$0.90 \leq p \geq 0.75$
4000ppb	1 (4)	6 (9)	5 (4)	5 (1)	3 (2)	$0.25 \leq p \geq 0.10$

^ Did not include Mg Control in the analysis because it would create artifical grouping, however the Mg group appears to be at later stages of developed then the Control and all of the dose groups

* Statistically significant ($p<0.05$)

Table 9 Monthly TKI of *Rana pipns* Exposed to Perchlorate

(e) Month 4

TKI Stage	1-9	10-12	13-15	16-18	19-22	
40ppb	1 (3)	1 (9)	3 (5)	2 (2)	3 (1)	$p \leq 0.999$
400ppb	2 (3)	7 (9)	5 (5)	3 (2)	2 (1)	$0.90 \leq p \geq 0.75$
4000ppb	1 (3)	3 (9)	2 (5)	8 (2)	5 (1)	$0.025 \leq p \geq 0.01$*

(f) Month 5

TKI Stage	1-9	10-12	13-15	16-21	
400ppb	4 (2)	7 (9)	4 (2)	4 (3)	$0.25 \leq p \geq 0.10$

(g) TKI Sample Size

	Month 0	*Month 1*	*Month 2*	*Month 3*	*Month 4*	*Month 5*
40 ppb	20	20	20	15	10	N/A
400 ppb	20	20	20	20	20	19
4000 ppb	20	20	20	20	19	N/A
Control	20	20	20	20	20	16

It is important to note that the magnesium group was not a part of Table 9 analysis however, it does appear to be more developed than all of the other dose groups. Including magnesium group would have created artificial grouping. The first month of TKI significance was month 1 with the 40 ppb group having more animals at advanced stages of development while most of the control group was still between TKI stages 4-6 (Table 11a). The accelerated development of the 40 ppb group continued through the second month. Month 4 shows that the 4000 ppb group is completing metamorphosis at a faster rate than the control. The results of the TKI show the accelerated development of the magnesium, 40 ppb and 4000 ppb groups, this is consistent with our previous data. It appears that these dose groups were both physically larger (tail, snout to vent, and total body length) and developed at a faster rate.

CHAPTER 5

DISCUSSION

Preliminary Study: Las Vegas Wash Water

One of the biggest difficulties encountered during the course of this study was the infestation of the parasite, *Dactylogyrus vastator*, in the 400 and 4 ppb groups. In the highest perchlorate dose group (400 ppb), all the animals died by the end of the fifth week of the study; whereas the animals in the 4 ppb group all died by the end of the twelfth week. It is unclear why the middle dose group was unaffected by this parasite.

Two possible explanations for this phenomenon have been evaluated. First, the parasite may have been present in the tadpoles upon arrival from the Trans-Mississippi Biological Supply Company. Following exposure to perchlorate, the animals may have had a suppressed immune system allowing the parasites to multiply and overtake the animals. Sublethal environmental change could stress larval or post metamorphic amphibians sufficiently that their immune systems become compromised; infections and opportunistic pathogens can then be followed by death (Carey & Bryant, 1995). Amphibian larvae and metamorphosing individuals might be more vulnerable to environmentally influenced disruption of immune function than adults. An evaluation of the life history of the parasite indicates that these are common parasites in aquatic organisms, mainly fish, and infest the gills leading to respiratory distress and eventually

death. Following a phone call to Trans-Mississippi Biological Supply, they have no documented infestations of parasites in this species.

Second, we postulated that the parasite was present in the Las Vegas Wash water collected from the LM-8 site. Since the highest dose group was serially diluted to make the lower dose groups (see appendix A for details) a density-dependent exposure to the parasites should also exist. This would explain the rapid die-off of the high dose group, but would not account for the absence of effects in the middle dose group. In January 2004, *Dactylogyrus vastator* was identified in the gills of fish obtained from a local surface body of water in close proximity to the water collection site at LM-8. This further supports the introduction of the parasite via the Wash water.

Heavy infestations of *D. vastator* cause loss of blood, erosion of epithelium and provide access for secondary bacterial or fungal infestations. Massive attrition due to monogean infections are common in crowded situations of fish culture ponds (Schmidt & Roberts, 2000). Most of the research on *D. vastator* involves fish species from the Middle Eastern and Eastern European region of the world. Inspection of fish species from Diyala River, Iraq found *D. vastator* along with various other parasites (Al-Shaikh, Mhaisen, Al-Khateeb, 1995). This river was sampled because the pollution in the river could have possible effects on fish tolerance to parasitic infections (Al-Shaikh *et al.*, 1995). Khalifa (1989) found *D. vastator* on both *B. gryus* and *C. carpio* from fish ponds as well as the Al-Tharthar Canal in Iraq. Molar (1995) performed research of the *D. vastator* and notes that parasitic infestations impair natural resistance in fish making them more susceptible to environmental stressors (such as perchlorate). In general, Schmahl

and Mehlhorn (1985) assert that fish are seldom heavily affected by monogeneans such as *D. vastator*. However, monogeneans can become a serious threat when fish are crowded in the ponds of fish farms or in tanks for scientific research.

Toxicological investigations of perchlorate on several other thyroid related endpoints support the possibility of a u-shaped dose-response curve (USEPA, 2002). However, it should be noted that our explanations of the possible relationships to parasitic infestations are highly speculative and this preliminary study was designed to assess the effects of perchlorate on development and metamorphosis in *R. pipiens* not parasitic susceptibility. Further research is needed to more thoroughly examine the susceptibility of tadpoles to parasitic infections following perchlorate exposure.

The only significant differences noted in the development occurred during the first month of development, when the 4 and 400 ppb groups were developmentally delayed when compared to the control group. Since the parasitic infestation occurred during the fifth week of the experiment in the 400 ppb group, most likely the developmental delays were due to the increased physiological stress placed on the animals during the early stages of parasitic infestation. Once the parasite was noted and identified, all animals were dead within several days within this group. At this time, animals from all the other groups were removed from the tanks and inspected for parasites; however, no parasites were found in any other dose groups. The same parasite, *D. vastator*, was then identified in the 4 ppb group at week twelve. It should be noted that we did see delays in the development of the 4 ppb group in the absence of any visible parasitic infection following the month one TKI, but these delays were not evident in the 40 ppb group. With the

confounding problem of the parasites, it is difficult to attribute the effects in the 4 ppb group to the perchlorate exposure, but this possibility cannot be ruled out.

The weekly measurements provided very consistent findings between the dose groups. Although there were significant differences in the animal sizes at the start of the study (0, 40 ppb > 4 ppb, 400 ppb), these differences were no longer detectable by the fourth week of the experiment. In fact, all statistical analyses performed on snout to vent length, total length, and tail length following week three demonstrated that no differences were detectable between any of the dose groups.

The hindlimb results were more interesting, as the hindlimbs measured in the 40 ppb group during weeks 11, 15, and 16 were significantly larger than the control group. Unfortunately, the 4 ppb group all died three weeks prior and could not be evaluated. These data, although limited, may support the possibility of perchlorate exhibiting a dose-response curve that demonstrates a hormetic effect. However, with the added confounds of the parasites in this study, it is very difficult to make any firm scientific evaluation as to the possible mechanisms associated with the observed effects.

Secondary Study: Laboratory Mixed Perchlorate Water

A general trend noted in the experiment was that the magnesium group was larger and developed at the fastest rate. The magnesium control group completed metamorphosis by the eleventh week of the experiment and also had the highest number of animals that completed metamorphosis. The 40 ppb and 4000 ppb group were the next two groups to complete metamorphosis, respectively. Major conclusions that were identified in our

research are based on the following observations: 1) the doses used were not high enough to cause dose-dependent growth nor developmental delays; 2) since literature supports the fact that magnesium is necessary for glycolysis, we believe that magnesium increased energy production and subsequently increased growth, development and oxygen consumption; 3) sufficient iodine was available for growth and development; 4) oxygen consumption was the most accurate biomarker to match perchlorate's established mechanism and is a more sensitive analysis when using environmentally relevant low doses of perchlorate and 5) differences in the densities of animals among the tanks does not appear to have delayed development. Each of thse five statements will be discussed individually below.

Effect of Dose of Deformities, Growth, Development and Survival

It appears that the concentrations of perchlorate used were not high enough to cause developmental delays nor growth differences. For example, the results of the rate of development do not agree with our hypothesis. The established mechanism of perchlorate involves interference with the tadpole's ability to store and uptake iodine resulting in a deficiency of thyroid hormones, which are necessary for development. Our doses of perchlorate were not high enough to cause delayed development. There is little research on the LOAEL that will cause an effect on amphibian thyroid hormone levels and subsequent development. The results of our study with respect to growth and delays are not consistent with perchlorate's established mechanism of action; one possible explanation is that our doses of perchlorate were not high enough to deplete the thyroid

hormones. This is supported by the fact that even the highest dose group completed metamorphosis faster than the control group.

There were no major differences or trends that could be identified from the survivorship data among tanks (see Table 4). This survivorship data were opposite what we hypothesized as we expected survivorship to be reduced in a dose dependent phenomenon. Based on the known mechanism of action of perchlorate, we expected tadpoles exposed to perchlorate would not have the necessary TH to develop and would thereby experience a higher mortality rate. Although we did not measure TH, the survivorship data indicates that the TH were not depleted because there was no notable mortality or developmental delays among tanks.

Growth, as measured by snout to vent, hindlimb, total body and tail length were similar to the developmental results. Following a temporal analysis, the magnesium and the 40 ppb group had larger total body, snout to vent and tail lengths than the other groups. During week 11 through 20, the 4000 ppb group had larger snout to vent length than the control. Following a staged-based analysis, most of the differences were no longer apparent. This indicates that once the animals reach the same stage of development, there are no statistical differences in snout to vent, hindlimb, total body or tail length but temporally, differences were apparent. A more accurate method for scientists to determine growth differences is a stage-based analysis. Temporal analysis is not as biologically relevant because one would expect differences when there is comparison at different stages. These stage-based growth measurements provide an accurate assessment of any differential growth that may have occurred.

Another advantage of using stage-based measurements is that once differences were noted in the temporal analysis it did not always remain significant. Even though independence was assumed between each week of temporal morphometric characteristics, we are unsure what percentage of the tadpoles were constantly measured. Tadpoles that were included in numerous weekly analysis will have inherit variability which will skew the data. This variability will create error between the temporal measurements that cannot be accounted for. Since it was not feasible to identify individual tadpoles and perform temporal autocorrelation statistics; independence was assumed allowing us to perform an ANOVA when in fact the samples were not completely independent.

All of the morphometric characteristics, except the tail length, increase in size at each subsequent stage of development (see Fig. 6). More developed animals tend to have a larger total body size, except when the tail starts reabsorbing (see Fig. 7). Our results were not consistent with these statements because the higher concentrations of perchlorate did not result in smaller animals. We postulate the tadpoles still had sufficient TH, which allowed for development and growth despite the fact TH was not actually measured. The dose of perchlorate administered was probably not high enough to limit or block thyroid hormone availability. In subsequent studies it would be beneficial to measure T_3, T_4 and TSH in order to confirm if TH is dropping in the perchlorate dose groups. See Future Studies section for a more through discussion about measuring TH and the problems with measuring these in amphibians.

Asymmetrical limb development is the second component of the morphometric analysis, which was quite unpredictable following a temporal analysis. Although there was statistical significances during various weeks of the temporal analysis, no trends could clearly be identified. When these same hindlimb data were analyzed using a stage based analysis; no difference was noted between the right and left limb length within each group. The symmetry of forelimb development was also analyzed based on stage and there also was no statistical difference within dose groups. Perchlorate did not cause any differential development of the hindlimb or forelimb within each group.

Our research did not indicate a standard linear dose response curve. The molecular mechanism of perchlorates effects is dependent on the NIS. If iodine is still available to form thyroid hormones, one would not expect a standard dose response curve because the basic assumption is not met. The assumption that has to be met in order to see a dose response curve is that perchlorate is depleting internal stores of iodine and interfering with the uptake of iodine at the NIS. In order determine the biochemical effects of perchlorate, it would useful examine the molecular mechanism and measure T_3, T_4, TSH, TRH, bound vs. unbound thyroid hormones; however, this can be very expensive and requires a large sample size due to their small body weight.

No meaningful statistics could be performed on the deformities because we failed to track the weekly deformities in the individual tadpoles. This problem will be addressed more thoroughly in the Weakness of Approach section. The deformities table appears to show 4000 ppb having the most total deformities, which was uniform with our hypothesis. The control group had the second highest percent of deformities followed by

the 40 ppb, 400 ppb, and the magnesium group had the least number of deformities. The control group having the second highest percent of deformities was contradictory to our hypothesis. However, the validity of these data limits they usefulness.

Goldman *et al.* (2002a) performed a similar experiment with a wider range of perchlorate concentrations. Goldman *et al.*'s perchlorate concentrations were 5 ppb, 18 ppb, 147 ppb, 1412 ppb, 14.4 ppm, 133 ppm, 425 ppm, 585 ppm, and 1175 ppm. Ammonium perchlorate reduced the percentage of tadpoles completing tail resorption, but ammonium perchlorate did not completely prevent tail resorption at concentrations in the ppm range. No significant effect in snout to vent length were observed in concentrations below 425 ppm; however, perchlorate reduced hindlimb growth in a concentration-dependent manner and 5 ppb was the lowest concentration capable of inhibiting forelimb emergence. The results from Goldman *et al.*'s work were very different from our experiment's results. Most of the results that Goldman *et al.* saw were at concentrations in the ppm range while our research was in the ppb range. The only similarity was when Goldman *et al.* saw reduced hindlimb growth at the 5 ppb dosage. Besides the differences in perchlorate concentrations there were other variations in the form of perchlorate used; Goldman *et al.* used ammonium perchlorate while our research used magnesium perchlorate. Goldman *et al.* used *Xenopus laevis* because its development is well studied, while we used *Rana pipiens,* a genus native to Nevada. Because of the differences in the life-cycle (*Xenopus* is a completely aquatic frog) it is very hard to compare these two species.

Research performed by Sparling *et al.* (2003) found no differences in morality or growth in the 2.2 to 50 ppm range of *Hyla versicolor*. However, inhibition of hindlimb formation and metamorphosis was apparent in the latter stages. When only considering those tadpoles that entered the climax stage, there were no differences in the number of days to metamorphosis among treatments. Sparling *et al.*'s work seems to match the morality and growth trends that we found despite the fact that their concentrations of potassium perchlorate were all well above concentrations in our research. Sparling *et al.* (2003) found inhibition of hindlimb that we did not found, however I believe that this was apparent in their work because of the higher perchlorate concentrations and due to dosing in the larval stages.

Magnesium

The magnesium group was noticeably more developed than the other groups, so much so that it could not be included in the developmental (TKI) statistical analysis as it would have created artificial groupings and zeros in the expected positions. Despite the fact that the magnesium group was excluded from the statistical analysis, clearly it developed faster than the other groups. The statistical analysis measuring development demonstrates that the 40 ppb group had accelerated development during Month 1 and 2. The 4000 ppb group also had accelerated development during Month 4.

Magnesium is necessary for the metabolism of ATP-ADP in the course of glycolysis (Scanlon & Sanders, 1999). During glycolysis, glucose enters the cell and is phosphorylated by ATP to form glucose 6-phosphate, which is catalyzed by hexokinase (Stryer, 1988). Hexokinase, like all other kinases, requires Mg^{2+} for activity (Stryer,

1988). Magnesium is an essential element for the neuromuscular system, bone formation, and is the activator for enzymes (especially involving phosphate transfer) in amphibians (Brown, 1964). Based on how magnesium is used in the body, the up-regulation of magnesium should increase energy production and could thereby increase metamorphosis and oxygen consumption. The effects of the magnesium ion appear to have possibly attenuated the perchlorate effects. The two groups with the largest concentrations of magnesium were the magnesium control and the 4000 ppb, each having 30 mg of magnesium per 80 L of water. These groups (along with the 40 ppb) completed metamorphosis faster than the control and the 400 ppb possibly directly related to magnesium concentration.

Magnesium was added to all of the tanks in the form of frog brittle, however no analysis was performed to assess how much magnesium was taken up by the tadpoles from the frog brittle. The magnesium concentration in the tanks are as follows:

Table 10 Magnesium Per Tank (80L) Concentration

Dose Group	Magnesium Concentration (mg)
Control	0
40 ppb	0.33
400 ppb	3
4000 ppb	30
Magnesium Control	30

Ninety mg of magnesium was added to the tanks from the frog brittle each week (Nasco, 2003). Although the frog brittle contains three times the magnesium contained in the magnesium control and 4000 ppb tank, we did not perform analyses showing how much

of the frog brittle is actually bioavailable. Even though magnesium was added to the tanks, that does not mean the frog brittle dissolved in the water and the tadpoles had the capacity to uptake it. If future studies use magnesium perchlorate, it is necessary to measure how much magnesium is absorbed by the tadpoles.

A second explanation for why most of the groups completed metamorphosis prior to the control is that amphibians tend to speed up metamorphosis when their pond surface area starts to shrink. It has been shown that tadpole growth rate decrease and development rate increases as the pond dries (decreases in area) (Newman, 1989; Denver 1997a, b). Gradually reducing the water level leads to accelerated development of spadefoot toad tadpoles (Denver, 1998; Denver *et al.*, 1998). Denver *et al.*, (1998) found that the developmental acceleration due to water level reduction appeared to be related to the reduced swimming volume and perhaps the proximity to the water surface. One potential mediator of environmentally accelerated development may be stress neuropeptide corticotropin-releasing hormone (Denver, 1997 a, b) which, can directly stimulate the release of thyrotropin by the tadpole pituitary (Denver, 1998; Denver & Licht, 1989). Thus the effect of water level reduction is to increase the levels of thyroid stimulating hormones through corticotropin-releasing hormone. This same phenomena might be extrapolated to chemical parameters, such as perchlorate. The increased stress as a result of chemical exposure, could increase the rate of metamorphosis similar to the results seen with a shrinking pond. Although our data were not collected with this hypothesis in mind, it is an area that future studies should address.

During Month 0 of the oxygen consumption, the control and magnesium had a higher routine metabolic rate than the perchlorate dose tanks (See Figure 11). This analysis was performed exactly one week after the start of the experiment, prior to the filter problems. One week of exposure to the magnesium created an increase in oxygen consumption. Based on magnesium's established involvement in energy ATP hydrolysis, we speculate that even one week of exposure to magnesium causes an increase in ATP which subsequently increases oxygen consumption. This increased energy production, due to the magnesium, created a measurable increased oxygen consumption. Although we did not measure TH, the perchlorate doses did not result in biomarkers consistent with decreases in thyroid hormones. One possible explanation that the 4000 ppb group did not consume as much oxygen as the magnesium control, despite the fact that both groups were receiving the same magnesium concentrations, is that the 4000 ppb group also had perchlorate effects. Based on perchlorate's thyroid depleting mechanism, the 4000 ppb group would be consuming less oxygen than the control, however the 4000 ppb group also had a magnesium effect that increased oxygen consumption. The combination of the perchlorate and magnesium resulted in the 4000 ppb consuming less oxygen than the magnesium control while the control group consumed more oxygen than the 4000 ppb group.

Iodine

Since iodine is essential for thyroid hormone production it is imperative that sufficient iodine is available to the tadpoles. Some of the scientific literature suggests that even if animals experience developmental delays from perchlorate, after a recovery

period the changes can be reversed once iodine becomes available (Siglin *et al.*, 2000). The Nasco frog brittle contained 48 mg per kg of iodine (Nasco, 2003). Each tank (80 L) received approximately 4.5 grams of brittle each week, which means that 0.216 mg of iodine per 80 L tank could be available in the tanks from the frog brittle. All 4.5 grams of brittle did not dissolve in the tanks and no analysis was performed determining how much of the iodine was bioavailable to the tadpoles nor how much iodine the tadpoles actually receive from the food. High amounts of elemental iodine can induce metamorphosis in amphibians, however, iodine needs to be approximately 300 times the amount found in equally effective levels of thyroxin (Pollard & Adams, 1988). If equal consumption of the frog brittle is assumed then the amount consumed per individual animal can be calculated in order to compare the iodine values among groups. During the second week of the experiment there were 62 tadpoles in the magnesium control, 102 tadpoles in the control, 40 tadpoles in the 40 ppb group, 111 tadpoles in the 400 ppb group and 85 tadpoles in the 4000 ppb group (See table 2). Dividing the 0.216 mg by the number of animals in each tanks results in:

Table 11 Iodine Available Per Animal In Each Tank (80L)

Dose Group	Iodine Concentration (µg)
400 ppb	1.95
Control	2.11
4000 ppb	2.54
Magnesium Control	3.48
40 ppb	5.40

The greatest difference between the iodine levels is only 2.7 times different, therefore there was enough iodine available for development, however it was not a excessive level that would increase the growth and development. The amount of iodine available per individual animal is minimal, therefore the difference in iodine levels will not produce a difference in the iodine dependent development.

Sensitive Biomarkers

The oxygen consumption results matched our hypothesis better than any of the other analyses. One explanation is that oxygen consumption is a more sensitive test that can detect differences before they become apparent in the growth or development or at lower doses than the developmental or growth measurements. Despite the low body weight of the tadpoles resulting in the oxygen consumption being at the lower ends of the detection, the results of the analysis show that it matches of hypotheses. The weight-adjusted oxygen was a more biologically accurate method for determining the routine metabolic rate of the tadpoles because the volume of oxygen consumed by an organism is weight dependent. The logarithms of the basal metabolic rate and standard metabolic rates are linearly related to the logarithms of body mass (Gordon, Bartholomew, Grinnell, Jorgensen & White, 1982). This relationship is described by the allometric equation $\dot{E}_m=aM^b$ where $\dot{E}_m$ is the rate of energy metabolism, a (the proportionality constant) is the metabolic rate of an animal of unit mass, M is mass, and b is the exponent. For frogs of the family Ranidae at 25 degrees Celsius b=0.75 (Gordon *et al.* , 1982). Both the magnesium control and the control groups consumed more oxygen than the other groups and therefore had a higher routine metabolic rate. The weight adjusted oxygen

consumption is not a straight dose dependent phenomenon as the 400 ppb tank was consistently consuming the least oxygen. These results demonstrate partial support of our hypothesis that those tadpoles exposed to the higher concentrations of perchlorate will have a reduced routine metabolic rate. Oxygen consumption is proxy for cellular respiration. Perchlorate's established mechanism of action is depletion of TH; a decrease in TH results in lethargic tadpole, who will consume less oxygen. Therefore, oxygen consumption maybe an effective indirect tool to evaluate the effects of perchlorate on tadpoles.

Consistent with previous findings, the 40 and 4000 ppb groups; which completed metamorphosis before the control and 400 ppb, had a higher metabolic rate starting in Month 1which continued until the groups completed metamorphosis. Those groups that completed metamorphosis more quickly also had a higher metabolic rate.

Tadpoles, regardless of the stage of development, have a low metabolic rate (per gram body mass) because they are mostly comprised of water. The wet weight of tadpoles increases from the first day of hatching until the onset of metamorphosis (Brown, 1964). There is a large difference in oxygen consumption depending on whether wet or dry weights were taken. Since the tadpoles in our research were not sacrificed after oxygen consumption, it was only feasible to take the wet weight. The ratio of wet weight: dry weight of the animals ranges from about 0.2 to 1.0 at the first day, to 0.05 to 1.0 by the tenth day of development (Brown, 1964). This ratio then remains rather constant until metamorphosis (Brown, 1964). These data indicate that the metabolic rate

of tadpoles at different stages of development (TKI stages 1-18) are relatively similar and can be compared except during the climax stages (stages 19-25) of development.

Those tadpoles that were exposed to higher doses of perchlorate experienced a lower metabolic rate. Based on perchlorates mechanism, iodine was being depleted within the amphibians who were not able to effectively make TH. The decrease in the TH resulted a in a lower metabolic rate. The metabolic rate was able to detect minor differences that were not as evident in the morphological measures or TKI developmental stages. If T_3 and T_4 increase oxygen consumption, we would expect perchlorate to decrease oxygen consumption because the animals are lethargic due to the lack of TH. Recall, this may occur prior to seeing adverse effects on growth and development.

Differences in Tank Densities

Another possible explanation for thedifferent rates of metamorphosis may be differences in the individuals (see Fig. 2), which would lead to differences in resources (food, living space, stress). Not only did the filter related deaths create differences in the number of animals per tank but also size selected the animals. The smaller animals experienced greater attrition due to the filter, leaving larger animals in the tanks. All tanks started with 114 or 115 animals, by week 2 there were 62 animals in the magnesium control, 102 animals in the control, 40 animals in the 40 ppb tanks and 85 animals the 4000 ppb tanks. Tanks that contained the largest animals would presumably develop faster than the smaller animals, however, all the tanks started with no statistical differences among developmental stage (see Fig. 12, week 1).

The second component of the filter errors involved differences in tank densities. The number of animals per tank leads to differences in resource availability which could create a situation completely unrelated of perchlorate; however, the literature shows that the differences between the tanks was small enough that it did not appear to account for differential growth and development. The stock density for larval leopard frogs maximum growth is 25-50 per 10 gallon aquarium (NMCRWRU, 2003), the 20 gallon tanks in our experiment should therefore hold 50-100 animals without growth or developmental delays. Table 2 shows that the number of animals in each tank was slightly above the critical numbers as identified by NMCRWRU (2003) when the experiment started, but by the second week of the experiment, the number of tadpoles were well within acceptable ranges. If density was the only variable acting on the tadpoles then one would expect the 40 ppb to complete metamorphosis the fastest because it had the lowest density. This did not happen, the magnesium group completed metamorphosis at the fastest rate.

Altwegg (2003) manipulated density of *R. lessonae* and examined the effects on growth and survival. High larval density, but not high juvenile density, led to smaller size at this age. Both larval and juvenile density led to reduced growth during the early juvenile stage, but the effect of larval density appeared stronger than the effect of juvenile density. Survival was not significantly affected by the density treatments. Altwegg (2003) used 25 individuals per 1,100 L fiberglass cattle tanks in the low density units, while high density units held 75 individuals per 1,100 L. Altwegg's low density units are equivalent to 2 tadpoles in 80 L aquaria while the high density units are equivalent to 6

animals in 80 L aquaria. It is difficult to compare his research to ours because Altwegg's densities are so much lower than our experiment. In our research the differences in individuals per aquaria were greatest with 40 tadpoles per 80 L aquaria versus 111 tadpoles per 80 L aquaria.

Semlitsch & Caldwell (1982) found that density and time had significant effects on body size at metamorphosis and days to metamorphosis of *Scaphiopus holbrooki*. In their experiment densities ranged from 1 individual per 2.5 L of water to 90 individuals per 2.5 L. The low density aquaria in Semlitsch & Caldwell's research contained 1 individual per 2.5 L of water which is equivalent to 32 individuals per 80 L of water. This value is very similar to our research which had 40 individuals per 80 L of water; however, the high density group has over twenty-five times more individuals than our experiment. An aquaria with 90 individuals per 2.5 L of water is equivalent to 2880 individuals per 80 L of water; 2880 is vastly different than the 111 animals in our largest sample size. The second lowest density unit in Semlitsch & Cadwell's research was 15 individuals per 2.5 L (equivalent to 480 individuals per 80 L). The differences in the number of animals in our research were around Semlitsch & Cadwell's control group. Therefore, it appears that differences in size or days to metamorphosis occurred due to the number of individuals per liter of water occured at densities much greater than were present in our research.

In another experiment by Semlitsch & Caldwell (1982), the mean number of days to metamorphic climax was positively associated with the initial density treatment: 27 days at the lowest density and 86 days at the highest density. The survival of tadpoles

decreased exponentially with initial density and the growth index of tadpoles also decrease exponentially with density. The lowest density aquaria for these later experiments held 3 individuals per 2.5 L (equivalent to 96 individuals per 80 L) of water while the highest density aquaria held 30 individuals (equivalent to 960 individuals per 80 L). These densities are also much higher than our experiment

Weaknesses of Approach

Our research focused on the effects of environmentally relevant concentrations of perchlorate on the growth and development of *R. pipiens*. Because we were using low doses, endpoints that can detect slight differences in growth and development, such as oxygen consumption, must be used. Measuring the thyroid hormones (T_3, T_4 and TSH) would have been very useful in detecting these subtle differences; however, because our research involved such small organisms, a large number of animals would be required. Approximately 20 grams of tissue is required for TH analysis and each tadpoles usually weighs under one gram.

Interferences based on magnesium on amphibian development seem possible, thus, it would have been beneficial to use a different form of perchlorate salt in order to assess the effects that perchlorate (and the associated cations) have on amphibian growth and development. Since both the iodine and magnesium were supplied in the frog brittle, it would have been helpful to know how much magnesium and iodine the tadpoles are actually utilizing.

It is important to remember oxygen consumption values are based on one repetition that was selected. Analysis accounting for numerous repetitions can be computed,

however this would compound the error. Each repetition would have an average slope representing oxygen consumption of the 5 animals in each trail, with each repetition having its own variance. The average of the averages, or a grand mean, could be calculated to perform the statistics, however this would compound the variance for each measure. Combining the means would potentially attenuate the difference between the dose groups because the standard deviations could increase for each group. This may result in decisions that are confounded by statistical error and may or may not be biologically relevant.

The final issue that should be discussed is the tracking of the deformities. Deformities were not accurately counted from one animal to the next during each week, thus there was no way of identify if a new deformity was appearing or if it was counted the previous week. This led to our inability to perform meaningful statistics and therefore we could not make any significant conclusions about which tanks experienced the highest percentage of deformities. Since there are no easy identification methods for such small developing tadpoles, we recommend that once a deformity is identified the animal be sacrificed to avoid recounting deformities. It is also suggested that a clear, apriori distinction between categories of deformities is designated.

Future Studies

As toxicologists try to locate the minimum dose of perchlorate that can affect the development and growth of amphibian populations, it is important to have a wide enough range of treatments including a positive control. The 40 ppb, 400 ppb and 4000 ppb of perchlorate concentrations did not produce the results we expected; clearly these

concentrations were not high enough to delay development and growth. As future toxicology studies attempt to identify the perchlorate concentrations associated with an adverse effect on amphibians, they need to include a concentration of perchlorate already known to have adverse effects. The LC50s identified by Goleman *et al.* (2002) would be effective high doses in amphibian perchlorate studies (510 ppm for a 5-day analysis and 233 ppm of perchlorate for a 70-day analysis).

Perchlorates mechanism of action is well established; however, the exact dose that causes developmental and growth delays remains to be pinpointed for amphibians. Deformities and morphological differences as a result of a complete block of thyroid hormones are only going to occur at high perchlorate doses, however when looking for subtle toxicity difference endpoints must be used, such as oxygen consumption. This subtle toxicity can easily be identified and noticed in T_3, T_4 and TSH levels. One should see a difference in these thyroid hormones before any physiological or developmental effects occur. The problem with performing a TH chemical analysis in tadpoles is that numerous tadpoles will have to be sacrificed in order to create an adequate sample to measure T_3 ,T_4 or TSH. Performing several tissue analyses of the thyroid hormones is not only expensive but requires a very large original sample size in order to have sufficient samples from such small animals. These large sample size may also lead to density related problems.

Besides the necessity of appropriate dose concentrations, there is also the issue of the timing of the dose. Amphibians have a robust demand of thyroid hormones during forelimb emergence. The thyroid hormones must be depleted during this specific time of

increased demand (TKI stage 16-21) in order to see perchlorate's intended effect. In order to ensure that this is indeed occurring, it would be beneficial to measure the amount of thyroid hormones available during this time frame.

The research performed herein presents an interesting question regarding the toxicity of the cations associated with the perchlorate ion. Perchlorate salts dissolve readily in an aqueous solution but the effect of potassium perchlorate versus sodium perchlorate or magnesium perchlorate has not been performed to my knowledge. It would be valuable information to identify if toxicological differences exist between these different forms.

Similar research performed by Goleman *et al.* (2002) had different conclusions than our research, one explanation is the form of perchlorate that was used. Goleman *et al.* (2002) used ammonium perchlorate while our research utilized magnesium perchlorate during the laboratory experiment. The role that magnesium plays in metamorphosis was raised in our study; it would be useful to completely understand the relationship between magnesium and amphibian development. Perhaps a study only using different concentrations of magnesium should be done to invest this relationship.

When future studies investigate the effects that low doses of perchlorate have on amphibian populations I suggest that they include oxygen consumption in their experimental design because our research showed it to be an sensitive biomarker. Because it takes a larger dose to see more pronounced developmental deformities or morphological differences, using endpoints that measure more subtle differences in the thyroid hormones might be more appropriate.

Conclusions

This research attempted to identify the concentrations of perchlorate that affect the growth, development and survival of amphibian populations. Ammonium perchlorate contamination has become an issue of increased concern in the southwestern United States. As we begin to understand the effects that perchlorate is having on amphibian populations, we might also begin to gain a more thorough understanding about why amphibian populations are declining.

The majority of the results indicate that our doses of perchlorate (40 ppb, 400 ppb and 4000ppb) used were not high enough to cause deformities, developmental delays nor growth impediments. Although thyroid hormones were not measured, the tests we performed indicate the perchlorate was not reducing iodine availability. Perchlorate's established mechanism of action is the prevention of thyroid hormone formation, which are necessary for amphibian growth the development. Although there was statistical significance between doses when the snout to vent, hindlimb, total body and tail length were analyzed on a temporal bases, most of these differences were no longer apparent following a staged based comparison.

The magnesium control was so advanced in development that it had to be excluded from the developmental statistical analysis. One possible explanation for the magnesium control group's accelerated development relates to magnesium's role in ATP-ADP metabolism during glycolysis. Owning to magnesium's incorporation during energy production, the increased magnesium concentration appears to have increased energy production and subsequently expedited growth and development.

The oxygen consumption results matched our hypotheses better than any of the other tests and appears to be a more sensitive biomarker at low doses of perchlorate. It is necessary to use these sensitive biomarkers in future studies because the more obvious developmental and growth delays were not apparent in the environmentally relevant range.

Despite the difference in tank densities that occurred at the beginning of the experiment, the literature indicates that the differences that occurred in our research where not large enough to cause any developmental or growth effects.

This research is important to the community because it offers insight on the impact that low dose, environmentally relevant concentrations of perchlorate may have on amphibian populations. Most of what we know about the thyroid gland was discovered in amphibians models. Amphibians are relevant species to study the effects of perchlorate because their thyroid system functions in much the same way that human thyroid glands function. Humans need thyroid hormones for metabolism, growth and development just like amphibians do. In fact, the structure of thyroid hormones in highly conserved across species. Amphibians are important bioindicators of ecosystem health. Changes in ecosystems affect multiple trophic levels; and small disturbances to amphibian populations could end up having more pronounced impacts on predator species. Research on amphibians allows changes in our environment to be recognized prior to seeing any effects in upper trophic levels such as humans.

APPENDIX A

FINAL REPORT FOR THE LAS VEGAS WASH WATER EXPERIMENT

Final Report:
The effects of Las Vegas Wash water containing elevated levels of perchlorate (CLO_4-) on development and metamorphosis in the Northern Leopard Frog (*Rana pipiens*).

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INTRODUCTION

The greater Las Vegas metropolitan area is a rapidly growing urban center in the southwestern United States, which serves as home to approximately 1.2 million permanent residents. The cities of Las Vegas, North Las Vegas and Henderson constitute the majority of the population in the Las Vegas Valley, which is surrounded by Mt. Charleston, Black Mountain, Sunrise Mountain and the Spring Mountains. The Las Vegas Valley is a 1600 square mile area drained by the Las Vegas Wash (hereafter referred to as the Wash), which flows directly into Lake Mead.

Several industrial areas exist along the Wash and have released or spilled environmental contaminants into this area. Of particular concern is the release of ammonium perchlorate, an oxidizer used in the production of rocket fuel. This has resulted in concentrations of perchlorate (CLO_4-) in the Wash water that far exceed the 18 ppb EPA limits .

Perchlorate, in the mammalian system, is known to actively inhibit the uptake of iodide by the thyroid gland and to deplete the internal stores of thyroidal iodide. This mechanism of action has been related to measurable adverse effects such as hypothyroidism, hypertrophy, and hyperplasia of thyroid follicular cells.

In amphibians, large doses of perchlorate salts (such as potassium and sodium perchlorate) are known to block metamorphosis. Many of the steps that regulate metamorphosis in amphibians are controlled by the presence of the appropriate level of

thyroid hormones at crucial times during development. Thus, it should be no surprise that high concentrations of these chemicals could alter normal development and metamorphosis. However, the effects of environmentally relevant concentrations of perchlorate in surface waters, such as the Wash, have not been accurately assessed. Thus, the major objective of this project is: To determine if Las Vegas Wash water containing elevated concentrations of perchlorate can alter the normal development and metamorphosis of the Northern Leopard Frog (*Rana pipiens*).

MATERIALS AND METHODS

Animals

A total of 215 Northern Leopard Frog tadpoles (*Rana pipiens*) (equivalent to stages 0-1 according to Taylor-Kollros Index) were purchased from Trans-Mississippi Biological Supply, St. Paul, Minnesota. Northern Leopard Frog tadpoles were separated into four dose groups of fifty-two each, and 7 animals were sacrificed for chemical analysis prior to dosing. Animals were held in 20 gallon aquaria containing 22 ± 2 degree C water which was changed weekly (for protocol, refer to Appendix A), aerated and subject to a 12 hour light: dark cycle. The experiment began when the tadpoles arrived on June 30, 2002 and commenced on October 21, 2002 when the vast majority of the tadpoles had undergone metamorphosis. Tadpoles were fed powdered frog brittle (NASCO, Ft. Atkinson, WI, USA) three times a week. A sample of frog brittle was removed at the beginning and end of the project, frozen and shipped to Clayton Laboratories for chemical analysis. The University of Nevada Las Vegas has a certified AALAC facility and the appropriate animal use and care protocols were submitted and approved prior to the beginning of this project (UNLV Protocol #R993-0502-166).

Water Collection and Water Analysis

Water samples were collected once every two weeks from the Las Vegas Wash at monitoring site LM-8 (N 36' 36'35.3", W 114' 14'.28.3") (also known as ECS 3 using Parsons nomenclature and the demonstration weir using local nomenclature) and Overton Beach (N 36' 26' 39.3, W 114' 20' 52.8") at Lake Mead (control location). The LM-8 site

is known to have concentrations of approximately 439 ± 198 ppb of perchlorate, while the Overton site is below the detection limit of 4 ppb (Joseph Leising, Personal Communication), and thus assumed to be at or very near to zero. Following water collection, a 1000 ml sample of water was shipped to Clayton Laboratories, where the exact concentration of perchlorate and iodide in the water was determined. After receiving the results of the initial water tests, the concentrations of perchlorate in each Las Vegas Wash sample was diluted with Overton Beach water to approximate 0, 4, 40, and 400 ug/L. Once the respective tanks were diluted, a 1000 ml sample of water was obtained and sent to Clayton Laboratories to determine exact concentrations of perchlorate and iodide in each tank (started 18 June 2002). In order to accurately assess the potential environmental impacts of perchlorate on development and metamorphosis of *Rana pipiens*, environmental concentrations of perchlorate were allowed to fluctuate within one standard deviation (439 ± 198 ppb) of the target range. The protocol was designed to mimic the environmental conditions and natural fluctuations that occur in the Las Vegas Wash. Thus, the two lower dose ranges were also allowed to fluctuate; however, these were directly proportional to the concentration of the highest dose.

Water was changed weekly in accordance with ongoing animal husbandry practices. The water was allowed to acclimate to room temperatures for a minimum of12 hours before the tanks were change. Any additional water collected but not immediately used, was place into capped 5 gallon buckets and stored at 4° C until needed. No water was held longer than two weeks, or allowed to stay in a tank for more than one week.

The water used for the control animals was acquired from Overton Beach at Lake Mead, well upstream from the inputs of the Las Vegas Wash, using the same methods described above. The Overton water was used as a control and also used to dilute the Las Vegas Wash samples to achieve the desired concentrations listed above.

Five gallon buckets were filled from Overton Beach water and labeled "O" for use in the control tank and in dilution of the low perchlorate doses. Five gallon buckets were filled from Las Vegas Wash site LM-8 and labeled "W," and used as the high dose.

From June 20 to July 1, 2002, an Orion Research 811 meter was used to determine pH, an Orion 810 meter was used to determine dissolved oxygen, and an Orion 115 meter was used for conductivity and temperature. From July 2, 2002 to October 18, 2002 conductivity, pH, temperature, and percent dissolved oxygen were calculated daily using YSI model 63 and YSI model 85 digital meter for each tank (for protocol, refer to Appendix B).

Oxygen Analyzer

Oxygen consumption was performed in order to determine the metabolic rate of tadpoles by monitoring their total oxygen consumption. Total oxygen consumption provides an accurate measure of metabolic rate, which is known to be related to thyroid hormones. Once a month, 10 tadpoles from each dose group were tested. Two chambers were set up with 5 animals from the same dose group in each chamber for month 1, 2, 3, and 4 however, in month 5 the analysis was performed with only 2 animals per chamber due to the fact most of the tadpoles had already undergone metamorphosis. The equipment we used to perform the analysis was Sable System's model ReadOx-4.

The first step in preparing the oxygen analyzer is to assemble the electrodes by covering the electrodes with the 0.001" or 0.0009" thick polyethylene membrane. The electrode is then placed into de-ionized water for calibration. Sodium Dithionite is used to zero the equipment by removing all of the oxygen from the de-ionized water that sodium dithionite is dissolved in. The channels are then spanned back to 150. The animals are then placed in the chamber filled with water from the appropriate dose group and the electrode is connected. The acquisition program was set for 30 minutes to obtain the data points.

The Oxygen Analyzer provided data that was used to construct an oxygen consumption slope, which was used to then calculate the tadpoles VO_2/hour. The ambient air temperature and barometer pressure remained relatively constant around 23 degrees Celcius and 0.95 mb. Using a table of Oxygen Solubility in Fresh Water at 23 degrees Celcius the dissolved oxygen is 8.56 mg/L. The conversion factor of 0.7 is used because we needed to convert from mg/L to ml/L. The elevation in Las Vegas changes the standard of 1 atm to 0.95 atm, which is used in the following formula to obtain the saturation point of oxygen.

$$8.56 \text{ mg } O_2/\text{ L } H_2O \text{ X } 0.7 \text{ ml } O_2/\text{ L } H_2O \text{ X } 0.95 \text{ mb} = 5.69 \text{ ml } O_2/\text{L}$$

The chamber volume remained constant in all of the experiments at 0.5 L and the PO_2 at sea level is 159 but with an altitude correction PO_2 is 151. The following calculation was used calculate VO_2/min.

$$\frac{\text{Sat}[O_2] \text{ X Chamber Volume}}{PO_2} \text{ X Slope X } 1000 = VO_2/\text{minute}$$

This number was then multiplied by 60 minutes to determine the VO_2/hour which is multiplied by the mass of the animals in the chamber to get the final computation of VO_2/hour.

Developmental Landmarks

Taylor Kollros Index

Once a month an assessment of metamorphosis was completed on the tadpoles according to normal stages of *Rana pipien* development as defined by Taylor, A.C., and Kollros, J.J (1956). Ten tadpoles from each tank were examined once a month and were euthanized by immersion in 3- aminobenzioc acid ethyl ester (MS-222 1g/L in distilled water). After they were euthanized, the tadpoles were placed under a microscope and the Taylor-Kollros Index (TKI) was used to determine the development stage of each animal. The index was specifically designed for this genus and used a 25 stage evaluation series targeting the same endpoints that have been shown to be disrupted by perchlorate exposure in amphibians.

Weekly Measurements

Each Monday, 20 tadpoles were measured from each dose group. Each tadpole's tail length, body length, snout to vent length, total body length and hind limb length (if applicable) were determined. The tadpoles were measured using an electronic caliper. Weekly, all of the tadpoles were monitored for one of the three types of including: edema, bent tail, and abnormal swimming as described by Golemen *et al.* 2002.

RESULTS

Animals

The animals arrived on June 20, 2002 and were randomly assigned to the different tanks. The first month of the TKI was performed the first week on the experiment on June 25, 2002. During the second Taylor Kolloros Index performed during the fifth week on July 22, 2002 we noticed that all of the animals in the high dose group had developed a parasite, all of the 400 ppb dose group died during week 5 of the experiment. The parasite was later identified as Dactylogyrus vastator. The low dose group (4 ppb) also experienced a rapid death rate starting on September 5 which was later attributed to the infestation the same parasite. All of the 4 ppb does group was dead by week 12. See Appendix C for a chart of when animals died from each dose group. The experiment for the other two dose groups concluded with the final sacrifice on October 21, 2002.

Water Collection and Water Analysis

Perchlorate and iodine concentrations were determined weekly in each tank. Our highest dose range fluctuated from approximately 200-500 ppb perchlorate, with a target range of 400 which is illustrated in Figure 1. Similarly, acceptable fluctuations in the low dose groups were noted. The dose range for iodide fluctuated from approximately 0-45 ppb which is seen in Figure 2. These fluctuations were within the acceptable ranges discussed with the funding agency at the onset of this project.

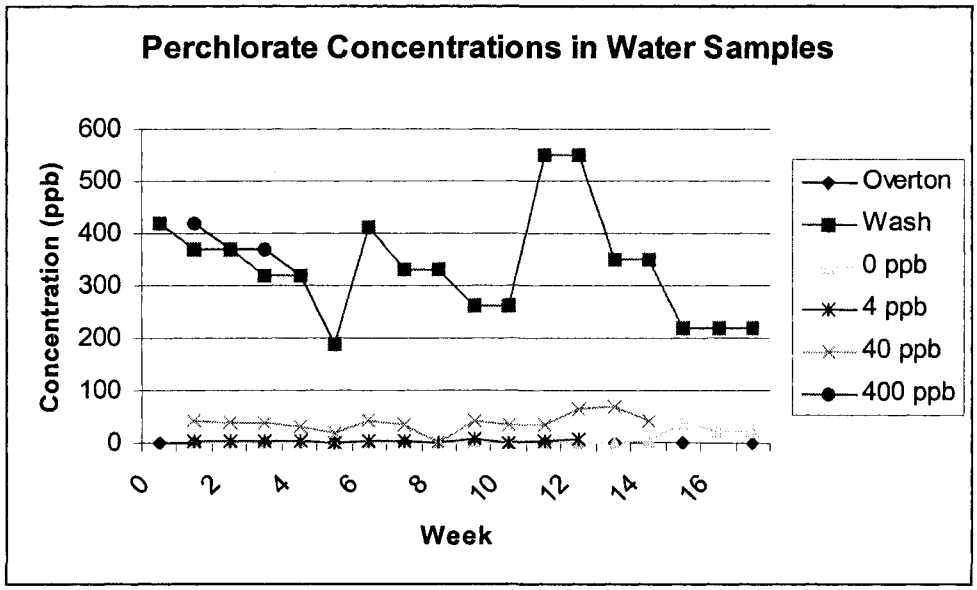


Figure 1. Weekly perchlorate concentrations in water samples taken from the Wash, Overton, 0 ppb, 4 ppb, 40 ppb, and 400 ppb tanks.

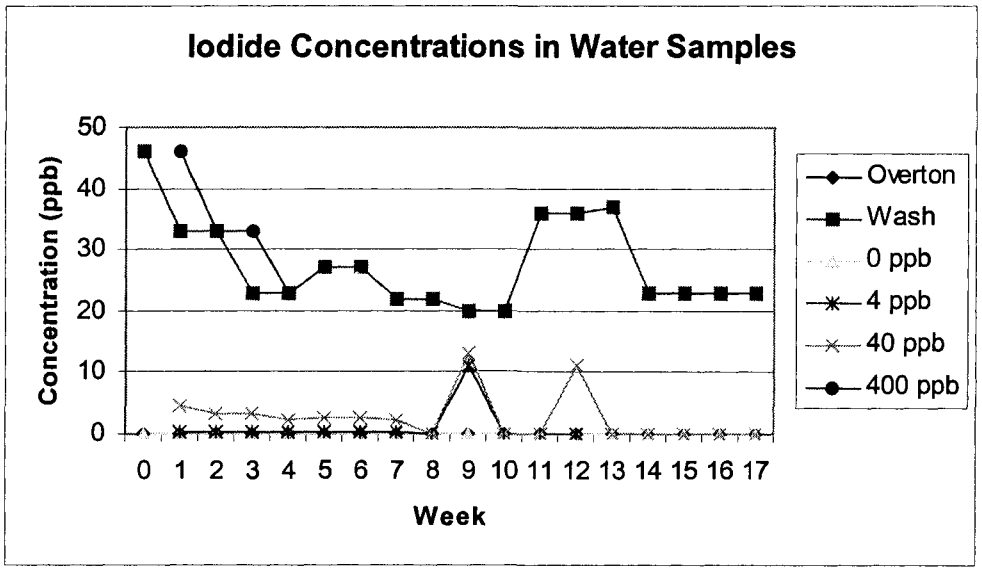


Figure 2. Weekly iodide concentrations in water samples taken from the Wash, Overton, 0 ppb, 4 ppb, 40 ppb and 400 ppb tanks.

Note that the experiment started on June 20, 2002 and Week 1 of the graph corresponds to the first full week of the experiment, the week of June 24, 2002.

See Appendix D for a chart of the Water Analysis analytical data for the perchlorate and iodine levels in Overton and Wash Water as well as the concentrations in the 4 dose groups

Daily determinations of pH, temperature and dissolved oxygen were monitored in all of the dose groups. Some discrepancies were noted in the data during the first three-weeks, but new equipment was purchased to remedy this problem. The range of the pH between weeks 3 through the end of the experiment was approximately 8.1 to 7.7 (Fig. 3). The range of the dissolved oxygen was between 60-75 % between weeks 3 through 17. The percent oxygen for the first two weeks is above 100 % due to conversions and the malfunctioning equipment (Fig. 4). Temperature ranged between approximately 18 and 21 degrees Celsius (Fig. 5). The conductivity was markedly higher in the highest dose group (400 ppb), with conductivity values below 1200 uS for all other dose groups (Fig. 6). Due to the fact the raw data set is so large, we did not included it all in the report, however it is available upon request.

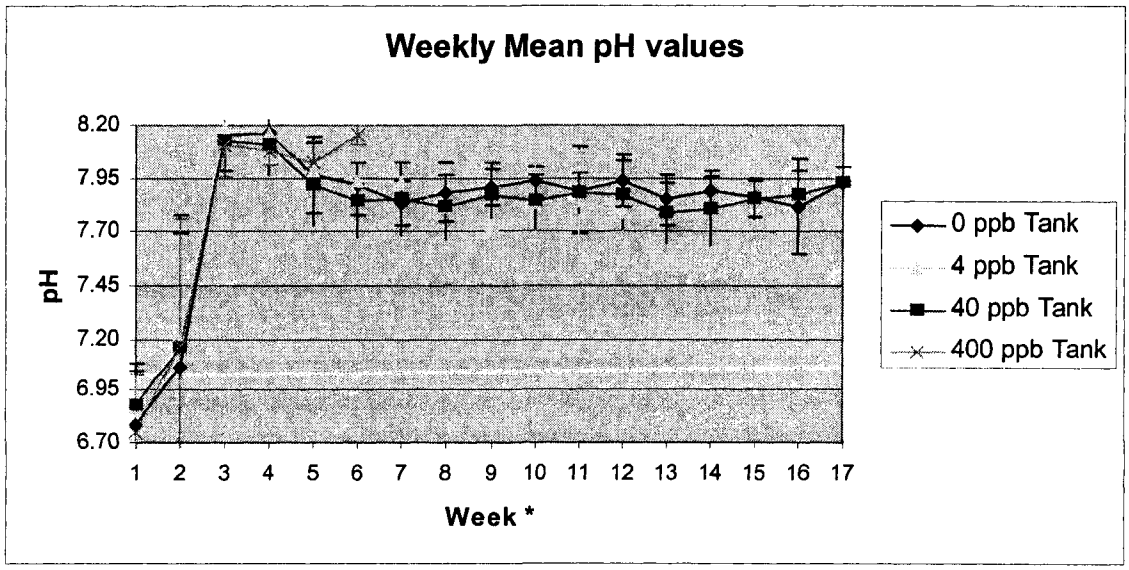


Figure 3. Weekly pH values for all dose groups based on seven-day averages.

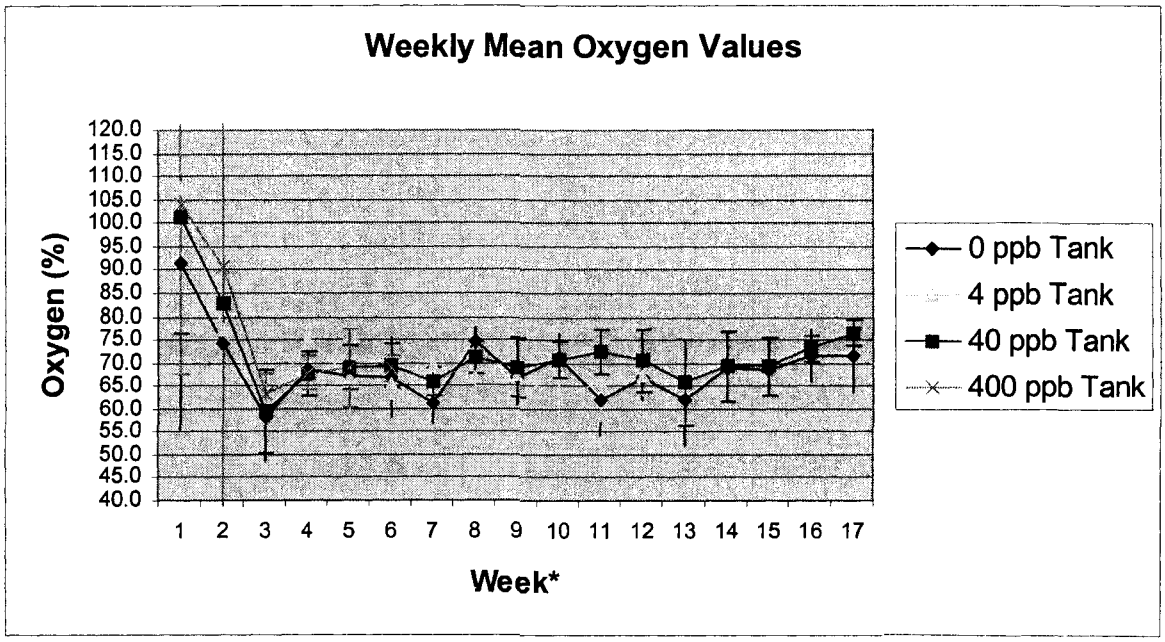


Figure 4. Weekly oxygen values for all dose groups based on seven-day averages.

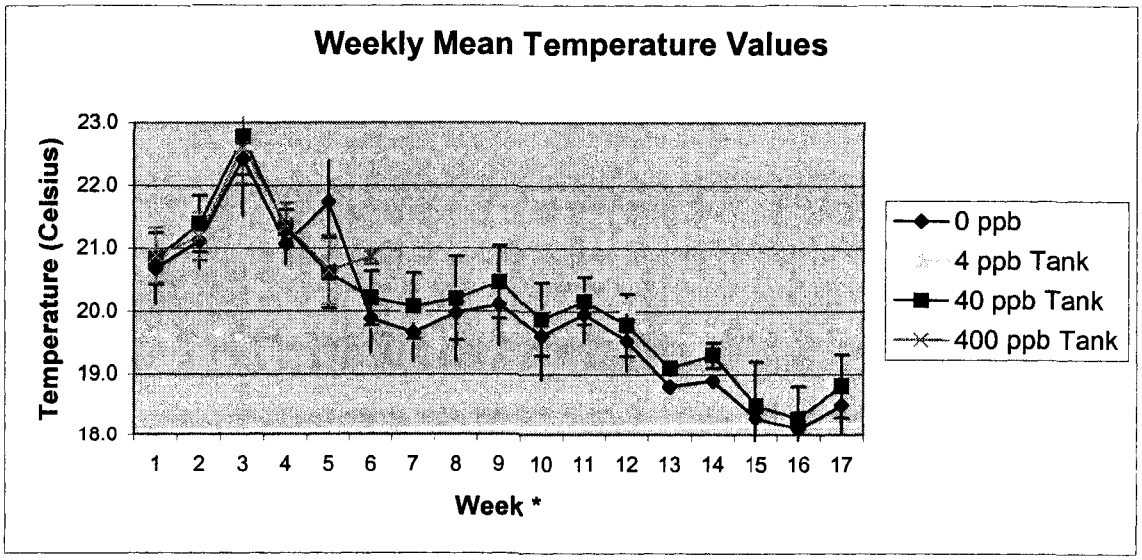


Figure 5. Weekly temperature values for all dose groups based on seven-day averages.

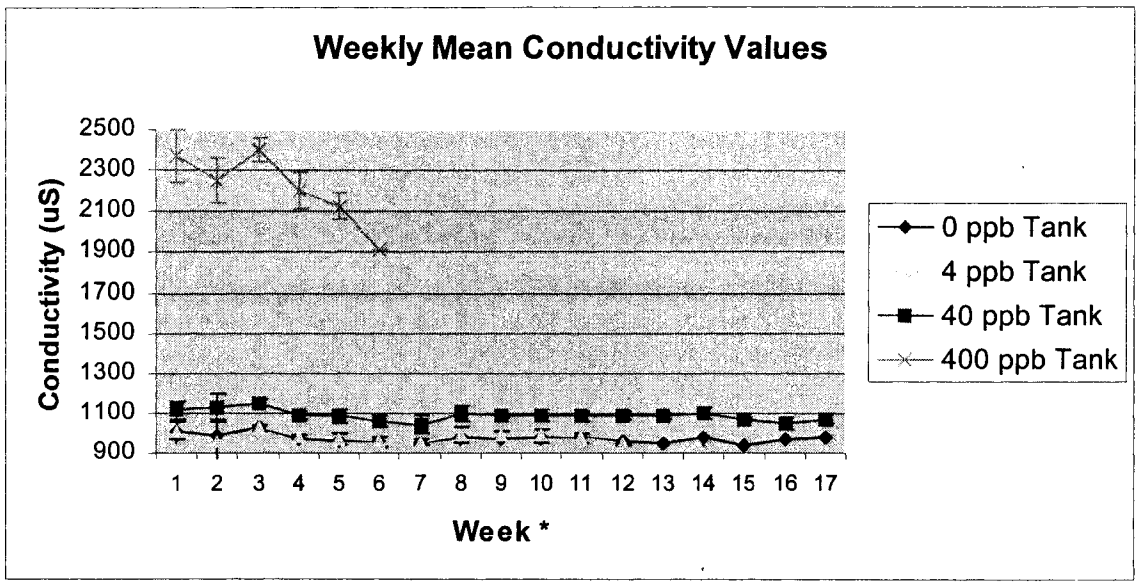


Figure 6. Weekly pH values for all dose groups based on seven-day averages.

Oxygen Analyzer

Total oxygen consumption was calculated for each group at monthly intervals, these data are shown in Appendix E. Figure 7 gives a summary of the total oxygen consumption per group including means and standard deviations for each dose group by month.

VO_2/grams x hour

Month

Concentration (ppb)	1	2	3	4	5
0	(n=7) 81+/- 31	(n=6) 147+/- 22	(n=3) 91 +/- 10	(n=6) 93+/- 26	(n=3) 94+/- 29
4	(n=6) 198+/- 121	(n=5) 132+/- 35	(n=4) 55+/-11	N/A	N/A
40	(n=4) 134+/-171	(n=6) 139+/-111	(n=4) 80+/-44	(n=6) 86+/- 14	(n=4) 103+/- 19
400	(n=6) 196 +/- 90	(n=4) 79 +/- 51	N/A	N/A	N/A

Figure 7. Volume of oxygen consumed per gram of tadpole per hour. Expressed as mean +/- standard deviation (sample size)

Developmental Landmarks

Taylor Kollros Index

The following are frequency distributions for the monthly Taylor Kollros Index (TKI). The x- axis is the stage of development and the y-axis is the dose group concentration with the frequency is tabulated in the body of the graphs for each month. The TKI stages were only significant during the first month of the experiment. The TKI for month one was performed on June 25, 2002, the first week of the experiment. The 4 ppb group exhibited delayed development compared to the control group (Chi Square, χ^2 =12.83, P = 0.025, Critical Value = 12.83). The 400 ppb group also exhibited delayed development when compared to the control group (Chi Square, χ^2 =17.47, P<0.005, Critical Value = 16.750.) For months two through five, no significant differences were noted in the stage of development (TKI) between the dose groups using the Chi Square test.

Stage of Development

Dose (ppb)

	1	2	3	4	5	6	
0	3	2	1	2	1	1	
4	7	1	1	0	0	0	P=.025 χ^2=12.83
40	5	1	0	2	2	0	
400	10	0	0	0	0	0	P=<.005 χ^2=17.47

Figure 8. Month1 Taylor Kollros Index for 0 ppb, 4 ppb, 40 ppb and 400 ppb dose groups. The range of the TKI for Month 1 was 1 through 6.

Stage of Development

	3	4	5	6	7	8	9	10	11

Dose (ppb)									
0	0	1	0	5	2	0	0	0	1
4	1	3	1	3	0	0	1	0	1
40	3	1	0	2	1	0	3	0	0
400	0	2	3	4	1	0	0	0	0

Figure 9. Month 2 Taylor Kollros Index for 0 ppb, 4 ppb, 40 ppb and 400 ppb dose groups.

Stage of Development

Dose (ppb)	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
0	0	0	1	2	2	3	0	0	1	0	0	0	1	0
4	1	1	2	1	0	0	1	2	1	0	0	0	0	1
40	0	0	0	1	2	1	3	0	1	0	0	0	1	1

Figure 10. Month 3 Taylor Kollros Index for 0 ppb, 4 ppb, and 40 ppb dose groups

Stage of Development

Dose (ppb)	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
0	1	0	1	1	1	1	1	0	0	0	2	0	1	1
40	0	0	1	1	0	2	1	1	0	2	2	0	0	0

Figure 11. Month 4 Taylor Kollros Index for 0 ppb and 40 ppb dose groups.

Stage of Development

Dose (ppb)			10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
0	0	0	1	0	1	0	0	1	1	0	1	1	1	1	0	2	
40	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	

Figure 12. Month 5 Taylor Kollros Index for 0 ppb and 40 ppb a dose groups.

Weekly Measurements

Appendix F provides a chart of the weekly measurements and sample sizes; for weeks 1-9 the sample size is 20 and weeks 10-17 the sample sizes vary due to mortality and complete metamorphosis. Appendix G provides a chart of the weekly means and standard deviations for each development endpoint. Due to the size of the raw data for the weekly measurements it was not included, only the means were illustrated but the complete data set is available upon request.

Weekly hind limb, snout to vent, total body and tail lengths are illustrated in Figures 13-16. Week 1 of the weekly measurement charts (Figures 13-16) refer to the first full week of the experiment, the week of June 24, 2002.

The hind limbs became visible around week 3 of the experiment and they had the greatest standard deviation between the animals in each dose group (Fig. 13). The hind limb length was significantly different during week 11 of the experiment when comparing the control group to the 40 ppb group (ANOVA, F= 3.527, p=0.038). Weeks 15 and 16 also showed a significant difference from the control group in the hind limb length, a t-test was performed instead of the ANOVA because two of the dose groups had experienced complete attrition during the later weeks of the experiment. During weeks 15 and 16, the 40 ppb group had significantly longer hind limbs than the control group (t-test p=0.038; t-test p=0.010 respectively). Appendix F includes a separate chart of the hind limb sample sizes, as hind limb samples sizes vary due to the fact these data are dependent upon the onset of the endpoint.

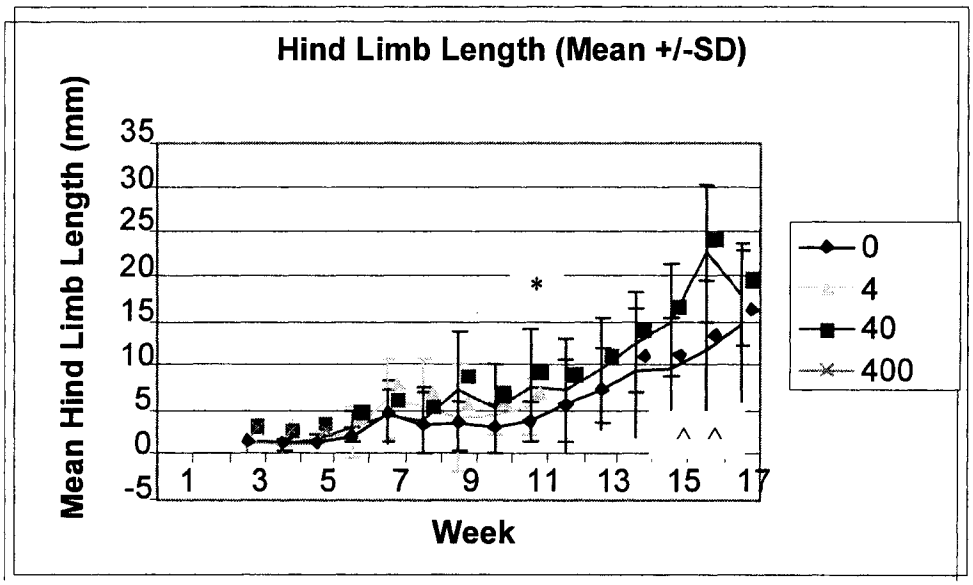
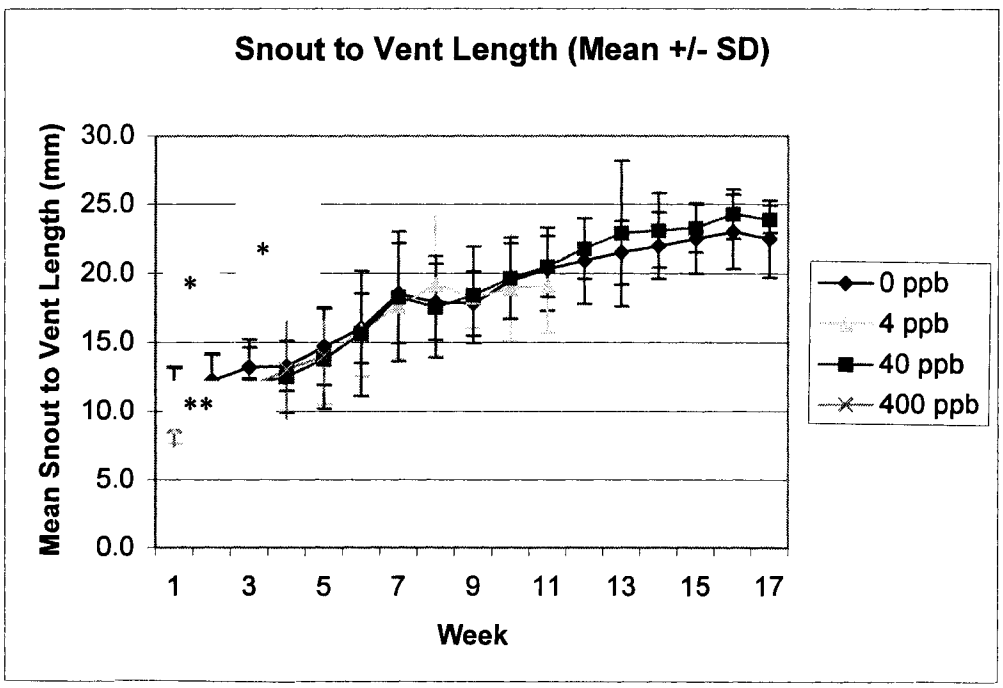


Figure 13. Weekly hind limb length as determined in all four-dose groups. (Week 11, 0 ppb v 40 ppb* post hoc p=0.029; Week 15, 0 ppb v 40 ppb ^, post hoc p=0.038; Week 16, 0 ppb v 40 ppb^ post hoc p=0.010)

The snout to vent length (see Figure 14) remained fairly consistent between the dose groups throughout the experiment. However, there was a significant different in the snout to vent length between groups during week one of the experiment, as the control group was significantly larger at the start of the study. Major differences were noted between the control group and 4 ppb group and 400 ppb group (ANOVA, F=10.96, p=0.00) (See Figure 14.) during week 1 on the experiment. During week three, significant differences were noted between the 4 ppb and 400 ppb group when compared to the 40 ppb group (ANOVA, F=3.29 p=0.025) (See Figure 14 for post hoc analysis). The snout to vent length was only significant during the first and third week of the experiment, after which time the measurements resulted in no longer significant.



* Post hoc significantly different from the control group; P < 0.05 Tukey test
^Post hoc significant different from the control group; P<0.05 T-test

Figure 14. Weekly snout to vent length as determined in all four-dose groups. (Week 1; 0 ppb v 4 ppb* post hoc p= 0.000, 0 ppb v 400 ppb* post hoc p=0.000, 40 ppb v 4 ppb** post hoc p=0.002, 40 ppb v 400 ppb** post hoc p=0.001: Week 2; 0 ppb v 4 ppb* post hoc p=0.002)

The total body length of the tadpoles were similar to the snout to vent results, with a steady consistent gain between dose groups (Fig. 15). There were statistical differences in the total body length during the first three weeks of the study, these differences were noted between the control group and 4 ppb group and 400 ppb group during the first week (ANOVA, F=16.2, p=0.00) as well as during the second week (ANOVA, F=5.6, p=0.02). Week three also presented differences in the total body length between the control group and the 4 ppb group (ANOVA, F=3.8, p=0.013) (See Figure 15).

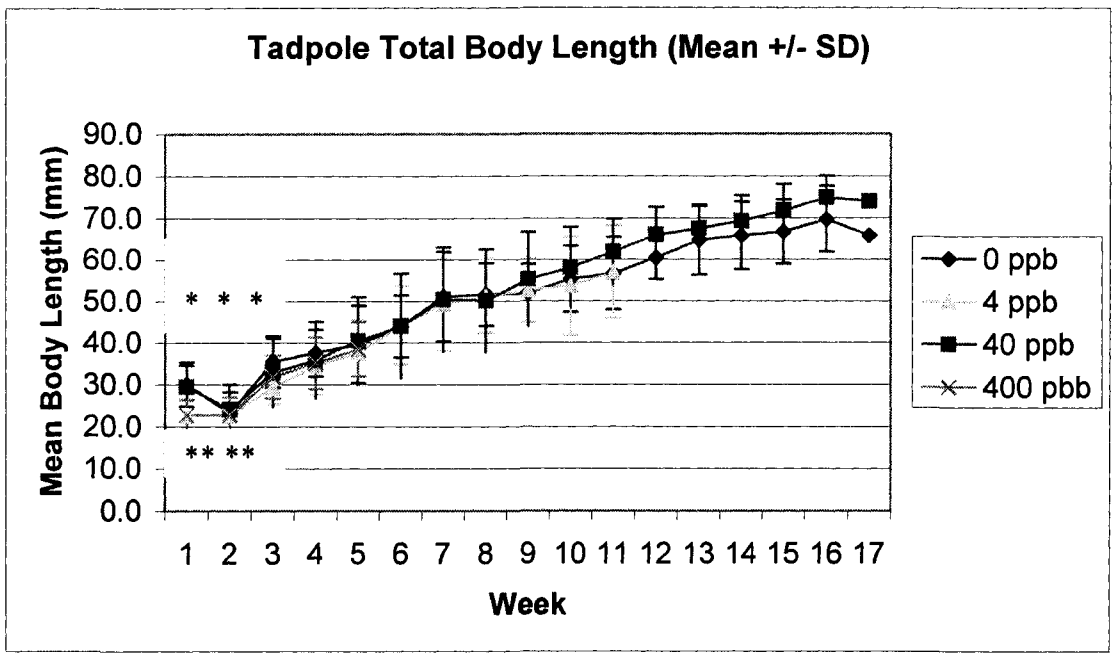


Figure 15. Weekly total tadpole length as determined in all four-dose groups. (Week1; 0 ppb v 4 ppb* post hoc p=0.000, 0 ppb v 400 ppb* post hoc p=0.000, 40 ppb v 4 ppb** post hoc p=0.000, 40 ppb v 400 ppb** post hoc p=0.000: Week 2; 0 ppb v 4 ppb* post

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* Post hoc significantly different from the control group; P<0.05 Tukey test
**Post hoc significantly different from the 40 ppb group; P < 0.05 Tukey test

hoc p=0.003, 0 ppb v 400 ppb* post hoc p=0.047, 40 ppb v 4 ppb** post hoc p=0.030: Week 3; 0 ppb v 4 ppb* post hoc p=0.007)

A steady increase in tail length was noted for all groups as the animals completed metamorphosis. The tadpole tail length was consistent with the previous developmental trends seen in the hind limb, snout to vent and total body measurements, as there was only a significant difference in the tail length during the first three weeks of the study (Fig. 16). During the first week major differences were noted between the control group and 4 ppb group and the control versus 400 ppb (ANOVA, F=15.2, p=0.00). During the first week there were also differences between the 4 ppb and 400 ppb groups when compared to the 40 ppb group (ANOVA, F=15.2, p=0.00). Week two also presented significance in the tail length between the control group and the 4 ppb group as well as significance in the 4 ppb group when compared 4 to the 40 ppb group (ANOVA, F=7.0, p=0.00) (See Figure 16). Tail length was also significant during week three when comparing the control group to the 4 ppb group (ANOVA, F=3.3, p=0.054, Fig 16.)

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* Post hoc significantly different from the control group; $P < 0.05$ Tukey test
** Post hoc significantly different from the 40 ppb group; $P<0.05$ Tukey test

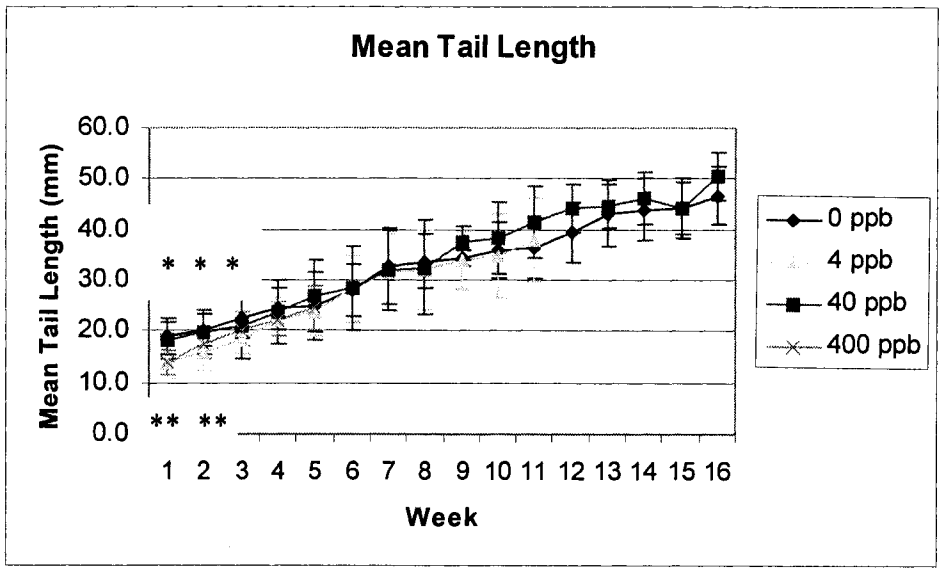


Figure 16. Weekly tail length following exposure to 0, 4, 40, 400 ppb perchlorate in water for all four-dose groups. (Week1; 0 ppb v 4 ppb* post hoc p=0.000, 0 ppb v 400 ppb* post hoc p=0.000, 40 ppb v 4 ppb** post hoc p=0.000, 40 ppb v 400 ppb** post hoc p=0.000: Week 2; 0 ppb v 4 ppb*, post hoc p=0.001, 40 ppb v 4 ppb** post hoc p=0.003: Week3; 0 ppb v 4 ppb*

* Post hoc significantly different from the control group; $P < 0.05$ Tukey test
** Post hoc significantly different from the 40 ppb group; $P<0.05$ Tukey test

Figure 17 is a graph of the total tadpole length when all of the tadpoles in each tank were measured on August 18, 2002; as opposed to only a sample of 20 being measured each week. The total tadpole lengths taken on the entire population did not show any significant differences following a Chi Square analysis when comparing the dose 4 ppb and 40 ppb groups to the control

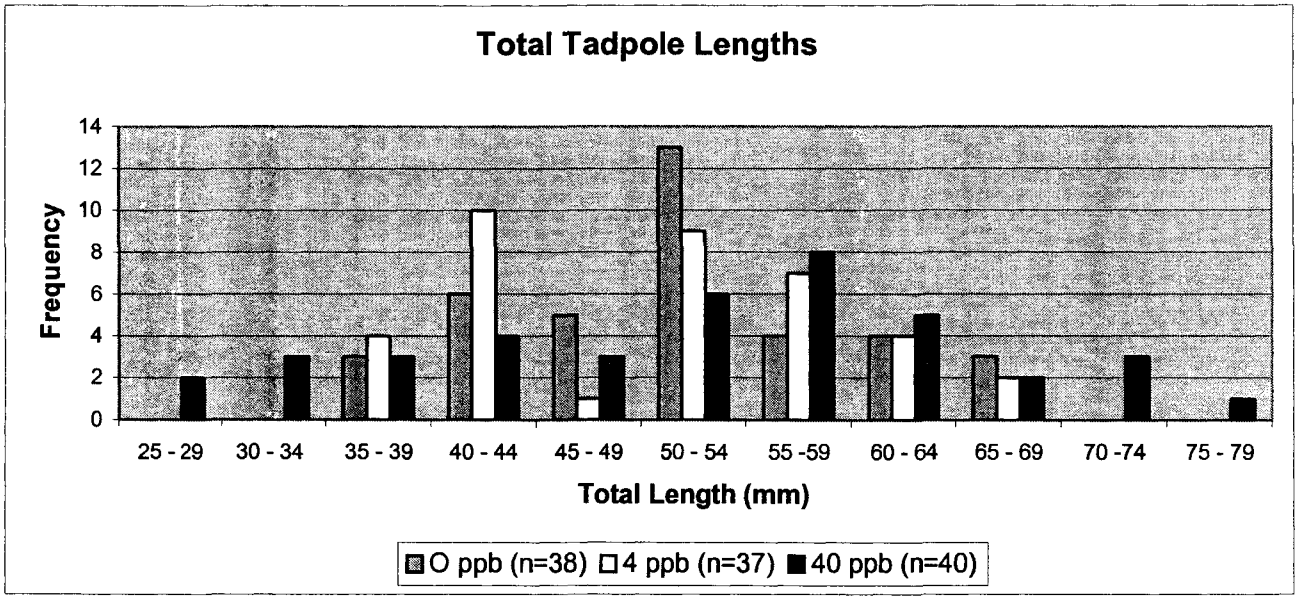


Figure 17. Frequency distribution of total tadpole length from the entire tadpole population in the three dose groups taken on August 18, 2002

DISCUSSION

One of the biggest difficulties encountered during the course of this study was the infestation of the parasite, *Dactylogyrus vastator*, in the 400 and 4 ppb groups. In the highest dose group (400 ppb) all the animals had died by the end of the fifth week of the study; whereas the animals in the 4 ppb group all died by the end of the twelfth week. It is unclear why the middle dose group was unaffected by this parasite.

Figure 18. *Dactylogyrus vastator* as identified on the 4, 400 ppb group

Two possible explanations for this phenomenon have been evaluated. First, the parasite may have been present in the tadpoles upon arrival from the Trans-Mississippi Biological Supply Company. Following exposure to the perchlorate, the animals may have had a suppressed immune system allowing the parasites to multiply and overtake the animals. An evaluation of the life history of the parasite indicates that these are common parasites in aquatic organisms, mainly fish, and infest the gills leading to respiratory distress and eventually death. Following a phone call to the tadpole supplier, they have not had any other parasitic problems in this species. However, tadpoles and frogs have shown immune suppression following exposure to other environmental contaminants (Zettergren et al).

Second, we postulate that the parasite was present in the Las Vegas Wash water collected from the LM-8 site. Since the highest dose group was serially diluted to make the lower dose groups (see methods or appendix for details) a density-dependent exposure to the parasites, which corresponds to dose also exists. This would explain the rapid die-off of the high dose group, but may not account for the absence of effects in the middle dose group.

In either case, toxicological investigations of perchlorate on several other thyroid related endpoints support the possibility of a u-shaped dose-response curve. The findings that support a u-shaped dose-response curve have been highlighted in the recent report to the Federal Register. However, it should be noted that our explanations of the possible relationships to parasitic infestations are highly speculative as this study was designed to assess the effects of perchlorate on development and metamorphosis in *Rana pipiens*. Further research is needed to more thoroughly examine the susceptibility of tadpoles to parasitic infections following perchlorate exposure.

The only significant differences noted in the TKI occurred during the first month of development, with the 4 and 400 ppb groups exhibiting a developmental delay when compared to the control group. Since the parasitic infestation occurred during the fifth week of the experiment in the 400 ppb group, most likely the developmental delays were due the increased demand placed on the animals by the early stages of parasitic infestation. Once the parasite was noted and identified, all animals were dead within several days within this group. At this time, animals from all the other groups were removed from the tanks and inspected for signs of parasites; however, no parasites were

found in any of the other dose groups. The same parasite, *Dactylogyrus vastator*, was then identified in the 4 ppb group at week twelve. It should be noted that we did see delays in the development of the 4 ppb group in the absence of any visible parasitic infection following the one month TKI, but these delays were not evident in the 40 ppb group. With the confounding problem of the parasites, it is difficult to attribute the effects in the 4 ppb group to the perchlorate exposure, but this possibility cannot be ruled out.

The weekly measurement data showed very consistent findings between the dose groups. Although there were significant differences in the animal sizes at the start of the study (0, 40 ppb > 4, 400 ppb), these differences were no longer detectable by the fourth week of the experiment. In fact, all statistical analyses performed on snout to vent length, total length, and tail length following week three demonstrated that no differences were detectable between any of the dose groups.

The hind limb results were more interesting, as the hind limb's measured in the 40 ppb group during weeks 11, 15, and 16 were significantly larger than the control group. Unfortunately, the 4 ppb group all died three weeks prior to this determination and could not be evaluated. These data, although limited, also support the possibility of perchlorate exhibiting a dose-response curve that demonstrates a hormetic effect.

With the added confounds of the parasites in this study, it is very difficult to make any firm scientific evaluation as to the possible mechanisms associated with the observed effects. To strengthen this study we would suggest repeating the experiment using known concentrations of perchlorate acquired from a laboratory source. Thus, we could

avoid the potential introduction of parasites from surface water sources. These data could then be used to better evaluate the consequences to tadpoles following environmental exposure scenarios such as those presented in the LV Wash and surrounding areas.

Report Appendix A

Perchlorate Project – Weekly Tank Water Change

Introduction

This procedure has been selected to take place on Thursday of every week. The 5-gallon containers of water to be used will have been set out the day before to acclimate to the tank room temperature. Approximately 5 white containers of Las Vegas Wash water will be used and approximately 12 orange containers of Overton Beach water will be used. This is the time that a count of the tadpoles in all four tanks (A, B, C, and D) will be done and recorded. Make sure that the daily water analysis has been done before changing the tank water.

Equipment

Protocol labeled "Preparation of Water in Tanks" [in logbook]
Logbook [on the shelf above the sink in tank room WHT 135]
4 empty five-gallon containers to hold the tadpoles during water change [from lab MPE 224]
fish nets [resting on top of each tank]
2 clear hoses [on top of the paper towel dispenser in the tank room]
4 large, white, rectangular plastic containers [in the "clean equipment room" of the animal care facility]
pencil and scratch paper
water thermometer [on the shelf above the sink in tank room]
Pyrex beaker [on shelf above the sink in tank room]
masking tape and marker [on shelf above the sink in tank room]

Procedure

1. Use the masking tape and marker to label the 4 empty (5 gallon) containers A, B, C, and D.

2. Use the beaker to scoop water from the corresponding tanks into the empty containers marked A, B, C, and D. Fill them approximately 6 inches full.

3. Count the tadpoles 1 at a time, placing them in the corresponding labeled container with water. At every 5 tadpoles, use the scratch paper to make a mark. Make a mark for each 5 tadpoles counted into the container.

4. When done counting the tadpoles, record the number in the logbook and move to the next tank. Repeat the counting procedure.

5. After counting all the tadpoles and logging the numbers in the logbook, set the containers with the tadpoles out of the way where they will not be disturbed.

6. Turn off the bubblers at the multiple power-outlet strip. Take the covers off of the bubblers. Remove the intake tube (tubes with the filters on the ends-they just snap out). Empty the bubblers and rest them on the tank stand.

7. Place 2 of the large, white, rectangular, plastic containers in front of one of the tanks.

8. Submerge one of the clear hoses in the tank until all the air is gone from the hose. Plug one end of the hose with your finger and quickly remove it and place it into the container in front of it. You must be careful not to let the other end come out of the tank.

**Note: the rectangular container is lower than the tank, so the hose will act as a siphon to remove the tank water.

9. Repeat #7 with the second hose and another rectangular container.

10. Watch both of the rectangular containers fill with water. When they are almost full, switch the hose to another empty container so you can dump the full one in the sink.

**Note: if the hose gets any air in it...the siphoning will stop. Just submerge the entire hose again, plug one end, and start again.

11. When the tank is almost empty, it can be lifted and the remaining water CAREFULLY dumped into the sink. Make sure the bubbler is securely away from the tank so you don't yank the cord and bubbler.

**Note: You MUST BE CAREFUL to keep the tank from touching the sink. The sink is ceramic and the corners of the tanks are glass.

12. Repeat this process to empty all four tanks.

13. For tank A, located at the highest level, you will either have to hold the hose and point it at a rectangular container that is catching the water, or you will have to place the rectangular container on top of a step stool or bucket. The hose will not reach the rectangular container if it is on the floor.

14. To fill the tanks, see the protocol labeled "Preparation of Water in Tanks" [located in the logbook].

15. Check the temperature of the new tank water with the temperature gauge [on the shelf above the sink]. Make sure the temperature is not radically different from the water the tadpoles are in. Check the tadpole water temperature. Example: if the water temperature

of the tadpoles is 20.1 degrees Celsius and the tank water is 19.5 degrees Celsius...then you can return the tadpoles to the tanks. If the temperature is more than a degree different, speak with Marsh Moon and get her approval to return the tadpoles. You may have to wait awhile for the new tank water to acclimate more.

16. Replace the bubblers, their uptake tubes, and fill their overflow tanks.

17. Turn the outlet strip for the bubblers back on and prime the overflow tank to get the bubblers started.

18. After the tanks have been filled according to the protocol and the water temperature has been checked and the bubblers are on...count the tadpoles 1 at a time, placing them in the corresponding labeled tank as the container you are counting from. At every 5 tadpoles, use the scratch paper to make a mark. Make a mark for each 5 tadpoles counted back into the tank.

19. Check the first count of tadpoles. Record both counts. If the margin of error is more than one or two...leave a note for Andrea so that she may count them again.

20. Take the rectangular, white, plastic containers to the far back room of the animal care facility and leave them on the metal counter next to the equipment washer.

21. Ask Marsha Moon or Jewel Sutton where a mop is located to wipe up the spills on the tank room floor.

22. Rinse out the hoses and place them back above the paper towel dispenser.

23. As per the "Preparation of Water in Tanks" protocol...take all of the empty 5-gallon containers to the MPE 224 Lab.

(SJMoloney, 7 July 2002)
APPROVED SLG, July 16, 2002

Report Appendix B

Perchlorate Project – Daily Water Analysis (new equipment)

Introduction

These procedures monitor the pH level, dissolved oxygen, conductivity, and temperature of the water from the tadpole tanks labeled A, B, C, and D that are located in WHT 135. Each test is to be done in triplicate (per tank), data recorded, and the Mean determined and recorded. (Mean-add 3 consecutive results from one of the tests [on one tank] and divide the sum by 3). All data should be recorded in the logbook that is located in the tank room, WHT 135. Date and initial the logbook daily after completing the tests.

Two instruments {the YSI model 85 for oxygen consumption and YSI model 63 for pH level, conductivity, and temperature} are located in Dr. Gerstenberger's Lab (MPE 224). They are in their own black, plastic cases. These instruments are expensive and should be handled with care. If there is no response in the LCD display of these instruments after pressing the "ON/OFF" button, there are extra batteries in one of the cases. Please be sure to return these instruments to MPE 224 when finished.

**Note: check in with Marsha Moon in animal care facility office, WHT 121, so that she will be available to open the facility on Saturdays and Sundays. The animal care facility office phone number is 895-3384.

Equipment

- YSI model 85 in black case [in lab-MPE 224]
- YSI model 63 in black case [in lab-MPE 224]
- Pyrex beaker (2000 mL) [above the sink in the tank room, WHT 135]
- distilled water
- logbook [above the sink in the tank room, WHT 135]
- standard YSI pH buffer with value of pH 7 (YSI 3822) [in the labeled, covered, small plastic container above the sink in the tank room (solution is green)]
- 100 mL graduated cylinder [above the sink in the tank room, WHT 135]
- paper towels
- pencil
- calculator [one is provided in the inside flap of logbook]
- tadpole tanks A, B, C, and D in WHT 135

Procedures

1. Fill the 2000 mL Pyrex beaker with distilled water from the sink in WHT 122 (next to the tank room). This sink has one spout with a white knob...this is the distilled water spout.

2. Remove the YSI model 85 from its case and press the "ON/OFF" button once. Set aside.

3. Fill the 100 mL graduated cylinder half full with the pH 7 buffer from the labeled plastic bottle, both found above the tank room sink.

4. Remove the YSI model 63 from its case and press the "ON/OFF" button once.

5. Press the "MODE" button until "pH" is displayed on its LCD screen. (model 63)

6. Remove the YSI model 63's probe from the side of the unit and rinse it in the beaker with distilled water. Carefully dry the probe.

7. Immerse the probe completely (to where the cord attaches) in the cylinder with the pH 7 buffer solution. (model 63)

8. Press the up and down arrows simultaneously one time. (model 63)
["CAL" will appear at the bottom of the LCD display and "STAND" will be flashing in the bottom left corner of display. The display will also read "7.00 pH"]

9. Press "ENTER" (model 63).

["CAL" will still display. "STAND" will still display, but it will stop flashing. The decimal within the "7.00 pH" will flash for a few seconds until the reading is stable.]

10. When the decimal stops flashing within the "7.00 pH" in the display, **press and hold** the "ENTER" button until "SAVE" is displayed (model 63).

["SLOPE" will appear on the left of the display and it will be flashing]

11. Press "MODE" and the YSI model 63 is ready for taking readings.

12. Remove the probe from the buffer solution, rinse it in the beaker of distilled water, and then place it in one of the tadpole tanks until the water is covering the entire probe.

13. Remove the other probe from its storage on the YSI model 85 instrument, rinse in the distilled water, and submerge this probe in the same tank as the model 63.

**Note: submerge both probes entirely and place them at the right, front corner of the tank, furthest away from the bubbler.

**Note: gently shake the probes in the water to remove any small air bubbles.

**Note: rinse both probes in distilled water in between different tank readings.

14. Under the appropriate tank heading in the logbook (A, B, C, or D) record the pH level when it has stabilized. (model 63)

15. Also record the temperature in degrees Celsius from this same display. (model 63)

16. Press "MODE" once and there will be a number reading in units of "uS" or "mS" and the degree Celsius sign **will NOT be flashing**. This is the conductivity reading. Record this reading. (model 63).

**Note: there is another numeric reading in units of "uS" or "mS" but the degree Celsius sign DOES flash...we do not want this reading...press mode again until you come to the appropriate mode.

17. Press "MODE" until you return to the "pH" reading again, remove the probe from the tank water until the reading starts to change and then replace it in the same tank for a second and third set of readings, repeating steps #14 thru # 17 each time.

18. Watch the model 85 display until it stabilizes within a point or two (example: between 41.2 % and 42.6 %) It will fluctuate some because of the oxygen movement created from the bubbler. This numeric reading shows as "%".

19. Record this number from the model 85 and, like the model 63, remove the probe for a few seconds and then replace it into the same tank for a second reading and then again for a third reading.

20. Rinse both probes in the distilled water and then place them in the next tank. Take readings in triplicate for all four tanks.

**Note: [The pH, the temperature in degrees Celsius, and the conductivity in uS or mS will always be read from the model 63.] [The % of dissolved oxygen will always be read from the model 85.]
**Note: while you are waiting for any of the readings to stabilize, you can pour the pH 7 buffer solution from the cylinder back into the labeled plastic container, rinse the cylinder in the sink, and place both back on the shelf above the sink.

**Note: When all of the readings are taken, rinse the probes in distilled water a final time and place them back into their storages on the sides of the instruments. Turn the instruments off. Place them carefully back into their cases and close, being careful not to crimp the cords. Return these two instruments to Dr. Gerstenberger's Lab-MPE 224.

21. Use the calculator to determine the Mean of all of the data and record. Initial and date the data page and return the logbook to the shelf above the sink in the tank room. Rinse the beaker and the set on same shelf.

**Note: clear any debris from the bubbler filters daily. If you have to, remove the tube with the filter and clean it off in the beaker with distilled water.

APPENDIX B

SAMPLE SIZE FOR TEMPORALLY BASED TOTAL BODY, SNOUT TO VENT AND TAIL LENGTH

Week	Mg Contol	Control	40 ppb	400 ppb	4000ppb
1	40	40	40	40	40
2	40	40	40	40	40
3	19	40	40	40	40
4	18	40	40	40	40
5	17	40	40	40	40
6	18	40	40	40	40
7	17	40	39	40	40
8	13	40	39	40	40
9	9	57	34	40	40
10	8	57	26	40	40
11	5	55	25	40	34
12		53	20	40	29
13		42	13	40	24
14		39	11	40	21
15		39	11	38	20
16		38	11	35	19
17		37	9	40	11
18		36	7	38	9
19		34	7	35	8
20		33	4	32	6
21		23	2	25	4
22		18		22	
23		18		20	
24		16		19	
25		13		19	
26		12		18	
27		14		17	
28		11		13	
29		10		12	
30		7		11	
31		6		11	
32		5		7	
33		5		7	

APPENDIX C

SAMPLE SIZE FOR TEMPORALLY BASED HINDLIMB

Week	Mg Contol	Control	40 ppb	400 ppb	4000ppb
4	10	0	3	1	0
5	16	16	24	14	8
6	18	9	23	7	13
7	17	11	28	8	16
8	13	12	28	10	22
9	9	16	28	15	26
10	8	18	23	27	34
11	5	45	25	20	25
12		40	20	30	28
13		42	11	30	20
14		27	10	37	16
15		28	10	32	19
16		27	9	26	13
17		28	8	39	9
18		24	6	32	7
19		28	6	32	8
20		27	3	28	5
21		22	2	24	4
22		17		21	
23		18		20	
24		16		16	
25		13		19	
26		12		18	
27		14		17	
28		11		13	
29		10		12	
30		7		11	
31		6		11	
32		5		7	
33		5		7	

APPENDIX D

STAGE BASED MORPHOMETRIC CHARACTERISTICS OF *RANA PIPIENS* EXPOSED TO PERCHLORATE

	Overall Sig.
Weight	0.24
Snout to Vent	0.835
Tail	0.142
Total	0.558
Right Forelimb	0.004**
Right Hindlimb	0.176

	Sample Size	Rank Sums
Mg Control	13	1178
Control	27	1600
40 ppb	27	1970
400 ppb	38	1892
4000 ppb	20	1235

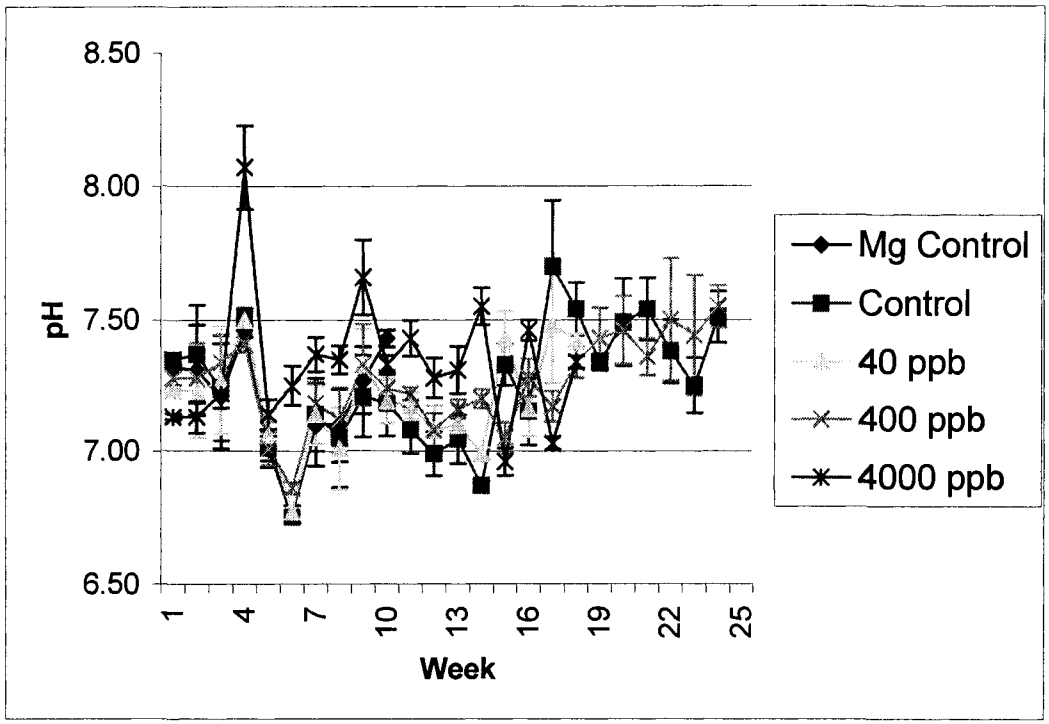
** Statistically significant ($p<0.05$) by Kruskal-Wallis examination

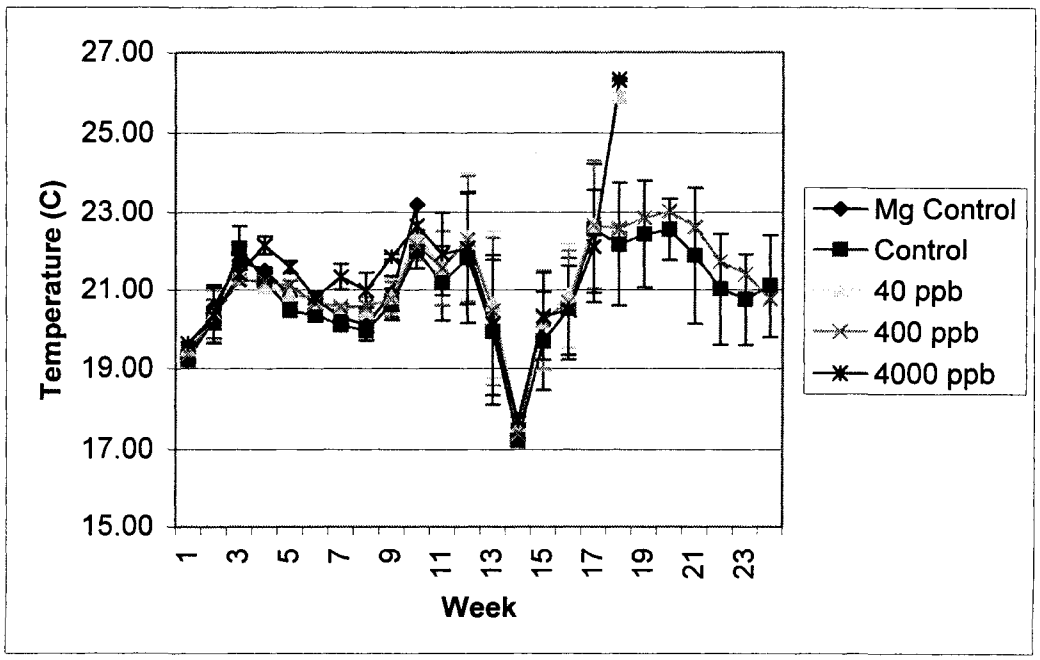
S.E.= ((N(N+1)/12)*((1/n)+(1/n)))^1/2

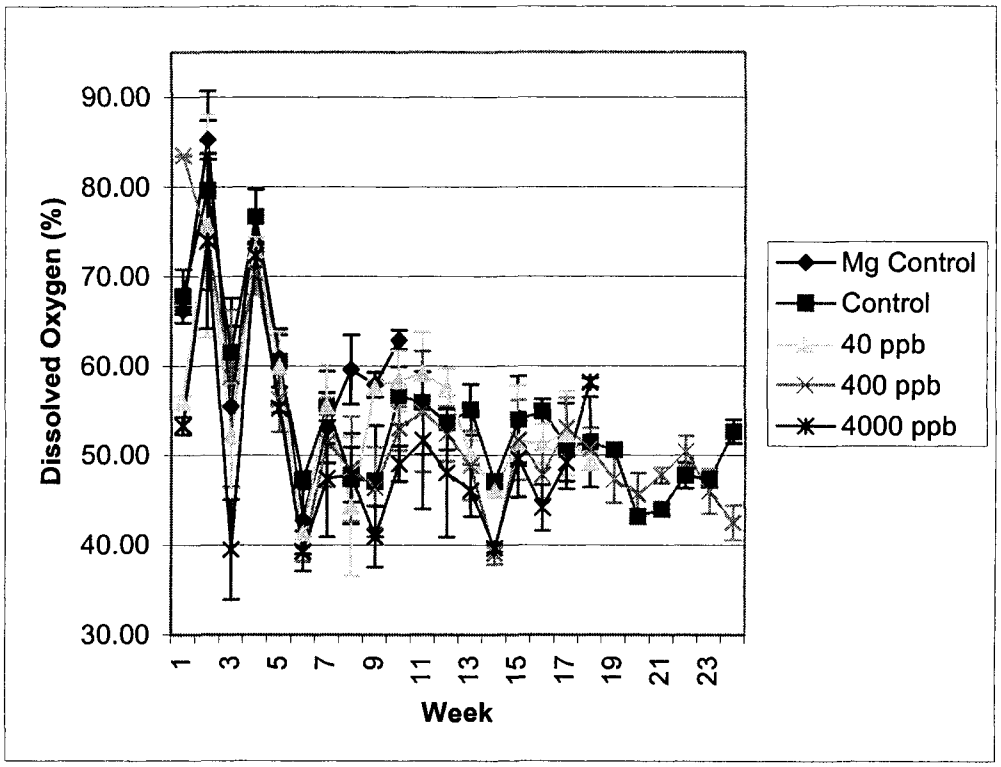
Nemenyi Post-hoc on Right Forelimb			
Comparison	S.E.	Difference	q
Mg v. Control	12.17	422	34.67
Mg v. 40 ppb	12.17	792	65.07
Mg v. 400 ppb	11.64	714	61.34
Mg v. 4000 ppb	12.91	57	4.41
Control v. 40 ppb	9.86	370	38.43
Control v. 400 ppb	9.12	292	32.01
Control v. 4000 ppb	10.68	356	34.17
40 ppb v. 400 ppb	9.12	78	8.55
40 ppb v. 4000 ppb	10.68	735	68.82
400 ppb v. 4000ppb	10.01	657	65.6
Critical value q 0.05, infinity, 5 is 3.858 Thus, the post hoc does not show any significance between groups			

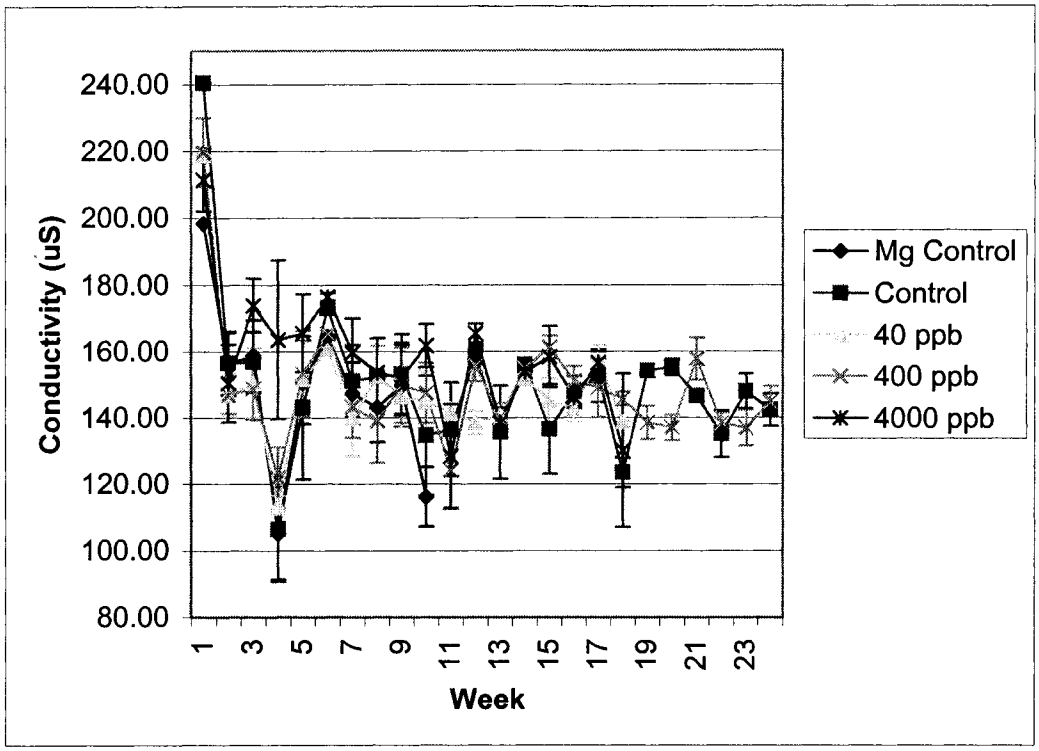
APPENDIX E

WEEKLY TANK WATER QUALITY









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